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Biofilm-based Healthcare-associated Infections

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Persister Cells in Biofilm Associated Infections

1

Brian P. Conlon, Sarah E. Rowe, and Kim Lewis

1 Introduction

Persisters were first identified by Joseph Bigger in 1944 (Bigger 1944). Bigger was the first to show that penicillin is a bactericidal antibiotic. He also found that addition of a lethal dose of penicillin to a population of *Staphylococcus aureus*, always resulted in a subpopulation of survivors. These surviving colonies could be regrown and the resulting population could also be lysed efficiently with penicillin to yield a subpopulation of survivors, or persister cells (Bigger 1944).

Persisters are dormant, phenotypic variants of regular cells, capable of surviving in the presence of high levels of bactericidal antibiotics. They can be pre-existing in a population, or their formation can be induced by environmental conditions (Balaban et al. 2004; Dorr et al. 2010). The relative abundance of persister cells in a population is dependent on the growth phase, with a particularly high number being observed in the stationary phase (Keren et al. 2004; Conlon et al. 2013). Persister cells often display multi-drug tolerance, meaning that an individual persister can survive for prolonged periods in the presence of a variety of antibiotics (Conlon et al. 2013). Persister formation can actually be induced by exposure to antibiotics of various classes (Dorr et al. 2010; Johnson and Levin 2013).

Persister cells have been identified in vivo and have been directly implicated in disease (Conlon et al. 2013; Helaine et al. 2014; Mulcahy et al. 2010). They are proposed to be the underlying reason for the tolerance demonstrated by bacterial biofilms. Targeting these dormant persister cells with novel antibiotics will allow us to efficiently treat chronic biofilm infections and eradicate bacterial populations (Conlon et al. 2013).

2 Mechanisms of Persister Formation

The *hipA* gene was the first gene demonstrated to have an impact on persister formation. Moyed and co-authors found that a particular allele of this gene, termed *hipA7*, led to at least a 100-fold increase in persister cells formation. In other words, antibiotic treatment of a population of *E. coli* harboring the *hipA7* gene, resulted in 100–1,000 times more survivors than an identical treatment of a wild-type population with a different *hipA* allele (Falla and Chopra 1998). Importantly, the minimal inhibitory concentration (MIC) of the antibiotics examined in this study, were identical for the wild-type and *hipA7* strains. A change in MIC suggests the development of resistance to an antibiotic, not apparent in a high persister (*hip*) phenotype.

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The *hipA* gene is the toxin portion of a toxin-antitoxin locus, *hipBA* (Falla and Chopra 1998). HipA is neutralized by its antitoxin HipB. It has been demonstrated that under cellular stress, the Lon protease degrades HipB allowing HipA to exert a toxic effect on the cell (Hansen et al. 2012; Tsilibaris et al. 2006). The toxicity of HipA is due to its kinase ability. It phosphorylates the glutamyl tRNA synthetase resulting in the accumulation of uncharged tRNA leading to activation of the stringent response, a shutdown of protein synthesis and subsequent persister formation (Germain et al. 2013).

The stringent response is activated under amino acid starvation (Durfee et al. 2008). Stalling of ribosomes due to accumulation of uncharged tRNA leads to activation of RelA which synthesize the alarmone (p)ppGpp (Durfee et al. 2008), and has a global impact on transcription, leading to downregulation of protein synthesis machinery and an induction of other genes including those involved in amino acid biosynthesis (Zhou and Jin 1998).

The stringent response has been heavily implicated in bacterial persister formation (Maisonneuve et al. 2013; Kaspy et al. 2013; Nguyen et al. 2011). Gerdes and co-authors have put forward a model whereby persister formation is governed by activation of the stringent response leading to antitoxin degradation by the Lon protease allowing free toxins to mediate persister formation (Germain et al. 2013).

Stochastic switching to slow growth results in persister cell formation and is governed by (p)ppGpp (Maisonneuve et al. 2013). Accumulation of ppGpp leads to the activation of the Lon protease (Balaban et al. 2004; Maisonneuve et al. 2011).

Degradation of the antitoxins is primarily mediated by the Lon protease in *E. coli* (Helaine et al. 2014; Hayes and Van Melderen 2011). The Clp protease has been shown to be responsible for antitoxin degradation in *S. aureus*, which lacks a Lon homologue (Donegan et al. 2010). Degradation of the antitoxin and subsequent freeing of the toxin can occur in response to specific environmental stimuli, leading to persister formation.

TA genes were first identified as addiction modules that play a role in plasmid maintenance (Ogura and Hiraga 1983). The antitoxin is far less stable than the toxin and is degraded by cellular proteases (Ogura and Hiraga 1983). TAs were subsequently discovered on bacterial chromosomes. Many studies have implicated chromosomal TAs to have a role in persistence which will be discussed in this section (Dorr et al. 2010; Falla and Chopra 1998; Hansen et al. 2012; Maisonneuve et al. 2011; Kim and Wood 2010; Moyed and Bertrand 1983).

There are 11 TA mRNA interferases in *E. coli* K12. Their involvement in persister cell formation was first suspected as overexpression of the toxin portion of the TA module led to cell shutdown and persister formation in vitro (Kim and Wood 2010; Donegan and Cheung 2009). They achieve this feat by cleavage of mRNA. Further investigation of the role of TAs in persister formation found that deletion of individual TAs did not lead to a measurable decrease in persister formation and it was hypothesized that redundancy between the TA modules may explain this discrepancy (Spoering et al. 2006).

A study that deleted 10 of these TA modules, in series, confirmed this to be the case (Maisonneuve et al. 2011). Deletion of 5 TAs led to a significant decrease in persister cells and the effect was heightened for each subsequent mutation, with the final Δ10 strain displaying a 100-fold reduction in persister formation. Furthermore the TA deletions were repeated in reverse order and, again, the phenotype was evident after five deletions and most pronounced after deletion of all 10 TA modules (Maisonneuve et al. 2011).

It should be mentioned that it is entirely possible that individual TA loci play an important role in persisters under certain specific environments or in certain strains. The fact that each TA locus in *E. coli* is unique and differentially regulated certainly suggests this (Gerdes et al. 2005). Norton et al. reported that mutation of the TA module PasTI had no phenotype when deleted in *E. coli* lab strain MG1655 but displayed a 100-fold reduction in persisters when deleted in a clinical isolate CFT073 (Norton and Mulvey 2012).

Dorr et al. showed that another toxin, *tisB* which is induced by the SOS-response could specifically induce persisters in response to fluoroquinolones (Durfee et al. 2008). They showed that ciprofloxacin induced the SOS system leading to increased persister formation and that this increase could be attributed to induction of the TisB toxin. TisB exerts its toxic effects by targeting the membrane causing a decrease in proton motive force which, at the right levels, can induce persister cell formation (Germain et al. 2013; Durfee et al. 2008). A *tisB* mutant also displayed a decrease in persisters but only to high levels of ciprofloxacin. The authors concluded that *tisB* likely mediates survival during extreme SOS stress when shutting down the cell, rather than attempting repair is more beneficial to survival.

3 Other Genes Implicated in Persister Cell Formation

A variety of studies and screens have identified a multitude of genes implicated in the persister phenotype. One such study identified the gene, *glpD*, involved in glycerol metabolism as having a significant impact on persister cell formation (Spoering et al. 2006). Interestingly, overexpression of *glpD* also induced persistence to ofloxacin and ampicillin suggesting that these persisters are multi-drug tolerant. Deletion of the *glpD* gene in a wild type background reduced levels of persisters to ciprofloxacin in stationary phase.

Various aspects of metabolism have been implicated in persister cell formation in numerous other studies (Bernier et al. 2013; Allison et al. 2011; Amato et al. 2014). Bernier et al. identified numerous amino acid auxotrophs that displayed an increase in persister cell formation (Bernier et al. 2013). Girgis et al. showed that mutation of methionyl tRNA synthase resulted in a 10,000-fold increase in persisters (Girgis et al. 2012). They also found that mutations in transketolase A (*tktA*) and glycerol-3-phosphate (G3P) dehydrogenase (*glpD*) increase persistence through metabolic flux alterations that increase intracellular levels of the growth-inhibitory metabolite methylglyoxal (Girgis et al. 2012). Another high-throughput screen in *Pseudomonas aeruginosa*, using a 5,000 gene knockout library, identified numerous genes involved in metabolism that had a significant impact on persister cell numbers (De Groote et al. 2009a). Another study in *Streptococcus mutants* identified persister genes associated with phosphate metabolism, phospholipid synthesis, purine metabolism, folate biosynthesis and energy metabolism (Leung and Levesque 2012). Persister levels increase as nutrients becoming limiting in the stationary phase (Keren et al. 2004). (p)ppGpp accumulation results in an increase in persister cell formation (Maisonneuve et al. 2013; Kaspy et al. 2013). Addition of sugars enables killing of persisters with an aminoglycoside (Allison et al. 2011). Together these findings demonstrate the central role metabolism plays in persister formation. This is important from the perspective of biofilm as these environments may be low in nutrients.

Another screen for persister genes utilized the Keio collection (Baba et al. 2006), an ordered deletion library of all 3,985 non-essential genes in *Escherichia coli*. A number of hits were confirmed and most of these were global regulators with relatively minor effects on persister levels (Hansen et al. 2008).

Similarly, studies of persisters in the pathogenic organism, *P. aeruginosa* identified a number of global regulators that altered persister levels although the precise mechanism by which they control persister formation remains unclear (Moker et al. 2010; Murakami et al. 2005; Viducic et al. 2006; De Groote et al. 2009b).

In general, although many genes in a variety of pathways appear to be involved in persister formation, they all lead to a common phenotype by achieving a level of dormancy that protects them from antibiotic treatment (Fig. 1.1).

4 Persisters and Biofilm

Bacterial biofilms have been implicated in an array of human diseases. A biofilm can protect bacterial cells from the host immune system, serving as a protective barrier against complement

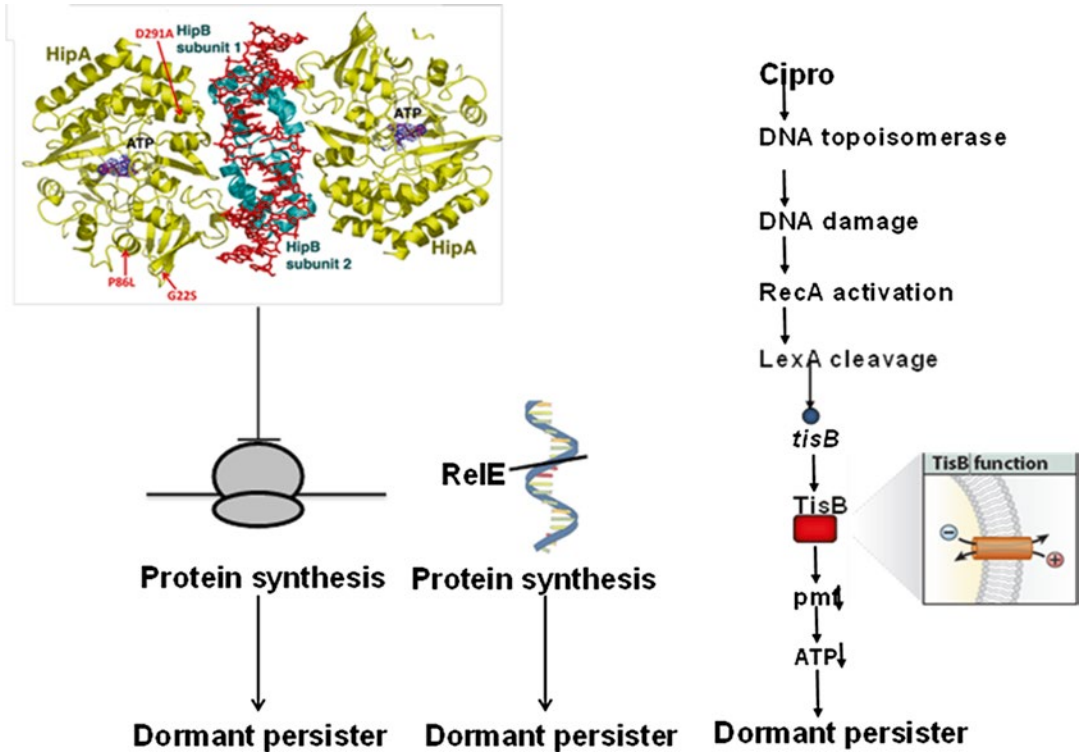

Fig. 1.1 Redundant pathways of persister formation

immunity and phagocytosis. The antibiotic tolerance of a microbial biofilm has also long been recognized and thought to be central to the difficulty in treating biofilm associated diseases (Costerton et al. 1987).

Biofilm associated infections such as a cystic fibrosis lung infection, infective endocarditis, wound infections and infections of indwelling devices are notoriously recalcitrant to antibiotic treatment, often leading to prolonged treatment regimens or extreme measures such as removal and replacement of an infected device or debridement of an infected wound (Ammons 2010).

It was hypothesized that biofilm protected indwelling cells from antibiotic penetration (Gristina et al. 1987). Upon detailed examination of this hypothesis, it was shown that antibiotics penetrate biofilm quite well (Vrany et al. 1997; Anderl et al. 2000; Darouiche et al. 1994; Jefferson et al. 2005). The tolerance of the biofilm was finally explained by the presence of persisters within the biofilm population (Lewis 2001). A bactericidal antibiotic will kill the majority of cells in a biofilm but persisters will survive. These persisters are shielded from the immune system by the biofilm matrix. Once antibiotic treatment is ceased, these persisters can revert to a growing state and repopulate the biofilm causing a relapse of infection (Fig. 1.2).

Persister cells are more abundant in the stationary phase of growth (Keren et al. 2004), presumably due to nutrient deprivation inducing cellular shutdown. Cells within a biofilm may be in a nutrient deprived state and thus the biofilm lifestyle may serve to induce persister cell formation. Indeed, *Staphylococcus aureus* biofilm populations tend to demonstrate far higher proportions of persisters than an exponential phase planktonic population (Conlon et al. 2013).

One recent study looked at what was known about persister formation in planktonic *E. coli*

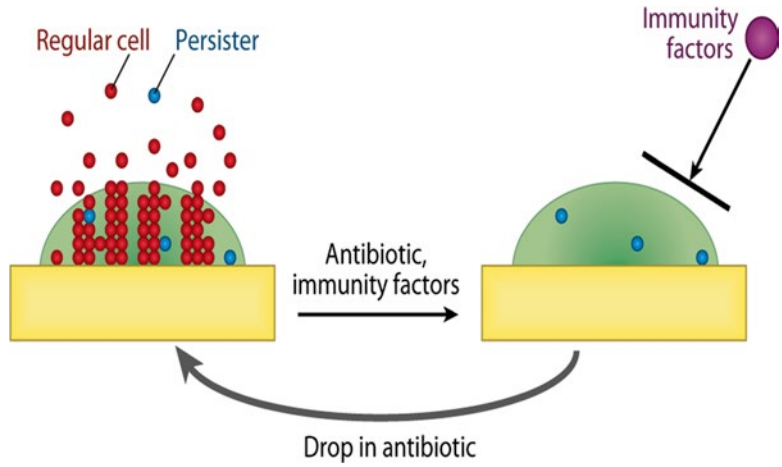

Fig. 1.2 A model of a relapsing biofilm infection

and applied it to the biofilm environment. What they found was that ppGpp was central to persister formation in the *E. coli* biofilm and persister cell formation was governed by nutrient transitions, again implicating metabolism as a central component of persister cell formation (Amato and Brynildsen 2014). The carbon source was altered and this was shown to impact persister levels in the biofilm through RelA and ppGpp (Amato and Brynildsen 2014). Interestingly, this ppGpp mediated persister formation did not implicate TA modules and was therefore independent of the proposed model involving (p)ppGpp accumulation, followed by Lon mediated degradation of antitoxins, which was previously discussed.

Another study, again using *E. coli* biofilm, conducted a screen to find genes involved in biofilm tolerance to the fluoroquinolone, ofloxacin (Bernier et al. 2013). Amino acid auxotrophs displayed higher levels of tolerance and this was biofilm specific. Again starvation plays a central role in the antibiotic tolerance phenotype. They also found that the SOS response played a central role in ofloxacin tolerance in biofilm associated persisters (Bernier et al. 2013). Interestingly, the group reported that induction of fluoroquinolones tolerance observed in biofilms compared to planktonic cells was independent of any of the TA mRNA interferases or TAs previously shown to be induced by the SOS response.

P. aeruginosa is an important opportunistic human pathogen. It can cause incurable biofilm associated infections in cystic fibrosis (CF) patients. Mulcahy et al. examined persister cell formation in longitudinal isolates from a number of cystic fibrosis patients and found that high-persister (hip) mutants emerged in 10 out of 14 cases studied (Mulcahy et al. 2010). Repeated exposures of the bacterial population to antibiotics selected for hip mutants over the course of disease progression. This directly implicates persisters in *P. aeruginosa* biofilm infections of the cystic fibrosis lung and the recalcitrance of this infection to antimicrobial treatment.

A similar investigation was performed with cancer patients with oral *Candida albicans* infection (Lafleur et al. 2010). *C. albicans* produces oral biofilms resulting in the disease known as oral thrush. Longitudinal isolates were studied from patients treated daily with chlorhexidine. The patients were divided into two groups, transient carriers and long-term carriers. Hip mutants were only identified in patients with long-term carriage again implicating persisters in this biofilm associated disease and drug tolerance (Lafleur et al. 2010).

A recent study involving a novel anti-persister antibiotic treatment further substantiates the role of persister cells in biofilm tolerance. The ability to kill a persister directly translates to the ability to target and kill the biofilm (Conlon et al. 2013).

5 Killing Persister Cells and Treating Biofilm Infection

Antimicrobial tolerance of the biofilm can be explained by the presence of dormant persister cells. Killing these persister cells would therefore lead to eradication of the biofilm. The problem with conventional antibiotics is that they rely on the corruption of active processes in the cell. Aminoglycosides corrupt protein synthesis leading to toxin peptide production (Davis 1987), B-lactams corrupt peptidoglycan biosynthesis leading to cell lysis (Tipper and Strominger 1965) and fluoroquinolones corrupt gyrase leading to double-strand breaks (Drlica and Zhao 1997). If the cell is dormant and these mechanisms are down-regulated or completely inactive, then the antibiotic does not kill and the persister cell survives. Within the biofilm, many such cells exist as energy may be limiting.

A mechanism to kill persisters within a biofilm was proposed by Allison et al. based on metabolite stimuli (Allison et al. 2011). Tolerance to aminoglycosides may be primarily due to low membrane potential in the persister cells. Aminoglycosides require a proton motive force for uptake. Adding specific sugars to the culture induced the uptake of gentamicin via generation of a proton motive force resulting in death of the persister cell. Importantly, they were able to show that this metabolite enabled eradication was not confined to *E. coli* and worked equally well against *S. aureus*. Addition of mannitol (*E. coli*) or fructose (*S. aureus*) to the treatment regimen allowed eradication of a biofilm in a mouse catheter infection model (Allison et al. 2011). More recently it has been demonstrated that L-arginine also facilitates biofilm eradication by gentamicin by affecting the pH. Again, the efficacy of treatment was demonstrated in a mouse catheter model of infection (Lebeaux et al. 2014).

It has also been shown that long term aminoglycoside treatment alone is capable of eradicating *P. aeruginosa* stationary phase cultures, implying a sufficient proton motive force is maintained in all cells while protein synthesis also remains active (Keren et al. 2012). This prolonged aminoglycoside treatment could inform improved dosing regimens in the clinic to deal with *P. aeruginosa* biofilm infections.

A persister specific compound capable of killing a persister without effecting regular cells was reported by Kim et al. in (2011). They demonstrated that this compound, termed C10, could eradicate a population of *E. coli* in combination with norfloxacin although the mechanism of action remains unknown.

Another approach to persister killing and biofilm eradication relied on the activation and corruption of a mechanism in the bacterial cell without the requirement for energy. The acyldepsipeptides are a family of antibiotics that bind the ClpP protease (Kirstein et al. 2009).

Proteolysis is an important aspect of protein homeostasis and quality control (Michel et al. 2006). It is a tightly regulated process involving the cooperation of ClpP, its associated unfoldases, ClpA, ClpX or ClpC and requires ATP (Michel et al. 2006). Under normal circumstances, a protein can be targeted for degradation, for example by an *ssrA* tag (Farrell et al. 2005). The unfoldases will unfold the protein at the expense of ATP and feed it into the catalytic chamber of ClpP where it is degraded (Farrell et al. 2005). ADEP4 is a synthetic acyldepsipeptides antibiotic that binds the ClpP protease, opening its proteolytic core and results in dysregulation of proteolysis (Brotz-Oesterhelt et al. 2005). This proteolysis no longer requires ATP, which may be at extremely low levels in persister cells (Dorr et al. 2010). A stationary phase population of *S. aureus* cells behaves like a persister population, meaning that it is completely tolerant to antibiotics (Conlon et al. 2013). Incubation of stationary phase *S. aureus* cells with ADEP4 for 24 h resulted in mass protein degradation (Falla and Chopra 1998). Over 400 proteins were significantly degraded by ADEP4 treatment, many of which were essential proteins. This coincided with death of the persister population. There was a caveat to ADEP4 however. The ClpP target is not essential so resistance occurs at high frequency. However, mutation of *clpP* resulted in an increased susceptibility to conventional antibiotics, rifampicin, linezolid and ciprofloxacin.

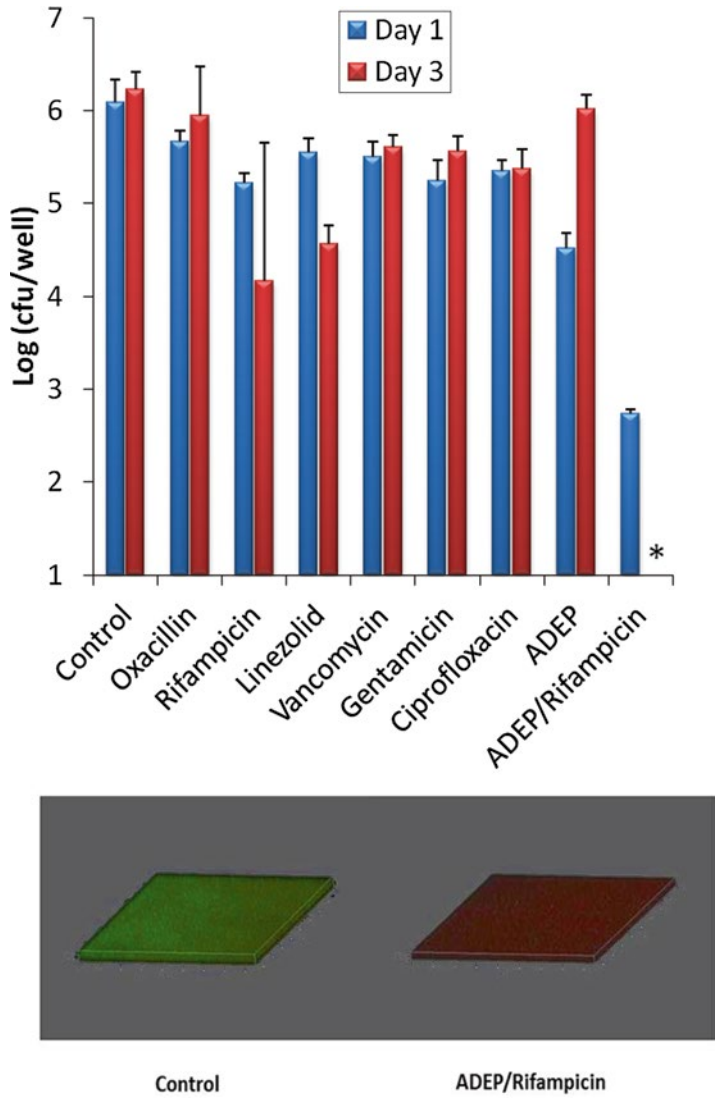

Fig. 1.3 Eradication of biofilm by ADEP4/rifampicin

Combining ADEP4 with rifampicin resulted in complete eradication of the population to the limit of detection in a variety of experiments (Conlon et al. 2013). Importantly, a *S. aureus* biofilm was also eradicated by an ADEP4/rifampicin combination (Fig. 1.3). Finally a biofilm infection was established in a mouse thigh and this too could be eradicated by the ADEP combination (Conlon et al. 2013). To date, antibiotic discovery has focused on how to kill fast growing cells while these recent studies provide much needed insight into how to eradicate a non-growing persister population.

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Fungal Biofilms in Human Disease

2

Craig Williams and Gordon Ramage

1 Introduction

Fungal biofilms are an important clinical problem. The widespread use indwelling medical devices, broad spectrum antibiotics and an aging and more immuno-compromised patient population has created an opportunity for yeasts and moulds to form infections in the form of biofilms. This chapter will discuss the diversity and importance of fungal biofilms in different anatomical areas, provide insights into the management of fungal biofilm infection, explain why biofilms may be difficult to treat with antifungal therapy, and discuss how our current level of knowledge may lead to different treatment interventions.

A biofilm is composed of microorganisms attached to surfaces or one another and enclosed within an extrapolymeric matrix. The biofilm mode of growth is the preferred form of growth of microorganisms and account for up to 65 % of all clinical infections. This mode of growth gives the organism a number of advantages including high level antimicrobial resistance which may cause problems for the clinician attempting to treat such infections (Donlan and Costerton 2002). Over recent years there has been a growing appreciation that pathogenic fungal species both have the ability to form biofilms and that these biofilms may impact clinical practice (Ramage et al. 2009; Sayed et al. 2012; Fanning and Mitchell 2012).

Fungi can be broadly divided into yeasts and moulds and in terms of the number of infections, *Candida albicans*, a normal commensal of human mucosal surfaces and opportunistic pathogen in immunocompromised patients, is the most clinically important of fungi species in terms of the production of clinically relevant biofilms. This dimorphic fungus exists in both yeast and hyphae forms which results in a structurally complex biofilm. This begins with yeast cells attaching to a relevant surface using defined adhesins, such as the agglutinin-like sequence protein Als3p and the GPI anchored cell wall protein Eap1p (Zhao et al. 2006; Li et al. 2007). The next step is the formation of a microcolony with yeast cells undergoing morphological switching to pseudo- and true-hyphae under the regulatory control of Efg1p (Ramage et al. 2002) which results in the rapid formation of a meshwork of hyphae interspersed with budding yeast cells. As the biofilm matures it becomes enclosed in a glucan rich polymeric matrix (Nett et al. 2010a) which provides protection from host defences and treatment with antifungal agents. Within the biofilm there are a range of niches and in hypoxic areas, Tye7p controlled

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up-regulation of glycolytic genes, which influence filamentation, occurs (Bonhomme et al. 2011). Flow of fluids across the surface of the biofilm may then result in the dispersion of daughter cells which attach to a new substrate and the cycle starts again (Uppuluri et al. 2010). This entire process is controlled by transcription factors, such as Bcr1p, Ace2p, Efg1p and Zap1p, which are involved in precisely regulated molecular pathways (Zhao et al. 2006; Finkel and Mitchell 2011; Nobile and Mitchell 2006; Fanning et al. 2012).

From a clinical point of view it is important as an understanding the basis of adherence, proliferation, maturation and dispersal both forms the basis for all other pathogenic fungal biofilm studies and signposts important potential new targets for clinical interventions in these infections. This chapter will review fungal biofilms and their clinical importance, discuss why these infections may be so difficult to treat, and provide evidence for potential novel strategies to improve clinical management.

2 Where May Fungal Biofilms Be Important?

2.1 Oral Cavity

The oral cavity represents one of the major portals of entry for microorganisms, and is a site in which the presence of multispecies microbial biofilms has been widely studied both in the presence and absence of foreign materials (Jakubovics 2010). Within the oral cavity, caries, periodontal disease, endodontic infection and mucosal infections all involve microbial biofilms (Beikler and Flemmig 2011) and *Candida* species are the important fungal pathogen (Rautemaa and Ramage 2011). The oral cavity is a good environment for biofilm growth for a variety of fungal species. In a study of 20 healthy individuals Fungal microbiome analysis of the oral cavity, using a pyrosequencing approach, identified 74 culturable and 11 non-culturable fungal (Ghannoum et al. 2010). *Candida* species were shown to be the most prominent genera in this group (75 %), followed by *Cladosporium* (65 %), *Aureobasidium* (50 %), *Saccharomycetales* (50 %), *Aspergillus* (35 %), *Fusarium* (30 %), and *Cryptococcus* (20 %). Overall 101 species were present and each individual had from 9 to 23 different fungal species. While these results demonstrate the potential diversity of fungi in the mouth there remains the possibility that DNA from these fungal species was ingested or inhaled.

Nevertheless, yeasts clearly exist within the oral cavity and form biofilms. Oral candidosis, best defined fungal biofilm infections of both soft and hard tissue in the mouth forms complex biofilms in association with host components and bacteria (Rautemaa and Ramage 2011; Dongari-Bagtzoglou et al. 2009). *Candida* species have been isolated from periodontal pockets, orthodontic appliances, enamel, dentures and mucosal surfaces (Dongari-Bagtzoglou et al. 2009; Ramage et al. 2004; Sardi et al. 2010; de Carvalho et al. 2006; Arslan et al. 2008). Where *Candida* species are isolated from subgingival mixed species biofilms in patients with severe chronic periodontitis (Canabarro et al. 2012) there is a clear correlation between disease severity and both the quantity and species of yeast cells isolated with *C. albicans* being found in high numbers from those with moderate and severe chronic periodontitis. However a causal relationship has yet to be demonstrated and the presence of *Candida* species may simply represent poor oral health.

Interestingly there seems to be a relationship between Yeasts and other bacteria in oral biofilms. Another metagenomic analysis of elderly patients showed that increased candidal load favoured the presence of oral streptococci (Bamford et al. 2009) a potential mechanism for this has been suggested in mixed *C. albicans* and *Streptococcus gordonii* mixed biofilms where growth is enhanced through specific cell-cell interactions involving both Als3p and the surface protein adhesion SspB (Silverman et al. 2010). In addition these physical interactions chemical interactions have also been shown to influence biofilm formation (Bamford et al. 2009). Complex biofilms are a key mode of survival within the oral cavity and this process may influence both oral and systemic health (Coulthwaite and Verran 2007).

When foreign materials such as dentures are present in the mouth biofilms are also important in the causation of denture stomatitis (Nett et al. 2010b). This is characterized by *Candida* species forming on a denture prosthesis (Pereira-Cenci et al. 2008) usually associated with the upper fitting denture, where the biofilm forms on the surface adjacent to the oral mucosa (Ramage et al. 2004). *C. albicans* is the most frequently isolated yeast from the denture, but *Candida glabrata*, *Candida dubliniensis*, *Candida tropicalis*, *Candida krusei* and a range of other *Candida* species have been isolated (Coco et al. 2008; Williams et al. 2011). The level of inflammation of the palate ranges from localised areas of erythema to diffuse areas of severe erythema (Newton 1962). In this condition again the species of yeast present seems to be important in that those with severe inflammation preferentially cultured *C. albicans* (Coco et al. 2008). Further in vitro analysis of these strains showed a positive correlation between the severity of disease and secreted aspartyl proteinase (Sap) release and expression when the organism was grown as a biofilm (Ramage et al. 2012), which has also been shown with strains isolated from type 1 diabetes patients (Rajendran et al. 2010). These proteolytic enzymes have been shown to be present in various in vivo studies (Naglik et al. 2003, 2004, 2006) but it is not possible to attribute a causal role as no single Sap plays a predominant role in mucosal invasion (Lermann and Morschhauser 2008; Naglik et al. 2008). It is possible however to suggest that Sap proteins play a role in proteolytic cleavage of the mucin Msb2, which activates the Cek1 MAPK pathway and induce filamentation (Puri et al. 2012) which is known to play a key role biofilm development and stabilisation (Ramage et al. 2002) which suggest a physical and regulatory role for proteolytic enzymes in *C. albicans* biofilm production.

It is clear that there is interaction between yeasts and bacteria, however synergistic interactions between different *Candida* species within a biofilm have also been proposed as a pathogenic mechanism. Coco et al (Coco et al. 2008) showed that *C. glabrata* and *C. albicans* are often co-isolated from patients, particularly those with severe inflammation. He suggested that as *C. glabrata* does not produce hyphae and therefore forms relatively structurally poor and unstable biofilms, that it was possible, in mixed yeast biofilms, that *C. glabrata* was using *C. albicans* as a structural scaffold to gain entry to the host. This has now been confirmed in another study where *C. albicans* appears to assist the invasive capacity of *C. glabrata* within an in vitro reconstituted epithelial biofilm model (Silva et al. 2011). Further studies using in vivo models to investigate the pathogenesis of denture stomatitis would be useful in this context (Nett et al. 2010b).

2.2 Upper Airways

Sinusitis (or rhinosinusitis) is defined as an inflammation of the mucous membrane lining the paranasal sinuses. It may be acute or chronic however subacute, and acute exacerbation of chronic diseases have also been described and as all types have similar symptoms it is often clinically difficult to distinguish these. Around 90 % of adults have had some symptoms of sinusitis at some time. There is a growing appreciation that chronic rhinosinusitis is typified by biofilm growth (Foreman et al. 2011; Keir et al. 2011; Ebbens et al. 2009a). While there is increasing evidence for the role of bacterial biofilms in this infection, there role of fungi remains controversial (Ebbens et al. 2009b). Paranasal sinus fungus balls have been described (Grosjean and Weber 2007; Karkas et al. 2012), which share some of the features of fungal biofilms (Harding et al. 2009; Mowat et al. 2009). In a recent study of 118 patients with chronic sinusitis, nasal discharge, headache and visual disturbance, over a 14 year period 23.7 % had a sphenoidal fungus ball in which *Aspergillus fumigatus* and *Aspergillus nidulans* hyphae were observed microscopically (Karkas et al. 2012). Other fungi have also been implicated including *Schizophyllum commune* (Chowdhary et al. 2013; Sa et al. 2012), *Trichosporon inkin* (Janagond et al. 2012), *Mucorales* (Mignogna et al. 2011), and *Fusarium* (Rombaux et al. 1996). In terms of infections associated with foreign bodies *A. fumigatus* infection within

the maxillary sinus associated with a zygomatic implant has been reported (Sato et al. 2010). Experimental studies have shown that *A. fumigatus* biofilms form in a primary human sinonasal epithelial model (Singhal et al. 2011) and in a sheep model of induced sinus biofilms *A. fumigatus* readily forms biofilms often associated with *Staphylococcus aureus* (Boase et al. 2011). These data suggest that fungal biofilms, alone or more likely in mixed species biofilms with other organisms, may play a role in sinus infection however there is little evidence to support the role of fungi in other upper airway biofilm infections such as otitis media (Bakaletz 2007; Martin et al. 2005; Yao and Messner 2001).

When foreign bodies are present, such as head and neck related prostheses polymicrobial biofilms containing *Candida* species have been reported (Ariani et al. 2012). Biofilms including *C. albicans* and *C. glabrata*, have also been extensively described in voice prosthesis biofilms (Buijssen et al. 2012; Ell 1996) where they have been shown to bind to salivary proteins (Holmes et al. 2006), and are often found co-aggregated with bacterial species (Kania et al. 2010). Clinically they restrict airflow (Elving et al. 2001), impede speech, swallowing and respiration (Sayed et al. 2012). Other fungal species such as *Fusarium solani* species complex (Honraet et al. 2005) and *Cryptococcus neoformans* (Bauters et al. 2001) have also been described.

The finding of fungal biofilms on speech prostheses may also be of relevance in terms of the pathophysiology of Ventilator associated pneumonia (VAP). Studies have shown that the isolation of *Candida* species isolated alone or in combination from respiratory secretions in those with suspected VAP are associated with increased mortality compared to those with only bacteria isolated, an unadjusted odds ration of 2.9 (Delisle et al. 2011). In addition *Candida* colonisation has been associated with an increased risk of isolation of multi-drug resistant bacteria (Hamet et al. 2012). The mechanisms for this are unclear but it is possible that yeasts form the basis of multi-species biofilms which effect the pathogenicity of other yeasts or bacteria contained in the biofilm. It is also possible that the incidence of fungi within these VAP samples may be due to previous treatment with broad-spectrum antibiotics, but in VAP following cardiac surgery 30.19 % of patients were culture positive for fungi, including *C. albicans* (16.97 %), *Pneumocystis jirovecii* (3.77 %), *C. glabrata*, *Candida sake*, *C. krusei*, *Geotrichum capitatum* and *Cryptococcus humicola*, (1.89 % each) (Serban et al. 2010).

Care bundles which include measures that may reduce or prevent the growth of biofilms such as oral decontamination with chlorhexidine, have dramatically reduced the rates of VAP in the intensive care setting (Stonecypher 2010; Caserta et al. 2012).

2.3 Lower Airways

Lower respiratory tract infection may be due to biofilm infection, the archetype of which is *Pseudomonas aeruginosa* in cystic fibrosis patients (Singh et al. 2000). It is also now recognised however that fungal biofilms present in the lung may also contribute to infection.

Filamentous fungi, mainly *A. fumigatus,* may cause a spectrum of respiratory disease including from a discrete lesion in a pre-existing cavity, aspergilloma, wheezing mediated by an immune response, allergic bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis (IA) (Denning 1998). A bronchopulmonary lavage (BAL) of these individuals often reveals the presence of numerous intertwined hyphae in the form of a complex multicellular structure when examined histologically (Jayshree et al. 2006), this is indicative of a biofilm phenotype (Harding et al. 2009; Mowat et al. 2009). The recently described Aspergillus bronchitis may also be biofilm associated and is characterized by bronchial casts containing mycelia forming compact masses (Young et al. 1970). It is clear that *Aspergillus* species form medically important biofilms (Gutierrez-Correa et al. 2012; Ramage et al. 2011) and understanding their clinical role in is crucial, as with all biofilms, these structures are highly resistant to antifungal therapy (Mowat et al. 2008; Seidler et al. 2008).

A number of fungal species including *Aspergillus* spp., *Scedosporium* spp. and *Exophiala* spp. have been isolated from different cohorts of CF patients (Blyth et al. 2010; Cimon et al. 2000; Kondori et al. 2011). Given the ubiquitous nature of moulds within the environment, and with thousands of conidia being inhaled every day (Richardson 2009), it is unsurprising that pathogenic fungi can adhere, colonise and form complex multispecies biofilms in lungs with abnormal clearance mechanisms such as CF however their pathogenic role has not yet been fully elucidated. A number of recent studies have reported that lung function declines more rapidly in patients co-infected with *A. fumigatus* and *P. aeruginosa when* compared to single-species infection (Amin et al. 2010; Gangell et al. 2011), this has also been reported with *Candida* species and *P. aeruginosa* (Chotirmall et al. 2010). Evidence is therefore increasing for the improved clinical management of these patients (Delhaes et al. 2012).

There is a suggestion that interactions in mixed eukaryotic prokaryotic biofilms (polymicrobial infections) in the CF lung may lead to adverse clinical outcomes (Leclair and Hogan 2010). It has been shown that *P. aeruginosa* is able to both form biofilms and kill *C. albicans* in the hyphal form but not the yeast form (Hogan and Kolter 2002) possibly through the release of a phenazine toxin (Gibson et al. 2009; Morales et al. 2010). Pseudomonas has also been shown to inhibit the morphological transition of yeast through a 3-oxo-C12 homoserine lactone (Hogan et al. 2004) which has also been demonstrated in studies of *A. fumigatus* biofilms (Mowat et al. 2010). Further evidence of eukaryotic/prokaryotic interaction comes from the fact that the release of farnesol, a quorum sensing molecule of *C. albicans* impacts by inhibiting its quinolone signalling and subsequent pyocyanin production in *P. aeurginosa* (Cugini et al. 2007). These studies highlight potential battles going on within a polymicrobial environment such as the CF lung, which plays a crucial role in the overall pathogenesis of disease (Peters et al. 2012) exemplified by studies in a Drosophila model of polymicrobial infection in which microorganisms from CF showed a different outcome depending on the presence or absence of *P. aeruginosa* (Sibley et al. 2008a, b).

3 Gastrointestinal and Urinary Tract

The mucosa of the gastrointestinal (GI) tract is heavily laden with bacterial microbiota, growing as healthy biofilm communities (Macfarlane and Dillon 2007). Clinically they present a problem, for example when they are located in the stomach of those with percutaneous endoscopy gastronomy (PEG) feeding tubes for enteral nutrition, or in the large intestines in diseases such as ulcerative colitis (Macfarlane 2008). *C. albicans* and *C. tropicalis* have been shown to colonise these PEG tubes and contribute to degradation of the polyurethane (Trevisani et al. 2005; Gottlieb et al. 1993). Clinically this may lead to diarrhoea, or possibly cause translocation of microbes across the epithelial barrier, leading to sepsis.

Candida spp. colonisation of the GI tract is common, accounting for 30–80 % in normal healthy adults (Damman et al. 2012). Chronic colonisation may lead to GI candidisasis, which in immunocompromised individuals may lead to systemic candidiasis. Whilst little direct work has focussed on fungal biofilm in the GI tract per se, this environment is largely a polymicrobial biofilm, and interactions between yeasts and bacteria are likely to exist and play a role in health and disease. In fact, it has been suggested that *Candida* colonization may enhance inflammation in the GI tract (Kumamoto 2011). Investigations of *Escherichia coli* and *C. albicans* co-infection have reported synergistic virulence when grown together (Klaerner et al. 1997). Interestingly, in vitro studies have revealed dynamic population changes of these two organisms within biofilms, with a proposed role for lipopolysaccharide (LPS) modulation of *C. albicans* (Bandara et al. 2009). Recently, experimental murine studies have reported that *C. albicans* are able to modulate the bacterial microbiota composition of non-pathogenic species following antibiotic exposure (Mason et al. 2012), suggesting that in health

there is a bidirectional relationship between bacteria and *C. albicans*, rather than simply competitive inhibition by bacteria.

The urinary tract is also a polymicrobial environment, with a diverse metagenome present that is capable of preventing bacterial vaginosis, yeast infections, sexually transmitted disease and urinary tract infections (Ma et al. 2012). High acidity from lactic acid bacterial metabolism is a key mediator of selective inhibition of other species (Gajer et al. 2012). Therefore, control of candidal biofilms may be best achieved through competitive inhibition by bacterial flora, such as lactobacilli, though no definitive studies have focussed in this area yet (McMillan et al. 2011). Nonetheless, it is suggested that 75 % of woman experience vulvovaginal candidiasis at some point in their life, suggesting that *Candida* species are important in this body site. *Candida* species have been associated with pyelonephritis, cystitis and prostatitis (Kauffman et al. 2011; Sobel et al. 2011). *Candida* biofilms have been detected on ureteral stents and have been shown to grow in this lifestyle on experimentally on vaginal mucosa (Reid et al. 1992; Harriott et al. 2010). Urinary catheters are also a significant risk factor in intensive care units for healthcare associated fungal infections (Yang et al. 2013). Moreover, they are commonly detected on intrauterine contraceptives (Chassot et al. 2008). Whilst relatively rare, reports of an aspergilloma also occurs within the urinary tract (Lee 2010; Muller et al. 2011).

3.1 Wounds

Non-healing wounds, such as diabetic foot ulcers (Seth et al. 2012) represent a significant clinical burden to patients, and are associated with the presence of microbial biofilms. *S. aureus* and *P. aeruginosa* are often isolated together in these patients and have been shown to have a non-random association within the wound site (Fazli et al. 2009). Evidence is emerging that pathogenic fungal species may play a role in these infections (Branski et al. 2009).

Wounds acquired in combat situations especially with persistent evidence of wound necrosis often contain fungi with mould isolates found in 83 % of cases (*Mucorales*, n = 16; *Aspergillus* spp., n = 16; *Fusarium* spp., n = 9), commonly with multiple mould species among infected wounds (28 %). Clinical outcomes included three related deaths (8.1 %), frequent debridements and amputation revisions (58 %) (Warkentien et al. 2012).

A metagenomic approach to venous leg ulcers reveals that *C. albicans, C. glabrata* and *Aspergillus* species are present, but intriguingly the authors report that individuals seem to have unique microbial profiles (Wolcott et al. 2009). A further retrospective molecular analysis of 915 chronic wound infections, pressure ulcers, diabetic foot ulcers, non-healing surgical wounds and venous leg ulcers, showed that 208 (23 %) of these contained pathogenic fungi (Dowd et al. 2011). Yeasts were the most abundant fungi (*Candida* spp.), but *Aureobasidium, Cladosporium, Curvularia*, *Engodontium, Malessezia*, *Trichtophyton,* and *Ulocladium* were also. Overall, fungal species represented over 50 % of the microbial burden in the majority of specimens examined but direct evidence that the fungi were present as biofilms is lacking.

There is a potentially interesting interaction between *Staphylococcus* and *Candida.* In the studies above there was a negative association however previous studies have shown a positive biofilm relationship between these organisms, with *S. aureus* using *C. albicans* hyphal biofilms as a scaffold through Als3p, in an analogous way to *S. gordonni* and *C. glabrata* (Silverman et al. 2010; Coco et al. 2008; Harriott and Noverr 2009). Synergistic interaction between these two organisms has been described with respect to mortality in a murine intraperitoneal model (Carlson 1982) which may be due to *S. aureus* upregulating lactate dehydrogenase (Peters et al. 2010).

3.2 Medical Devices

Broad-spectrum antibiotics, parenteral nutrition, immuno-suppression due to chemotherapy and radiotherapy, and disruption of mucosal barriers due to surgery, are among the most important predisposing factors for invasive fungal infection (Odds 1988). *Candida* species predominate and

are the fourth most common cause of bloodstream infection in patients requiring intensive care and the most common etiologic agent of fungal related biofilm infection.

Indwelling medical devices, such as intravascular catheters, become colonized with *Candida spp*. allowing the development of adherent biofilm structures from which cells can then detach and cause an acute fungemia and/or disseminated infection. Experimental studies have reported that cells detaching from the biofilm have a greater association with cytotoxicity and mortality than equivalent planktonic yeasts (Uppuluri et al. 2010). Investigations have therefore begun to investigate whether the biofilm phenotype does play a defined clinical role. In an initial retrospective investigation using multivariate analysis to analyse the risk factors associated with patients with candidaemia it was reported that inadequate antifungal therapy (OR 2.35, P = 0.03), APACHE III scores (OR 1.03, P < 0.001) and biofilm formation (OR 2.33, P = 0.007) were independent predictors of mortality (Tumbarello et al. 2007). Analysis of mortality with biofilm forming ability demonstrated that both *C. albicans* (P < 0.001) and *C. parapsilosis* (P = 0.007) correlated with increased mortality. In a subsequent prospective case-control study by the same group it was shown that *Candida* bloodstream infections caused by biofilm forming isolates could be independently predicted by the presence of central venous catheters, urinary catheters, total parenteral nutrition and diabetes mellitus (Tumbarello et al. 2012). The hospital length of stay and cost of antifungal therapy were also greater in those with biofilm forming isolates, and these patients had a greater risk of hospital mortality (OR 1.77). However in these studies biofilm formation was defined by XTT and spectrophotometric transmittance, rather than a biofilm biomass and/or dry weight (Taff et al. 2012; Kuhn et al. 2003) which may bias the data towards non-albicans species, such as *C. glabrata*, which do not form hyphae (Kuhn et al. 2002a). In fact, it was reported in this study that *C. albicans* biofilm production was significantly less frequent (26.2 % n = 122) than non-albicans species (61.1 % n = 85) (p < 0.001) (Tumbarello et al. 2012), an observation also reported elsewhere (Pannanusorn et al. 2012).

One of the first documented episodes of biofilm related disease associated with *C. glabrata* was in terminally ill patients with intravenous catheters (Valdivieso et al. 1976). Interestingly, although the patients ultimately died, the candidaemia was treated by the removal of the catheter, a practice reported elsewhere in the same era (Berkowitz et al. 1979). Historically, biofilm associations have also been reported in patients with endocarditis (Heffner and Franklin 1978), prosthetic joints (Goodman et al. 1983), peritoneal dialysis (Cecchin et al. 1984), venous catheters (Paige et al. 1987), cannulation (Komshian et al. 1989), ventriculoperitoneal shunts (Walter et al. 1990), in addition to other indwelling devices. Not surprisingly, the presence of an indwelling catheter was a defined risk factor for the development of *C. glabrata* candidaemia (Fortun et al. 2012).

C. parapsilosis is another important species of *Candida* that has been shown to play an important clinical role in biofilm infections. Biofilm development by these organisms is similar to *C. albicans*, sharing the key transcriptional biofilm regulator Bcr1 (Ding et al. 2011). Indwelling catheters in the neonates patient group are an important risk factor for this organism (Pammi et al. 2013). However, prosthetic knees, hip joints and breast implants have also been implicated (Wada et al. 1998; Fox and Lee 2012; Younkin et al. 1984), in addition to a substantial literature on its role on endocarditis of bioprosthetic valves (Wallner et al. 2012; Garzoni et al. 2007). *C. tropicalis* has also received attention in relation to biofilm formation in vitro and in vivo, having been shown to be important in bioprosthetic heart valves and catheter related disease (Mansur et al. 1996; Negri et al. 2012).

Other yeasts and filamentous fungi biofilm related infections have also been increasingly described, including *Aspergillus* (Escande et al. 2011), *Cryptococcus* (Walsh et al. 1986), *Coccidioides* (Davis et al. 2002), *Zygomycetes* (Singh et al. 2011), *Blastoschizomyces* (D'Antonio et al. 2004), *Malassezia* (Cannizzo et al. 2007). *Aspergillus* species have been reported to cause serious biomaterial related biofilm infections, involving catheters, joint replacements, cardiac pace makers, heart valves, and breast augmentation implants (Escande et al. 2011; Langer et al.

2003; Rosenblatt and Pollock 1997; Jeloka et al. 2011; Golmia et al. 2011). *C. neoformans* has been shown to colonize and subsequently form biofilms (Ajesh and Sreejith 2012), cardiac valves (Banerjee et al. 1997), peritoneal dialysis fistulas (Braun et al. 1994), ventricular shunts (Walsh et al. 1986), and prosthetic hip joints (Johannsson and Callaghan 2009). *Malassezia pachydermatis* has been isolated from patients undergoing parenteral nutrition and upon catheters (Cannizzo et al. 2007; Curvale-Fauchet et al. 2004), *Blastoschizomyces capitatus* has been associated with catheter-related fungemia (D'Antonio et al. 2004), and recurrent meningitis has been associated with a *Coccidioides immitis* biofilm at the tip of a ventriculo-peritoneal shunt tubing (Davis et al. 2002). Finally, *Trichosporon* species can cause biofilm-related infections (Agirbasli et al. 2008; Di Bonaventura et al. 2006; Pini et al. 2005), including cardiac grafts (Krzossok et al. 2004), catheters (Ruan et al. 2009), and breast implants (Reddy et al. 2002).

Fungal biofilms are also associated with building fabrics and hospital infrastructure (Richardson 2009; Short et al. 2011; Siqueira et al. 2011; Anaissie et al. 2002).

3.3 Clinical Management

It is clear from the literature that a wide variety of fungi have the capacity to form biofilms on a range of anatomically diverse sites. Arguably the most important reason for their clinical importance is our inability to manage these infections effectively, leading to unacceptably high rates of morbidity and mortality. The following section discusses conventional and novel methods for effective clinical management of fungal biofilms.

4 Conventional Antifungal Approaches

Undoubtedly the most effective and logical way of dealing with clinically important fungal biofilms is to either inhibit their development, use mechanical force to disrupt them or simply remove and replace an implicated medical device. The European Society for Clinical Microbiology and Infectious Disease (ESCMID) have recently produced guidelines discussing the role of catheter associated infection and their clinical management (Cornely et al. 2012). The guidelines indicate that where possible the catheter should be removed. This is supported by clinical data, such as a prospective randomized trial comparing fluconazole to amphotericin B deoxycholate, where removal of a catheter within the first 24 h of candidaemia resulted in a shorter duration of candidaemia (Rex et al. 1995). Conversely, when comparing echinocandins to liposomal amphotericin B the removal of the catheter showed no improved time to mycological eradication, possibly due to the effectiveness of both antifungal agents against biofilms (Nucci et al. 2010). A recent meta-analysis from seven prospective randomized clinical trials has provided some clarity to this, reporting that removal of central venous catheter is associated with decreased mortality (OR, 0.50, 95 % Cl, 0.35–0.72, $p = 0.0001$) (Andes et al. 2012).

Where removal of the catheter is not possible, the use of antifungal therapy should be considered, though unlike bacterial biofilm infections, there are currently no guidelines for treating *C. albicans* associated biomaterial infections with chemotherapeutic agents (O'Grady et al. 2011). However, limited evidence exists for in situ use of antifungal lock therapy (ALT) in fungi. A recent review has highlighted a limited number of case studies that advocate the potential for ALT where clinically appropriate (Walraven and Lee 2013). This small analysis reported 11 studies (20 cases) where *C. albicans* was the most frequently treated ($n = 9$), followed by *C. parapsilosis* ($n = 5$), *C. glabrata* ($n = 4$), *C. tropicalis* ($n = 1$), *C. guillermondii* ($n = 1$), *C. lipolytica* ($n = 1$), *Rhodoturula* ($n = 1$) and *Malassesia furfur* ($n = 1$). Amphotericin B deoxycholate was used most frequently, and was effective in 76.9 % of cases (Arnow and Kushner 1991; Johnson et al. 1994; Krzywda et al. 1995; Benoit et al. 1995; Viale et al. 2001; Angel-Moreno et al. 2005; Wu and Lee 2007). Liposomal amphotericin B was also effective in 60 % (three of five cases) (Castagnola et al. 2005; Buckler et al. 2008).

Caspofungin was used, but only once and was effective against *C. lipolytica* (Ozdemir et al. 2011). In one case ethanol was used as the solitary ALT solution rather than antifungals, which was shown to be effective (Blackwood et al. 2011). ALT was most commonly used for 14 days when negative blood cultures were observed. These studies collectively provide evidence to demonstrate that antifungal ALT for biofilm associated infections is worth considering, with emphasis on using either echinocandins, liposomal amphotericin B or amphotericin B lipid complexes (Cornely et al. 2012), this recommendation relates to evidence from early in vitro studies, where these compounds were shown to be highly effective against *C. albicans* biofilms (Bachmann et al. 2002; Kuhn et al. 2002b). More recently, a number of important studies have been conducted in vitro with various *Candida* species to test the potential in ALT, which have compared a range of antifungal compounds (Walraven and Lee 2013). In these models, caspofungin and micafungin has been shown to have excellent activity, though complete eradication of the biofilm was not demonstrated (Cateau et al. 2008, 2011). Time-dependant killing analysis has recently reported that liposomal formulations of amphotericin B kill significantly quicker than echinocandins (Ramage et al. 2013). Rather oddly, in an independent in vitro ALT study it was reported that azoles (itraconazole, voriconazole and fluconazole) were more effective than both caspofungin and amphotericin B (Ko et al. 2010). This study highlights how biofilm study design can negatively impact interpretation of data during in vitro studies, as it is universally accepted that azoles have little effect on mature fungal biofilms in vitro (Ramage et al. 2013), and in vivo (Andes et al. 2004; Kucharikova et al. 2010).

Animal studies have shown that *C. albicans* biofilms in implanted catheters respond to both caspofungin and amphotericin B formulations (Lazzell et al. 2009; Schinabeck et al. 2004; Mukherjee et al. 2009). Fluconazole (10 mg/ml) on the other hand was unable to salvage any treated catheters, whereas liposomal amphotericin B (10 mg/ml) led to a 100 % success rate (Schinabeck et al. 2004). Finally, comparison of amphotericin B deoxycholate (3.33 mg/ml) with caspofungin (6.67 mg/ml) produced a 81.3 and 100 % catheter salvage success rate, respectively (Shuford et al. 2006).

This has important implications for other fungal biofilm infections, particularly those associated with indwelling devices or on anatomically 'hard-to-reach locations', such as heart valves and orthopaedic joints (Falcone et al. 2009; Dutronc et al. 2010). For *Candida* endocarditis retrospective data suggest that combined antifungal treatment with surgery gives the best outcomes, with prosthetic valve infections having poorer outcomes than native valve infection (Falcone et al. 2009). Prognosis is poor, with 1-year mortality greater than 50 %, combined with substantial relapse rates (Ellis et al. 2001). Valve replacement should be performed as soon as possible, though if prevented liposomal amphotericin B and caspofungin can be used (Boland et al. 2011). There is however an account of successfully treating *A. fumigatus* prosthetic valve endocarditis with oral voriconazole (Reis et al. 2005). Less evidence is available for the treatment of infected hip joints, though it has been reported that voriconazole and amphotericin B have been used together in bone cement to treat a *C. albicans* infected hip (Deelstra et al. 2013). Amphotericin B and fluconazole have also been used to treat a *C. neoformans* infection of a prosthetic hip joint, but this was unsuccessful due to poor penetration through the biofilm (Johannsson and Callaghan 2009). There are suggestions however that fluconazole may have a role in the treatment of candidal prosthetic joint infection (Kelesidis and Tsiodras 2010).

Wound fungal biofilms are managed with surgical debridement (Warkentien et al. 2012). In severe wounds, such as those occurring from combat trauma, liposomal amphotericin B, voriconazole and posaconazole have been used, often as combinational therapy, although the clinical outcomes were variable. Nevertheless, it has been reported that in the management of a case of fungal osteomyelitis combined use of voriconazole and terbinafine along with surgical debridement was able to successfully control a

Scedosporium inflatum infection and salvage the limb (Cetrulo et al. 2012).

These studies suggest that wound fungal biofilms may have a different structural composition, as they respond to azoles more effectively than other fungal biofilms. Many of these infections are polymicrobial, and undergo repeated debridement with topical antiseptics. Moreover, wound dressings containing antimicrobial molecules are used, so it is not surprising that fungal wound biofilms respond to azole therapy in this context.

5 Concluding Remarks

From review of the available literature it is evident that fungal biofilms do play a significant role in clinical medicine. Over 20 different genera of fungi have been implicated in some way of another in clinical biofilm infections, most notably the *Candida* genera. Fungi have been demonstrated to form biofilms on both hard and soft tissue, and upon implanted medical devices. Diagnosing the presence of a fungal biofilm is difficult, with reliance on clinical skill and judgement, along with some key mycological considerations. Removal and replacement of medical devices, or surgical debridement of soft tissue, where appropriate, represents the first line in clinical management, followed by antifungal management. Treatment outcomes vary to conventional antifungal agents, which are largely dictated on by the accessibility of the infection site. Liposomal formulations of amphotericin B and echinocandin antifungal agents show the greatest efficacy against fungal biofilms, whereas azoles are highly ineffective against mature biofilms. Developing methods to augment antifungal activity have been demonstrated experimentally, such as matrix degrading molecules, natural products and microbially derived molecules. Moreover, our knowledge of the how adaptive resistance within the biofilm has revealed therapeutic targets, potentially through the pharmacological depletion of specific molecules involved in these processes. Collectively, these approaches provide a viable platform to successfully manage fungal biofilms of clinical importance. However further consideration needs to be given to how interactions between prokaryote and eukaryote in polymicrobial biofilm infections impact clinical management.

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Biofilm-Infected Pressure Ulcers: Current Knowledge and Emerging Treatment Strategies

3

Louise Suleman and Steven L. Percival

1 Introduction

Pressure ulcers, otherwise known as decubitus ulcers, are a type of chronic wounds that are commonly seen in the healthcare environment, particularly in the elderly. The formation of pressure ulcers, as the name suggests, is due to the pressure of body weight on areas of the skin for extensive periods of time or excessive friction, a common feature in those with reduced or no mobility. The epidemiology of pressure ulcers is very much dependent upon the clinical setting, with prevalence ranging from 18.1 % in general hospitals (Vanderwee *et al.* 2007) and 27 % in long-term care facilities (LTCFs) respectively (Capon *et al.* 2007). Not surprisingly, pressure ulcers present healthcare organisations with significant economic burden, with the cost of treatment increasing with the severity of the wound (Dealey *et al.* 2012; Brem *et al.* 2010).

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The loss of barrier function in pressure ulcers increases the risk of microbial colonisation, which may lead to the development of a biofilm within the wound (Donelli and Vuotto 2014). James and colleagues reported that approximately 60 % of all chronic wounds contain a microbial biofilm (James *et al.* 2008). However, it is more likely that all chronic wounds contain biofilms considering the nature of microbial survivability. Microbial biofilms are complex communities of microorganisms that attach to surfaces and secrete extracellular polymeric substances (EPS). The secretion of EPS acts as a biochemical barrier, protecting the biofilm from both mechanical and shear forces, and more importantly, the penetration of antimicrobial substances. Microorganisms that reside in a biofilm are known to be tolerant to the action of a wide range of antimicrobials when compared with their planktonic counterparts. Thus, the presence of a biofilm in pressure-ulcers, introduces a different dynamic to the way we should approach the treatment and management of these wounds.

This chapter will review what is known about the mechanisms behind the development of pressure ulcers and how the presence of a microbial biofilm affects the normal healing process. Furthermore, the risk factors associated with biofilm-infected pressure ulcers, and both current and potential future treatment options will be discussed.

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2 Pressure Ulcers

2.1 Clinical Manifestation and Characterisation

Pressure ulcers are localised areas of tissue damage due to prolonged mechanical pressure and increased shear stress, particularly over bony prominences, leading to both superficial and deep-tissue injury. There are several risk factors associated with the development of pressure ulcers (see Table 3.1), both intrinsic and extrinsic, with factors such as reduced mobility, poor nutrition and pressure, regarded as the strongest predictors of pressure ulcer formation (Reynolds and Chow 2013).

Pressure ulcers can be classified as either superficial or deep-tissue ulcers. Superficial ulceration is limited to the skin layers and primarily caused by shear stresses in the skin layer. Deep-tissue ulcers are where tissue breakdown extends to the muscle layers, which predominantly occur over bony prominences in the presence of sustained pressure. Pressure ulcers can be clinically characterised into four stages of wound progression according to the National Pressure Ulcer Advisory Panel (NPUAP) and European Pressure Ulcer Advisory Panel (EPUAP) (EPUAP 2009):

- **Stage I (Non-blanchable erythema)**: Classified as intact skin with the presence of non-blanchable redness.
- **Stage II (Partial thickness)**: The partial loss of dermis, presenting as an open wound with a pink wound bed. No slough is present at this stage. The presence of serum-filled blisters may also be a feature at this stage.
- **Stage III (Full thickness *skin* loss)**: Loss of full thickness skin without visible signs of bone or muscle. This stage may include the presence of slough. Pressure ulcers at stage III can vary in depth depending on the anatomical location; for instance, pressure ulcers on the nose or ear, whereby subcutaneous fat is limited, will result in more shallow depth ulcers.
- **Stage IV (Full thickness *tissue* loss)**: Loss of full thickness tissue resulting in the exposure of bone, muscle or tendon. The presence of tunnelling and slough is also a common feature. Furthermore, the depth of the ulcer, as with stage III pressure ulcers, may vary depending on anatomical location. Due to the extension of the ulcer to muscle and other supporting structures, stage IV pressure ulcers can lead to the development of osteomyelitis.

Table 3.1 Intrinsic and extrinsic risk factors associated with the development of pressure ulcers

Risk factors	
Extrinsic factors	Intrinsic factors
Pressure	Limited mobility (wheelchair-bound, spinal cord injury)
Friction	Poor nutrition
Shear stress	Increased age
Moisture	Reduced blood flow (Diabetes mellitus)

2.2 Aetiology of Pressure Ulcers

Some of the theories explaining the pathogenesis of pressure ulcers have been associated with localised ischemia, reperfusion injury, impaired lymphatic function and sustained mechanical deformation of cells (Berlowitz and Brienza 2007). Although we understand that prolonged pressure can lead to the development of pressure ulcers, the pathways that lead to the mechanical breakdown of underlying tissue are still poorly understood (Bouten *et al.* 2003). Despite this, some studies have attempted to elucidate potential mechanisms that may play a role in these pathways.

An *in vitro* study into the effect of mechanical pressure on skeletal muscle constructs revealed that cell death occurred as early as 1–2 h after initial pressure induction, highlighting the theory of pressure-induced deformation of cells leading to cell death (Breuls *et al.* 2003).

The application of pressure to an area of skin is known to increase cutaneous blood flow in healthy individuals, known as pressure-induced vasodila-

tion (PIV) (Fromy *et al.* 2000). However, in the context of ageing and diabetes, the mechanism of PIV is thought to be impaired, and a decrease in cutaneous blood flow increases the risk of ulceration (Köitka *et al.* 2004; Fromy *et al.* 2010). Fromy and colleagues (2012) explored the mechanosensory mechanisms that may be involved in the detection of pressure in mice and humans. More specifically, this research group targeted the acid-sensing ion channel 3 (Asic3), an ion channel largely expressed on sensory neurons and located at nerve endings where mechanical stimuli are detected. The authors recorded a lack of detectable PIV and significantly reduced cutaneous blood flow in *Asic*$^{-/-}$ mice. Similarly, the use of *Asic3* inhibitors, either subcutaneously injected or applied topically, abolished PIV in both rats and human subjects due to pressure detection failure (Fromy *et al.* 2012).

Another interesting dynamic on the possible pathways that lead to tissue destruction is the involvement of autophagy. Autophagy is a cellular lysosomal degradation pathway that is primarily involved in the recycling of cellular components for energy in periods of starvation. In addition to this, autophagy can be more specific, with the targeted removal of damaged proteins or invading bacteria or viruses within a cell. Autophagy is therefore an important process in cellular housekeeping and impairment in this process can ultimately lead to cell death. Autophagy is mechanosensitive and has been shown to be up-regulated in response to mechanical stress (King *et al.* 2011). King (2012) later hypothesised that autophagic responses to mechanical stress could be involved in an array of pathological processes from heart-related conditions to deep-tissue ulcers (King 2012). With this in mind, autophagy could be an intriguing cellular pathway to investigate the pathology of pressure ulcers. Impairment of the autophagy pathway in certain individuals may lead to increased cellular death in response to mechanical stress, but also may play a role in the ineffective control of invading microorganisms, a feature that could be costly in infected-pressure ulcers.

2.3 Non-healing Pressure Ulcers: Where Does It All Go Wrong?

2.3.1 Physiological Tissue Repair

Before it is possible to elucidate the possible mechanisms behind impaired cutaneous healing in chronic wounds such as pressure ulcers, it is first important to understand the physiological responses to tissue injury. Cutaneous wound healing comprises of four major stages:

- Haemostasis: Upon initial injury, the production of platelets leads to the formation of a fibrin clot. This stage occurs in the presence of haemorrhage but is not a necessary factor in wound healing.
- Inflammation: The release of chemotactic and vasoactive mediators initiates the recruitment of inflammatory cellular mediators such as neutrophils. Neutrophils are the first inflammatory cell type to infiltrate the wound, and are responsible for the clearance of invading microorganisms and foreign particles. Monocytes are recruited to the wound and become activated macrophages that attach to the extracellular matrix. This attachment aids the release growth factors and cytokines including colony-stimulating factor 1 (CSF-1), tumour necrosis factor alpha (TNF-α), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), ultimately encouraging granulation tissue formation.
- Proliferation: This phase includes re-epithelialisation, whereby epidermal cells at the wound edge proliferate and migrate across the wound.
- Tissue regeneration and vascularisation: The development of a provisional extracellular matrix acts as a scaffold for cell migration. New granulation tissue beings to form in the wound bed in the presence of fibroblasts and macrophages, which secrete the growth factors necessary for the production of granulation tissue and angiogenesis (Epstein *et al.* 1999; Diegelmann and Evans 2004).

2.3.2 Impaired Wound Healing: The Theories

Despite the known aetiology of pressure ulcers, the reasons as to why these wounds fail to heal or take excessively long periods of time to heal is still unclear. Despite this, several theories have been proposed. In a pathological setting the normal responses to tissue damage are impaired and in chronic wounds, a persistent state of inflammation is observed. In the case of pressure ulcers, excessive infiltration of neutrophils into the wound site is a hallmark biomarker (Diegelmann and Evans 2004).

The pathogenesis of pressure ulcers is, in part, thought to be due to imbalances in the secretion of extracellular matrix (ECM) degrading proteases and the counteracting release of their inhibitors (Menke *et al.* 2007; Widgerow 2011). The characteristic influx of neutrophils in pressure ulcers leads to the release of neutrophil-derived proteases such as elastase and matrix metalloproteinases (MMPs), which contribute to ECM degradation (Nwomeh *et al.* 1999). For instance, neutrophil-derived elastase is known to degrade growth factors including PDGF and transforming growth factor-β (TGF-β), both beneficial growth factors for the release of ECM components from resident fibroblasts (Eming *et al.* 2007). The disproportionate release of proteases in chronic wounds, particularly pressure ulcers has been documented. Yager and colleagues (1996) detected 10-fold to 25-fold increases in the gelatinases MMP-2 and MMP-9 in the wound fluid of patients with pressure ulcers when compared to healing wounds. Furthermore, collagenase was significantly increased in pressure ulcers compared to acute surgical wounds (Yager *et al.* 1996).

The implications of infection within chronic wounds are vast, including delayed wound healing, increased patient discomfort and increased rates of morbidity and mortality to name a few (Reynolds and Chow 2013).

With evidence to suggest a diverse polymicrobial profile of infected-pressure ulcers, the effect of these microorganisms in the pathogenesis of non-healing wounds was carefully considered. Bjarnsholt and colleagues (2008) hypothesised that it was the bacterial burden of a wound that prevents a chronic wound from healing, in particular the Gram-negative bacteria *Pseudomonas aeruginosa*. More specifically, it is the ability of this microorganism to form a biofilm within the wound, which effectively is able to resist the action of antimicrobials, evade host immune responses and release virulence factors (Bjarnsholt *et al.* 2008). In the context of biofilm-infected pressure ulcers, the colonisation of microorganisms and the establishment of a biofilm may correlate with pressure ulcer progression. However, it may be that host responses such as the release of proteases and inherent defective bacterial clearance may also affect biofilm formation (see Fig. 3.1).

3 Microbial Biofilms

3.1 Biofilm Formation

Biofilms are complex communities of microorganisms that attach to both abiotic and biotic surfaces and secrete EPS, in which these microorganisms reside (Donlan 2002). Biofilm formation comprises of several stages including initial reversible attachment, followed then by irreversible attachment, colonisation and dispersion. The attachment of a microorganism to a surface is facilitated by such factors as increased shear forces, bacterial motility and the electrostatic forces between both the cell and the surface. The reversible attachment stage of biofilm formation is thought to be an equilibrium of both adherent and planktonic microorganisms (Lindsay and Von Holy 2006). Factors such as the presence of fimbriae and flagella, hydrophobicity and EPS production, play a significant role in the irreversible attachment of microorganisms to a surface (Donlan and Costerton 2002; O'Toole *et al.* 2000). For instance, the Gram-negative opportunistic pathogen *Klebsiella pneumoniae* has been show to express type 3 fimbriae, which actively facilitates cellular attachment to a surface (Murphy and Clegg 2012).

Following the establishment of a 'mature' biofilm, individual microorganisms can detach from

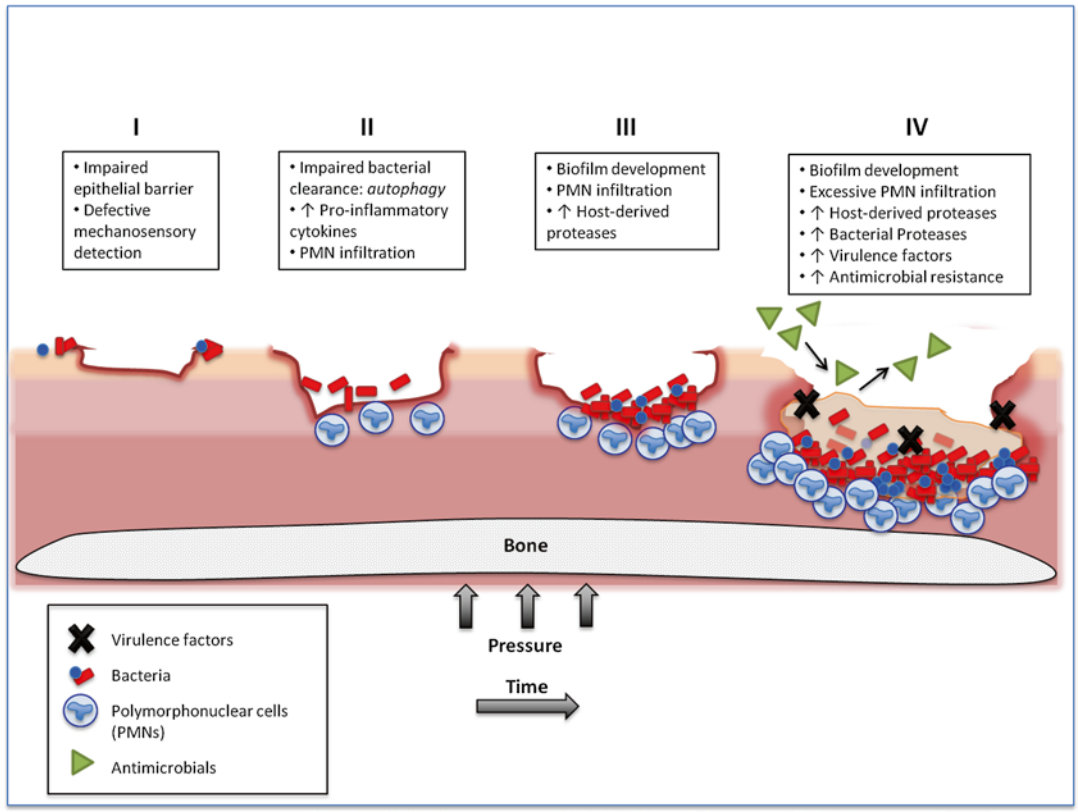

Fig. 3.1 Proposal for the development of biofilm-infected pressure ulcers

the biofilm, translocate and colonise a new site. This mechanism is known as 'dispersal' and is a process whereby microorganisms within a relatively static, slow-growing micro-community, become highly differentiated and motile (McDougald *et al.* 2012). This mechanism is thought to be regulated by a second messenger molecule known as cyclic-di-GMP (c-di-GMP) (Karatan and Watnick 2009). C-di-GMP effectively controls the transition of the bacterial cell from a biofilm to planktonic phenotype through its intracellular decrease, leading to dispersal (McDougald *et al.* 2012).

3.2 Intercellular Communication

Bacteria in biofilms have shown the ability to communicate with each other through biochemical signalling known as 'quorum sensing'. In doing so, the bacteria in a biofilm are able to sense the density and numbers of a biofilm (Lindsay and Von Holy 2006). Not all biofilms release the same chemical signals. For example, Gram negative bacteria release molecules called acyl-homoserine lactones, where as Gram positive bacteria release peptide molecules (Lindsay and Von Holy 2006). Some of the well-studied quorum sensing molecules are associated with *P. aeruginosa* biofilm quorum sensing such as N-(3-oxo-dodecanoyl)-L-homoserine. In addition, the N-(3-oxo-dodecanoyl)-L-homoserine quorum sensing molecule has been reported to increase *P. aeruginosa* biofilm virulence and repress host immune responses (Driscoll *et al.* 2007). Given that *P. aeruginosa* has been implicated in a number of pathological processes, particularly cystic fibrosis, these quorum sensing molecules have since been the target for drug development using quorum sensing inhibitors (Hentzer *et al.* 2003).

3.3 Resistance Mechanisms

Microbial biofilms have gained great attention in recent years, particularly in a clinical setting, due to their association with many pathogenic processes and importantly their increased resistance to antimicrobial action. Within these biofilms, the transfer of genetic material between microbial cells, a process known as plasmid exchange, occur at a higher rate, thus increasing the likelihood of developing both naturally occurring and antimicrobial-induced resistance (Hausner and Wuertz 1999). The mechanisms by which microorganisms are able to resist the action of antimicrobials have been proposed (Francolini and Donelli 2010; Percival *et al.* 2011) and include:

1. **Incomplete or slow penetration** of antimicrobial agents through the surface (EPS) layers of the biofilm;
2. **An altered microenvironment** such as nutrient depletion or low levels of oxygen may affect antibiotic action;
3. **A resistant phenotype** in a portion of the biofilm population. These resistant cells are known as 'dormant cells' or 'persister cells', and are characterised by a slower growth rate compared to other microorganisms in the biofilm and greater resistance to the action of antimicrobials.

3.4 Host Response to Biofilms

A great deal of research has been dedicated to understanding host immunity in the control and tolerance of both commensal and pathogenic microorganisms. However, a large portion of this research has been focussed upon bacteria within the planktonic state. It is now generally accepted that the growth of microorganisms in both environmental and clinical settings is within a biofilm. Thus research into the host immune response toward microorganisms within a biofilm has gained precedence.

Microorganisms within a biofilm have the capabilities to evade host immune responses. For instance, the EPS matrix of *P. aeruginosa* biofilms has been shown to protect against interferon-γ-(IFN-γ)-mediated macrophage killing (Leid *et al.* 2005). Furthermore, the presence of polymorphonuclear leukocytes (PMLs) has been shown to enhance *P. aeruginosa* biofilm formation and development *in vitro* through the deposition of neutrophil-derived polymers, actin and DNA, acting as a scaffold for microbial colonisation and biofilm formation (Walker *et al.* 2005). The polymicrobial nature of many environments may also lend itself to enhance biofilm formation. The yeast *Candida albicans* has demonstrated successful biofilm formation in the presence of peripheral blood mononuclear cells (PBMCs) in an cytokine-rich environment (Chandra *et al.* 2007).

3.5 Biofilm Detection

The detection of biofilms in a clinical environment, particularly in wounds, is a relatively undefined area. There are few methods available for the identification of biofilms in a clinical setting. The presence of infection is initially determined through clinical signs including fever and persistent inflammation. It is at this point when blood tests, swabs or scrapings are then taken and assessed for microbial growth using traditional microbiological plate counting. Although a cost effective method, plate counts do not determine whether the microorganism has been taken from a biofilm, nor does it reveal the developmental stage of the biofilm. Furthermore, the results of such tests may take up to 72 h, depending further on the cultivability of the microorganism (Bryers 2008). In addition, microorganisms within a biofilm are thought to reside in a viable but non-culturable (VBNC) state. The emergence of small colony variants (SCVs) is a good example of one of the barriers to overcome in the efficient detection of a biofilm in a clinical setting. SCVs are a sub-population of biofilm bacteria, which produce small colonies, develop resistance to antimicrobial action and can evade detection due to their slow growth rate (Neut *et al.* 2007). Given that SCVs have a reduced metabolic rate, they can be easily missed during routine microbiological cultures, which are commonly grown on agar

plates for 48 h (Neut *et al.* 2007). In this case, there is opportunity for false-negative results, which may wrongly indicate that no infection is present.

Biofilms can usually be identified using microscopy methods including scanning electron microscopy (SEM), confocal laser scanning microscopy or atomic force microscopy (AFM) (Lindsay and Von Holy 2006), however this method of detection is more commonly used in research environments. Therefore it is essential that rapid and accurate diagnostic tests are developed to aid the detection of clinical biofilms. Sophisticated techniques such as fluorescence *in-situ* hybridisation (FISH) or fluorescent probes specific to a nucleotide sequence with bacterial RNA or DNA is used to identify live and dead bacteria in complex biological samples.

3.6 Evidence of Polymicrobial Biofilm-Infected Pressure Ulcers and Other Chronic Wounds

The characterisation of infected pressure ulcers is an area of research that has been some what neglected. Probable reasoning behind this could be due to the diversity of chronic wound subtypes; thus a 'one fits all' approach is adopted.

Despite this, there still remain some studies that have identified polymicrobial-infected pressure ulcers. The most commonly isolated bacteria in chronic wounds include *Staphylococcus aureus (93.5 %)*, *Enterococcus faecalis (71.1 %)*, *P. aeruginosa (52.2 %)* and coagulase negative *Staphylococci* (CoNS) *(45.7 %)* (Gjødsbøl *et al.* 2006). More recent studies have extended this research in an attempt to gain a more comprehensive understanding of the microbial profile of wounds. Dowd and colleagues (2008) used a combination of pyrosequencing, denaturing gradient gel electrophoresis (DGGE) and full ribosome shotgun sequencing to determine the major microbial players in diabetic foot ulcers, venous leg ulcers and pressure ulcers. The most common wound isolates in this study included *Staphylococcus*, *Pseudomonas*, *Peptoniphilus*, *Enterobacter*, *Stenotrophomonas*, *Finegoldia* and *Serratia* spp. More interestingly however, was the population profile of each type of chronic wound, with pressure ulcers housing more obligate anaerobes (62 %) than both diabetic and venous ulcers (Dowd *et al.* 2008). Further research has shown *Streptococcus*, *Corynebacterium*, *Staphylococcus*, and *Pseudomonas* to be among the most common primary genera of bacteria isolated from the pressure ulcers of 49 patients, which were identified using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) (Smith *et al.* 2010).

Though the presence of microorganisms within chronic wounds has been known for some time, it is the microorganisms that reside in a biofilm within the wound that is of great importance regarding chronic wound pathogenicity. With this in mind, James and colleagues (2008) investigated the presence of biofilms in both acute and chronic wounds, using both scanning electron microscopy (SEM) and the molecular technique, DGGE. The authors discovered a significant difference in the presence of biofilms between chronic and acute wounds, with 60 % of chronic wounds containing a biofilm when compared to just 6 % in acute wounds ($p < 0.001$). Furthermore, DGGE revealed that these biofilms were in fact polymicrobial (James *et al.* 2008). A study by Kirketerp-Møller and colleagues (2008) also detected the presence of biofilms in chronic wounds using peptide nucleic acid-based fluorescence *in situ* hybridisation (PNA-FISH). Using this method, the structural organisation of bacteria within a chronic wound could be determined, and thus the aggregation of bacteria into microcolonies within an alginate matrix, with very few planktonic cells, was observed. In addition, the authors found no correlation between the bacterial species identified using traditional culture techniques and PNA-FISH, with wound colonisation results showing >60 % *S. aureus* and <30 % *P. aeruginosa* using culture methods, and only 15 % *S. aureus* and 70 % *P. aeruginosa* using PNA-FISH (Kirketerp-Møller *et al.* 2008). Fazli and colleagues (2011) identified aggregated bacteria (indicative of biofilm) in venous leg ulcer wound biopsies using PNA-FISH. Furthermore, the number of neutrophils in

biopsies containing *P. aeruginosa* was significantly higher than biopsies containing *S. aureus* (Fazli *et al.* 2011). This research supports the earlier proposed theory by Bjarnsholt and co-workers, implicating *P. aeruginosa* biofilms in the persistent inflammatory response evident in chronic wounds (Bjarnsholt *et al.* 2008).

3.7 Understanding the Effect of Biofilms in Pressure Ulcer Healing

3.7.1 *In Vivo* Models

There are few *in vivo* models that represent biofilm-infected pressure ulcers. Nakagami and colleagues (2011) assessed the effect of wild-type *P. aeruginosa* PAO-1 strain and the quorum sensing-deficient strains Δ*lasI*Δ*rhlI* and Δ*lasR*Δ*rhlR* in a murine ischemic-induced pressure ulcer model. After 3 days post-infection, the authors observed an infiltration of inflammatory cells at the wound site, however there was no significant difference in inflammatory response between the three strains. Furthermore, results showed increased bacterial motility in the Δ*lasR*Δ*rhlR* strain when compared to PAO-1, which correlated with a larger wound area (Nakagami *et al.* 2011). Although the true effect of quorum sensing on the pathogenesis of pressure ulcers was not clearly defined, this study highlighted an important role for bacterial motility in the deficient healing of pressure ulcers.

The majority of research into the effect of biofilms in chronic wounds has been conducted in various other wound models. For example, the presence *S. aureus* biofilms in wounded New Zealand rabbit ears has shown sustained low grade inflammation, decreased granulation tissue formation and reduced re-epithelialisation (Gurjala *et al.* 2011). Trøstrup and colleagues (2013) highlighted the differences in the innate immune response to biofilm infection between two different strains of laboratory-bred mice. In this study, the researchers modelled *P. aeruginosa* biofilm-infected burn wounds in BALB/c and C3H/HeN mice, whereby they observed that C3H/HeN mice controlled *P. aeruginosa* biofilm infection more effectively than BALB/c mice (Trøstrup *et al.* 2013). Thus differences in immunoregulatory responses, bacterial clearance and tolerance upon infection may explain why some individuals reside in perpetuate inflammation, effectively preventing wound closure. Not all *in vivo* biofilm-infected wound models have been successful. Kanno and colleagues (2010) infected full thickness wounds with *P. aeruginosa* biofilm in a murine model. The authors visualised biofilm presence on the wounds at as early as 8 h however there were no differences in epithelialisation when compared to uninfected controls (Kanno *et al.* 2010).

Despite the fact that several studies have now determined the polymicrobial nature of biofilm-infected wounds, there still remains scope for determining the interactions between the microbial species within a polymicrobial biofilm and how they affect wound dynamics.

Harriott and Noverr (2009) performed an *in vitro* study into polymicrobial biofilms consisting of *S. aureus* and *C. albicans*. In this study, *S. aureus* was shown to develop substantial biofilms in the presence of *C. albicans* when compared to monoculture biofilms, with *C. albicans* acting as a scaffold for *S. aureus* formation. Furthermore, this polymicrobial biofilm showed a phenotypic change in *S. aureus* as well as increased resistance to the antibiotic vancomycin (Harriott and Noverr 2009). A more recent study developed further on this bacteria-fungus interaction by investigating the mechanisms behind *C. albicans* and *S. aureus* binding in a biofilm. Using both *in vitro* and *in vivo* methods, Peters and colleagues (2012) revealed that *S. aureus* was able to bind to a receptor on *C. albicans* referred to as Als3p (Peters *et al.* 2012). In the context of impaired wound healing, a study into the effect of a *P. aeruginosa*, *S. aureus*, *Finegoldia magna* and *E. faecalis* polymicrobial biofilm in an *in vivo* wound mouse model showed significantly lower percentage wound closure when compared to *P. aeruginosa* mono-species biofilm alone (Dalton *et al.* 2011).

With increased structural stability of the biofilm and enhanced virulence, there remains scope

for further research into the effects of clinically relevant polymicrobial biofilms in chronic wound pathogenesis.

4 Treatment Strategies

4.1 Current Treatment for Pressure Ulcers

The treatment of pressure ulcers is very much dependent on the severity of the wound, anatomical location of the wound, the mobility of the patient and of course the patient's general health (NICE 2005). Thus the use of such treatment strategies as repositioning, debridement and negative pressure therapy (NPT) have been used at various stages of pressure ulcer development (Levine *et al.* 2013).

4.1.1 Repositioning

Repositioning is the most common strategy in the prevention and treatment of pressure ulcers. Many studies assess the effectiveness of various methods of repositioning such as frequency or the angle of repositioning. For example, the repositioning of patients aged 80 and over with stage I and II pressure ulcers, using the 30° tilt with three-hourly per night, showed to be more clinically effective when compared to routine prevention of six-hourly repositioning using a 90° lateral rotation (Moore *et al.* 2011). Whilst widely used however, there remain discrepancies in the amount of evidence to support repositioning in the prevention of pressure ulcers. Rich and colleagues (2011) assessed the association between frequent repositioning and the prevention of pressure ulcers in a 3-year cohort study. The authors established a lack of correlation between frequent repositioning (two-hourly) and the prevention of pressure ulcers in elderly, bedbound, hip fracture patients (Rich *et al.* 2011).

4.1.2 Wound Dressings

A variety of wound dressings are used in the treatment of chronic wounds and in doing so it is essential that these dressings can effectively manage wound bioburden and protease-containing exudate. However, the selection of a wound dressing used should be dependent on the clinical characteristics of the wound; thus assessing factors such as exudate levels, presence of necrotic tissue, presence of tunnelling, microbial bioburden and the necessity of protection from the external environment. Wound dressing options such as hydrocolloids, hydrogels and foams should be used in pressure ulcer treatment, as opposed to more traditional dressings such as gauze (NICE 2005). In a study involving 27 spinal-cord injury patients exhibiting pressure ulcers, the effectiveness of a hydrogel dressing was compared with topical gauze with povidone-iodine. Although healing rates in the patients treated with hydrogel was higher than those treated with gauze, the results were deemed to be non-significant. Nevertheless, there was a significant difference in the epithelialisation of pressure ulcers treated with hydrogel compared to those treated with gauze (Kaya *et al.* 2005).

4.1.3 Negative Pressure Therapy (NPT)

Negative pressure therapy (NPT), whereby a vacuum is applied to a sealed wound dressing, is a method used to reduce microbial bioburden, increase blood flow and reduce oedema, essentially promoting wound closure (Levine *et al.* 2013). However the claims of bioburden management and the control of oedema have been disputed (Mouës *et al.* 2011).

4.1.4 Nutrition and the Use of Vitamins

The effect of dietary intake on pressure ulcer healing has been recorded. A study by Ohura and colleagues (2011) focussed on the effect of nutrition on pressure ulcer healing. In a randomized study, patients with stage III/IV pressure ulcers on an increased calorie intake showed a significant decrease in wound size when compared to the control group (Ohura *et al.* 2011). Furthermore, either the topical or oral treatment of pressure ulcers with vitamins and minerals has been shown to have a positive effect on wound healing. A very early randomised controlled trial by Taylor and colleagues (1974) showed a

reduction in the area of pressure sores in patients treated with 500 mg of ascorbic acid (vitamin C), twice daily for 4 weeks (Taylor *et al.* 1974).

4.1.5 Debridement

The method of periodic debridement is thought to be an effective way to remove necrotic tissue, cellular debris, microorganisms and biofilms (Wolcott *et al.* 2009). Debridement of pressure ulcers can be achieved using several strategies such as:

Biological: The application of larvae or maggots to the wound, effectively removing necrotic tissue.

Autolytic: The use of naturally occurring enzymes that breakdown dead tissue.

Chemical: Chemical compounds such as hypochlorite help to debride a wound.

Enzymatic: Collagenase and papain are just a few enzymatic debriding preparations that are used to degrade necrotic tissue.

Mechanical: Mechanical debridement encompasses a variety implements from the use of wound dressings to the surgical removal of dead tissue.

Despite this array of debridement methods, there are no clinical trials to support which of these methods are the most effective in the debridement of wounds (Dryburgh *et al.* 2008; Levine *et al.* 2013).

4.2 Effectiveness of Current Treatment Strategies on Biofilms: Fine-Tuning Established Techniques

Biofilms have not only been shown to impede wound healing and sustain an inflammatory phenotype within the wound, they also remain unaffected from routine wound-care treatment strategies such as a single debridement. For instance, Seth and colleagues (2012) assessed the effect of several debridement methods, including lavage and Silvadene, on a rabbit ear biofilm-infected wound model. Although initial debridement therapies showed disruption of a *P. aeruginosa* biofilm, disruption was only temporary and the biofilm reformed after 24 h (Seth *et al.* 2012).

Already established treatments such as NPT, have been tested against *P. aeruginosa* biofilms *in vitro*. Ngo and colleagues (2012) demonstrated a small but significant reduction in *P. aeruginosa* biofilm after NPT and an even greater reduction when combined with silver-impregnated foam, where a reduction in biofilm thickness and diffusion distance was observed (Ngo *et al.* 2012). Seth and colleagues (2013a) explored the effectiveness of species-specific bacteriophage therapy against *S. aureus* in a New Zealand rabbit ear model. The authors observed no changes in wound healing using the bacteriophage therapy, however when combined with sharp debridement, significant improvement in wound healing when compared to either bacteriophage therapy or sharp debridement alone was observed (Seth *et al.* 2013a). Further studies by the same researchers also tested the efficacy of ultrasound treatment on *P. aeruginosa* biofilms in the rabbit ear model. Non-contact, low frequency ultrasound (NLFU) improved wound healing and significantly reduced bacterial counts. Furthermore, NLFU significantly reduced inflammatory cytokine expression. This research is the first *in vivo* study to provide evidence for the use of ultrasound therapy in the control of biofilm-infected wounds (Seth *et al.* 2013b).

The incorporation of antimicrobials such as silver into wound dressings has demonstrated effectiveness in the control of wound bioburden, particularly biofilms (Percival *et al.* 2007, 2008). The use of ionic silver alginate/carboxymethyl cellulose dressing has been shown to significantly increase wound closure in patients with chronic venous and pressure ulcers over a 4-week period in a randomised open label study (Beele *et al.* 2010).

4.3 Future Treatment Options

4.3.1 Anti-biofilm Agents and Topical Antimicrobials

There is a plethora of claims towards the 'anti-biofilm' properties of many agents such as xylitol, lactoferrin and ethylenediaminetetraacetic acid (EDTA), however although

promising results within *in vitro* models has been reported, the evidence for their efficacy within *in vivo* models is greatly lacking (Wolcott and Rhoads 2008; Ammons and Copié 2013). A very recent study by Gawande and colleagues (2014) demonstrated the efficacy of a novel, naturally occurring enzyme-based gel on chronic wound – associated microorganisms (Gawande *et al. 2014*). This novel gel, Dispersin-B® -KSL-W, contains the juvenile periodontitis-associated *Aggregatibacter actinomycetemcomitans*-derived enzyme Dispersin-B®, which inhibits biofilm formation and disperses preformed biofilms. In addition to this, the gel contains a broad-spectrum cationic antimicrobial decapeptide named KSL-W. Together, the authors demonstrated that the novel Dispersin-B® -KSL-W gel significantly reduced log counts in methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, CoNS, *A. baumannii* and *K. pneumonia*, when compared to the a control and the commercially available Silver-Sept™ wound gel. This study highlights a potential role for the combination of naturally occurring enzymes and broad-spectrum antimicrobials in the treatment of biofilm containing wounds in pressure ulcers (Gawande *et al.* 2014).

4.3.2 Quorum Sensing Disruption and Inhibitors

Quorum sensing within bacterial biofilms is associated with biofilm development and enhanced virulence, making it an intriguing target for pharmacological action (Hentzer *et al.* 2003). The proposed targets in the quorum sensing system include the signal generator, the quorum-sensing molecule and the signal receptor. In many cases, it is the signal receptor that is targeted by using molecules that block these receptors and disrupt signalling pathways (Rasmussen and Givskov 2006). Christensen and colleagues (2012) showed that *P. aeruginosa* biofilms in an *in vivo* mouse model could be disrupted by the use of the antibiotic tobramycin and several quorum sensing molecules including furanone and horseradish juice extract. Synergy was seen between both treatments and the presence of quorum sensing inhibitor molecules, increased the susceptibility of the *P. aeruginosa* biofilm to tobramycin (Christensen *et al.* 2012). In the context of chronic wounds, Schierle and colleagues (2009) demonstrated reduced epithelialisation in a murine chronic wound animal model in the presence of *S. aureus* and *S. epidermidis* biofilm. Furthermore, the introduction of the quorum sensing disruptor RNAIII inhibiting peptide (RIP) significantly restored re-epithelialisation when compared to the antibiotic oxacilin, due to reduced quantifiable bacterial burden. In addition, the staphylococcal biofilm signalling-associated gene TRAP appeared to play an important role in the reduction of wound closure, which was demonstrated by a weak effect on re-epithelialisation in $TRAP^{-}$ bacterial mutants (Schierle *et al.* 2009).

4.3.3 Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) is the application of a photoactive dye coupled with irradiation that brings about microbial cell death. The antimicrobial effect of PDT has been well documented. The efficacy of this treatment in the eradication of biofilms has been investigated *in vitro* and *in vivo*. Di Poto and colleagues (2009) investigated the action of PDT and antibiotic treatment on the eradication of *S. aureus* biofilms *in vitro*. PDT using a cationic porphyrin resulted in a significant inactivation of *S. aureus* and when combined with vancomycin, showed almost complete eradication of the bacteria (Di Poto *et al.* 2009). A small body of work exists for the treatment of biofilm-infected wounds in animal models. The use of PDT using porphyrin resulted in a 98 % reduction in *E. coli* in excisional wounds of mice (Hamblin *et al.* 2002). Further to this, *S. aureus*-infected leg wounds in mice were successfully treated using a similar PDT, resulting in complete eradication of *S. aureus* in three of five mice (Gad *et al.* 2004). In humans, the treatment of two autoimmune ulcers in an Italian patient using PDT resulted in significant wound closure from 4.0 to 1.8 cm diameter. Given that these ulcers were previously shown to be highly recalcitrant to systemic antibiotics and the application of wound dressings; it is thought that the successful use of PDT could be through such mechanisms as enhanced keratinocyte migration, immunomodu-

latory activity and antimicrobial effectiveness (Motta and Monti 2007). A further case study by Clayton and Harrison (2007) involved a patient with a 19.6 cm^2 chronic venous leg ulcer colonised by MRSA that had not responded to treatment with potassium permanganate, silver nitrate, bacteriostatic dressings or larval therapy (Clayton and Harrison 2007). Twice weekly PDT (5-ALA photosensitiser and a 633 nm red light source) was topically applied to the patient's leg ulcer over 4 weeks. The patient tolerated the PDT well reporting no discomfort and the ulcer was shown to improve significantly. More recent research has demonstrated a significant reduction in bacterial load when treated with the cationic photosensitiser PPA904 [3,7-bis(N,N-dibutylamino) phenothiazin-5-ium bromide] in patients with chronic leg ulcers when compared to the placebo controls. In this phase IIa, randomized, blinded and single treatment study, PPA904 was applied topically to the wound before applying 50 J cm^{-2} of red light for 15 min. Results showed complete wound closure in 50 % of patients that underwent PDT when compared to 12 % of patients on placebo (Morley *et al.* 2013).

5 Future Perspectives

Whilst there are a growing number of studies that provide convincing evidence towards the control of bioburden in chronic wounds, the numbers of *in vivo* models for biofilm-infected pressure ulcers are few and far between. It is important to recognise that infection in chronic wounds, particularly pressure ulcers, is polymicrobial; thus the investigation of inter-species interactions within biofilms will provide insight into whether certain polymicrobial profiles may be more virulent. For instance, it would be interesting to investigate at which stages of pressure ulcer progression microbial biofilms are present. Indeed, the microbial profile at the various stages of pressure ulcer development may be indicative of any pathogenic correlation between pressure ulcer stage and microbial profile. Furthermore, the interactions between polymicrobial biofilms and cellular components of the host immune response to biofilms will help direct pharmacological intervention. Whilst there are differences in the aetiologies of various chronic wound types, it is thought that the primary problem of defective healing remains the same (Bjarnsholt *et al.* 2008). Despite this, it is imperative that physiologically relevant *in vivo* models for these various wound types are utilised to determine not only the effect of biofilms but also additional factors such as inherent barrier function impairment, ageing and cellular mechanisms such as autophagy on wound repair. Nevertheless, there remain promising opportunities in the control of bioburden in pressure ulcers.

Key Points

- Factors such as sustained pressure, increased shear stress and moisture are known contributors in the formation of pressure ulcers. Despite this, pathways leading to tissue destruction are poorly understood. Research into the mechanosensory receptors and cellular degradation pathways such as autophagy, present exciting and interesting concepts into pressure ulcer pathology.
- The formation of a biofilm within a wound is thought to play a major role in impaired wound closure in chronic, non-healing wounds, and indeed, polymicrobial communities have been identified in pressure ulcers.
- There remains a lack of sufficient *in vivo* biofilm-infected pressure ulcer models, with various chronic wound models used as a 'one fits all' in the determination of the effect of biofilms in pressure ulcers.
- Treatment strategies such as photodynamic therapy (PDT) show promising opportunities in the control of bioburden in pressure ulcers. However the appropriate use of a combination of strategies such as anti-biofilm agents and debridement may be a more effective method of wound management.

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Microbial Biofilms and Adverse Reactions to Gel Fillers Used in Cosmetic Surgery

4

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1 Introduction

Chronic bacterial infections are characterized by a slow pathologic progression compared to acute infections and the symptoms are often vague (Hall-Stoodley et al. 2012). On the other hand these chronic infections are very difficult if not impossible to cure with antibiotics (Burmolle et al. 2010). Increasing evidence suggests that the chronicity of persistent bacterial infections is due to bacterial biofilm formation as opposed to the planktonic bacteria seen in the acute infections (Bjarnsholt 2013; Costerton 2007; Hall-Stoodley et al. 2012).

Chronic infections and the chronic inflammatory response are usually characterized by an adaptive immune response, dominated by mononuclear leukocytes and IgG antibodies. In some chronic infections the inflammatory response is characterized by a chronic inflammatory response as well as a continuously recruitment of PMNs. Some of these can develop in patients suffering from a disease or condition causing deficiencies in the primary defense barriers (the innate immunity), which may be disruption of anatomic sites (*skin, mucous membranes, cilia*), physiologic causes (*temperature, low pH*), or inflammatory barriers with phagocytic defects, polymorphonuclear granulocytes and macrophages. Such deficiencies can be divided into congenital abnormalities, presence of foreign bodies and acquired chronic diseases.

A classic example is the chronic lung infection in patients suffering from the genetic disorder cystic fibrosis (CF). These patients have an increased viscosity of the periciliary fluid, the mucus, in the airways, impairing normal mucociliary clearing of paranasal sinuses and lungs (Koch and Høiby 1993). The viscous mucus is the perfect habitat for bacterial growth and biofilm development and facilitates persistent bacterial infection (Bjarnsholt et al. 2009a; Johansen et al. 2012). The presence of foreign bodies including artificial joints and indwelling catheters also support the advent of chronic biofilm infections (Bjarnsholt et al. 2013), and we have found that the same is also true for injected tissue fillers (Alhede et al. 2014; Bjarnsholt et al. 2009b; Christensen et al. 2013). Acquired chronic diseases such as diabetes mellitus, arteriosclerosis of leg arteries, and smoking-induced chronic obstructive pulmonary disease (COPD) also create perfect habitats for chronic bacterial infections as seen in COPD patients, who are prone to chronic lung infections or patients suffering from non-healing wounds, which are chronically infected with

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bacteria (Bjarnsholt et al. 2008; James et al. 2008; Kirketerp-Moller et al. 2008).

2 Cosmetic Surgery: A Historical Overview

Cosmetic surgery is a way for people to improve or enhance an otherwise healthy and normal body through shaping and sculpting the body to achieve certain beauty ideals.

The field of plastic/cosmetic surgery was officially organized in the United States in 1931 with the formation of the American Society of Plastic and Reconstructive Surgeons. As technology advanced along with a rise in surgical procedures, cosmetic surgery evolved as a special branch. Common procedures are liposuction, fat transplantation, rhinoplasty, eyelid surgery, face-lift, brow lift, breast augmentation and abdominoplasty. These are steadily increasing in popularity with more than 11 million cosmetic surgeries performed in the US in 2006 (http://www.medscape.com/viewarticle/542448) and 15.1 million today (American Society of Plastic Surgeons).

In the old days facial cosmetic surgery meant real surgery such as face-lift, brow-lift, nose correction or fat transplantation. Injection therapy started in the late twentieth century with paraffin, which was used to add volume to the tissue (like in saddle nose deformity after syphilis). The treatment was used from 1903 to 1920, after which it was abandoned due to material migration and nodules (paraffinomas) (Haiken 1997). During 1863–1876 silicone injections were used for augmentation of breast and other soft tissues, but the FDA never approved it. The gel was found to disappear from the injection site, it could not be demonstrated where it went, and local inflammatory reactions with granulomas were noted. However, silicone gel is still being used 'off label' for facial injections, predominantly in the US (Chasan 2007).

Today focus in facial cosmetic surgery is on the injection of botolinum toxin (Botox), which paralyses nerves responsible for wrinkles, and injection of gel fillers, which add volume to soft tissue sites.

3 Usage of Tissue Fillers

Augmentation of soft tissues by injection of gel fillers is an increasingly popular procedure for patients seeking cosmetic facial enhancement. The procedure has been used in the past administering paraffin (Haiken 1997) and silicone (Chasan 2007), but it was not until 1981, when the first product, collagen gel (Zyderm), was approved by the FDA, that the procedure became popular (Narins et al. 2008). Gradually other fillers appeared on the market – first and foremost fillers based on the degradable hyaluronic acid (HA), which was FDA approved in 2003 (Narins et al. 2003) – and over the years several hundred fillers were developed, many of which disappeared again. Various versions of biological as well as synthetic fillers were constructed with the purpose of giving them as long duration of effect as possible without compromising safety. Only seven different types of filler are used today (Sadove 2009):

(a) **Degradable fillers** (hyaluronic acid gel and collagen gel). Degrade from 3 to 9 months (Narins et al. 2003, 2008).
(b) **Slowly degradable fillers** (degradable fillers containing slowly degraded microparticles, no = 2). Degrading time approximately 2–5 years (Jansen and Graivier 2006; Sadove 2009).
(c) **Non-degradable fillers** (polyacrylamide hydrogel, silicone gel (off-label) and a degradable collagen gel filler containing non-degradable microparticles) (Gabel et al. 1987; Lemperle et al. 2003; Pallua and Wolter 2010).

The different filler types differ with respect to composition, tissue interaction and longevity, and they appear to differ with respect to type of adverse reaction as well.

4 Adverse Events Using Tissue Fillers

Adverse events of long duration following injection with the different filler types have typically been described by their clinical presentation (tissue hardening, nodules, lumps, cysts, sterile abscesses) (Alijotas-Reig et al. 2009; Bergeret-Galley et al.

2001; Christensen et al. 2005, 2006; Christensen 2007; Daines and Williams 2013; DeLorenzi 2013; Dijkema et al. 2005; Requena et al. 2011). The exact aetiology of these reactions is still not completely understood for all fillers. A systematic search for bacterial RNA or DNA has only been made for polyacrylamide gel (Bjarnsholt et al. 2009b).

Nodule formation is the most common complication with significant morbidity to the patient in both the short- and the long term. It may appear at any time from days to years after treatment and cause discomfort and pain.

Quickly biodegradable fillers like collagen and HA gels may induce severe complications, usually abscess formation (Van et al. 2010), but these will normally disappear spontaneously in a few months or earlier, if hyaluronidase is given (DeLorenzi 2013). Slowly degradable or non- resorbable fillers, on the other hand, may give rise to severe reactions that show little or no tendency to spontaneous improvement (Alijotas-Reig et al. 2009; Bergeret-Galley et al. 2001; Daines and Williams 2013).

Rare long-term serious adverse events associated with filler use include the development of tissue hardening and necrosis, cysts and fistulas, displacement, sensory dysfunction and scarring (Alijotas-Reig et al. 2009; Bergeret-Galley et al. 2001; Christensen et al. 2005, 2006; Christensen 2007; Daines and Williams 2013; DeLorenzi 2013; Dijkema et al. 2005; Requena et al. 2011; Van et al. 2010).

Reports on adverse events to all types of fillers have appeared in steadily increasing numbers over the years corresponding to the increased usage of the higher number of different fillers, we have today. The incidence rate is difficult to determine, as the majority of publications on adverse reactions are case reports, and often neither patient nor treating doctor (which is rarely the same, who has injected the gel) know which filler they have been treated with.

In the 1960s to 1980s the majority of these reactions were described as granulomas, nodules or indurations to silicone gel and from 1999 similar complications were seen following injection with two other non-degradable fillers, Artecol and Dermalive (the latter out-of-use today), both characterized by non-degradable micro-particles (Christensen et al. 2005).

When polyacrylamide hydrogel was introduced in Europe and Australia in 2001 as a non-degradable hydrogel filler with a very similar tissue appearance and interaction to that of hyaluronic acid gel (Christensen et al. 2006; Christensen 2009a), the adverse reaction pattern changed: Excessive swelling, fistulation and purulent secretion could be seen in early stage lesions, especially if steroid was administered, and granulomas, nodules or indurations similar to those seen after the other non-degradable fillers were typically observed in delayed lesions (Christensen et al. 2013).

Histopathologically, early lesions with swelling, which were typically seen after injection with polyacrylamide gel, comprised an abundance of large phagocytising scavenger cells such as macrophages and foreign-body giant cells surrounding the homogenous gel deposits (Bjarnsholt et al. 2009b; Christensen et al. 2005, 2006, 2013; Christensen 2009b). Late lesions whether originating from tissue injected with silicone, polyacrylamide gel or gels with microparticles, were firmer in consistency and showed a different pattern. Macrophages and giant cells were primarily present in gels with microparticles, which they were trying to engulf (Christensen et al. 2005; Christensen 2009a), and in silicone granulomas, distended by the foreign material (Christensen et al. 2005; Christensen 2009a). Common to them all, however, was the presence of fibrosis and chronic inflammatory cells, mainly lymphocytes (Bjarnsholt et al. 2009b).

5 Allergy vs. Infection

Treatment options in the early days ranged from local injections with hyaluronidase over steroids and 5-fluoro-uracile to laser removal and finally surgery. The adverse reactions were generally considered bouts of delayed hypersensitivity or allergy to the injected product, and although they were never symmetrical and the tissue contained no eosinophilia it is still widely recognized that only some reactions (mainly those following

polyacrylamide gel injections) are due to biofilm infection with commensal bacteria from the injection site (Bjarnsholt et al. 2009b). Nodules or granulomas to other fillers are still mainly considered purely immune-mediated (Alijotas-Reig et al. 2009, 2012, 2013).

6 Identification of Bacteria in Patient Samples

The presence of bacteria causing these adverse events has been investigated in only a few studies. The first study examined samples from eight patients which had different fillers injected at different sites. By fluorescence in situ hybridization using peptide nucleic acid probes (PNA FISH) targeting bacteria, bacterial biofilms were observed by microscopy in seven out of the eight samples (Bjarnsholt et al. 2009b). In a follow-up study samples from 54 patients with adverse events due to filler injection and 24 controls were investigated (Christensen et al. 2013). Using a combination of Gram stain histology, culture, PNA FISH and PCR bacteria were identified in 53 out of the 54 samples and in none of the controls. All of the bacteria identified using microscopy were positioned in small aggregates (see Fig. 4.1). This study strongly suggests that bacteria play a main role in the adverse events seen after injected tissue fillers.

7 Tissue Fillers and Bacterial Growth

As mentioned above solid evidence has been provided that bacteria grow within tissue fillers from patients with adverse events (Christensen et al. 2005, 2013; Christensen 2007). It is still argued, however, that tissue fillers do not support bacterial growth (Alijotas-Reig et al. 2010). To investigate this further researcher incubated different relevant bacteria (including *S. epidermidis* and *P. acnes*) with different tissue fillers in an in vitro setup (Alhede et al. 2014). They found that non-degradable as well as slowly degradable and degradable fillers were able to nest bacterial biofilms (see Fig. 4.2), and none of the fillers showed growth inhibitory patterns. Furthermore, the bacteria readily formed biofilm aggregates, which showed high tolerance towards antibiotics already within the first day (Alhede et al. 2014).

It has also been suggested that chemotaxis could play a role for biofilm development in connection with the injection of tissue fillers. The same authors as arguing fillers cannot support bacterial growth argued that fillers do not serve as a chemo-attractant for bacteria (Alijotas-Reig et al. 2010), but since the bacteria are likely injected into the skin along with the filler during needle penetration, this argument is irrelevant speaking about in vitro observations.

8 Evidence from an Animal Model

An in vivo study was necessary to determine whether bacteria and subsequently biofilms would be a likely cause of the adverse events in fillers, and a mouse animal model was developed. Here contaminated needles were used to injecting the filler (to simulate punching through a microcolony on the skin) in the neck of a mouse. As little as 40 single bacteria was sufficient to establish gel infection at that site. However, long term infection could only be established with the permanent filler polyacrylamide. After 7 days no bacteria were detected within the HA gel, and only a limited amount was found in a third gel, which contained slowly degradable microspheres of calcium hydroxy-apatite.

The clinical symptoms on the above mentioned adverse events (often culture-negative bacteria creating a low grade chronic inflammation) have convinced many that adverse events to fillers are caused by an allergic or autoimmune reaction towards the filler, and accordingly, many have been treated with steroids or non-steroidal anti-inflammatory drugs to suppress the inflammatory process. This, on the other hand, has often worsened the inflammatory response, resulting in the formation of fistulas and abscesses, and therefore, steroid treatment against the infected gels in the mice was studied as well. It was found that

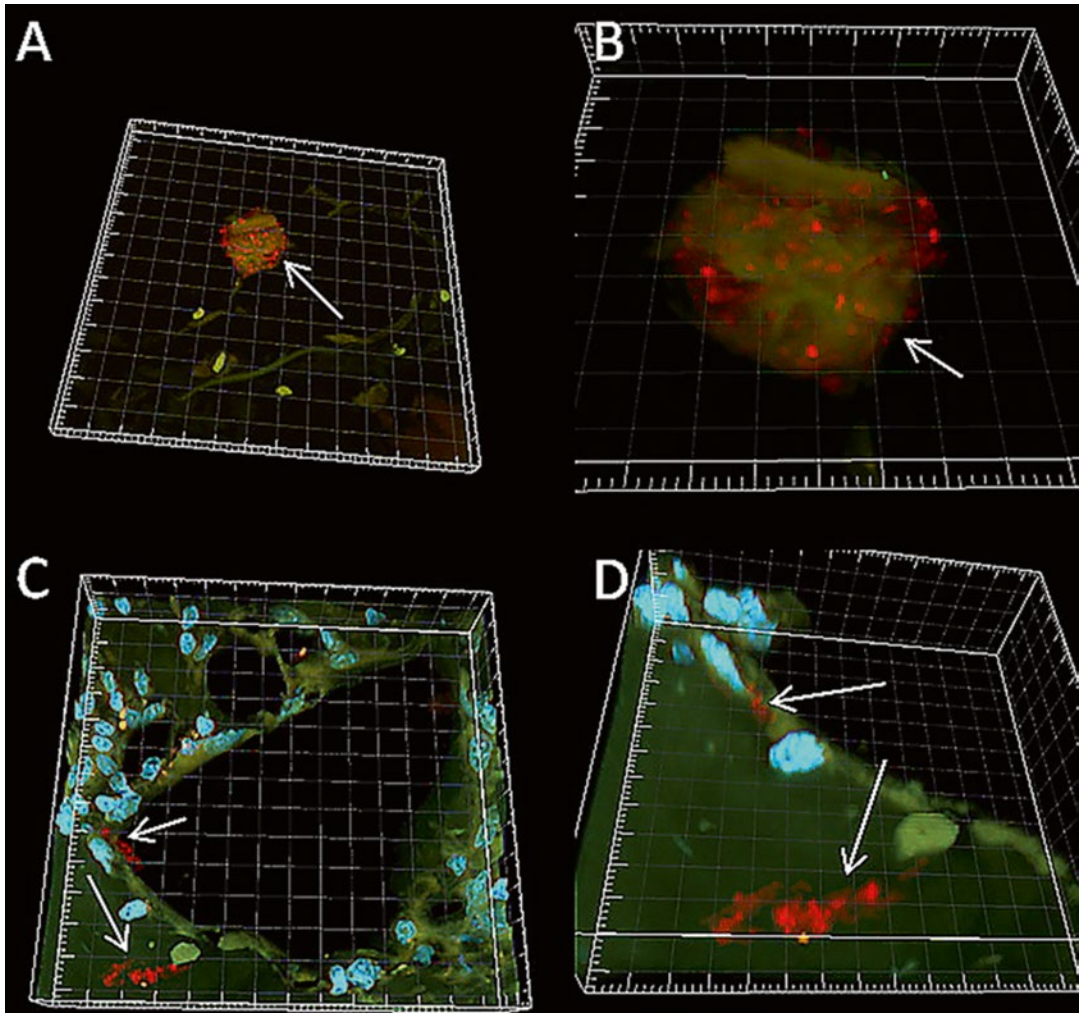

Fig. 4.1 Aggregates of bacteria (*Propionibacterium acnes*) in a lip biopsy showing a Grade 2 lesion 6 months after gel injection. The bacteria were visualized as small red microspheres by confocal laser scanning microscopy using PNA FISH (*arrows*). Macrophage and granulocyte nuclei were seen as large blue DAPI positive blue dots. Frame B is an enlargement of frame *A*, and frame *D* is an enlargement of frame *C*. The slightly opaque yellow-green material is PAAG. *A* and *C*: ×600, B and D: ×1,000 (Adapted from Christensen et al. 2013)

mice carrying contaminated gels with a low dose (40 μg per gram bodyweight) of the steroid Triamcinolone Acetonide for 6 days did not influence the bacterial load within the gel, but a higher dose treatment (100 μg per gram bodyweight) gave a response similar to that seen in patients treated with steroid: bacterial growth within the gel increased, and the mice were left in a poor condition.

As the bacteria *P. aeruginosa* were able to grow as tolerant biofilms within the non-degradable polyacrylamide gel in vivo, it was further tested whether infection could be prevented by prophylaxis or whether an established infection could be successfully treated with antibiotics. The group having received prophylactic treatment with tobramycin (100 μg/g) 2 h prior to injection of contaminated PAAG was able to completely eliminate the bacteria within the gel, whereas the non-treated group was inadequate in clearing the bacteria. In contrast, a delayed long term treatment (i.e. once a day for 7 days initiated 7 days post

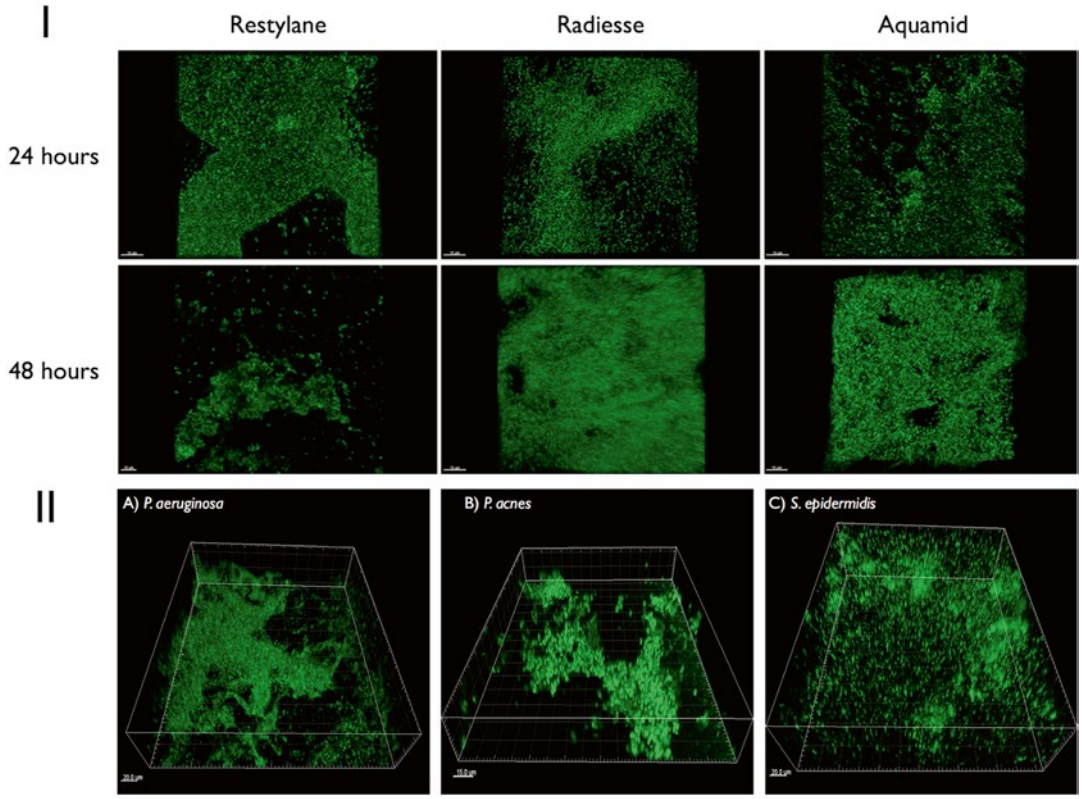

Fig. 4.2 (**I**) **In vitro growth of *P. aeruginosa* in tissue filler gels**. Microscopic images of tissue filler gels inoculated with GFP-tagged *P. aeruginosa*. The pictures were obtained with the use of a Zeiss.Z2 microscope with the connection of LSM 710 CLSM with Zeiss Zen v.6.0 software with a 63×/1,4 oil objective and prepared in Imaris. The databar represents 15 μm in all images. (**II**) **Microscopic images of PAAG inoculated with *P. aeruginosa*, *P. acnes* or *S. epidermidis* in the silicone setup**. The bacteria were grown for 24, 48 and 72 h, respectively, as biofilm on the PAAG. The gel and bacteria inoculated into chamber cut in the silicone sheets and incubated in a moisture chamber at 37 °C. The pictures were obtained with the use of a Zeiss.Z2 microscope with the connection of LSM 710 CLSM with Zeiss Zen v.6.0 software with a 63×/1,4 oil emersion objective and prepared in Imaris. Biofilm appears after (*A*) 24 h for *P. aeruginosa*, (*B*) 48 h for *P. acnes* and (*C*) 72 h for *S. epidermidis* (Adapted from Alhede et al. 2014)

injection) with tobramycin reduced the bacterial load but it did not clear the bacteria, suggesting biofilm infection;.

Similar in vivo experiments performed with the skin bacterium *S. epidermidis* showed that prophylactic treatment with rifampicin (Rifadin 5 μg/g) of the infected gels prevented the bacteria to settle and establish an infection. This is in contrast to the late treatment starting after 7 days, where an established infection occurred (although with lower bacterial load than the non treated mice).

These above mentioned experimental findings are in accordance with a newly published clinical retrospective study showing that administrating prophylactic antibiotics (a single oral dose of azithromycin (Zitromax) and moxifloxacin (Avelox)) to subjects with tissue fillers significantly reduced the incidence of inflammation/infection from 7 to 3 % of the patients (Nygart et al. 2014). It would be interesting to see, if prophylactic antibiotics can reduce the number of adverse reactions to other fillers as well.

It seems clear that the best option is to prevent the bacteria from settling into biofilms. Once the biofilm has formed, even the highest doses of antibiotics are not enough, and the only solution to the problem is surgical removal with a risk of scarring (Dr. Vibeke Breiting, personal communication) or syringe-assisted suction of the biofilm-infected gel (39).

9 Perspectives

We believe that it is beyond any doubt that adverse reactions to polyacrylamide gel are caused by bacteria living in a biofilm environment (Alhede et al. 2014; Bjarnsholt et al. 2009b; Christensen et al. 2013), and as a consequence injectors give prophylactic antibiotics with a dramatic effect on the incidence of adverse reactions to this gel (Nygart et al. 2014). Extreme care should be taken to prevent contamination during the injection. With bacteria as the most likely cause of adverse event to filler injections steroids should be discouraged as a first choice of treatment, systemically and locally. Local steroid injections or systemic treatment will exacerbate the condition and give the bacteria better conditions (25). It must thus be the last treatment option and only used in combination with an antibiotic, which is known to be specific in eradication of the contamination bacteria.

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Role of Biofilms in Breast Implant Associated Infections and Capsular Contracture

5

Jose L. del Pozo and Cristina Auba

1 Introduction

Breast implants are used for cosmetic breast enlargement, correction of asymmetries and congenital defects and for reconstruction after mastectomy for breast cancer or cancer risk reduction. According to the American Society of Plastic Surgery, by 2013, breast augmentation had become the leading type of cosmetic plastic surgery in the USA, with 290,224 procedures performed that year (http://www.plasticsurgery.org/ 2014). Breast implants may be placed between the pectoralis major muscle and the breast gland (subglandular), or under the muscle (submuscular). The surgical approach may be through an inframammary, periareolar, transaxillary, transareolar, or transumbilical incision (Fig. 5.1). The two most commonly used types of breast implants are silicone gel and saline implants (Fig. 5.2). Silicone implants contain silicone gel within a silicone polymer shell and saline implants contain saline within an outer silicone polymer shell. Saline implants are expandable and are filled at the time of insertion by the surgeon. Breast reconstruction can be performed by using implants (implant-based breast reconstruction), autologous tissue or a combination of both (i.e., latissimus dorsi plus implant). Whilst autologous breast reconstruction used to be the treatment of choice, the last decade has seen a shift from autologous towards implant-based breast reconstruction, which is now used in 37 % of all breast cancer patient in USA. Implant-based breast reconstruction include tissue expander plus implant (i.e., two-stage) or a direct-to-implant reconstruction (i.e., single-stage) (Salzberg 2012; Pittet et al. 2005; Cordeiro 2008). The one-stage approach has experienced a growth by the increase of skin sparing or nipple sparing mastectomies (which have been proven to be oncologically safe procedures), the increased indication of prophylactic mastectomies, the proven safety of silicone breast implants and the development of biological matrices and synthetic meshes. Thus, immediate implant-based breast reconstruction offers psychological benefit to patients, decreasing the number of surgical procedures.

Women who receive a breast implant may have complications after implant placement (Gabriel et al. 1997) (Table 5.1). Complications have a significant negative impact on patient satisfaction, hospital length of stay, and associated costs. Despite improvements in implant design and surgical techniques, these complications still represent a significant challenge for clinicians. Breast implant

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infections are a significant source of morbidity for breast cancer patients, with immediate breast implant reconstruction presenting the highest rate of complications. The incidence of infection after breast reconstruction with tissue expanders and implants is reported to be as great as 35 % in this subset of patients (Craft et al. 2012). Cellulitis, periimplant infection, fistula presence and/or implant exposure often mandate immediate device removal. However, there been some reports of attempted implant salvage in certain patients.

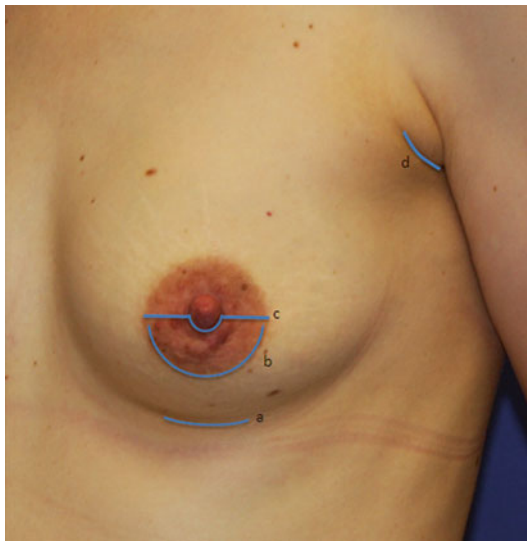

Fig. 5.1 Most common used surgical approaches for breast implantation. Inframammary (**a**), periareolar (**b**), transareolar (**c**) and transaxillary (**d**)

Other potential complication is capsular contracture. The host forms a capsule as a foreign-body reaction around the breast implant. Contracture of this capsule around a soft implant leads to a painful, tight capsular contracture. It has been postulated that contracture might be related to a chronic infection with propionibacteria, coagulase-negative staphylococcus and other skin organisms. Rates of capsular contracture may range between 1 and 33 % (Prince et al. 2012). Cause of capsular contracture and, accordingly, treatment and prevention, remains to be elucidated (Del Pozo et al. 2009; Gundeslioglu et al. 2005; Dobke et al. 1995; Virden et al. 1992; Carpaneda 1997).

2 Breast Implant Associated Infection

2.1 Incidence

Breast implant related infection is one of the leading cause of morbidity that complicates breast implantation representing around 2 % of interventions in most series (Gabriel et al. 1997; Armstrong et al. 1989; Capozzi 1986; Cocke 1994; De Cholnoky 1970; Mladick 1993; Rheingold et al. 1994). However, it has been reported that infection may occur in up to 35 % of reconstruction cases (Craft et al. 2012). Most infections occur in

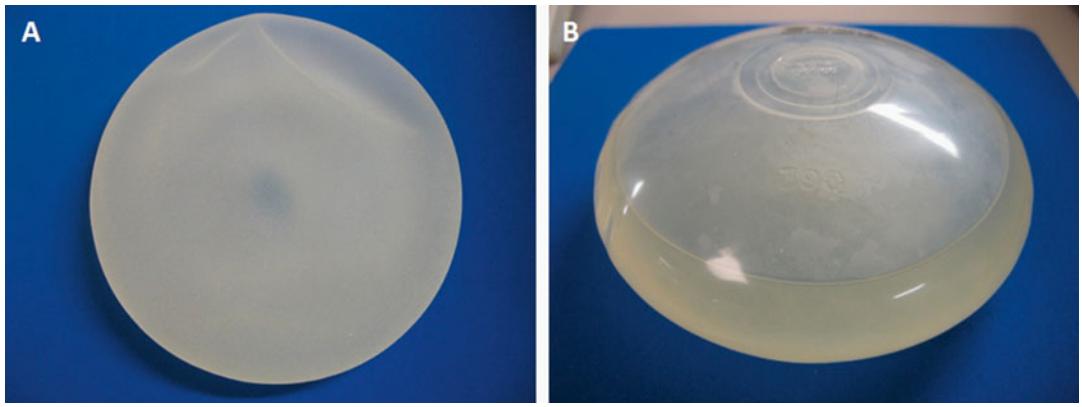

Fig. 5.2 Breast implant models. (**a**) Gold standard for breast implant: silicone gel contained within a silicone rubber envelope. (**b**) Inflatable saline implant (filled by the surgeon after insertion)

Table 5.1 Risk factors for and complications after breast implant placement

Risk factors	Short-term complications	Long-term complications
Surgical technique	Seroma	Late breast implant infections
Lymph-node dissection	Haematoma	Capsular contracture
Pre-existing tissue scarring	Wound dehiscence	Silicone granuloma
Radiation therapy	Skin and soft tissue infection	
Adjuvant chemotherapy	Acute breast implant infections	
Elevated body mass index	Implant displacement	
Diabetes	Hypertrophic scar	
Smoking	Capsular contracture	
Postmenopausal status	Implant rupture	
Prior breast operation		
Steroid therapy		
Increased American Surgical Association (ASA) class		
Amount of blood loss		
Need for surgical drains		
Nipple-sparing mastectomy		

the immediate post-surgical period, but they can also present many years after surgery (De Cholnoky 1970). Hospital-associated costs of breast implant associated infections have been estimated to be around $4,000 per episode (Olsen et al. 2008).

There are several strategies trying to prevent breast implant associated injections: a sterile environment, better surgical techniques, and antimicrobial prophylaxis. Need for antimicrobial prophylaxis in breast surgery is not well defined. Although most guidelines do not routinely recommend prophylaxis for breast surgeries, they do in cases of implant placement (Tejirian et al. 2006).

2.2 Risk Factors

Risk factors for breast implant associated infections have not been clearly assessed. Rates of infection after reconstruction surgeries are significantly higher than after cosmetic procedures. Immediate implant placement after mastectomy increases infection risk when compared with delayed placement (De Cholnoky 1970), and depends on the degree of tissue scarring and skin atrophy resulting from cancer surgery and radiation therapy. Surgical technique and patient's comorbidities are the most reported risk factors for infection (Capozzi 1986; Jabaley and Das 1986; Nahabedian et al. 2003; Schatten 1984; Hester et al. 1988; Schlenker et al. 1978; Eyssen et al. 1984; Slade 1984). Probability of infection was demonstrated to be higher in women with lymph-node dissection in a study (Nahabedian et al. 2003). Nipple-sparing mastectomy were identified as a risk factor in other study (Kato et al. 2013). Adjuvant chemotherapy was also associated with a significantly higher rate of infection (Vandeweyer et al. 2003). Other preoperative factors include increased American Surgical Association class, prior breast surgeries, diabetes mellitus, elevated body mass index, postmenopausal status, history of smoking, steroid therapy, higher amount of blood loss during surgery, and need for surgical drains after surgery (Washer and Gutowski 2012).

Acellular dermal matrices have become an increasingly common component of implant-based breast reconstruction introducing a new risk factor for infection. Acellular dermal matrices are biotechnological tissues prepared from human, bovine or porcine tissue from which the cellular components that cause rejection and inflammation have been removed. The resulting structurally intact tissue matrix provides the biological scaffold necessary for normal tissue in-

growth and cellular repopulation, as well as the biochemical components needed to initiate angiogenesis and revascularization. Since implant or expander are only partially covered by pectoralis muscle, acellular dermal matrices provides coverage at the inferolateral pole allowing the placement of a direct implant at the time of the reconstruction, and eliminating the need for recruiting the serratus anterior or other muscles in the case of the expander. Thus, this technique avoids or shortens the tissue expansion/implant reconstructive process, avoids mastectomy flap contraction during the latency period of expansion, provides an additional layer of tissue between the skin and the implant, and offers an additional option for immediate, single-stage breast implant reconstruction (Macadam and Lennox 2012). There have been reported low rates of complications associated with the use of acellular dermal matrices. In 790 breast reconstructions performed with acellular dermal matrices in 439 patients, major complications occurred at an incidence of less than 2 % including infection (1 %) and capsular contracture (0,5 %) (Salzberg 2012). In other study that reviewed 331 immediate single-stage reconstruction with acellular dermal matrices reported an elevated rate of skin necrosis requiring reoperation (9 %) and 3 % of infection (Colwell et al. 2011). An alternative to implant-based breast reconstruction using acellular dermal matrix is the titanium-coated polypropylene mesh approved in Europe in 2008. Although only limited clinical data are available, a multicenter study of 231 cases has recently shown acceptable complication rates (i.e., 6 % infection and 2.2 % capsular contracture) (Dieterich et al. 2013). However, concerns about their use remain unclear because of the significant costs associated with these products.

2.3 Microbiology

The breast is not a sterile organ; it contains endogenous flora, derived from the nipple and is similar to normal skin microflora (i.e., coagulase-negative staphylococci, diphtheroids, lactobacilli, alpha-haemolytic streptococci and anaerobes, mainly *Propionibacterium acnes*) (Fig. 5.3).

Gram-positive organisms (i.e., *Staphylococcus aureus* and streptococci) and gramnegative bacteria are the most common pathogens associated with early post-implant infections (Feldman et al. 2010). Late infections are most commonly due to coagulase negative staphylococcus and *Propionibacterium* spp. (Washer and Gutowski 2012). The syndrome of late developing, massive, odourless, severe effusion with negative routine cultures may indicate that special acid-fast cultures and stains should be requested (Eliopoulos and Lyle 1999). *Mycobacterium fortuitum* is the most common atypical mycobacteria associated with breast implant infection (Macadam et al. 2007; Vinh et al. 2006), but multiple other species have been reported (i.e., *Mycobacterium avium*, *Mycobacterium abscessus*, *Mycobacterium conceptionense*, *Mycobacterium thermoreistible* and *Mycobacterium chelonae*). Other uncommon organisms have also been reported including *Streptomyces* species, *Pasteurella multocida*, *Brucella* species, *Granulicatella adiacens* (del Pozo et al. 2008), and rarely fungal infections.

2.4 Pathogenesis

Contamination of breast implants with patient's skin microflora during surgery is the origin of most implant infections. Other less common pathogenic mechanisms include: a contaminated implant, a contaminated surgical environment, skin-penetrating accidents, local soft tissue infections, breast trauma or massage, or seeding of the implant from remote infection (De Cholnoky 1970; del Pozo et al. 2008; Brand 1993) (Fig. 5.4). Once colonized, implants have a high risk for symptomatic infection due to formation of biofilms on its surface (del Pozo and Patel 2007). Bacteria that attach to a surface and grow as a biofilm and are protected from killing by innate host defenses and antimicrobial agents. This often leads to failure of antimicrobial treatments, and the need to remove the implanted device to cure infection.

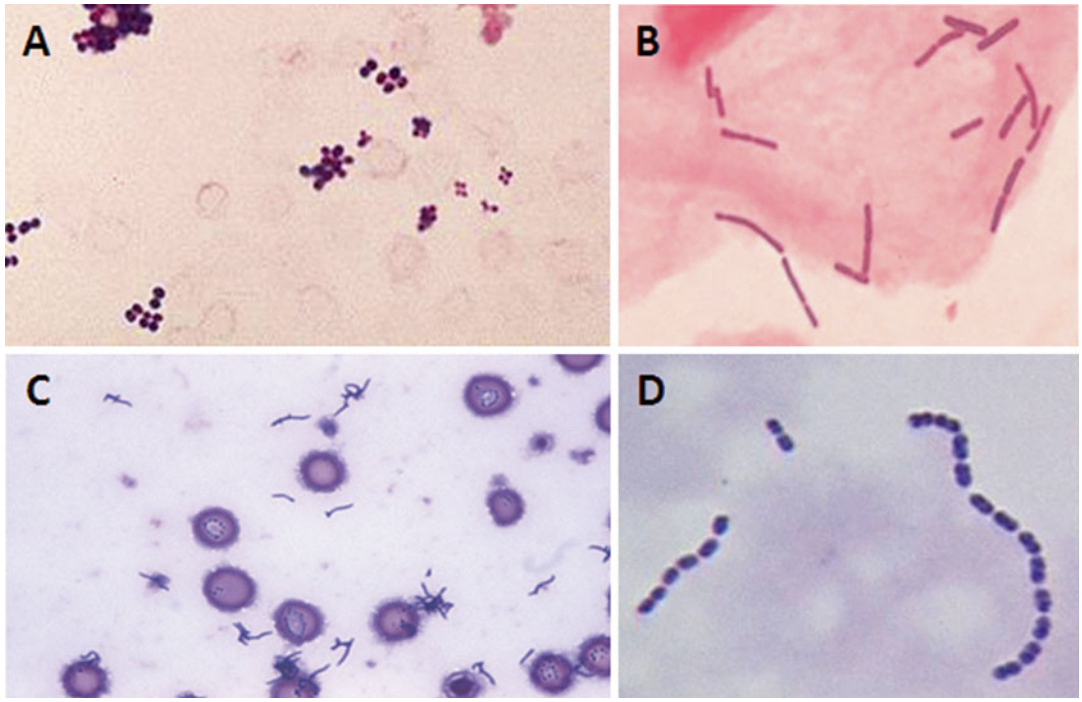

Fig. 5.3 Breast usual microflora. The human breast contains endogenous flora, derived from the nipple: (**a**) coagulase-negative staphylococci; (**b**) diphtheroids, lactobacilli; (**c**) anaerobes, mainly *Propionibacterium acnes*; and (**d**) *beta-haemolytic* streptococci

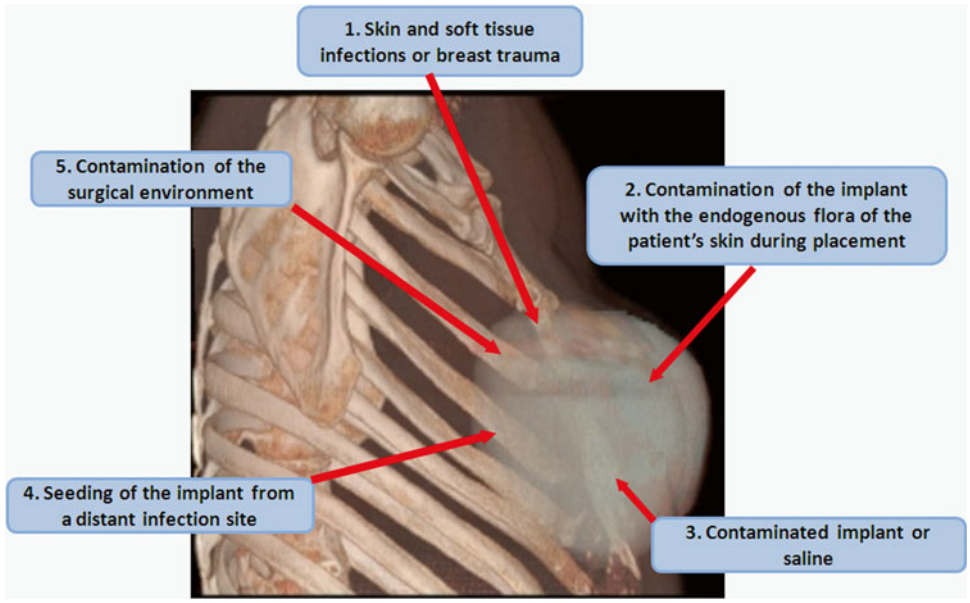

Fig. 5.4 Pathogenesis of breast implant infections

2.4.1 Role of Bacterial Biofilms in Breast Implant Infections

Implant-associated infection pathogenesis involves interactions between the microorganism, the implant and the host (Darouiche 2004). Microorganisms growing in biofilms cause breast implant infections, and these biofilms are the essential factor in the persistence of infection (Fig. 5.5). A small number of skin bacteria (i.e., staphylococci) can colonize an implant during surgery and persist despite of the presence of functionally intact granulocytes and/or antimicrobial agents. Finally, a complex community of sessile bacteria develops on the implant surface. This community exist within a polymer matrix containing fluid channels that allow for the flow of nutrients and waste, and are able to display cell-to-cell signaling (i.e., *quorum* sensing) (Donlan 2001). Bacterial biofilms exhibit dramatically reduced susceptibility to killing by antimicrobial agents as compared to free-floating (planktonic) cells of the same microorganism (Ceri et al. 1999). A variety of potential antimicrobial resistance mechanisms have been proposed (del Pozo and Patel 2007) including: existence of altered growth rate inside the biofilm (i.e., persister cells), quorum sensing signaling systems, antimicrobial destroying enzymes, stress response to hostile environmental conditions, overexpression of genes and restricted antimicrobial penetration through the biofilm matrix.

2.5 Clinical Presentation

Two-thirds of infections occur during the first month after implantation acute infections are usually associated with fever, pain, and erythema or sinus tract (Armstrong et al. 1989; Ahn et al. 1996) (Fig. 5.6). In addition to local signs of infection, patients may present with toxic shock syndrome if the infection is due to toxin-producing *S. aureus* or Streptococcus species (Tobin et al. 1987; Kohannim et al. 2011) (Fig. 5.7). Late infections may present months or years after implantation with symptoms such as breast pain with or without inflammatory skin changes or drainage, prolonged wound healing, or extrusion or implant movement. Late infection usually results from secondary bacteraemia or an invasive procedure at a distant location (Eliopoulos and Lyle 1999; del Pozo et al. 2008; Ablaza and LaTrenta 1998; Hunter et al. 1996; Petit et al. 1998; Niazi et al. 1996; Bernardi and Saccomanno 1998; Gnanadesigan et al. 1994; Gibney 1987).

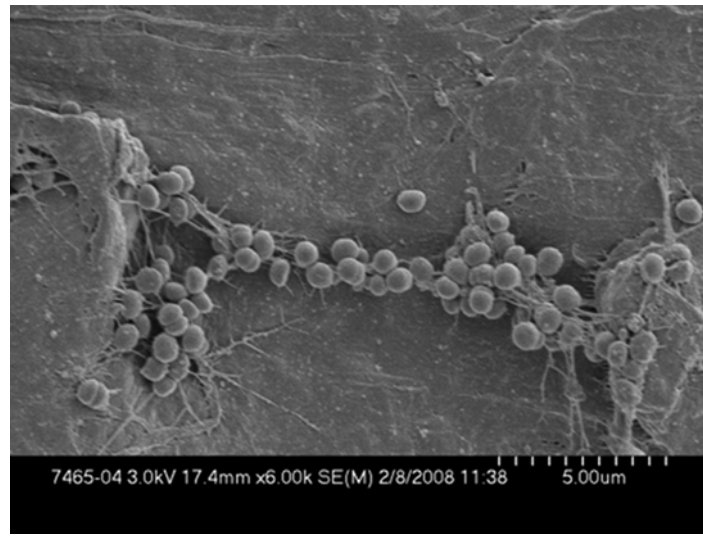

Fig. 5.5 Scanning electron microscopy of the surface of a colonized breast implant showing cocci

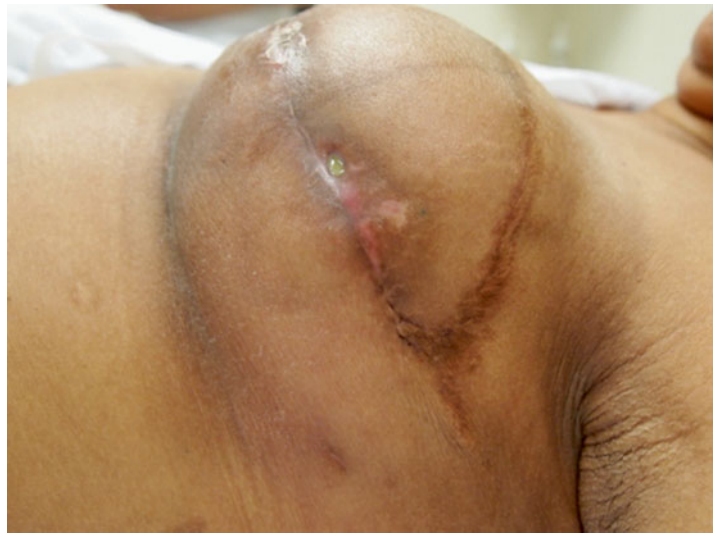

Fig. 5.6 Acute breast implant associated infection. Fifty-three year-old female patient with left breast ductal carcinoma treated with radical modified mastectomy, radiotherapy and reconstruction with latissimus dorsi plus implant. Seven months postoperatively, she presented an acute breast implant associated infection with draining pus through the scar. Two stage surgical approach was carried out: in the first surgery, the implant removal, cavity washing and partial capsulectomy were performed. Three months later, the patient underwent a new surgery for implant placement

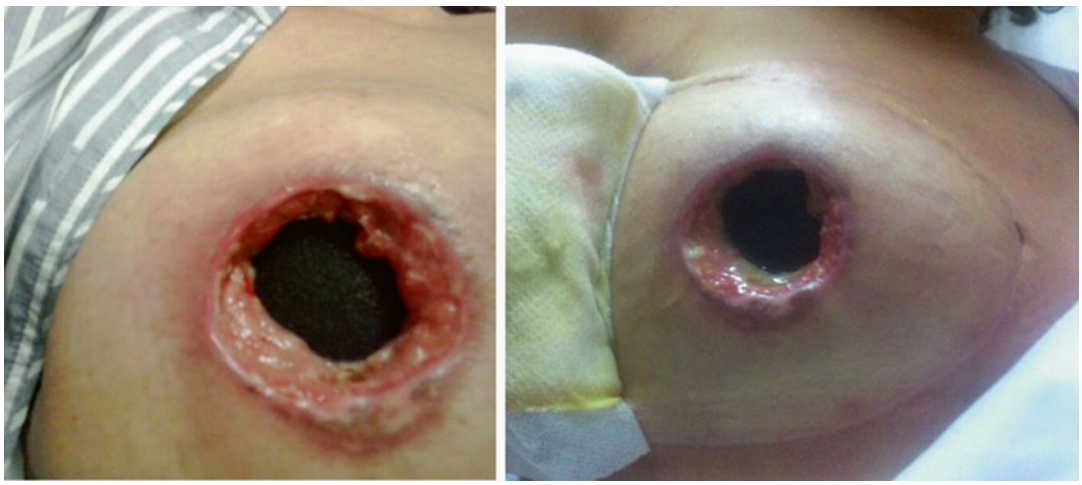

Fig. 5.7 Acute breast implant associated infection with implant exposure. Forty-five year-old female patient with a right breast reconstruction with DIEP flap. An implant was placed under the flap for breast augmentation. Six months later, nipple areola complex (NAC) was performed with a skin graft from inguinal area. Two weeks later the patient presented an acute infection limited to the NAC area but affecting the entire thickness of the flap with implant exposure. Antibiotics were administered endovenously and a new surgery was carried out in which the implant was removed and partial capsulectomy was performed. The patient is waiting for the placement of a new implant not before at least 3 months after the last surgery

2.6 Diagnosis

Differentiation among cellulitis and implant infection is essential for management. Ultrasonography can be used to confirm the presence of a fluid collection around the implant (Fig. 5.8). However, absence of a fluid collection does not rule out implant infection. Aspiration guided by ultrasonography may be done if fluid is present. Exudate should be sent for Gram stain, acid-fast

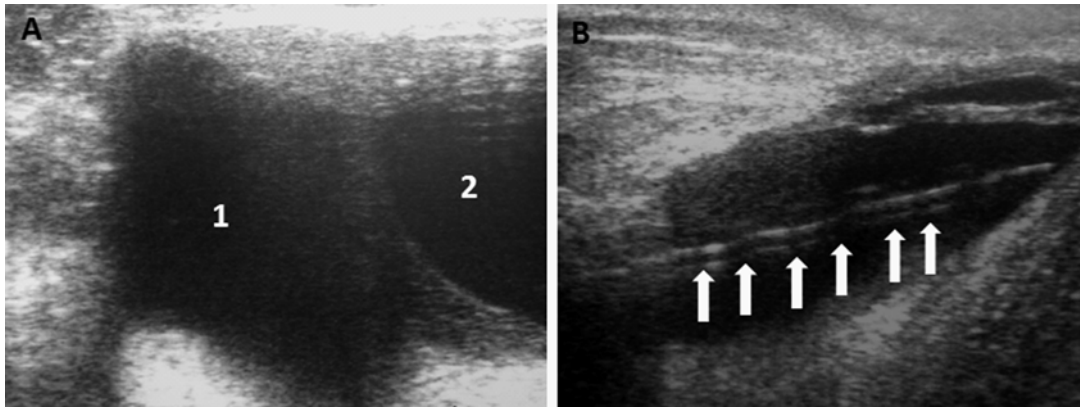

Fig. 5.8 (**a**) Breast echography showing a fluid collection (*a1*) around the implant (*a2*). (**b**) Catheter (*arrows*) placed to drain the fluid

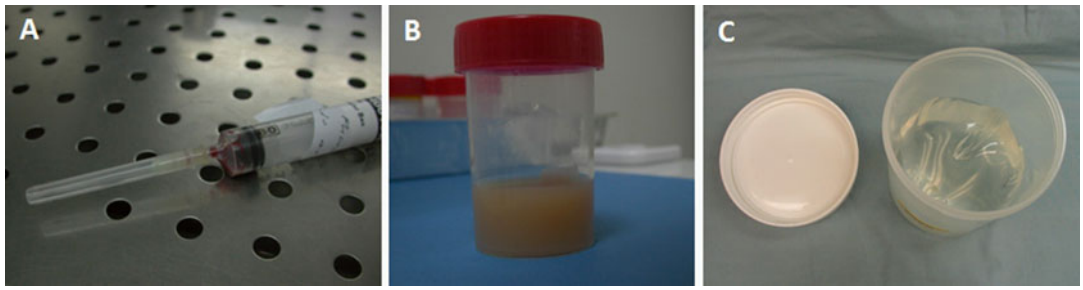

Fig. 5.9 Fluid around implant collected with syringe (**a**) or into sterile cup (**b**) for bacterial culture (**c**). Breast implant (even if ruptured) placed in autoclaved 1 L polypropylene straight-side wide-mouth jar using sterile technique

stain, and aerobic, anaerobic, and acid-fast cultures. Among patients with sepsis, blood cultures should be obtained. If a surgical device removal is done, the implant should be sent for culture (Fig. 5.9).

2.6.1 Implant Culture

A new technique which uses a combination of vortexing and sonication to sample biofilm bacteria on the surface of prosthetic surfaces was described in 2007 (Trampuz et al. 2007). It was shown that this technique was more sensitive than periprosthetic tissue culture for the diagnosis of prosthetic joint infection (Trampuz et al. 2007). As there is a lack of sensitivity in the available diagnostic tools of breast implant associated infection (Pittet et al. 2005), we hypothesize that this vortexing-sonication technique might be applied to explanted breast implants providing a more sensitive tool to detect bacterial biofilms.

2.7 Treatment

Treatment of an implant-associated infection usually involves implant removal, but salvage by systemic antibiotics may be sometimes possible. However, there are no randomized controlled trials that directly compare implant removal with salvage. In clinical situations with severe infectious condition in the absence of an identified source of infection, removal of the implants should be strongly considered. There are no established guidelines for empiric antimicrobial therapy for breast implant associated infections. After implant removal, systemic antimicrobials should be given for 14 days for standard bacterial infections and for several months for mycobacterial infections (Washer and Gutowski 2012). Immediate reimplantation is not advocated, and the delay to proceed will depend on the causative organisms and the duration of antimicrobial therapy.

Reimplantation may be considered 3–6 months after ending the antimicrobial treatment and an antimicrobial-free period, to assure there is a complete resolution of infection (Pittet et al. 2005). Spear et al. (2004) reported a 77 % implant salvage rate among 24 patients with capsulectomy, device exchange, and an extended course of antimicrobials. The new implant should be placed in a different plane (i.e., submuscular) if it is possible.

However, because implant removal is costly and often devastating for the patient, treatment with antimicrobials without implant removal is occasionally attempted and may be a valid approach for some patients (Spear et al. 2004). There remains controversy regarding indications for device explantation compared with attempted salvage. Several strategies for device salvage have been reported. Some authors have used capsule curettage, partial or total capsulectomy, and device exchange with or without postoperative antimicrobial irrigation (Prince et al. 2012; Spear et al. 2004; Peled et al. 2012; Chun and Schulman 2007). It has been reported successful implant salvage with a protocol of systemic antimicrobials for 6 weeks combined with exchange of the implant (Laveaux et al. 2009) or with implant salvage with use of an implant sizer and negative-pressure wound management (Kendrick and Chase 2008). Prince et al. (2012) reported 60 patients with breast implant related infection. Forty-three patients underwent attempted implant salvage with a reported successful rate of 76 %. Yii and Khoo (2003) reported on 14 patients with breast implant infections who underwent attempted implant salvage. Salvage was successful in 64 %. *S. aureus* infection was associated with a poorer salvage rate ($p=0.023$). Courtiss et al. (1979) reported 29 patients with an implant infection, with 45 % treated successfully with antimicrobials and wound drainage. We retrospectively analyzed all consecutive patients diagnosed with breast implant associated infection that were treated in a conservative way in our hospital (Mauleón et al. 2012). We analyzed if a conservative treatment based on long-term antimicrobial use could be a sure and effective alternative in these patients. Clinical cure with breast implant retention was achieved in 84 % of the patients with a non-exposed implant, while 100 % of the patients with implant exposure required removal. In our experience, breast implant exposure was highly predictive of failure if a conservative treatment was intended. So, if implant is not exposed a conservative treatment based in long-term administration of antimicrobials could be a valuable therapeutic option. Other risk factors for failed implant salvage have been reported in literature. Infection with atypical pathogens (i.e., mycobacteria, fungi), gram-negative rods, or methicillin-resistant *S. aureus* (Reish et al. 2013), and history of radiotherapy (Spear and Seruya 2010). A well-defined algorithm for the management of breast implant related infection would be a valuable option in the management of these patients. Salvage of the infected breast implant might be a challenging yet viable option for a subset of patients.

3 Breast Capsular Contracture

3.1 Introduction

Breast implant capsular contracture is a complication of unknown origin that may occur after breast implantation. This is one of the most common and frustrating complication with a reported incidence as high as 17–74 % according to most series (Gabriel et al. 1997; Burkhardt et al. 1986). Capsular contracture etiology is unclear and probably multifactorial. It has been suggested that biofilms might play a role in the pathogenesis of this complication.

3.2 Classification

Capsular contracture is clinically classified according to the Baker classification as follows: Grade I: Breast absolutely natural, soft. Grade II: Minimum contracture, palpable not visible. Grade III: Moderate contracture, palpable and visible. Grade IV: Severe contracture, hard, painful with distortion visible.

In the absence of large prospective studies with long-term follow-up, the exact incidence of capsular contracture after breast implantation is unknown. Capsular contracture (grades II–IV

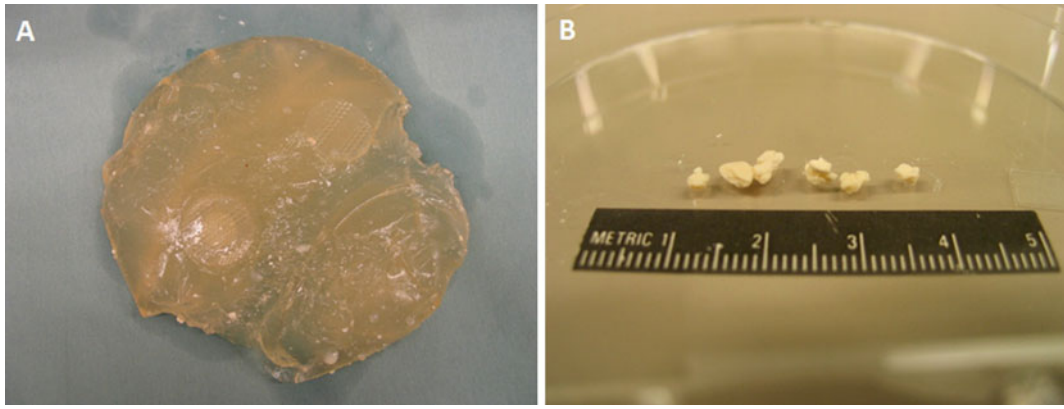

Fig. 5.10 Ruptured explanted implant due to capsular contracture (**a**) with presence of small calcifications (**b**)

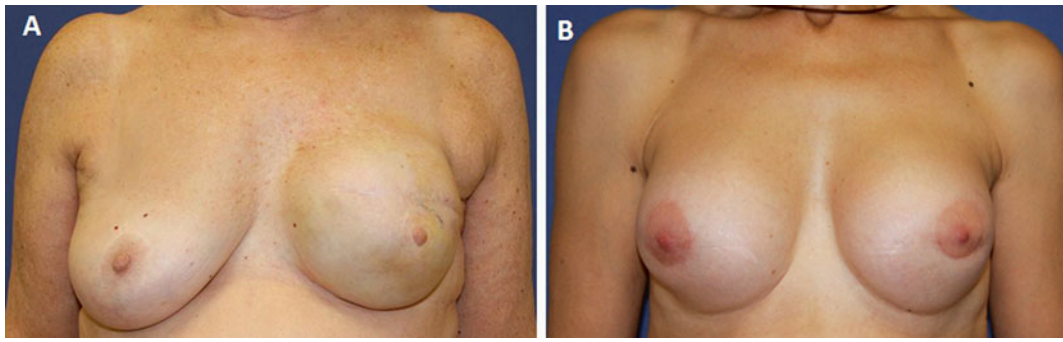

Fig. 5.11 (**a**) *Left* capsular contracture (Baker III) after breast reconstruction with expander plus implant. (**b**) *Right* capsular contracture (Baker III) after augmentation mammaplasty. Distortion at the inferolateral quadrant is visible

according to Baker) has been observed among 4 % with a 2-year follow-up period for an incidence rate of 1,7 per 1,000 implant-months and 2,8 per 1,000 patient-months (Henriksen et al. 2003; Little and Baker 1980).

3.3 Pathogenesis

Formation of a collagenous sheath usually follows the placement of prosthesis. The body forms a fibrous capsule around the implant that is too large to be phagocytized and too inert to produce a toxic reaction (Nemecek and Young 1993) (Fig. 5.10). This capsule serves to keep the implant in proper position, but in some women, the capsule tightens and hardens, resulting in what is termed capsular contracture (Fig. 5.11). This capsular contracture is associated with unwanted cosmetic appearance, firmness of the breast, breast distortion, and pain. A number of factors, including foreign body reaction and implant related infection, have been suggested to play a role in the genesis of this complication (Araco et al. 2006). A retrospective review of 307 patients (Reiffel et al. 1983) reported a statistically significant lower incidence of capsular contracture in patients with saline implants. It has also been shown that submuscular placement of an implant reduces the incidence of capsular contracture (Woods et al. 1980; Vazquez et al. 1987). In the same way, some studies have reported that textured silicone surfaces significantly delay or decrease the rate and incidence of capsular contracture when compared with smooth textures (Ersek 1991). Several studies, suggest a role of subclinical infection in the pathogenesis of capsular contracture (Del Pozo et al. 2009; Virden

et al. 1992; Pajkos et al. 2003). Local skin flora (e.g., *P. acnes*, coagulase-negative staphylococci, and *Corynebacterium* species) may get access to the implant surface during placement. It has been suggested that biofilms formed on the implant, stimulate fibrosis and, ultimately, causes capsular contracture (Dobke et al. 1995; Virden et al. 1992; Pajkos et al. 2003; Netscher 2004).

There is evidence that staphylococci accelerate development of capsular contracture in experimental animal models. Shah et al. (1981) evaluated infection with *Staphylococcus epidermidis* as a cause of capsular contracture in a silicone implant rabbit model; all implants inoculated with *S. epidermidis* developed capsular contracture. Likewise, using a guinea pig model, Kossovsky et al. (1984) demonstrated capsular contracture around silicone implants inoculated with *S. aureus*. Darouiche et al. (2002) reported a significant decrease in the rates of contracture when silicone implants impregnated with minocycline-rifampin were tested in a rabbit model.

Several studies have reported culturing explanted breast implants. Virden et al. (1992) recovered bacteria from 55 silicone devices, including breast implants and tissue expanders, from 40 patients. Bacteria, predominantly *S. epidermidis*, were cultured from 56 % of implants surrounded by contracted capsules and 18 % of implants without capsular contracture ($p<0.05$). Dobke et al. (1995) cultured 150 explanted silicone breast implants from 87 patients using prolonged incubation with continuous agitation. Bacteria were detected on 76 % of implants surrounded by contracted capsules and 28 % of those without capsular contracture ($p<0.05$). Netscher et al. (1995) cultured tissue surrounding 389 implants removed for reasons other than infection. A correlation between positive cultures and capsular contracture was demonstrated. In other study, capsule and implant samples obtained during explantation were broth cultured (after maceration and sonication for 20 min) (Pajkos et al. 2003). A total of 48 implant and/or capsular samples were studied from 27 patients. Analysis of capsules demonstrated that 17 of 19 samples obtained from patients with significant contracture (i.e., Baker grade III/IV) were positive, compared with only one of eight samples obtained from those with minimal or no contracture ($p<0.05$). the microorganism most frequently isolated were coagulase-negative staphylococci. In another study (Rieger et al. 2009), 41 % of 22 breast implants removed because capsular contracture and cultured were positive for coagulase-negative staphylococcus, *P. acnes*, or both. We prospectively studied 45 consecutive patients who underwent breast implant removal for reasons other than infection at the Mayo Clinic over an 8-month period (Del Pozo et al. 2009). Removed breast implants were processed using a vortexing/sonication procedure and then subjected to semiquantitative culture. Twenty-seven of the 45 implants collected were removed due to significant capsular contracture, among which 9 (33 %) had >20 CFU bacteria/10 ml sonicate fluid; 18 were removed for reasons other than significant capsular contracture, among which only 1 (5 %) had >20 CFU/10 ml sonicate fluid ($p<0.05$). *Propionibacterium* species, coagulase-negative staphylococci, and *Corynebacterium* species were the microorganisms isolated in all cases. The results of this study demonstrated that there was a significant association between capsular contracture and presence of bacteria on the implants. By contrast, in a study involving 49 removed implants from 31 women, electron microscopic examination did not show any microorganism (Rudolph and Woodward 1983). Of 139 implants removed in other study (Ahn et al. 1996), only 47 had a positive culture, so the association was not demonstrated.

In summary, several, but not all, studies have revealed an association between bacteria in breast implants and clinically significant capsular contracture. However, most of these studies did not use appropriate techniques to sample implant-associated biofilms; instead, conventional culture methods, methods inadequate to recover *P. acnes*, and/or prolonged implant sonication were used. In addition, most of these studies yielded quantitative bacteria counts associated with implants; accordingly, some of these findings might represent contamination. If bacteria contribute to and/or

cause capsular contracture, new strategies to prevent and treat this devastating complication will need to be studied.

3.4 Treatment

There are many studies performed in order to prevent the onset of capsular contracture but, although promising results, little is set for its application on clinical practice. Capsulectomy/capsulotomy continues being the gold standard treatment in severe cases although the future may undergo non-invasive techniques, at least in mild stages of disease. If the implant position is subglandular, a total capsulectomy should be performed (Collis and Sharpe 2000; Prantl et al. 2006, 2007). If the implant position is subpectoral, an anterior capsulectomy should be performed at a minimum. After creation of a new implant pocket and thorough irrigation is necessary. This is done in preparation for replacement with a textured implant, as there is evidence that textured implants may reduce the rate of recurrence of capsular contracture (Collis and Sharpe 2000). In an effort to evaluate the impact of bacterial colonization on the incidence of capsular contracture it could be an option to culture explanted implants and/or capsules in order to administer antimicrobial treatment if positive results. In a prospective, randomized controlled clinical trial (Burkhardt et al. 1986), the use of local antimicrobial agents in or around breast implants was analyzed in 124 patients undergoing augmentation mammaplasty. Antimicrobial treated patients had sevenfold reduced early postoperative onset of Baker III to IV capsular contracture. Another study (Adams et al. 2006) assessed the efficacy of breast irrigation with bacitracin, cephalexin and gentamicin in 35 patients undergoing breast implantation. This intervention was associated with a lower incidence of capsular contracture when compared with historical series not using antimicrobial irrigation. In summary, antimicrobial agents appear to prevent capsular contracture, indirectly suggesting that bacteria might be involved in the pathogenesis of this entity.

4 Conclusions

Breast implants are increasingly used for several purposes (i.e., cosmetic breast enlargement, correction of defects and reconstruction after mastectomy). Breast implant associated complications may have a significant morbidity, some mortality, and an increase in hospital length of stay and associated costs. These complications represent a significant challenge, being breast implant-related infections and capsular contracture the two most common ones. Biofilms play a key role in both complications. Implant associated infections often mandate immediate device removal, however, there been some reports of attempted implant salvage in a subset of patients. It has been postulated that capsular contracture might be related to a chronic infection with propionibacteria, coagulase-negative staphylococcus and other skin organisms. Cause of capsular contracture and, accordingly, treatment and prevention, remains to be elucidated.

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Innovative Strategies for Combating Biofilm-Based Infections

6

Roberta J. Melander and Christian Melander

1 Introduction

The identification of novel targets and compound-scaffolds to combat biofilms is a valuable tool in the fight against infectious diseases, many of which are now known to be biofilm-mediated. Continuing research into the environmental cues and genetic elements that play a part in the regulation of biofilms, such as quorum sensing, two-component signal transduction systems, cyclic-di-GMP signaling, and indole-signaling, along with an evolving knowledge of the composition of biofilms, has led to numerous innovative strategies for combating biofilms.

Strategies to inhibit biofilm formation, and/or eradicate established biofilms, have the potential to have a profound impact on human medicine by enhancing the efficacy of antibiotics that are otherwise ineffective against biofilm bacteria. Controlling biofilm development via non-microbicidal mechanisms should limit the pressure on bacteria to evolve resistance as compared to the selective pressures exerted by conventional bactericidal entities. Here, we aim to provide an overview of the various strategies that have been explored for the inhibition or eradication of bacterial biofilms, focusing on strategies that operate via non-microbicidal mechanisms. These approaches range from: effecting phenotype shifts through controlling cell-signaling pathways with small molecules, to enzymatic approaches to biofilm eradication by matrix degradation. Such strategies have the potential, when paired with conventional antibiotics that will kill planktonic bacteria, to eliminate established biofilm infections.

1.1 Biofilms

Biofilms, defined as highly organized surface-associated communities of bacteria encased within an extracellular matrix, play a significant role in infectious disease. Biofilms contribute to both pathogenesis and antibiotic/host immune resistance, and have a considerable impact on many fields, including medicine, food, environment, and industry. The impact of biofilms upon infectious disease is a particular cause for concern given the fact that The National Institutes of Health (NIH) estimates that 80 % of all bacterial infections occurring in the human body are biofilm related, and it is estimated that 17 million new biofilm infections arise each year in the U.S., resulting in up to 550,000 fatalities (Quave et al. 2012). Biofilms play a role in lung infections of cystic fibrosis (CF) patients, burn wound infections, ear infections, bacterial endocarditis, chronic wound infections, and tooth decay (Musk and Hergenrother 2006). In addition to increased mortality rates, biofilm mediated infections impart a

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considerable economic burden; with device related biofilm infections adding over one billion dollars to US hospitalization costs annually (Shirtliff and Leid 2009). The total economic impact of biofilms to the agricultural, engineering, and medical sectors of society has been estimated at billions of dollars annually (Davies 2003).

Bacteria within a biofilm present a number of characteristics that lead to difficulties in their eradication. They are phenotypically distinct from their planktonic counterparts, particularly in regard to growth rates and gene expression (Donlan and Costerton 2002). Biofilms are inherently insensitive to antibiotics, exhibiting upwards of 1000-fold higher resistance than planktonic bacteria (Rasmussen and Givskov 2006). Furthermore, the higher cell densities found within a biofilm (10^{11} CFU/mL compared to 10^{8} CFU/mL for bacteria in the planktonic state) vastly increase the opportunity for horizontal gene transfer, with transfer rates of up to 1,000-fold higher than for planktonic cells (Hausner and Wuertz 1999), leading to an increased likelihood of the emergence of strains with increased resistance or altered virulence profiles (Quave et al. 2012).

1.2 Effectiveness of Conventional Antibiotics in Eradicating Biofilms

There are many factors that lead to the increased resistance bacteria exhibit towards antibiotics while in a biofilm state. These include changes in gene expression related to antibiotic resistance, such as upregulation of genes responsible for efflux pumps (Davey and O'Toole 2000). The biofilm matrix, which is predominantly comprised of self-produced extracellular polymeric substances (EPS) including carbohydrates, proteins, lipids and extracellular DNA, affects penetration of certain antibiotic classes into the biofilm (Mah and O'Toole 2001; Flemming et al. 2007). The reduced growth rate exhibited by cells embedded deep within the biofilm that experience a lack of nutrients and oxygen also likely plays a role in resistance to antibiotics (Mah and O'Toole 2001), as bacterial cells with reduced metabolic activity are inherently more recalcitrant to antimicrobial therapies (Spoering and Lewis 2001). This reduced efficacy is due to the fact that almost all conventional antibiotics target one of five biosynthetic processes occurring in actively growing bacteria: the biosynthesis of proteins, RNA, DNA, peptidoglycan and folic acid (Hurdle et al. 2011). In the absence of genetic resistance mechanisms, conventional antibiotics efficiently kill growing and dividing bacterial cells, but are very inefficient at killing non-multiplying bacteria (Coates and Hu 2008), leading to minimal inhibitory concentrations (MICs) of conventional antibiotics against biofilm-residing bacteria being 100-1,000-fold higher than against planktonic bacteria (Hoiby et al. 2010a).

Despite this recalcitrance, certain conventional antibiotics have demonstrated activity against bacterial cells growing in the biofilm state. For example, rifampin has activity against staphylococcal biofilm cells, particularly when used in combination with one or more additional antibiotic including: fusidic acid, vancomycin and ciprofloxacin (Saginur et al. 2006). Colistin has demonstrated activity against non-dividing *Pseudomonas aeruginosa* biofilm cells that reside at the center of a biofilm; however metabolically active cells can acquire resistance to polymyxins and other cationic peptides rapidly due to modification of their outer membrane. Therefore, colistin appears to only be a viable option for treating *P. aeruginosa* biofilm infections when used in combination with another antibiotic such as ciprofloxacin (Hoiby et al. 2010a). Even more problematic is the fact that several studies have shown that the presence of sub-MIC levels of some antibiotics, as experienced at the beginning or end of a dosing regimen, or by cells deep within the biofilm throughout the regimen, can induce in vitro biofilm formation in a range of bacteria. Almost all the most commonly used antibiotic classes including: aminoglycosides, ß-lactams, fluoroquinolones, glycopeptides, rifamycins, tetracyclines exhibit this phenomenon. This has prompted a need to develop alternative strategies to combat biofilms (Kaplan 2011).

2 Small Molecule Strategies for Combating Biofilms

Several approaches to the design of small molecules that either inhibit the formation of biofilms, or cause established biofilms to disperse, have been investigated. Many of these approaches involve designing small molecules to interfere with the bacterial communication pathways that control the formation and maintenance of biofilms. Additionally, many natural products possess anti-biofilm activity and have been used as structural inspiration in medicinal chemistry programs to identify anti-biofilm compounds. These strategies are detailed below.

2.1 Disruption of Quorum Sensing Pathways

Quorum sensing (QS) involves intercellular communication between bacteria via the production of diffusible small molecules, and allows the community to make coordinated alterations in gene expression based upon population density (Camilli and Bassler 2006). There exist a number of different quorum sensing molecules that have been shown to play a role in the formation of biofilms in numerous species of bacteria. These include: acyl homoserine lactones (AHLs) in Gram-negative bacteria, autoinducer peptides (AIPs) employed by Gram positive bacteria, and autoinducer-2 (AI-2) molecules derived from 4,5-dihydroxy-2,3-pentanedione (DPD), which are interspecies signals employed by both Gram-negative and Gram-positive bacteria (Irie and Parsek 2008). The approach of interfering with QS pathways to modulate biofilm formation has been one of the most popular approaches to combating biofilms and a number of small molecules that modulate QS pathways have been developed.

Acyl Homoserine Lactones (AHLs)

AHLs are the predominant QS signal employed by Gram-negative bacteria, and more than 70 species of bacteria are known to communicate via AHL-mediated QS, with specificity mediated via variation in the length and oxidation state of the acyl side chain (for example, compounds **1**–**3**) (Fig. 6.1) (Amara et al. 2009). In the well-studied QS system of *Vibrio fisheri*, AHL synthesis occurs when the *luxI* gene is activated, producing the AHL synthase enzyme LuxI. When the AHL reaches a threshold intracellular concentration, it binds to the transcriptional activator LuxR, leading to activation of the *luxR* operon. AHLs freely diffuse in and out of bacterial cells allowing the AHL concentration to correlate to the bacterial concentration, which enables population density-based control of gene expression and therefore control of various processes such as biofilm formation and maintenance (Finch et al. 1998). Homologous systems to the LuxI and LuxR proteins exist in several Gram-negative bacteria, including LasI and LasR in the medically important biofilm-forming bacterium *P. aeruginosa* (Parsek and Greenberg 2000). The role of AHL based QS in biofilm development was first demonstrated by the discovery that a *P. aeruginosa*

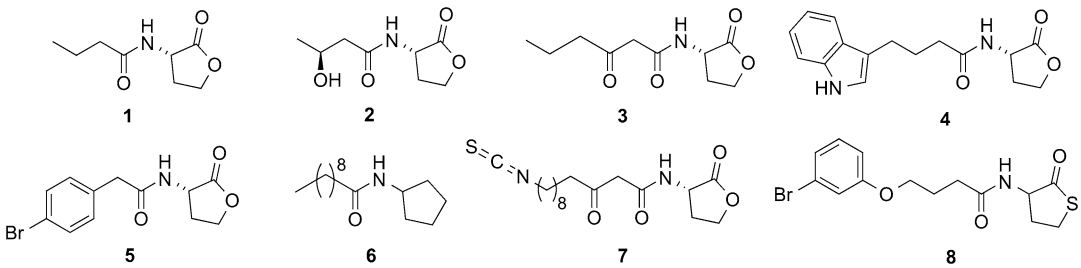


Fig. 6.1 Native AHLs from *P. aeruginosa* **1**, *V. harveyi* **2**, and *V. fisheri* **3**, and synthetic AHL analogues **5**–**8**

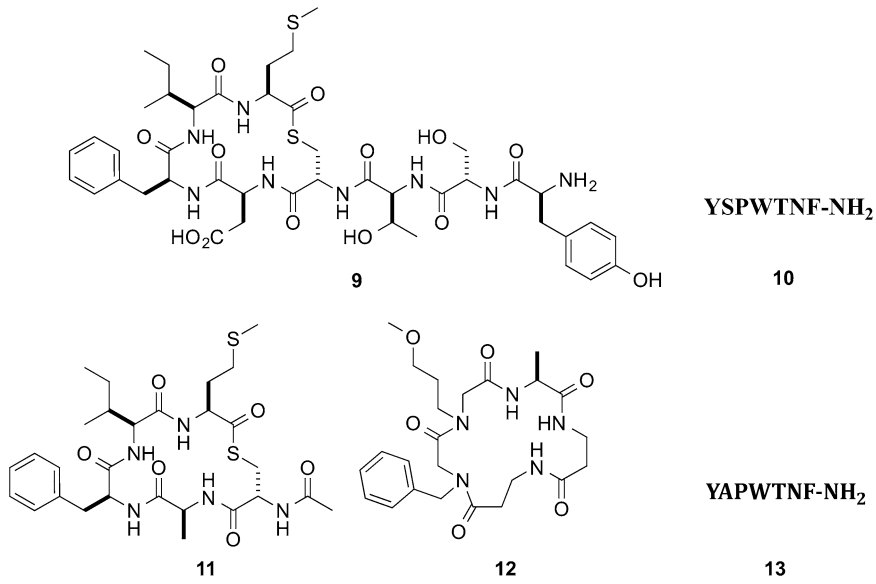


Fig. 6.2 *S. aureus* AIP-1 **9**, RIP **10**, and synthetic AIP analogues **11–13**

lasI mutant forms biofilms with altered morphology and reduced thickness compared to the wild type strain, and that the mutant biofilms are sensitive to biocides. Exogenous addition of the synthetic QS ligand to the mutant strain resulted in biofilms that resembled the wild-type and restoration of biocide resistance (Davies et al. 1998).

AHLs are prone to hydrolysis at physiological pH and the ring-opened product is QS inactive, and they have also been reported to possess immunomodulatory activity (Yates et al. 2002). This has led to the design of several synthetic AHL analogues with the aim of improving stability and in vivo properties. Recently, the Blackwell group have documented the synthesis and identification of a number of natural and unnatural AHLs with the ability to modulate QS in *P. aeruginosa* and *Agrobacterium tumefaciens,* and demonstrated that two of the most active AHL analogues inhibit biofilm formation in *P. aeruginosa* PAO1. Significant inhibition of biofilm formation was observed for a green fluorescent protein (GFP) producing PAO1 strain in the presence of synthetic AHLs **4** and **5** (Fig. 6.1) at 50 μM (Geske et al. 2005). The *N*-acyl cyclopentylamide analogue, C10-CPA **6**, completely prevents biofilm formation by GFP labeled *P. aeruginosa* PAO1 at 250 μM under flow conditions (Ishida et al. 2007).

A series of inhibitors of the *P. aeruginosa* transcriptional regulator LasR bearing electrophilic functional groups, including isothiocyanates, bromoacetamides, and chloroacetamides, were designed to react with Cys79 in the LasR binding pocket. Isothiocyanate containing ligands were able to covalently and selectively bind Cys79 and inhibit quorum sensing, with compound, **7** (Fig. 6.1), inhibiting PAO1 biofilm formation by almost 50 % at a concentration of 50 μM (Amara et al. 2009). More recently, the thiolactone **8** was reported by the Bassler group and reduces *P. aeruginosa* biofilm height by almost threefold at 100 μM, and brings about a reduction in nematode death in a *Caenorhabditis elegans P. aeruginosa* infection assay at 50 μM (O'Loughlin et al. 2013).

Autoinducing Peptides (AIPs)

QS in Gram-positive bacteria is predominantly mediated by autoinducing peptides (AIPs) (Lyon and Novick 2004). The accessory gene regulator (*agr*) operon, which plays an important role in

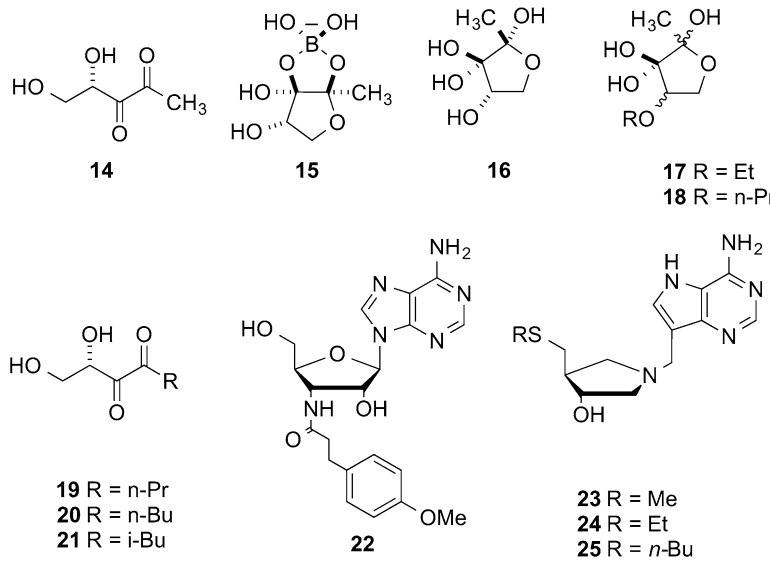


Fig. 6.3 DPD **14**, native AI-2 s from *V. harveyi* **15**, and *S. typhimurium* **16**, and synthetic compounds **17**–**25** that interfere with AI-2 QS

biofilm formation in *Staphylococcus aureus*, contains the *agrD* gene, which encodes AgrD, the precursor of the *S. aureus* AIP **9** (Fig. 6.2). The *S. aureus* AIP binds AgrC, a histidine kinase, leading to the expression of a small non-coding RNA known as RNA-III, which subsequently down-regulates genes that encode adhesins required for biofilm formation (Bordi and de Bentzmann 2011). The RNA-III activating protein (RAP) activates TRAP (target of RNA-III activating peptide) via phosphorylation, leading to increased cell adhesion and biofilm formation (Fux et al. 2003). The RNA-III inhibiting peptide (RIP) **10** inhibits phosphorylation of TRAP leading to reduced biofilm formation, and RIP has been investigated as an anti-biofilm agent, successfully preventing infections in multiple animal models (Giacometti et al. 2003). AIP analogues have also been investigated as anti-biofilm agents; the truncated peptide **11** is a potent AgrC QS antagonist (Chan et al. 2004), while the peptidomimetic **12** promotes biofilm formation in *S. aureus* (Fowler et al. 2008), and the synthetic RIP derivative FS3 **13** (Fig. 6.2) enhances the efficacy of tigecycline in a rat model of staphylococcal vascular graft infection (Simonetti et al. 2013).

Autoinducer-2 (AI-2)

Autoinducer-2 (AI-2) is the collective term for a number of autoinducing signals derived from DPD **14** (Fig. 6.3). The synthase that drives DPD production is conserved in over 55 bacterial species (Waters and Bassler 2005) and AI-2 has been proposed as a putative universal quorum sensing mechanism shared by both Gram-negative and Gram-positive bacteria. The structures of AI-2 from *Vibrio harveyi* (S-THMF borate **15**) and *Salmonella typhimurium* (R-THMF **16**) are depicted in Fig. 6.3 (Miller et al. 2004).

Several AI-2 analogues have been developed, including the C4-alkoxy-5-hydroxy-2,3-pentanediones **17** and **18** (Fig. 6.3), which activate the AI-2 pathway more potently than DPD, exhibiting submicromolar EC_{50} values in a *V. harveyi* reporter strain (Tsuchikama et al. 2012). Several C1-substituted DPD analogues act as antagonists of AI-2-based QS, with propyl-DPD **19** and butyl-DPD **20** exhibiting IC_{50} values ten-

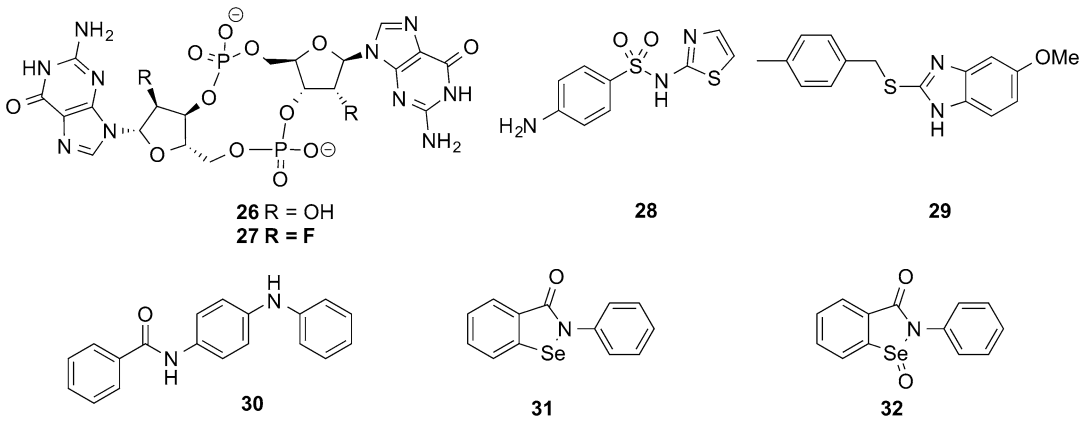


Fig. 6.4 c-di-GMP **26**, 2′-F-c-di-GMP **27**, and small molecules **28**–**32** that interfere with c-di-GMP signaling

fold below that of DPD (Lowery et al. 2008). Another C1-substituted analogue, isobutyl-DPD **21** significantly inhibits maturation of *Escherichia coli* biofilms and also brings about near complete clearance of pre-formed *E. coli* biofilms when administered in combination with gentamicin (Roy et al. 2013).

Another approach to interfering with AI-2 signaling is to design compounds that inhibit synthesis of the signal itself. The biosynthesis of DPD begins from *S*-adenosyl methionine (SAM), therefore a number of nucleoside analogues have been evaluated as potential inhibitors, with the finding that the adenosine analogue **22** (Fig. 6.3) blocks AI-2-based QS without affecting bacterial growth. This compound was subsequently shown to affect biofilm formation in several *Vibrio* species (Brackman et al. 2009). Another enzyme involved in the biosynthesis of both AI-2 and AHLs is 5′-methylthioadenosine nucleosidase (MTAN), which catalyzes the *N*-glycosyl hydrolysis of SAM and other adenosyl derivatives (Ronning et al. 2010; Gutierrez et al. 2009). Transition state analogues **23**, **24**, and **25** (Fig. 6.3) inhibit MTAN activity in cell lysates of a virulent *Vibrio cholerae* strain with nanomolar IC_{50} values, and inhibit QS induction in *V. harveyi* reporter strains. Analogues **23** and **25** also inhibit MTAN in *E. coli*, resulting in inhibition of AI-2 production, while compound **25** reduces biofilm formation in *E. coli* and *V. cholerae* by 18 and 71 % respectively at a concentration of 1 μM without inhibiting planktonic growth (Gutierrez et al. 2009).

2.2 Small Molecule Disruption of Other Signaling Pathways

c-di-GMP

Bis-(3′5′)-cyclic di-guanylic acid (c-di-GMP) **26** (Fig. 6.4) is a ubiquitous second messenger bacterial signaling molecule. Diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), which are responsible for the synthesis and breakdown of c-di-GMP, respectively (Yan and Chen 2010) are regulated by various environmental and intracellular signals such as oxygen, light, and small molecules, and play a role in the transition between the planktonic and biofilm lifestyle in *P. aeruginosa* (Tamayo et al. 2007). It has been observed that exopolysaccharide (a component of the biofilm matrix) synthesis is regulated by c-di-GMP in various proteobacterial species including *V. cholera, P. aeruginosa, Pseudomonas fluorescens, A. tumefaciens, E. coli,* and *Salmonella enterica* (Ryjenkov et al. 2005), while dispersion of bacteria from a mature biofilm is also thought to be regulated by c-di-GMP (Tamayo et al. 2007).

Interfering with c-di-GMP signaling is therefore another attractive target for the control of biofilm

formation. Modification of the 2′ position of c-di-GMP generates analogues with selectivities for different classes of c-di-GMP binding proteins, and the 2′-fluoro analogue **27** exhibits higher affinity for DGCs than does the native ligand (Zhou et al. 2013). Screening of a 1,120 compound library with known biological activities identified sulfathiazole **28** (Fig. 6.4), which inhibits DGC activity and inhibits *E. coli* biofilm formation with an IC_{50} value of 5.8 μM without significantly inhibiting bacterial growth (Antoniani et al. 2010). The benzimidazole **29** was identified from a high-throughput screen (HTS) of approximately 66,000 compounds and natural-product extracts from the Center for Chemical Genomics at the University of Michigan for compounds that affect induction of a *V. cholerae* c-di-GMP-inducible transcriptional fusion. Compound **29** is a broad-spectrum inhibitor of biofilm formation, significantly inhibiting biofilm formation by *P. aeruginosa* (CF-145), *Klebsiella pneumoniae, Erwinia amylovora,* and *Shigella boydii*, at 100 μM and by MRSA USA300, and *S. aureus* Newman at 25 μM, without affecting bacterial growth. IC_{50} values for inhibition of biofilm formation by *P. aeruginosa* and *V. cholerae* were reported to be 45.9 and 32.3 nM, respectively, however compound **29** did not disperse preformed biofilms (Sambanthamoorthy et al. 2011).

Another small molecule, compound **30**, was identified from a high-throughput screen for inhibitors of DGCs and shown to significantly reduce c-di-GMP levels in *V. cholerae* and inhibit biofilm formation by *V. cholerae* and *P. aeruginosa* (Sambanthamoorthy et al. 2012). In other studies, ebselen **31** and ebselen oxide **32** were shown to reduce DGC activity by covalently modifying cysteine residues, leading to inhibition of c-di-GMP-receptor binding and subsequent inhibition of biofilm formation in *P. aeruginosa* (Lieberman et al. 2014).

Indole Signaling

Indole is another putative universal intercellular signal molecule (Lee and Lee 2010) that plays a role in the control of many behaviors including biofilm formation (Lee et al. 2007b). Eighty-five species of bacteria have been documented to produce indole, with both indole-positive and indole-negative strains of bacteria altering various behaviors upon the extracellular presence of indole (Lee and Lee 2010). *E. coli* produces high concentrations (>600 μM) of extracellular indole when cultured in rich medium and indole has been shown to decrease biofilm formation in *E. coli* in a non-toxic manner (Lee et al. 2007a). Indole is readily converted by oxygenases found in several bacterial species to a number of oxidized indole derivatives, such as hydroxyindoles **33**–**35**, isatin **36**, and isoindigo **37** (Fig. 6.5), which have been investigated for their effects on biofilm formation. 5-Hydroxyindole **33** and 7-hydroxyindole **34** inhibit biofilm formation by enterohemorrhagic *E. coli* (EHEC) by 11-fold and 27-fold respectively at a concentration of 1 mM, compared to 18-fold inhibition by indole at the same concentration (Lee et al. 2007a). Indole-3-acetaldehyde, **38**, which is produced by the plant pathogen Rhodococcus sp. BFI 332, inhibits biofilm formation by EHEC without affecting planktonic growth, while the spent medium of *Rhodococcus* sp. BFI 332, from which **38** was identified, was shown to have an inhibitory effect on biofilm formation by two Staphylococcal species, *S. aureus and Staphylococcus epidermidis* (Wood et al. 2008).

The plant secondary metabolites 3-indolylacetonitrile (IAN) **39** and indole-3-carboxyaldehyde (I3CA) **40** (Fig. 6.5) reduce biofilm formation by *E. coli* O157:H7 by 11-fold and 24-fold respectively at 100 μg/mL, compared to a threefold reduction brought about by indole. In contrast to indole and the hydroxyindoles, which promote biofilm formation by *P. aeruginosa* PAO1, **39** and **40** weakly inhibit biofilm formation by this bacterium, effecting a 1.9-fold and 2.3-fold reduction in respectively (Lee et al. 2011a). A follow up study to identify indole derivatives with increased anti-virulence activity compared to IAN **39**, identified the simple synthetic indole derivatives, 7-fluoroindole (7FI) **41**, and 7-formylindole **42**, which reduce *P. aeruginosa* biofilm formation by fourfold and fivefold respectively at concentrations of 1 mM (Lee et al. 2012).

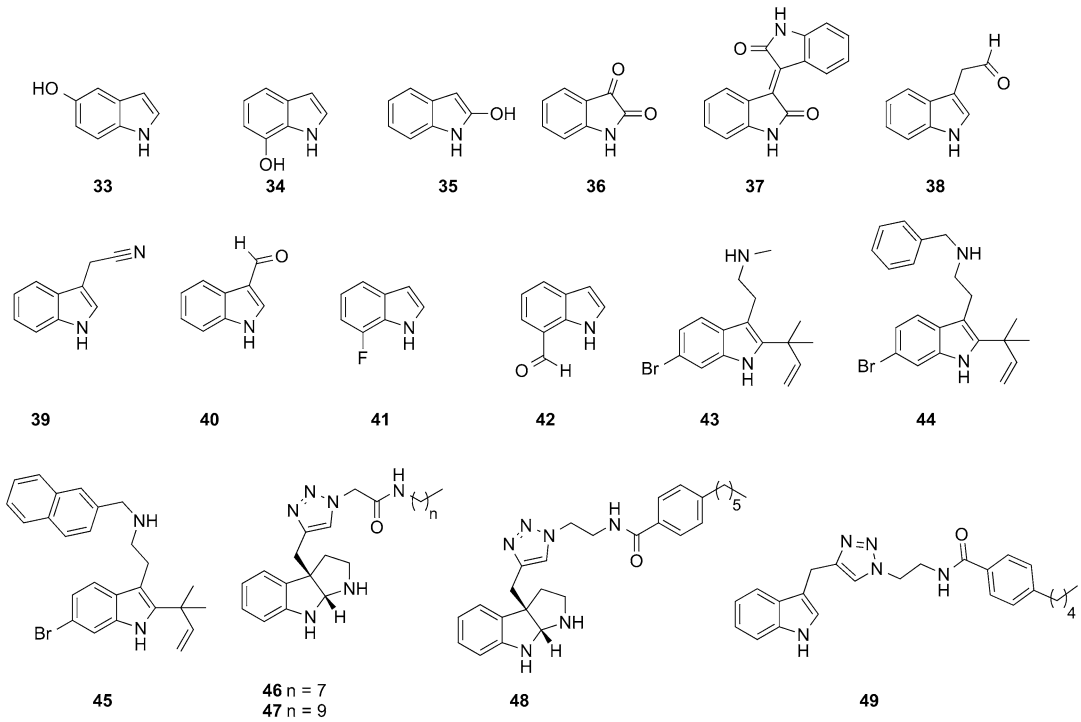


Fig. 6.5 Hydroxyindoles **33**–**35**, naturally occurring indole derivatives, **36**–**39**, and synthetic indole derivatives including simple derivatives **41** and **42** and flustramine derivatives **43**–**49**

The pyrroloindoline and indole containing monobrominated secondary metabolites from the bryozoan *Flustra foliacea* (Peters et al. 2003) have been used as scaffolds for the design of compounds to interfere with indole signaling. One metabolite, desformylflustrabromine (dFBr) **43** (Fig. 6.5) inhibits biofilm formation by *E. coli* and *S. aureus* with IC_{50} values of 70 μM and 174 μM respectively, however this compound exhibits microbicidal effects on planktonic growth at these concentrations (Bunders et al. 2011b). Synthetic manipulation of various regions of the dFBr scaffold led to the identification of **44,** which inhibits biofilm formation by *S. aureus* and *E. coli* with IC_{50} values of 5.9 μM and 53 μM respectively, and compound **45**, which inhibits biofilm formation by *S. aureus* and *E. coli* with IC_{50} values of 7.7 μM and 15.6 μM respectively. Both compounds elicit their effects through a non-microbicidal mechanism. Mechanistic studies with both compounds in wild-type and knockout *E. coli* strains revealed that the activity of these indole derivatives is dependent on the same factors as the activity of indole itself, namely temperature, the transcriptional regulator SdiA, and tryptophanase, suggesting that the observed anti-biofilm activity may be occurring through modulation of indole-based signaling pathways (Bunders et al. 2011b; Minvielle et al. 2013b). Other flustramine derived small molecules with non-microbicidal anti-biofilm activity include compound **46**, which inhibits biofilm formation by *Acinetobacter baumannii* with an IC_{50} value of 193 μM, compound **47**, which inhibits biofilm formation by *E. coli* with an IC_{50} value of 36 μM, and compounds **48** and **49**, which exhibit Gram-positive anti-biofilm activity, inhibiting biofilm formation by *S. aureus* (Bunders et al. 2011a; Minvielle et al. 2013a).

Two-Component Systems

Two component signal transduction systems (TCS) are a class of regulatory systems found mainly in prokaryotes that allow the organism to sense and

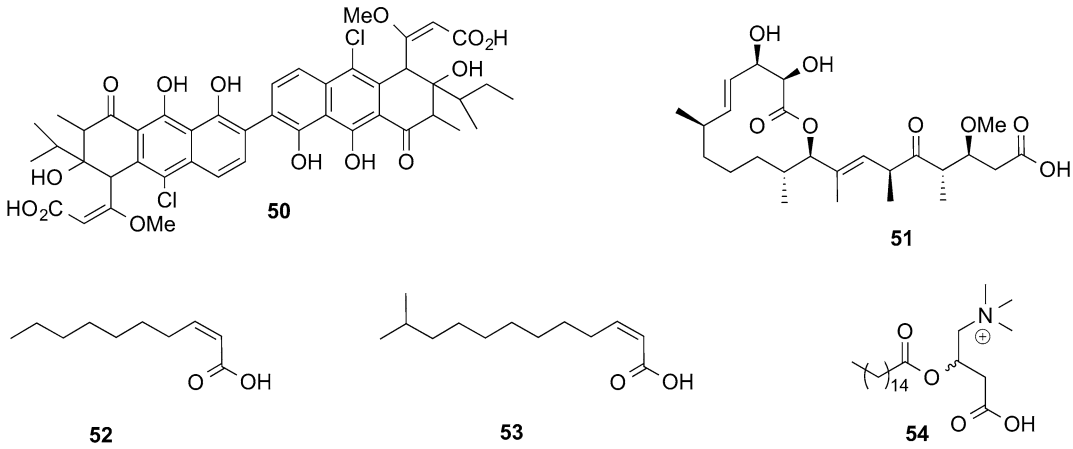


Fig. 6.6 Small molecules that interfere with two-component system signaling (**50** and **51**), and fatty acid derived small molecules that modulate biofilm formation (**52**–**54**)

respond to changes in their environment. The prototypical TCS consist of a histidine kinase and a response regulator. In response to an extracellular signal, the histidine kinase undergoes autophosphorylation at a conserved histidine residue. The phosphorylated histidine kinase subsequently transfers the phosphate group to a conserved aspartate residue on the response regulator that results in activation of an effector domain and ultimately leads to the response, which typically involves altered gene expression (Stock et al. 2000). TCS regulate the expression of genes that control numerous behaviors including biofilm formation and maintenance (Gotoh et al. 2010). Examples of TCS involved in biofilm regulation include, AgrAC (*S. aureus*) (Gotoh et al. 2010) LytSR (*S. aureus*) (Sharma-Kuinkel et al. 2009), BfmRS (*A. baumannii*) (Tomaras et al. 2008) BfmRS (*P. aeruginosa*) (Petrova et al. 2011), GacAS (*P. aeruginosa*) (Parkins et al. 2001) and VicRK (*S. mutans*) (Reck et al. 2011).

The role of TCS in the control of biofilm formation and maintenance, and the fact that it has been demonstrated that both the histidine kinase and response regulator are targetable by small molecules makes them attractive targets for the development of anti-biofilm compounds (Worthington et al. 2013). Walkmycin C **50** (Fig. 6.6), a member of the walkmycin family of natural products produced by *Streptomyces* sp. strain MK632-100 F11, inhibits autophosphorylation of the histidine kinase WalK (YycG) from both *B. subtilis and S. aureus,* and was subsequently shown to inhibit the in vitro autophosphorylation activity of purified VicK (an orthologue of WalK that is involved in sucrose dependant biofilm formation) from *Streptococcus mutans* with an IC_{50} of 2.87 μM. At sub-MIC levels, walkmycin C causes the formation of abnormal biofilms and a reduction in biofilm mass (37.6 % of the control at 0.63 μg/mL) in *S. aureus* (Eguchi et al. 2011). Walkmycin C also inhibits the histidine kinase CiaH in *S. mutans*, which plays a role in sucrose-dependent biofilm formation, with an IC_{50} of 4.87 μM (Qi et al. 2004). The bacterial secondary metabolite carolacton **51**, affects the viability of *S. mutans* biofilms at nanomolar concentrations, killing bacterial cells that are within a biofilm state while exhibiting only minor effects on the growth of planktonic bacteria. Carolacton was subsequently shown to affect the expression of several TCS in *S. mutans* biofilm cells, two of which (VicKRX and ComDE) were shown to play essential roles in the response to carolacton. It was shown that this response is mediated through the serine/threonine protein kinase PknB, which the authors posited could be due to phosphorylation of VicR by PknB Although the effect of carolacton upon *S. mutans* biofilms is microbicidal, the selectivity toward

biofilm bacteria over planktonic bacteria represents an alternative strategy for the small molecule control of biofilms and validates targeting of TCS as a means of controlling bacterial biofilms (Reck et al. 2011; Kunze et al. 2010).

Other Signaling Molecules

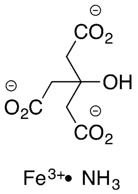
The fatty acid signaling molecule, cis-2-decenoic acid **52** (Fig. 6.6), which is produced by *P. aeruginosa,* has been shown to induce the dispersion of established biofilms of several bacterial species when assayed using microcolony disaggregation and measurement of the number of cells released into the bulk culture medium as the readout of biofilm dispersion (Davies and Marques 2009). The ability of cis-2-decenoic acid **52** to disperse pre-formed biofilms of *A. baumannii* and *S. aureus* has also been assayed with a crystal violet reporter assay, which measures the biofilm mass remaining as a function of compound concentration. However, under these conditions less than 10 % dispersion was observed at a concentration of 400 μM (Su et al. 2011). Another fatty acid, cis-11-methyl-2-dodecenoic acid **53**, known as diffusible signal factor (DSF), produced by *Xanthomonas campestris*, is able to disaggregate cell flocs formed by *X. campestris* (Dow et al. 2003). The fatty acid-derived molecule palmitoyl-DL-carnitine **54** (Fig. 6.6) inhibits biofilm formation by *P. aeruginosa* PAO1, *E. coli*, and *Listeria monocytogenes* with IC_{50} values of 13.2, 3.00 and 5.85 μM respectively, but does not disperse preformed biofilms (Wenderska et al. 2011; Nguyen et al. 2012).

Certain D-amino acids produced by bacteria in the stationary phase are thought to be a native signal for biofilm disassembly in *B. subtilis,* and exhibit inhibitory effects upon biofilm formation by several bacteria including *B. subtilis, S. aureus* and *P. aeruginosa*. This activity is thought to be a result of disruption of the connection between an extracellular matrix protein and the bacterial cell, which is posited to prevent growth of initial foci into larger assemblies of cells. D-Tyrosine, D-leucine, D-tryptophan, and D-methionine synergistically inhibit biofilm formation by *B. subtilis*, exhibiting activity at 10 nM in combination, while only displaying activity at micromolar to millimolar concentrations when administered alone. The analogous L-amino acids, and other D-amino acids including D-alanine and D-phenylalanine were inactive. Against *S. aureus*, the combination of D-phenylalanine, D-proline, and D-tyrosine exhibit greater biofilm inhibitory activity than the combination of D-tyrosine, D-leucine, D-tryptophan, and D-methionine (Kolodkin-Gal et al. 2010; Hochbaum et al. 2011).

2.3 Metal Ions and Chelation

Some metal ions play an important role in biofilm formation, stimulating cell–cell adhesion and aggregation (Abraham et al. 2012), and the effect of metal ion chelation upon biofilm formation has been investigated as a strategy to combat biofilms. Ethylenediaminetetraacetic acid (EDTA) was shown to disperse *P. aeruginosa* biofilms and result in 1,000-fold enhanced killing by gentamicin (Banin et al. 2006), while a combination of EDTA and minocycline effectively reduced in vitro and ex vivo staphylococcal catheter colonization (Raad et al. 2003).

A screen of 4,500 compounds belonging to the University of Illinois Marvel Library Compound Collection (MLCC) led to the identification of ferric ammonium citrate (FAC) **55** (Fig. 6.7) as a non-toxic inhibitor of *P. aeruginosa* PA14 biofilms, exhibiting an IC_{50} value of approximately 60 μM. No toxicity was observed even upwards of 500 μM and it was shown that neither the ammonium nor citrate ions were responsible for the observed anti-biofilm activity. The subsequent analysis of other iron salts (ferric chloride, ferric sulfate, ferrous sulfate) revealed biofilm inhibitory profiles that were comparable to that of FAC and it was also demonstrated that upon switching to an iron-rich growth media established *P. aeruginosa* biofilms could be disrupted and cleared in continuous flow experiments (Musk et al. 2005). High iron levels have been shown to suppress extracellular DNA (eDNA) release and structural development of biofilms in *P. aeruginosa*, and it is thought that



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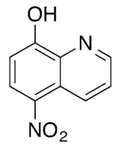

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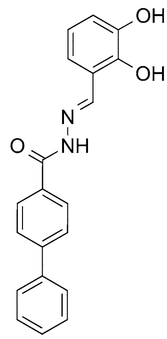

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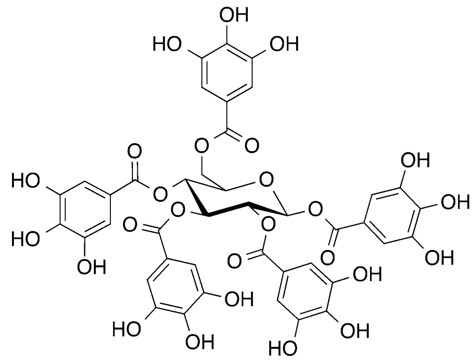

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Fig. 6.7 Small molecules **55**–**58** that modulate biofilm formation by chelating metal ions

biofilm formation by *P. aeruginosa* can only occur in a narrow iron concentration range (1–100 μM) (Yang et al. 2007). Iron chelated by either picolinic acid or acetohydroxamic acid prevents biofilm formation by *P. aeruginosa* PA14 and also by 20 clinical isolates of *P. aeruginosa* (Musk and Hergenrother 2008). Additionally, at sub-MIC levels the antibiotic nitroxoline **56** inhibits *de novo P. aeruginosa* biofilm synthesis with little microbicidal activity toward planktonic bacteria. The thickness of preformed biofilms was also reduced by 40 % by nitroxoline at 8 μg/mL, and iron and zinc complexation was shown to underlie the activity of nitroxoline (Sobke et al. 2012).

A high-throughput screen of 66,095 small molecules by Junker and Clardy identified several compounds that inhibit PAO1 biofilms with IC_{50} values below 20 μM, the structures of some of which lead the authors to posit that they act via metal chelation. The most active compound identified, **57** (Fig. 6.7), exhibits an IC_{50} value of 530 nM in minimal media, making it one of the most potent biofilm inhibitors reported to date (Junker and Clardy 2007).

The plant derived sugar 1,2,3,4,6-penta-O-galloyl-b-n-glucopyranose (PGG) **58**, which inhibits biofilm formation by *S. aureus* by preventing surface attachment of cells and reducing the production of the important matrix component polysaccharide intercellular adhesin (PIA), is a strong iron chelator and it has been shown that addition of $FeSO_4$ restores biofilm formation and PIA production, indicating that metal ion chelation is the mechanism of biofilm inhibition by PGG (Lin et al. 2012).

2.4 Natural Products and Natural Product Analogues

Natural products provide a diverse array of chemical structures and possess a plethora of biological activities. Natural products that inhibit or disperse bacterial biofilms provide a starting point for medicinal chemistry programs from which more efficacious compounds can be developed, and may even lead to the identification of new anti-biofilm targets. Much of the natural product inspiration for these programs has come from compounds isolated from plants and marine organisms.

2.4.1 Plant Natural Products

Interest in studying natural products derived from plant sources for the discovery of new biologically active compounds is not uncommon, and many traditional medicines have been rooted heavily in the use of plant extracts (Lai and Roy 2004). The antibiotic properties of garlic have long been known and can be attributed to the disulfide allicin **59** (Fig. 6.8) (Slusarenko et al. 2008). *P. aeruginosa* biofilms treated with garlic extracts in vitro exhibit enhanced susceptibility to treatment with tobramycin and to grazing by polymorphonuclear (PMN) leukocytes, and

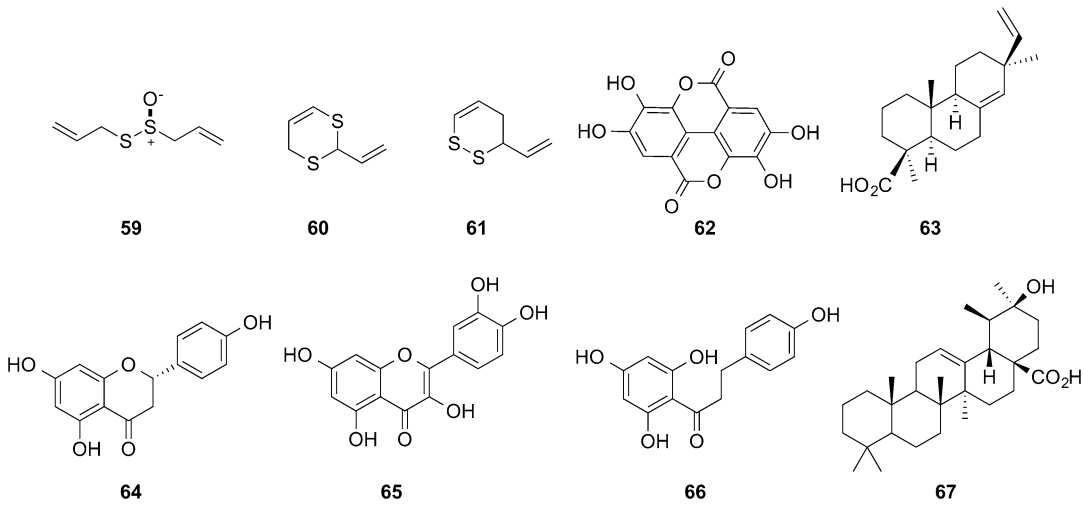

Fig. 6.8 Plant natural products that modulate biofilm formation

garlic extracts have also been shown to clear pulmonary *P. aeruginosa* infections in mouse models when dosed prophylactically (Bjarnsholt et al. 2005). Compounds identified as being responsible for these activities include the dithianes **60** and **61**, which have been shown to modulate QS in LuxR systems and do not display microbicidal activity (Persson et al. 2005).

Extracts from *Rubus ulmifolius* (Elmleaf blackberry), which are rich in the polyphenol ellagic acid **62** and its glycosylated derivatives, inhibit biofilm formation by *S. aureus* at concentrations of 50–200 μg/mL, and ellagic acid alone also possesses anti-biofilm properties (Quave et al. 2012). The resin acid 4-epi-pimaric acid **63**, which was isolated from *Aralia cachemirica* L. (Araliaceae), inhibits biofilm formation by *S. mutans* on a saliva-coated surface at sub-MIC concentrations (4 μg/mL) (Ali et al. 2012). Resveratrol, which is found in the skin of grapes and berries, inhibits biofilm formation by *V. cholerae* (Augustine et al. 2014). Several flavonoids from citrus species, including naringenin **64**, and quercetin **65**, are antagonists of AHL and AI-2-mediated cell–cell signaling in *V. harveyi*, and also inhibit biofilm formation by *V. harveyi* and *E. coli* by over 80 % at 50 μM (Vikram et al. 2010). The antioxidant phloretin **66** (found in apple tree leaves) reduces biofilm formation by EHEC O157:H7 by 89 % at 25 μg/mL without affecting the growth of planktonic cells and also without affecting commensal *E. coli* K-12 biofilms. This is significant as the eradication of commensal bacteria by conventional antibiotics is a major problem and can result in increased susceptibility to infection by opportunistic pathogens, making antibacterial strategies that selectively eliminate pathogenic bacteria a potential future therapeutic avenue for human medicine. Phloretin represses several genes including those encoding toxins, AI-2 importer genes, and curli genes, and reduces fimbria production in EHEC biofilm cells (Lee et al. 2011b). The triterpene ursolic acid **67** (Fig. 6.8), which was identified from a screen of 13,000 compounds, inhibits *E. coli* biofilm formation at 22 μM without affecting growth, upregulating genes responsible for several processes tied to biofilm formation including: chemotaxis, motility, and heat shock (Ren et al. 2005).

2.4.2 Marine Natural Products

Halogenated Furanones

Much research has centered on the biological activities and effects that halogenated furanones (HFs) have on bacterial QS (Hentzer et al. 2002; Manefield et al. 2002; Ren et al. 2001). HFs were isolated from the marine red algae *Delisea pulchra*

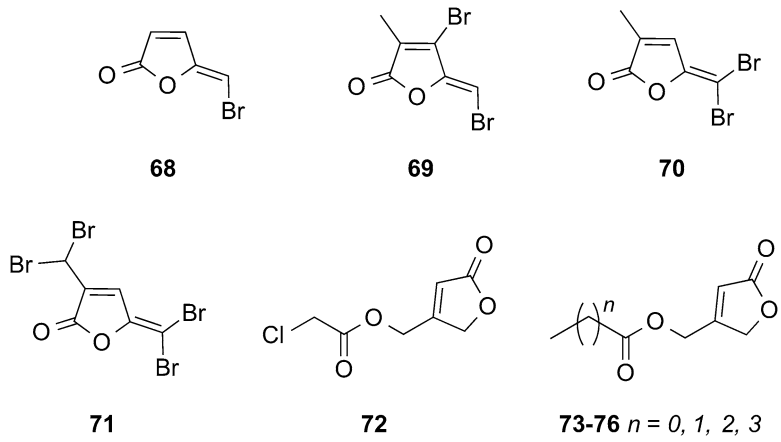

Fig. 6.9 Halogenated furanones and derivatives that modulate biofilm formation

and exhibit structural similarities to AHLs, possessing a non-polar aliphatic carbon "tail" of varying lengths attached to a relatively polar "head". Naturally occurring HFs inhibit biofilm formation and swarming in *E. coli* and *B. subtilis* at 20–60 μg/mL (Ren et al. 2001, 2002), Synthetic manipulation of the furanone scaffold has resulted in a number of HF analogues anti-biofilm activity. HFs have been shown to inhibit luminescence in *V. fischeri*, to affect the expression of numerous genes involved in virulence factor production and biofilm formation in *P. aeruginosa* (Hentzer et al. 2003; Rasmussen et al. 2000) and to penetrate *P. aeruginosa* biofilm matrices and interfere with bacterial QS, without any associated microbicidal properties (Hentzer et al. 2002). HF **68**, which lacks a side chain and possesses a vinyl bromide, is one analogue that exhibits such activity. Biofilms grown in the presence of HF **68**, display noticeable changes in biofilm maturation and structure, similar to those observed in *P. aeruginosa* mutants lacking the *lasI* system. The furanone causes rapid bacterial detachment, resulting in a loss of biomass in comparison to untreated biofilms, while other experiments have shown that furanone treated biofilms are sensitized to tobramycin (Hentzer et al. 2003). HF **68** also exhibits activity against Gram-positive biofilms, effecting a 63 and 76 % reduction in growth of *S. mutans* and *Streptococcus intermedius* biofilms respectively at a concentration of 60 μM (Lonn-Stensrud et al. 2007). HFs **69**, **70**, and **71** inhibit *E. coli* biofilm formation by up to 80 % at concentrations of 224 μM (**69** and **70**) and 141 μM (**71**) (Han et al. 2008), while furanones **72–76**, which contain an exocyclic ester functionality and are non-brominated, are effective inhibitors of *P. aeruginosa* biofilm formation (Kim et al. 2008) (Fig. 6.9).

Despite this promising activity, there are some issues with halogenated furanones that currently limit their therapeutic application, including toxicity, carcinogenic properties, and instability under aqueous conditions (Hentzer and Givskov 2003) Future medicinal chemistry efforts will be necessary to overcome some of these limitations.

2-Aminoimidazoles

The marine alkaloids oroidin **77** and bromoageliferin **78** (Fig. 6.10) are nitrogen-dense small molecules characterized by the incorporation of one or more 2-aminoimidazole (2-AI) sub-units that are produced by marine sponges of the family Agelasidae (Braekman et al. 1992). They are believed to a play a role as a chemical anti-feeding defense mechanism against predators, and were first reported to inhibit biofilm formation by the Gram-negative marine α-proteobacterium *Rhodospirillum salexigens* in 1997, exhibiting IC_{50} values of 169 μM and 2.43 nM respectively (Yamada et al. 1997).

These alkaloids have been used as structural inspiration for the development of simplified, synthetically accessible small molecules with anti-biofilm activity. Two simplified analogues of bromoageliferin: TAGE (*trans*-bromoageliferin) **79** and CAGE (*cis*-bromoageliferin) **80** (Fig. 6.10) inhibit biofilm formation by *P. aeruginosa* via a non-microbicidal mechanism, while TAGE also disperses established *P. aeruginosa* biofilms (Huigens et al. 2007). Installation of dibrominated acylpyrrole moieties similar to those present in bromoageliferin onto the TAGE scaffold results in compound **81**, which exhibits increased biofilm inhibitory activity against *P. aeruginosa*, with low micromolar IC_{50} values; however **81** does not disperse pre-formed biofilms with the same efficiency as TAGE (Huigens et al. 2008).

Oroidin **77** exhibits a similar level of activity to TAGE and CAGE for inhibition of biofilm formation by *P. aeruginosa* (Richards et al. 2008a), and a structure-activity-relationship (SAR) study of the oroidin scaffold was subsequently conducted thorough the construction of a 50-member library of analogues. Any modifications to the 2-AI head of the molecule completely abolished activity establishing the importance of this moiety for anti-biofilm activity. The most active molecule discovered was dihydrosventrin (DHS) **82** (Fig. 6.10), a reduced analogue of the oroidin family alkaloid sventrin, which has also been isolated from marine sponges. DHS inhibits the formation of biofilms by *P. aeruginosa*, *A. baumannii,* and *Bordatella bronchiseptica* with mid-micromolar IC_{50} values, and also disperses preformed biofilms of the same three species (Richards et al. 2008c). The construction of a library of second-generation analogues led to the identification of the *para*-bromo phenyl derivative **83**, which exhibits more potent non-microbicidal anti-biofilm activity against *A. baumannii* than DHS ($IC_{50} = 27$ μM, $EC_{50} = 41$ μM) (Richards et al. 2008d).

Other oroidin analogues with potent anti-biofilm activity include those in which the native amide bond directionality is reversed (Richards et al. 2008b). Several such analogues that possess an aliphatic tail group (**84**–**86**), are low micromolar inhibitors of *P. aeruginosa* biofilm development, exhibiting at least four times more potent activity than oroidin. The most active reverse amide 2-AI (**85**) is also an extremely effective dispersion agent, eradicating pre-formed *P. aeruginosa* biofilms with mid-micromolar EC_{50} values. Several of the reverse amide class of 2-AIs, including compound **86**, also inhibit biofilm formation by *A. baumannii* and a biotinylated analogue of compound **86** was used to identify the molecular target of this class of molecules in *A. baumannii* as BfmR (Thompson et al. 2012), the response regulator component of the BfmRS TCS. BfmR plays an important role in a biofilm formation and *bfmR* mutants exhibit significantly reduced biofilm-forming ability and altered planktonic cellular morphology (Tomaras et al. 2008), thus making BfmR an attractive anti-biofilm target.

The incorporation of a triazole moiety into the oroidin analogues has resulted in a number of highly active compounds (Rogers and Melander 2008), with the most active compounds possessing a substituted unsaturated aryl pendant group. Compound **87** (Fig. 6.10), which possesses broad-spectrum biofilm inhibition and dispersion properties, exhibiting low micromolar-high nanomolar IC_{50} values against *P. aeruginosa*, *A. baumannii,* and *S. aureus,* also exhibits synergism with several antibiotics for the dispersion of biofilms of several bacterial strains, and re-sensitizes planktonic bacteria of drug-resistant strains of *S. aureus* and *A. baumannii* to the effects of conventional antibiotics (Rogers et al. 2010a).

Aryl-2-AI derivatives (Fig. 6.10) have also proven to be active anti-biofilm compounds, with compound **88** effectively inhibiting *E. coli* biofilm formation (Bunders et al. 2010). Steenackers and co-workers subsequently reported a related series of aryl 2-AI compounds, the parent compound of which, compound **89**, displays moderate biofilm inhibitory activity against *S. typhimurium* and *P. aeruginosa*. Incorporating substituents on the phenyl ring of **89** resulted in increased biofilm inhibition activity, and introduction of substituents at the 5-position of the 2-AI ring resulted in a further slight increase in activity, with the most active analogues **90**–**96**

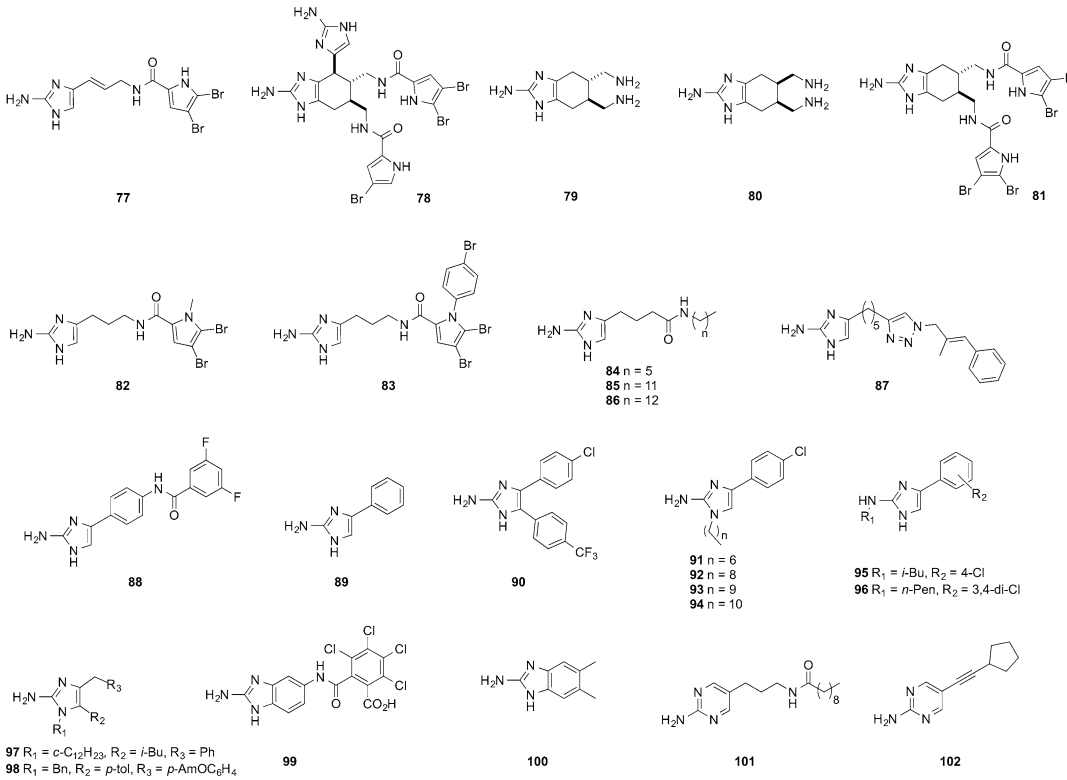

Fig. 6.10 2-AI containing marine natural products oroidin **77** and bromoageliferin **78**, synthetic 2-AI derivatives **79**–**98**, 2-ABI derivatives **99** and **100**, and 2-AP derivatives **101** and **102**

exhibiting low micromolar IC_{50} values for the inhibition of biofilm formation by *S. typhimurium* and *P. aeruginosa* (Steenackers et al. 2011a, b). A related series of 1,4,5-trisubstituted naamine alkaloids, including compounds **97** and **98**, also inhibit biofilm formation by *S. typhimurium and P. aeruginosa* with micromolar IC_{50} values (Ermolat'ev et al. 2010).

Scaffolds related to the 2-AI heterocycle that have been investigated for anti-biofilm properties include the 2-aminobenzimidazole (2-ABI) and 2-aminopyrimidine scaffolds (2-AP) (Fig. 6.10). The 2-ABI **99** is highly active against Gram-positive bacteria, both inhibiting and dispersing biofilms via a mechanism that was inhibited by the presence of Zn (II), and the compound was subsequently shown to bind Zn (II) (Rogers et al. 2009). A series of related 2-ABIs was later studied against *P. aeruginosa*, leading to the identification of compound **100**, which inhibits biofilm formation with an IC_{50} value of 4 μM and disperses pre-formed biofilms with an EC_{50} value of 92 μM (Frei et al. 2012). 2-APs are less active inhibitors of biofilm formation than their 2-AI counterparts, particularly against Gram-negative bacteria and none reported thus far disperse pre-formed biofilms. However compounds **101** and **102** do inhibit biofilm formation by *S. aureus* (Lindsey et al. 2012).

Other Marine Natural Products

Other natural product scaffolds of marine origin that possess anti-biofilm activity include: the diterpene alkaloid (−)-ageloxime D **103** (Fig. 6.11), which was isolated from the marine sponge *Agelas nakamurai,* and inhibits biofilm formation by *S. epidermidis* without affecting bacterial growth (Hertiani et al. 2010), and the structurally simple bacterial metabolite ethyl *N*-(2-phenethyl) carbamate **104** (Fig. 6.11),

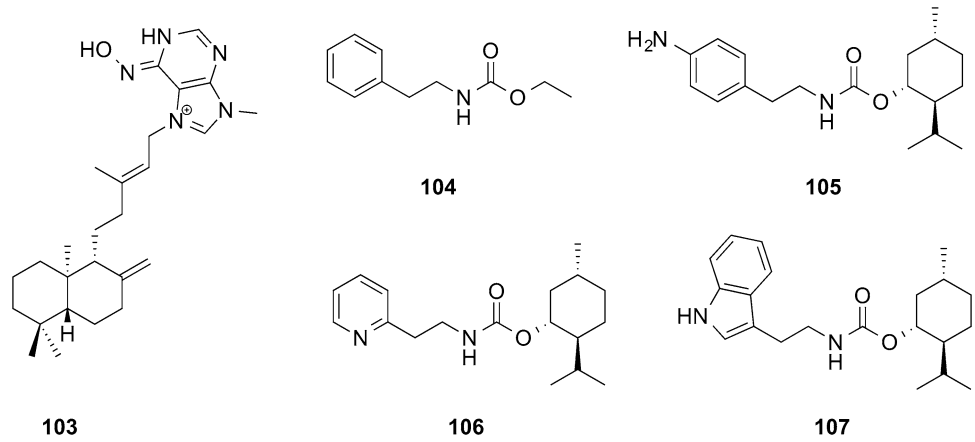

Fig. 6.11 Other marine natural products that modulate biofilm formation; (−)-ageloxime D **103**, naturally occurring carbamate **104** and synthetic derivatives **105**–**107**

which was isolated from the culture medium of the marine bacterium SCRC3P79 (Cytophaga sp.), and possesses moderate anti-biofilm activity against *R. salexigens* (Yamada et al. 1997). Compound **104** was subsequently shown to be moderately active in inhibiting biofilm formation by several medically relevant strains of bacteria, and construction of a library of readily accessible analogues based upon compound **104** led to the identification of a number of compounds with increased activity, including the menthyl derived compounds **105**–**107**, which inhibit biofilm formation by several strains of *S. aureus* with IC_{50} values in the mid to low micromolar range (Rogers et al. 2010b).

3 Macromolecule Approaches

3.1 Enzymatic Degradation of Matrix Components

Given that the matrix typically accounts for over 90 % of the dry mass of a biofilm and forms the basis of the three-dimensional structure of the biofilm, immobilizing the cells and allowing for cell-cell communication, degradation of the matrix is an attractive approach to eradicating biofilms. As mentioned earlier, bacteria within a biofilm produce EPS that constitute the matrix. Enzymatic degradation of the biomolecules that constitute the EPS is an innate phenomenon employed by several diverse bacterial species, and involves the secretion of enzymes such as glycosidases, proteases, and DNases that degrade various components of the EPS (Kaplan 2010). Examples of endogenously produced matrix degrading enzymes include the *S. aureus* DNase thermonuclease, the glycoside hydrolase dispersin B, and alginate lyase, which is produced by *P. aeruginosa*. These enzymes, and many others, are used by the bacteria to initiate dispersion of the biofilm, which contributes to bacterial survival and disease transmission (Kaplan 2010), and several of these enzymes have been investigated as potential therapeutic agents.

Dispersin B inhibits the formation of biofilms by several medically relevant bacterial species including *S. aureus*, *S. epidermidis*, and *E. coli*, and disperses *S. epidermidis* and *E. coli* biofilms (Izano et al. 2008; Kaplan et al. 2004; Itoh et al. 2005). Dispersin B also sensitizes *S. epidermidis* biofilm cells to the action of antimicrobials (Izano et al. 2008; Donelli et al. 2007) and is active in vivo, lowering the rate of catheter colonization by *S. aureus* in combination with triclosan in a rabbit model of infection (Darouiche et al. 2009). Alginate lyase degrades the polysaccharide alginate and enhances the microbicidal activity of aminoglycosides against *P. aeruginosa* biofilms in vitro (Alkawash et al. 2006; Alipour et al. 2009; Lamppa and Griswold 2013).

Alginate lyase has demonstrated in vivo efficacy, enhancing the clearance of mucoid *P. aeruginosa* when coadministered with amikacin in a rabbit model of endocarditis (Bayer et al. 1992).

Biofilms formed in the presence of DNase exhibit reduced biomass and decreased antibiotic tolerance (Tetz and Tetz 2010), and the use of nucleases to combat biofilms has been explored against a number of bacterial strains. An extracellular DNase (NucB), produced by *Bacillus licheniformis,* induces rapid biofilm dispersal against several species of bacteria including *B. subtilis, E. coli,* and *Micrococcus luteus* (Bayer et al. 1992), while *S. aureus* also produces a nuclease, known as Nuc, that exhibits biofilm inhibitory activity (Kiedrowski et al. 2011) Recombinant human DNase I (rhDNase) inhibits biofilm formation by both *S. aureus* and *S. epidermidis,* disperses *S. aureus* biofilms, and increases the susceptibility of both *S. aureus* biofilm cells to chlorhexidine gluconate and povidone iodine, and *P. aeruginosa* biofilm cells to aminoglycosides in vitro (Alipour et al. 2009; Kaplan et al. 2012). rhDNase also displays activity in vivo, increasing the survival of *S. aureus*-infected *C. elegans* when administered in combination with tobramycin (Kaplan et al. 2012). rhDNase I (also known as dornase alfa and marketed as Pulmozyme by Genentech) is used in the clinic for the treatment of pulmonary disease in cystic fibrosis (CF) patients (Parsiegla et al. 2012), in which biofilm mediated *P. aeruginosa* infections are a major contributing factor to lung tissue damage (Hoiby et al. 2010b). Administration of Pulmozyme leads to reduced demand for antibiotics and improved lung function in CF patients (Frederiksen et al. 2006).

Endogenous proteases also play a role in biofilm dispersal (Boles and Horswill 2008) and exogenous addition has been investigated for the dispersal of established biofilms. For example, the *S. epidermidis* serine protease Esp inhibits *S. aureus* biofilm formation and eradicates preformed *S. aureus* biofilms. Esp also enhances the susceptibility of *S. aureus* biofilms to the antimicrobial peptide human beta-defensin 2 (hBD2), and exhibits activity in vivo, eliminating human nasal colonization by *S. aureus* (Iwase et al. 2010). Proteinase K exhibits anti-biofilm activity against *S. aureus*, believed to be by effecting an increase in proteolytic activity (Park et al. 2012). Finally, the metalloprotease serratopeptidase (SPEP) from *Serratia marcescens*, a widely used an anti-inflammatory therapeutic, inhibits biofilm formation by *P. aeruginosa* and *S. epidermidis,* enhances the activity of ofloxacin against biofilms of these bacteria (Selan et al. 1993), and inhibits biofilm formation by *L. monocytogenes* (Longhi et al. 2008).

3.2 The Use of Antibodies to Combat Biofilms

Another non-small molecule approach to eradicating biofilms is the use of antibodies. Monoclonal antibodies (mAbs) that bind the *P. aeruginosa* cell surface anchored exopolysaccharide Psl, which plays a role in the formation and maintenance of biofilms by acting as a scaffold for other biofilm initiating components (Jackson et al. 2004; Digiandomenico et al. 2012), were identified from a screen of an M13 phage-based human antibody library. Lead mAbs inhibit host cell attachment by *P. aeruginosa* and impart significant protection in multiple animal models of *P. aeruginosa* infection including a mouse acute lethal pneumonia model and a thermal injury model (Digiandomenico et al. 2012). Antibodies to the partially de-*N*-acetylated form of the staphylococcal surface polymer poly-N-acetylglucosamine (PNAG), which promotes biofilm formation, increase killing of *S. aureus* by human neutrophils, while passive immunization of mice with anti-dPNAG-DT rabbit sera results in increased clearance of *S. aureus* from the blood compared to mice treated with normal rabbit sera (Maira-Litran et al. 2005).

4 Conclusion

The contribution of the biofilm phenotype to the pathogenesis and host/antibiotic resistance of numerous bacterial infections, combined with the ineffectiveness of conventional antibiotics in eradicating such infections, has led to a serious

need for alternative strategies to target bacteria residing within a biofilm. Numerous different approaches to this problem have been investigated, including the design or identification of small molecules that interfere with bacterial communication and signaling pathways and inhibit biofilm formation or cause biofilm dispersion, and the use of enzymes to degrade specific components of the matrix thereby causing the biofilm to disperse. In combination with conventional antibiotics these strategies have the potential to treat biofilm-based infections and have a significant impact in the fight against infectious disease.

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Antimicrobial Polymers for Anti-biofilm Medical Devices: State-of-Art and Perspectives

7

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1 Overview

The advances achieved in the area of synthetic polymers has allowed making significant improvements in the medical field, where polymers have become the materials of choice for manufacturing a wide range of products among which implantable medical devices are the prevailing ones.

The development and application of new, high-performance polymeric materials together with significant therapeutic innovations have enabled the healing of damaged parts of the body and the survival of a large number of patients.

Both natural and synthetic polymers are currently used in biomedical applications. Synthetic polymers offer several advantages over the natural ones. In fact, the versatility of the macromolecular chemistry allows tailoring polymer physico-chemical properties (in terms of molecular weight, composition, functionality, mechanical features, degradation rate) to fulfill different applications. In addition, polymers can be easily functionalized with bioactive molecules and processed in different shape and size.

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Therefore, numerous types of medical devices, including intravascular catheters, urinary catheters, heart valves, orthopaedic implants, dental implants and contact lenses, are currently made, all or in part, with polymers.

Despite the enormous progress in the optimization of operative procedures for the implantation of medical devices, clinical complications are still associated with their use, the most common being the onset of local and systemic infections.

When a biomaterial is implanted in the body, a biological response of the organism occurs causing the covering of the medical device surface with a conditioning film, rich in proteins, polysaccharides and cells, which plays an important role in the early stages of microbial biofilm formation, as it alters the surface properties of the biomaterial thereby influencing the magnitude and speed of microbial adhesion (Fitzpatrick et al. 2005).

The European Center for Disease Prevention and Control reported in 2008 that approximately four million patients per year are estimated to acquire an HAI in European hospitals. The number of deaths occurring as a consequence of these infections is estimated to be at least 37, 000. In US hospitals, about two million people acquire bacterial infections each year with a 5 % of mortality. Approximately 50,000 deaths per year are related to catheter infections. This problem is multifold. On one hand, these infections are associated with prolonged hospitalization and growing

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medical costs. On the other hand, the massive use of antibiotics to treat these infections is promoting the development of bacterial resistance at an alarming rate. For these reasons, a number of different strategies for the prevention of biofilm formation on medical devices have been proposed in the last three decades.

This chapter will be focused on the application of antimicrobial polymers to develop antibiofilm medical devices. First of all, a classification of the types of antimicrobial polymers will be introduced. Then, a survey of the antimicrobial agent-releasing medical devices that in the last decades have been evaluated experimentally or in clinical trials will be carried out. Next, the most innovative approaches to develop intrinsically antimicrobial polymers (biocidal polymers) able to kill microorganisms without releasing antimicrobial agents will be presented and discussed. Finally, a discussion of the future direction of antimicrobial polymers as biomaterials will complete the chapter.

2 Antimicrobial Polymers

The development of antifouling or antimicrobial polymers aims predominantly at the prevention of microbial adhesion and biofilm formation. Such materials either repel microbes (antifouling) or kill bacteria (antimicrobial) present in the surface proximity (Fig. 7.1).

Antifouling polymers are interesting since they avoid the use of drugs but their efficacy is strongly dependent on the bacterial species. As microbial surfaces are mainly hydrophobic and negatively charged, to be antifouling polymers should be either (i) hydrophilic; (ii) negatively charged; or (iii) with low surface free energy (Fig. 7.1) (Chen et al. 2010).

Among hydrophilic polymers, poly(ethylene glycol) (PEG) is surely the most investigated (Banerjee et al. 2011) for biomedical applications given its resistance to protein adsorption, non immunogenicity and antithrombogenicity. PEG antifouling ability is believed to be related to both hydration and steric effects (Chen et al. 2006). PEG antifouling properties depend on its molecular weight, degree of branching and surface packing density.

Negative charged polymers that have shown to exhibit an activity in reducing bacterial adhesion onto medical devices surfaces are heparin (Appelgren et al. 1996) and albumin (An et al. 1996). Particularly, the heparin coating of CVCs and dialysis catheters resulted in a significant reduction of catheter-related infections (Abdelkefi et al. 2007; Jain et al. 2009). Recently, a heparin-like polyurethane possessing negatively charged sulfate groups has been shown to counteract the adhesion of *Staphylococcus epidermidis* (Francolini et al. 2014).

Low surface energy polymer coatings are especially used in marine biofouling (Callow and

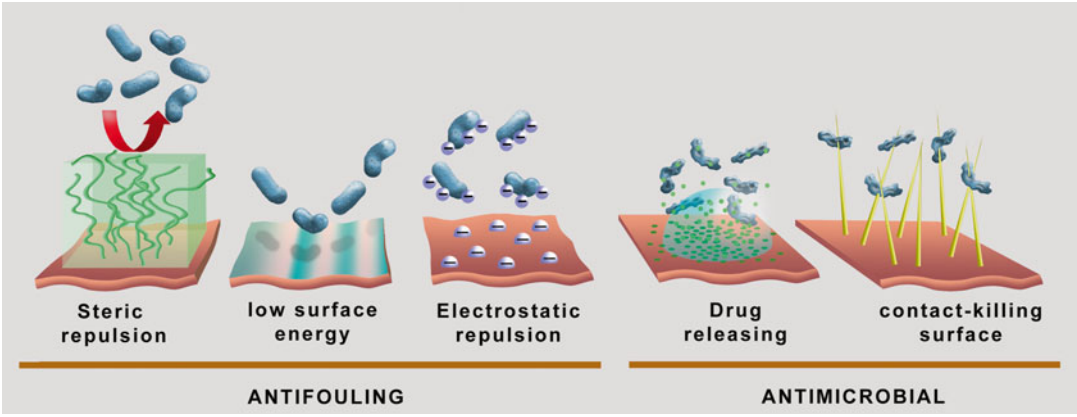

Fig. 7.1 Classes of antifouling or antimicrobial polymers

Fletcher 1994). Silicone elastomers and fluoropolymers have received most attention for potential use as antifouling coatings (Lindner 1992). Recently, Yasani et al. (2014) obtained antifouling polymers from styrene monomers containing siloxane, fluoroalkyl and/or ethoxylated side chains blended with elastomer matrices, either poly(dimethyl siloxane) or poly(styrene-b-(ethylene-co-butylene)-b-tyrene).

As far as antimicrobial polymers are concerned, they can be developed either (i) by impregnation with antimicrobial agents (antibiotics, disinfectants, and so on) or (ii) by introducing in the polymer backbone or side-chain functionalities exerting antimicrobial activity.

In the first approach, the polymer acts as a carrier for the antimicrobial agent that once released from the polymer exerts its action (antimicrobial agent-releasing polymers). In the second approach, bactericidal functionalities, such as quaternary amine compounds, are introduced in the polymer to obtain intrinsically antimicrobial polymers (biocidal polymers). Biocidal polymers do not release antimicrobial substances but exert their killing action when microorganisms contact the surface.

In the following sections, these two classes of antimicrobial polymers will be discussed in detail.

3 Antimicrobial-Releasing Polymers for Anti-biofilm Medical Devices

Antimicrobial agents can be applied to the polymer by: (a) physical adsorption (Darouiche et al. 2005; Piozzi et al. 2004; Donelli et al. 2002); (b) impregnation into the polymer matrix (Francolini et al. 2004; Schierholz et al. 1997); (c) complexation (Francolini et al. 2010, 2013) or (d) conjugation (Woo et al. 2002). Physical adsorption is by far the most used strategy for coating medical devices with antimicrobial agents since it is facile, inexpensive and potentially applicable for a wide-range of drugs. To improve drug affinity for the polymeric device surface, often intermediate layers are needed, such as the cationic surfactants tridodecylmethylammonium chloride (TDMAC) and benzalkonium chloride (BKZ) used for minocycline/rifampin and heparin coating, respectively.

Drug impregnation into the polymeric device bulk material matrix is done directly prior to injection molding or extrusion and can be performed only in case of not thermolabile drugs.

In this section, the main anti-biofilm medical devices releasing antibiotics, disinfectants, or metals that have been evaluated experimentally or in clinical trials are reviewed and discussed. Most of the reviewed systems are commercially available and used in clinical application.

3.1 Central Venous Catheters

Central venous catheters (CVCs) are essential devices for introducing fluids, intravenous nutrition and systemic therapies into the body. The major concern associated with the use of CVCs is the substantial risk of infectious complications that has been estimated from 11 % to 14 % with an attributable mortality that can exceed 25 % (Lynch and Robertson 2008). Device colonization by microbial pathogens can follow either an extraluminal route, arising from skin at the catheter insertion site, or an intraluminal route from the catheter hub or contaminated intravenous fluids. Usually, an extraluminal source of infection is more frequent in short-term catheterization, whereas an intraluminal source predominates with prolonged implantation times (Mermel 2011).

According to the guidelines for the prevention of intravascular catheter-related infections published by the Centers for Disease Control and Prevention in 2011, despite the use of maximal sterile barrier precaution and skin cleaning with antiseptic agents, the implantation of antimicrobial-coated CVCs is recommended in patients whose catheter is expected to remain in place > 5 days (O'Grady et al. 2011). Currently, several antimicrobial-coated CVCs are available on the market in Europe and the USA (Table 7.1). In the majority of the antimicrobial-coated CVCs listed in Table 7.1, the antimicrobial agents are

Table 7.1 Examples of antimicrobial-coated CVCs available on the market

CVC type	Manufacturer	Year of first clinical trial	Notes
First-generation chlorhexidine and silver sulfadiazine-coated CVC	Vygon Ltd, Ecouen, France Arrow International, Inc, PA, USA	1996	The antimicrobial agents are present only on the external CVC surface
Minocycline and rifampin-coated CVC	Cook Critical Care, Inc, IN, USA	1997	Both the internal and external surfaces are coated. The catheter trade name is Cook spectrum. The antibiotics are bond to the catheter surface through a TDMAC coating
Benzalkonium chloride-coated CVC	Becton Dickinson (UK) Ltd, Swindon, U.K.	2000	Both the internal and external surface is coated
Benzalkonium chloride and heparin-coated CVC	Baxter Healthcare Corporation, Irvine, CA, USA	2000	Both the internal and external surfaces are coated. The catheter trade name is AMC Thromboshield™
Silver ion-impregnated CVC	Multicath Expert, Vygon Ltd, Ecouen, France	2002	The catheter is made in polyurethane and is based on Agion® silver ion release technology
Silver, platinum and carbon-impregnated CVC	Vantex CVC, Edwards Life Science, CA, USA	2003	Also called Oligon CVC, where Oligon is the name of the polyurethane/antimicrobial agent mix. The antimicrobial agents are impregnated in the polymer bulk and therefore present on both the external and internal CVC surface
Second-generation chlorhexidine and silver sulfadiazine-coated CVC	Arrow International, Inc, PA, USA	2004	The concentration of chlorhexidine is three times higher than the first-generation catheters. Silver sulphadiazine is present on the external and chlorhexidine on the intraluminal catheter surface
Miconazole and rifampin-coated CVC	Vygon Ltd, Ecouen, France	2004	Both the internal and external surface is coated

adsorbed onto the catheter surface. Only in the CVCs treated with silver (silver, platinum and carbon (SPC)-impregnated CVCs and silver ion (Ag^+)-impregnated CVCs), the antimicrobial agents are impregnated in the polymer bulk.

From a historical point of view, the CVCs coated with chlorhexidine/silver sulfadiazine (CH/SS) or minocycline-rifampin (MR) are the first medicated catheters approved for clinical trials and still to this day in clinical use.

As far as the CH/SS-coated catheters are concerned, they were originally marked with both antimicrobial agents on the external surface (first generation catheters). Clinical studied performed on these catheters demonstrated they were efficacious just for short-term (less than 15 days) catheterization (Casey et al. 2008; Walder et al. 2002).

The second-generation CH/SS-coated CVCs have a concentration of chlorhexidine three times higher than the first-generation catheters. In addition, both catheter surfaces are coated with antimicrobials, silver sulphadiazine being present on the external surface and chlorhexidine on the intraluminal surface, extention sets and hubs.

The first multicentre randomized double-blind trial (Brun-Buisson et al. 2004) to test the efficacy of these catheters was carried out on 363 intensive care unit (ICU) patients, of which 175 receiving an uncoated CVC and 188 a CH/SS-coated CVC. The mean duration of catheterization was ca. 11 days in both groups. An incidence of catheter colonization in the treated group (seven patients, 3.7 %) lower than the control group (23 patients, 13.1 %) was observed. Also a slight reduction of CVC-related BSIs was

recorded when using CH/SS-coated CVCs, even if in a context of a low baseline infection rate. Similar results were obtained by Ostendorf et al. (2005) in haemato-oncological patients. Finally, Rupp et al. (2005) showed that CH/SS-coated catheters were less likely to be colonized at the time of removal compared with control catheters and did not promote antiseptic resistance.

With regard to minocycline and rifampin-coated catheters, a low rate of colonization by staphylococci was found in a prospective randomized double-blind controlled multicentre trial in which MR-coated catheters were implanted in ICU patients requiring a triple-lumen CVC for more than 3 days (Leon et al. 2004). However, a decrease in CR-BSIs or reduced length of stay was not observed. In contrast, other two randomized clinical trials on either long-term non-tunnelled (Hanna et al. 2004) or tunneled (Darouiche et al. 2005) MR-coated CVCs have shown their efficacy in reducing CR-BSIs up to ca. 60 day catheterization.

In a 2013 Cochrane review, the in vivo activities of MR-coated and CH/SS-coated CVCs were compared (Lai et al. 2013). MR coating resulted able to reduce catheter colonization and CR-BSIs significantly more than CH/SS coating. However, there was no difference between the two groups in mortality attributed to CR-BSIs and the rate of premature catheter removal. The weakness of MR-coated catheters is that they do not possess activity against *Pseudomonas aeruginosa* which contributes to ca. 5 % of CR-BSIs and *Candida* spp. contributing up to 12 % of CR-BSIs (Raad et al. 2008).

To counteract CVC colonization by *Candida* spp., the antifungal drug miconazole was incorporated together with rifampicin into polyurethane CVCs. The in vitro activity of catheters was evaluated against 158 clinical isolates of catheter-associated infections (Schierholz et al. 2000). The first clinical trial performed to assess the efficacy of rifampicin/miconazole (RM)-coated CVCs showed a significantly reduced colonization with respect to uncoated CVCs for a catheterization period of ca. 7 days (5 % vs. 36 %) (Yucel et al. 2004). Similarly, a more recent cohort study showed a statistically significant reduction in the incidence of CR-BSIs for short-term catheterization (Lorente et al. 2008).

To provide protection against *P. aeruginosa*, Raad et al. (2012) are developing a novel antimicrobial CVC coated with a combination of chlorhexidine and minocycline-rifampin (CH-MR). These catheters are produced by dipping polyurethane CVCs (Cook Medical, Bloomington, IN, USA) first in a concentrated chlorhexidine solution (40 mg/ml) for 4 h and then in a mixed solution of minocycline (15 mg/ml) and rifampin (30 mg/ml) for 1 h. CH-MR coating was shown to provide better protection against methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa*, and *Candida* spp. and a prolonged antimicrobial durability than CH/SS and MR coatings (Jamal et al. 2014).

Other antimicrobial coatings tested in alternative to MR and CH/SS are represented by silver alone and benzalkonium chloride (BKZ).

Several types of silver-coated CVCs are available (Table 7.1), including silver-impregnated CVCs and silver, platinum and carbon-impregnated CVCs (iontophoretic). Silver ion-impregnated CVCs are based on Agion® technology that consists in the incorporation of silver-containing zeolites in the CVC polymer matrix. The activity of these CVCs has been evaluated in two clinical trials (Kalfon et al. 2007; Stoiser et al. 2002), the most recent of which (Kalfon et al. 2007) performed on 577 ICU patients requiring a CVC for more than 3 days found similar rates of colonization for silver-impregnated CVCs and standard uncoated CVCs (14.7 % vs. 12.1 %).

Silver, platinum and carbon-impregnated CVCs are instead manufactured by using an antimicrobial material called Oligon that is a polyurethane combined with silver, platinum and carbon black. The efficacy of this coated catheter is still under debate (Moretti et al. 2005; Bong et al. 2003; Corral et al. 2003; Ranucci et al. 2003). The most recent prospective, randomized, multi-centre clinical trial involving a large number of patients (539) did not find an efficacy of Oligon silver CVCs higher than untreated CVCs, in terms of prevention of catheter colonization and reduction of CR-BSIs (Moretti et al. 2005).

Benzalkonium chloride (BKZ)-coated CVCs have been also largely investigated for their potential efficacy in reducing CVC colonization. Benzalkonium chloride is a cationic surfactant initially used to increase the affinity of the catheter surface towards anionic bioactive molecules, especially heparin (HP). In the early 1990s, Mermel et al. (1993) reviewed the antimicrobial activity of (BKZ-HP)-coated Swan-Ganz pulmonary artery catheters showing that the magnitude of activity against individual microorganisms was strongly correlated to their in vitro susceptibility to benzalkonium chloride. In 2000, a prospective randomized controlled clinical trial, carried out on 102 ICU children receiving a (BKZ-HP)-coated central venous line and 107 children receiving an uncoated line, showed a significant reduction in the incidence of infection (4 % vs. 33 %) and thrombosis (0 % vs. 8 %) in the group receiving (BKZ-HP)-coated catheters with respect to the control group.

In conclusion, the use of antimicrobial-coated CVCs, especially CH/SS or MR coated, should be promoted especially in patients with high risk of infection. Despite their acquisition cost is higher than that of untreated CVCs, the use of such coated catheters may potentially decrease hospital costs. Recently, Halton and colleagues (2009) evaluated the cost effectiveness of MR-coated catheters, silver/platinum and carbon-impregnated catheters, and CH/SS-coated catheters. Of these, MR-coated catheters generated the greatest health benefits and cost savings preventing 15 infections per 1,000 catheters.

3.2 Urinary Catheters

Urinary tract infections (UTIs) represent worldwide 30–40 % of hospital-acquired infections, the source of which being represented in 80 % of cases by catheterization (Donelli and Vuotto 2014; Biering-Sorensen 2002; Saint and Chenoweth 2003). Catheter-associated urinary tract infections (CAUTIs) is the most common type of hospital-acquired infection, about 449 334 patients year being estimated to suffer from this infection in US hospitals (Klevens et al. 2007). In addition, up to 4 % of patients with UTIs are estimated to develop a blood stream infection (Nazarko 2008).

There are several different types of urinary catheters: single-use, intermittent catheters, indwelling catheters and external catheters. Indwelling urinary catheters remains in place for many days or weeks and are therefore associated with a higher risk of infection. These catheters are usually inserted into the bladder through the urethra (urethral catheters) and sometimes through a tiny hole in the abdomen (suprapubic catheters). The most common type of indwelling urinary catheter is the Foley catheter that is constituted by two lumens, one of which is opened at both ends and allows urine to drain out into the drainage bag. The other lumen has a valve at the outside end and a small balloon at the tip filled with sterile saline solution to held the catheter in position.

Bacteria or fungi can enter the urinary tract in catheterized patients mainly in three ways: (i) Transfer of microorganisms from the urethra to the bladder at the time of catheter insertion. When the urinary tract is not affected by specific diseases, these bacteria are promptly cleared by the defense mechanism of bladder mucosa; (ii) Bladder contamination by migration of bacteria through the thin mucous sheath present at the urethral mucosa-catheter interface by capillary action (extraluminal route). This contamination route accounts for 70 % of CAUTIs and mainly involves intestinal bacteria; (iii) Bacterial migration through the catheter lumen as a consequence of the contamination of the drainage bag system (intraluminal route). This source of contamination reaches up to 15 % of infections in groups of hospitalized patients (Kalsi et al. 2003).

Both Gram-negative and Gram-positive bacteria as well as fungi have been reported as causative agents of CAUTIs. CAUTIs tend to originate from one species of bacteria (such as *S. epidermidis*, *Enterococcus faecalis* and *Escherichia coli*) while in case of long-term catheterization mixed-population biofilms are usually involved. *E. faecalis*, *P. aeruginosa*, *E. coli* and *Proteus mirabilis* are known to be the most common causative agents of CAUTIs (Macleod and Stickler 2007; Stickler 2008).

Silver is doubtless the antimicrobial coating of choice for urinary catheters, given the specific activity of silver versus Gram-negatives that are the microorganisms more frequently isolated in CAUTIs. Saint et al. (1998) carried out a meta-analysis of eight clinical studies that compared the UTI rates in uncoated and silver-coated catheters. Four of the reviewed trials concerned the use of catheters coated with silver alloy whilst the other four used catheters containing silver oxide. The pooled results showed that silver alloy was significantly more effective in reducing asymptomatic bacteriuria than silver oxide. Few years later, also Davenport and Keeley (2005) confirmed that the use of silver-alloy-coated hydrogel catheters can reduce CAUTIs by up to 45 %, the greatest reduction being in postoperative patients, ICU patients and burn patients. The silver alloy-coated catheters were shown to reduce the risk of asymptomatic and symptomatic UTIs for 1 week of catheterization, but at a lesser degree for longer catheterization time (Schumm and Lam 2008).

In addition to silver alloy-coated latex catheters, nitrofurazone-coated silicon catheters are currently marketed. In 2006 Johnson et al. (2006) published a systematic review summarizing the results of 12 clinical trials performed in the period 1986–2004. Three trials investigated the in vivo activity of nitrofurazone-coated silicon latex catheters while the remaining nine trails investigated silver alloy-coated latex catheters. In comparison with uncoated catheters, both types of antimicrobial-coated catheters reduced the development of asymptomatic bacteriuria in 30-day catheterization. So far, no trials have directly compared nitrofurazone and silver alloy-coated latex catheters. Recently, the antimicrobial activity of a nitrofurazone-coated all-silicone catheter (Rochester Medical Group) and a silver alloy-coated latex-hydrogel catheter plus (C. R. Bard, Inc) was compared in vitro (Johnson et al. 2012). Nitrofurazone-coated catheters significantly outperformed silver alloy-coated catheters for inhibitory activity, according to both inoculum broth and catheter sonicate counts, whether compared directly or against the corresponding control catheters.

Other antimicrobial agents used for urinary catheter coating include the couple minocycline/rifampin. Bladder catheters impregnated with minocycline and rifampicin were tested in a prospective, randomized clinical trial conducted by five academic medical centers (Darouiche et al. 1999). Patients who received the antimicrobial-impregnated catheters had significantly lower rates of gram-positive bacteriuria than the control group but similar rates of gram-negative bacteriuria (46.4 % vs. 47.1 %) and candiduria.

Among microbial species mainly involved in CAUTIs, *P. mirabilis* exhibits a significant number of virulence factors including the production of urease, swarming ability and adhesiveness to the urinary tract epithelium (Jones et al. 2004; Stickler and Morgan 2008). It's well-known that the *P. mirabilis* urease production and the consequent urine alkalization due to the ammonia release cause the precipitation of crystals of calcium and magnesium phosphates giving rise to a microcrystalline layer on the catheter surface which is then rapidly coated by a dense polymicrobial biofilm mainly constituted by *P. mirabilis*, *E. coli* and *P. aeruginosa*. Encrustation and biofilm development further cause the slowing of the urine flow within the catheter till occlusion (Broomfield et al. 2009; Morgan et al. 2009; Stickler and Morgan 2008). Jones et al. (2005) reported that inflating the retention balloons of Foley catheters with triclosan solutions (1–10 mg/ml) can help preventing the encrustation process. However, the issue is not yet solved. Indeed, all types of Foley catheters including silver- or nitrofurazone-coated devices are vulnerable to this problem (Stickler 2014). Therefore, the prevention of CAUTIs remains an area of active and ongoing research.

3.3 Orthopaedic Implants

Infection is a significant complication of total joint arthroplasty and remains a major challenge in orthopedic settings. Thanks to the improvement in the surgical procedures, the risk for orthopedic device-related infections is nowadays less than 1–2 %. However, the number of total hip and knee

arthroplasty has been estimated to increase (Kurtz et al. 2007). Early prosthetic joint infections (PJIs), usually caused by staphylococcal species especially *S. aureus* and *S. epidermidis*, can be fast diagnosed and this allows clinicians starting promptly an antibiotic therapy. Late chronic infections occur more than 1–2 years after prosthesis implantation and are due to either hematogenous seeding of the prosthesis from other infected body sites or a late manifestation of an infection acquired during prosthesis insertion. These infections are challenging to predict.

Debridement procedures with prosthesis retention associated with antibiotic therapy are not always efficacious in eradicating PJIs. Therefore, in many cases, prosthesis removal by one- or two-stage exchange surgery is needed (Campoccia et al. 2006). A one-stage exchange procedure involves excision of prosthetic components and bone cement, debridement of the surrounding bone and soft tissues, prosthesis removal, and implantation of a new prosthesis (Osmon et al. 2013). Following one-stage exchange, patients are medicated with a pathogen-specific intravenous antimicrobial therapy. The two-stage exchange surgery is the gold standard for treatment of chronic PJIs after total hip replacement in the USA (Cooper and Della Valle 2013) and involves removal of all infected prosthetic components and cement, debridement of infected tissues and placement of antibiotic-impregnated cements and temporary static or articulating spacers. Antibiotic-loaded cement spacers are needed to deliver locally antibiotics until a permanent prosthesis is placed, minimize soft-tissue contraction, preserve patient mobility and facilitate the prosthesis reimplantation. Patients are then treated with parental antibiotics for 6 weeks followed by a period without antibiotics to ensure that the infection is resolved (Cooper and Della Valle 2013). Finally, a new prosthesis is implanted.

In the last 40 years, a number of antibiotic-loaded acrylic bone cements and spacers has been developed to prevent or treat PJIs. Particularly, self-curing polymethylmethacrylate (PMMA), able to solidify at room temperature, has been extensively used in ophthalmology for intraocular lenses, in dentistry as dental filler, and in orthopedics for prosthesis fixation (Magnan et al. 2013).

In European countries, antibiotic-loaded cements are approved for use in primary total joint arthroplasty, as a preventive strategy, since there is evidence of their efficacy in reducing infection rate (Engesaeter et al. 2006). On the contrary, in the USA antibiotic-loaded cements have been FDA approved only for use in the second stage of a two-stage total joint revision (Jiranek et al. 2006), to eradicate PJIs. Indeed, Garvin and Hanssen (1995) showed an increase in PJI eradication rate from 82 % (unloaded PMMA) to 91 % (antibiotic-loaded PMMA) when used in the two-stage exchange surgery.

In Table 7.2, a list of antibiotic-loaded cements commercially available in Europe or USA is reported.

Commercial bone cements consist of two primary components, the powder polymer (also containing benzoyl peroxide as an initiator and zirconium dioxide or barium sulphate as radiopaque agents) and the liquid monomer. These two components are mixed prior use to permit cement solidification that usually occurs in few minutes. Different polymers can be used to obtain the cement. According to the manufacturers, (i) Palacos R+G (Heraeus), Palamed G (Heraeus), Cobalt G-HV (Biomet), Refobacin Bone Cement R (Biomet) and SmartSet GHV (DePuy) are made of ca. 80–85 % of methylmethacrylate-methyl acrylate copolymer; (ii) DePuy CMW1, CMW2, CMW3 and Vancogenx (Orthodynamics UK) possess ca. 82–85 % of polymethylmethacrylate, (iii) SmartSet GMV possesses 65 % of polymethylmethacrylate and 18 % of methylmethacrylate-methyl acrylate copolymer; (iv) VersaBond (Smith and Nephew) possesses ca. 43 % of methylmethacrylate-methyl acrylate copolymer and ca. 47 % of polymethylmethacrylate; (v) Cemex-Genta (Tecres/Exactech) contains ca. 83 % of polymethylmethacrylate (with 3 % styrene); (vi) Simple P possesses ca. 75 % of methylmethacrylate-styrene copolymer and 15 % of polymethylmethacrylate.

Dall et al. (2007) compared in vitro the handling, mechanical properties, gentamicin elution,

Table 7.2 Examples of antibiotic-loaded cements commercialized in Europe or US

Antibiotic	Product name (manufacturer)	Antibiotic amount per 40 g of cement	Notes
Gentamicin (G)	**Palacos R + G** (Heraeus)	0.5–1 g	Palacos, Cobalt, Cemex-Genta, DePuy 1 and VersaBond AB are FDA approved and sold in US
	Cobalt G-HV (Biomet)		
	Cemex-Genta (Tecres/ Exactech)		
	Palamed G (Heraeus)		
	Refobacin Bone Cement R (Biomet)		
	DePuy CMW1, CMW2, CMW3, CMW Endurance and CMW2000 with gentamicin (DePuy)		The first five listed products possess 0.5 g of antibiotic while the remaining 1 g
	VersaBond AB (Smith and Nephew)		
	SmartSet GHV and GMV (DePuy)		
	Palacos LV + G (Heraeus)		
Tobramycin (T)	**Simplex P with Tobramycin** (Stryker Orthopaedics)	1 g	In 2003, Simplex P with Tobramycin became the first cleared antibiotic-impregnated bone cement in the United States
Gentamicin (G) + Vancomycin (V)	**Vancogenx** (Orthodynamics UK)	1 g each for Vancogenx	Vancogenx is the first CE marked commercially available bone cement containing both gentamicin and vancomycin antibiotics
	Copal G + V (Heraeus)	0.5 g G and 2 g V for Copal G + V	
Gentamicin + Clindamycin	**Refobacin revision** (Biomet)	1 g each	These products are especially indicated by manufacturers for use in revision surgery after a prosthetic joint infection caused by gentamicin and clindamycin sensitive bacteria
	Copal G + C (Heraeus)		
Erythromycin + Colistin	**Simplex P with Erythromycin & Colistin** (Stryker Orthopaedics)	0.5 g Erythromycin and 0.1 g (3,000,000 IU) Colistin	A sustained release occurs in the first 72 h after implantation. The short release time and the small amount of Colistin minimize possible toxic effects

shrinkage and viscosity of Palacos R + G (PRG) and Refobacin Bone Cement R (RBC) showing that the two cements had comparable mechanical properties, eluted similar amounts of gentamicin. In a randomized study, Hallan et al. (2006) compared Palamed G versus Palacos R with gentamicin in total hip replacement finding no differences between the two groups, in terms of initial fixation of the femoral component and clinical results at 2 years.

Gentamicin is doubtless the most used antibiotic for cement impregnation given its broad spectrum antimicrobial activity and excellent water solubility. In 2003, Neut et al. (2003)

compared the release of gentamicin from six commercially available gentamicin-loaded bone cements (Palacos R, Palamed G, CMW1, CMW3, CMW Endurance, and CMW2000) after vacuum- or hand-mixing of the two cement components (powder polymer and liquid monomer). Results showed that vacuum-mixing reduced cement porosity leading to a reduction in antibiotic release as compared to hand-mixing. Three years later, the same research group showed that the addition of fusidic acid or clindamycin to gentamicin-loaded bone cement increased the antimicrobial effect against a collection of 38 clinical isolates. The combination of gentamicin and fusidic acid was effective against a higher number of gentamicin-resistant clinical isolates (Neut et al. 2006).

The loading of an additional antibiotic to gentamicin-loaded cement can not only widen the spectrum of activity but also enhance the release of drugs by increasing interconnectivity between antibiotics into the cement. Ensing et al. (2008) demonstrated that gentamicin release from the cement Copal G+C, containing gentamicin (1 g) and clindamycin (1 g), was higher (75 % in 672 h) than from Palacos R+G (4 % in 672 h) containing 0.5 g of gentamicin.

Vancomycin has been also widely investigated for loading in bone cements in combination with gentamicin (Bertazzoni et al. 2014; Corona et al. 2014; Masri et al. 1998). Corona et al. (2014) compared the performance of two industrially prefabricated cement spacers containing either gentamicin alone or a combination of gentamicin and vancomycin, finding no differences between the two type of spacers in terms of infection control rate, complications and quality of life. On the contrary, when used in total knee arthroplasty, vancomycin-loaded cement showed in vivo antibacterial activity superior than oxacillin-loaded cement (Ueng et al. 2012).

Tobramycin was also used for bone cement impregnation. Simplex P with tobramycin is the first cleared antibiotic-impregnated bone cement in the USA in 2003. The efficacy of this antibiotic-loaded cement was demonstrated in vitro (Scott et al. 1999) and in vivo (Sterling et al. 2003). Tobramycin was also loaded in commercially available unloaded PMMA cements together with daptomycin (Kaplan et al. 2012).

With the aim to identify new antibacterial agents to be loaded in PMMA, bone cements loaded with chitosan or chitosan derivatives have been lately investigated (Tan et al. 2012a, b). Particularly, quaternized chitosan-loaded PMMA was shown to be able to inhibit biofilm formation by MRSA and displayed superior physical properties and osteogenic activity compared to either gentamicin-loaded or unloaded cement (Tan et al. 2012a).

Cementless prostheses are becoming increasingly popular but require alternative prophylactic strategies than the use of antibiotic-loaded bone cements. Several studies have been published concerning the coating of cementless prostheses with antibiotics or antiseptics (Neut et al. 2011; Vester et al. 2010; Moojen et al. 2009; Schmidmaier et al. 2006). Particularly, implant coating with antibiotic-loaded biodegradable polymer layers seems to be a promising approach since the coating protects the drug and quickly degrades in vivo to ensure osseointegration. In this approach, polyhydroxy acids, such as poly(D,L-lactide) or poly(lactic-co-glycolic acid), are usually employed (Neut et al. 2011; Vester et al. 2010). Fusidic acid and rifampin co-loaded poly(lactic-co-glycolic acid) nanofibers were layered on titanium implant by electrospinning and reported to be able to prevent the adherence of MRSA in an in vivo model (Gilchrist et al. 2013).

Titania (TiO_2) nanotubes with fixed length (200 nm) and variable diameters (from 80 to 200 nm) loaded with gentamicin were recently shown to be able to inhibit bacterial adhesion and biofilm formation by both standard strains and clinical isolates of *S. epidermidis* and *S. aureus* (Lin et al. 2014). Similarly, vancomycin-loaded titania nanotubes, fabricated on the titanium surface through electrochemical anodization, showed good antibacterial efficacy both in vitro and in vivo (Zhang et al. 2013).

Also zinc (Li et al. 2013) and silver (Cheng et al. 2014) were incorporated into nanostructured titania to prevent orthopedic implant-related

infections. Particularly, the incorporation of silver (Ag) nanoparticles into titania nanotubes permitted a sustained silver release in vitro for 30 days and long-lasting antimicrobial activity in an in vivo model (Cheng et al. 2014). In order to immobilize silver and hydroxyapatite (HA) on metallic implant surfaces, recently Saidin et al. (2013) used polydopamine as an intermediate layer. Indeed, the cathecol groups in polydopamine were able to bind simultaneously stainless steel, silver and HA.

As an alternative to common antibiotics, Holmberg et al. (2013) proposed the use a bio-inspired antimicrobial peptide (AMP), deriving from parotid secretory protein, to coat titanium implants for dental applications. Similarly, Kazemzadeh-Narbat et al. (2013) anchored an AMP to titanium surface through a non-cytotoxic multilayered coating. The coating showed controlled and sustained AMP release and was highly effective against both Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria. Finally, Kaur et al. (2014) layered a broad-spectrum lytic bacteriophage alone or in combination with linezolid (a potent protein synthesis inhibitor) on orthopaedic wires and tested this coating in terms of ability to prevent the adherence of a MRSA strain. Maximum reduction in bacterial adherence was obtained with the bacteriophages-linezolid coating. This strategy has the potential to significantly limit the emergence of resistant strains.

4 Wound Dressings

The skin, the largest organ of the body, possesses as a primary function that of being a protective barrier against the environment. Loss of the integrity of large portions of the skin as a result of injury or illness may lead to major disability or even death. On the base of the repair process, wounds can be classified as acute or chronic wounds. Acute wounds are tissue injuries that heal completely within few weeks contrary to chronic wounds which heal slowly and often reoccur.

Wound healing is a complex biological process of growth and tissue regeneration comprising haemostasis, inflammation, migration, proliferation and maturation phases. In order to promote wound healing, skin needs to be covered with a dressing after damage. Many requirements should be possessed by an ideal dressing material. It should be non toxic and nonallergic, maintain a moist environment at the wound/dressing interface, remove excess exudates, allow gaseous and fluid exchanges, be nonadherent to the wound and easily removable without trauma, provide bacterial protection and promote wound healing.

Wound dressings are generally classified on basis of their action as (i) passive products, (ii) interactive products and (iii) bioactive products. Passive dressings (gauze and tulle) are simple covers allowing wound healing. Polymeric films and foams are part of interactive products. They are mostly transparent, permeable to water vapor and oxygen but impermeable to bacteria. Bioactive dressings are constituted by materials either possessing endogenous activity or delivering substances active in wound healing. Most of modern materials employed to delivery active agents include proteoglycans, hydrocolloids, hydrogels, collagens, polyurethane foam and films, silicone gels, alginates, chitin and chitosan (Francesko et al. 2013).

Chitin and chitosan, this latter obtained by partial deacetylation of chitin, are biocompatible, biodegradable and nontoxic polysaccharides, possessing antimicrobial and hydrating action. In bioactive dressing applications, they have been shown to facilitate repair of different tissues accelerating the wound healing process (Dai et al. 2011; Francesko and Tzanov 2011; Pillai et al. 2009).

Incorporation of drugs in bioactive dressings can be useful in promoting wound healing, by removing necrotic tissue and helping tissue regeneration, but also in preventing or treating infection. In the case of bacterial contamination, wound healing can be indeed seriously compromised. Percival et al. have evidenced that, especially in chronic wounds, microbial biofilms induce chronic inflammation delaying wound healing (Percival et al. 2012). Different antimicrobials have been

incorporated into modern wound dressings such as dialkyl carbamoyl chloride, povidone iodine, chlorhexidine, polyhexamethylene biguanide, antibiotics and silver (Boateng et al. 2008). Antimicrobial topical application permits to achieve a sustained drug release at the site of infection, limit the amount of needed drug, reduce development of antibiotic resistance and use antimicrobial agents not available for systemic use (Fallon et al. 1999; Moehring et al. 2000).

In the literature, different chitosan/silver systems were developed for use in treatment of different wound types. Particularly, novel composite scaffolds based on α chitin/nanosilver (Madhumathi et al. 2010) and β chitin/nanosilver (Kumar et al. 2010) were found to possess excellent antibacterial properties against *S. aureus* and *E. coli*.

To avoid bacterial contamination and promote the granulation tissue and smooth wound surface formation, sulfadiazine (AgSD) and hyaluronic acid (HA) were entrapped alone or together into polyurethane (PU) foams (Cho et al. 2002). PU foam containing both HA and AgSD showed a good drug release behavior and low adhesion of fibroblast cells. In addition, this foam showed excellent wound healing effect without any inflammation or yellow cluster.

The clinical performance and safety of a commercial sustained silver-releasing PU-based foam dressing (Contreet Foam) in the treatment of diabetic foot ulcers (Rayman et al. 2005) and venous leg ulcers (Karlsmark et al. 2003) were recently investigated. Both studies demonstrated that Contreet Foam dressing is safe, very efficient to hinder bacterial colonization and to promote wound healing process. Also hydrophilic silver-coated alginate dressings resulted active as barrier to invasive bacteria. Particularly, the ACTICOAT antimicrobial barrier dressing had better antibacterial properties than either of the existing silver-based products (silver nitrate, silver sulfadiazine and mafenide acetate) (Yin et al. 1999).

In order to avoid frequent removals of wound dressing, which may be painful and dangerous to the patient increasing the risk of secondary contamination, bioresorbable dressings have been also developed. Bioresorbable dressings based on collagen and chitosan were able to promote granulation tissue formation and epithelialization (Mogosanu and Grumezescu 2014; Lee et al. 2012; Alsarra 2009). However, antibiotic release from these hydrophilic materials was rather fast, resulting in a short antibacterial effect (Ruszczak and Friess 2003). To overcome this drawback two different strategies have been employed: (i) drug loading enhancement by formation of covalent bonds or ionic interactions between drug and polymer matrix (Sripriya et al. 2004); (ii) hydrophilic drug incorporation in hydrophobic synthetic polymers. As for this latter strategy, many works have reported the development of biodegradable fiber-based wound dressings for antibiotic release. Wound-dressing electrospun nanofibrous matrices can indeed have high gas permeation and protection of wound from infection and dehydration.

Bioresorbable nanofibers based on poly(lactide-co-glycolide) (PLGA) were fabricated by using an electrospinning process by Katti et al. (2004). The authors have demonstrated that PLGA nanofibers can be tailored to desired diameters through modification in processing parameters and loaded with cefazolin. Although drug-loaded nanofibers increased their diameter with respect to the unloaded one, they were still in the nanometer range showing potential as antibiotic delivery systems for the treatment of wounds.

Elsner and Zilberman (2009) have developed composite core/shell fibers loaded with mafenide acetate, gentamicin sulphate and ceftazidine pentahydrate. These composite fibers combined a dense polymer core and a drug-loaded porous shell structure. The fabrication process produced a structure with good mechanical properties as well as the desired drug release profile. The same authors have investigated other composite wound dressings based on a polygluconate mesh and a porous poly(DL-lactic-co-glycolic acid) binding matrix (Elsner et al. 2011). Those novel dressings were prepared by dip-coating woven meshes in inverted emulsions, followed by freeze-drying. The physical properties of the wound dressing (water

absorbance and water vapor transmission rate) as well as ceftazidine release profile could be controlled by changes in the inverted emulsion's formulation. The good mechanical properties with desired physical properties and controlled release of antibiotic from the binding matrix made potentially useful the composite structures as burn and ulcer dressings.

Finally, Thakur et al. (2008) have successfully fabricated a dual drug release electrosopun scaffold containing an anesthetic, lidocaine (LH), and an antibiotic, mupirocin. It was found that the presence of the two drugs in the same polymer matrix altered the release kinetics of at least one drug. Indeed, LH was eluted through a burst release mechanism while mupirocin was released simultaneously with LH through a diffusion-mediate mechanism. The authors concluded that the fabrication of a polymeric dressing with dual drug release kinetics could have potential application for wound therapy since the LH release can provide an immediate relief of pain while the antibiotic one an extended antibacterial activity.

5 Biocidal Polymers

Biocidal polymers are defined as intrinsically antimicrobial polymers containing bactericidal functionalities, among which quaternary amine compounds or phosphonium salts are the most popular (Kenawy et al. 2007). These polymers are "contact killing" since they are able to exert the killing action when microorganisms contact the surface. Since they do not release antimicrobial substances, they do not exhaust their activity, at least in principle (Lewis and Klibanov 2005; Tiller et al. 2001).

The characteristics that a biocidal polymer should possess are (Kenawy et al. 2007): (i) facile and inexpensive synthesis; (ii) good chemical stability, particularly in the temperature range required for use; (iii) water insolubility, in some applications such as water sterilization systems and medical device manufacturing; (iv) ability to not decompose giving toxic products, in the biomedical and food packaging applications.

To develop biocidal polymers, two strategies can be mainly pursued: (i) the covalent binding of an antimicrobial molecule to a polymer matrix (Woo et al. 2002); (ii) the preparation of a monomer having intrinsic antimicrobial activity to be polymerized or copolymerized with a second monomer (Kanazawa et al. 1994, 1995).

As far as the first strategy is concerned, different classes of antimicrobial agents have been linked to polymers. Particularly, hydantoins, oxazolidines and imidazolidines have been successfully introduced in both nylon and polyester fabrics (Ren et al. 2008; Lin et al. 2001, 2002) obtaining materials with antimicrobial activity without negatively affecting polymer mechanical properties. Biocidal polyurethanes containing semifluorinated and 5,5-dimethylhydantoin pendant groups were prepared by Makal et al. in (2006). After polyurethane activation with hypochlorite to convert near-surface amide groups to chloramide, the polyurethane resulted effective against both Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa* and *E. coli*) bacteria.

Park et al. (2004a) bonded three different antimicrobial acids, specifically 4-aminobenzoic acid, salicylic acid and 4-hydroxy benzoic acid, to a ethylene-co-vinyl alcohol copolymer. After functionalization, the copolymers became more flexible and active against Gram-positive bacteria. The copolymer containing salicylic acid was the most active.

Also antibiotics were covalently bonded to polymers. Levofloxacin, penicillin, ampicillin were linked to polydimethylsiloxane (Kugel et al. 2010) and polytetrafluoroethylene (Aumsuwan et al. 2007, 2008). To confer antimicrobial activity against both Gram positive and Gram negative bacteria, penicillin V and gentamicin were simultaneously linked to polypropylene (Aumsuwan et al. 2009). The antimicrobial properties of the resulting polymers were tuned by varying the penicillin V/gentamicin ratio. Woo et al. (2000) covalently bonded ciprofloxacin to a biodegradable polyurethane based on polycaprolactone diol and hexane diisocyanate. This polymer was shown to be able to release the drug in presence of cholesterol esterase up to 30 days while its activity against *P. aeruginosa* lasted for 10 days.

The second strategy to obtain biocidal polymers, that is by polymerization of an intrinsically active monomer, is doubtless the most versatile strategy. Most of the used synthetic monomers are acrylic derivatives of pharmacologically active compounds. The most extensively studied polymer matrices are those containing either quaternary ammonium salts, quaternary pyridinium, biguanide or phosphonium salts. All these polymers belong to the class of cationic polymers.

5.1 Cationic Polymers

Cationic polymers (CPs) carry positive charges in the main chain or side chain and possess remarkable potentiality in the drug delivery field. Indeed, they have been widely studied in gene delivery since they can form electrostatic complexes with anionic biomolecules, such as nucleic acids and proteins. In addition, they are considered very promising as biocidal agents thanks to their broad spectrum of antimicrobial activity.

Generally, these polymers are water-soluble due to the high polarity. Low molecular weight cationic antimicrobials, such as benzalkonium chloride and chlorhexidine, are currently employed in many commercial products. Thanks to the wide range of obtainable materials, the use of CPs is rapidly growing not only in the medical field but also in industrial applications.

The antibacterial action of cationic polymers is related to their ability to establish strong electrostatic interactions with the bacterial cell membrane. Indeed, the outer bacterial cell membrane is negatively charged and often stabilized by divalent cations such as Mg^{2+} and Ca^{2+}. The membrane negative charge is due to the presence of teichoic acid and polysaccharides in Gram positive bacteria or lipopolysaccharides and phospholipids in Gram negative bacteria. Given this different membrane composition, CPs efficacy could be different versus Gram positive and Gram negative bacteria. However, a high binding affinity of cationic antimicrobial agents, both at low and high molecular weight, for both Gram positives and Gram negatives has been demonstrated.

Although the action mechanism of CPs is still under debate, it is generally believed that they exert their action by cell wall and/or membrane disruption, causing breakdown of the transmembrane potential, leakage of cytoplasmic material and finally cell death (Kenawy et al. 2007; Munoz-Bonilla and Fernandez-Garcia 2012). The mechanism follows a sequence of events such as polymer absorption on the bacterial cell wall, diffusion through the membrane, disintegration of the cytoplasmic membrane. It is known that the higher the charge density of the polymer, the greater its antimicrobial efficacy. However, it has also been demonstrated that a too high positive charge is cytotoxic and hemolytic (Timofeeva and Kleshcheva 2011).

Different parameters affect the antimicrobial properties of CPs including polymer chain flexibility, molecular weight, hydrophobic and hydrophilic balance, presence of amino groups and pKa.

An important feature is the polymer molecular weight. Particularly, for molecular weights lower than 50,000 g mol^{-1}, an increase in antimicrobial activity was observed with increasing polymer molecular weight (Kenawy et al. 2007). In contrast, molecular weights above 120,000 g mol^{-1} negatively affected the antimicrobial activity (Ikeda et al. 1986). This dependence of antibacterial properties on molecular weight is due to the poor permeability of high-molecular weight polymers through the cell membrane of the target organism.

Quaternization of the polymer cationic groups by alkylation reactions serves to stabilize the positive charge in different environmental conditions. In these cases, the polymer antimicrobial activity was found to be dependent on the length of alkyl chain introduced in the cationic groups. In particular, long alkyl chains seem to significantly enhance the biocidal properties of the polymer, even if often there is a threshold value dependent on the specific system. For example, to create a surface that kills airborne bacteria on contact, Lewis and Klibanov (2005) covalently attached to glass slides (4-vinyl-*N*- alkylpyridinium bromide)-based polymers possessing alkyl chains with length ranging from C0 and

C12. The antibacterial properties of the coating depended on the alkyl chain length and specifically a remarked reduction in the number of adherent *S. aureus* was obtained for a C6 chain length. This finding highlighted that the introduction of hydrophobic chains, on one hand, increases polymer hydrophobicity promoting interactions with bacterial cell wall, but, on the other hand, causes a variation of polymer hydrophilic/hydrophobic balance that can lead to aggregation phenomena of the alkyl chains decreasing the antimicrobial activity.

Therefore, a suitable hydrophilic/hydrophobic balance is of fundamental importance for the activity of cationic polymers. Indeed, to well interact with the bacterial membrane, CPs must possess both a hydrophobic region and a hydrophilic positive charge. Amphiphilic random or block copolymers have shown to have a good antibacterial efficacy and low toxicity for human cells with respect to homopolymers (Timofeeva and Kleshcheva 2011). The poor toxicity of these copolymers is probably due to an increase of their selectivity versus bacterial cells.

For this reason, in the last decade numerous amphiphilic cationic polymers have been synthesized by employing hydrophobic monomers or neutral macromonomers and cationic monomers. In the following sections, some classes of the recently developed amphiphilic cationic polymers are reported.

5.1.1 Cationic Polymers Bearing Quaternary Amino Groups

Acrylic or methacrylic derivates possessing quaternary amino groups, including PDMAEMA (poly[2-(*N*,*N*-dimethylamino)ethyl methacrylate]), are widely investigated as biocidal agents (Rungsardthong et al. 2001; van de Wetering et al. 2000). The chemistry and structure of organic groups bonded to nitrogen, the number of nitrogen atoms as well as the counterion remarkably affect the antimicrobial activity of quaternary ammonium compounds. Generally, organic substituents are alkyls, aryls, or heterocyclics. However, to better interact with the lipid bilayer of the cell wall at least one of the substituents should be a long alkyl chain (Sauvet et al. 2000). In Fig. 7.2, the chemical structures of some DMAEMA-monomers containing quaternary ammonium salts with different length of the alkyl group are reported.

Lu et al. (2007) tested the activity of the DMAEMA-based monomers reported in Fig. 7.2 against *S. aureus* and *E. coli*. The minimum bactericidal concentration (MBC) values of monomers with longer chains (dodecyl bromide (DB) or hexadecyl bromide (HB)) were 12–24 μg/mL compared to > 50 mg/ml than monomers with shorter chains. Interestingly the authors found that the polymers poly(DMAEMA-BC) where BC is benzyl chloride and poly(DMAEMA-BB) where BB is butyl bromide exhibited greater bactericidal activities than their precursory monomers, but poly(DMAEMA-DB) and poly(DMAEMA-HB) showed contrary results.

The antimicrobial activity of silicone rubber with a covalently coupled 3-(trimethoxysilyl)-propyldimethyloctadecylammonium chloride (QAS) coating was studied in vitro and in vivo by Gottenbos et al. (2002). The coating was able to reduce the viability of adherent staphylococci from 90 % to 0 %, and of Gram-negative bacteria from 90 % to 25 %. The coated silicone rubbers were also subcutaneously implanted in rats for 3 or 7 days, and seeded pre- or postoperatively with

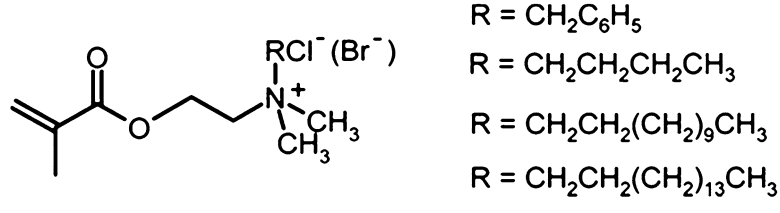

Fig. 7.2 Chemical structures of some DMAEMA-based monomers containing quaternary ammonium salts with different length of the alkyl group

S. aureus. In preoperative seeding, the QAS-coated silicone rubbers resulted in a significant reduction in infection rate compared to uncoated ones. In contrast, postoperative seeding resulted in similar infection incidences on both implant types.

Kenawy et al. (2006) reported the synthesis and antimicrobial activity of a series of cross-linked copolymers with quaternary ammonium and phosphonium groups. The copolymers showed good antimicrobial activity against *S. aureus*, *E. coli*, *Bacillus subtilis, Aspergillus flavits, Fusarium oxysporion* and *C. albicans.*

A variety of amphiphilic quaternary dimethyl-ammonium compounds bearing n-alkyl and oxyethylene groups have been designed and synthesized as antimicrobial additives for self-decontaminating polyurethanes by Harney et al. (2009). The migration of the compounds to the polyurethane surface was driven by the hydrophobicity of the additive within the hydrophilic polyurethane resin. The coating provided a 7-log reduction of *S. aureus* and *E. coli*.

Very recently, a cationic water-soluble polyacrylamide bearing tertiary amino groups was successfully used to complex usnic acid, a potent antimicrobial agent. The polymer/drug complexes possessed greater antimicrobial activity against *S. epidermidis* than both the free drug and the polymer alone (Francolini et al. 2013).

An interesting approach to give an higher biocompatibility of the quaternary ammonium compounds is the preparation of PEGylated-copolymers. Venkataraman et al. (2010) obtained PEGylated amphiphilic copolymers with different quaternized amine side chains by copolymerization of DMAEMA and PEGMA. Then, the tertiary amino side chain moieties were quaternized with different types of functional halides. The resulting polymers with shorter chains possessed lower MIC than those having long chains. In addition, the hemolysis resulted to be influenced by chain hydrophobicity.

Besides in side-chain, quaternary ammonium groups can be also introduced in the polymer backbone, resulting in the so called polyelectrolytes as those synthesized by Cakmak et al. (2004) by polycondensation of epichlorohydrin with bis-tertiary or secondary ammine or benzyl amine.

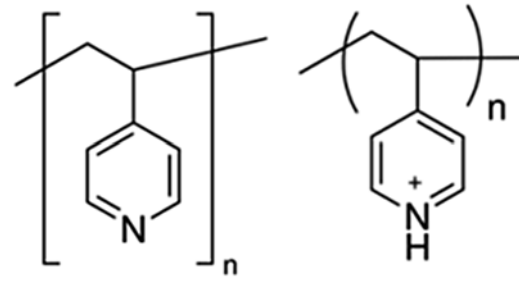

Fig. 7.3 Chemical structure of poly(4-vinyl pyridine) (*left*) and poly(vinyl pyridinium) salt (*right*)

5.1.2 Cationic Polymers Bearing Aromatic or Heterocyclic Structures

A class of cationic polymers is characterized by a neutral backbone and aromatic or heterocyclic structures in side chain. The most studied cationic polymers derive from poly(4-vinyl pyridine) (Fig. 7.3), polystyrene (PS) and imidazole (Anderson and Long 2010).

The intrinsically antimicrobial property of quaternized poly(vinyl pyridine) has been evidenced also in block or random copolymers with PS (Park et al. 2004b). The antimicrobial activity of N-hexylated poly(4-vinyl pyridine) as well as its biocompatibility have been improved by copolymerization with hydrophilic monomers as poly(ethylene glycol) methyl ether methacrylate (PEGMA) (Sellenet et al. 2007). Indeed, with increasing surface wettability, the antibacterial activity of copolymers was 20 times higher than that of the quaternized homopolymer. The introduction of PEGMA also prevented the hemolysis of polymers (Allison et al. 2007).

To obtain a suitable balance between antimicrobial activity and poor toxicity, Sambhy et al. (2008) synthesized several amphiphilic pyridinium-methacrylate copolymers by varying the ratio between positive charge and alkyl chain length on the polymer. It was observed that for polymers having the same charge/alkyl chain ratio, the spatial separation enhanced both biocidal action and reduced toxicity versus human cells.

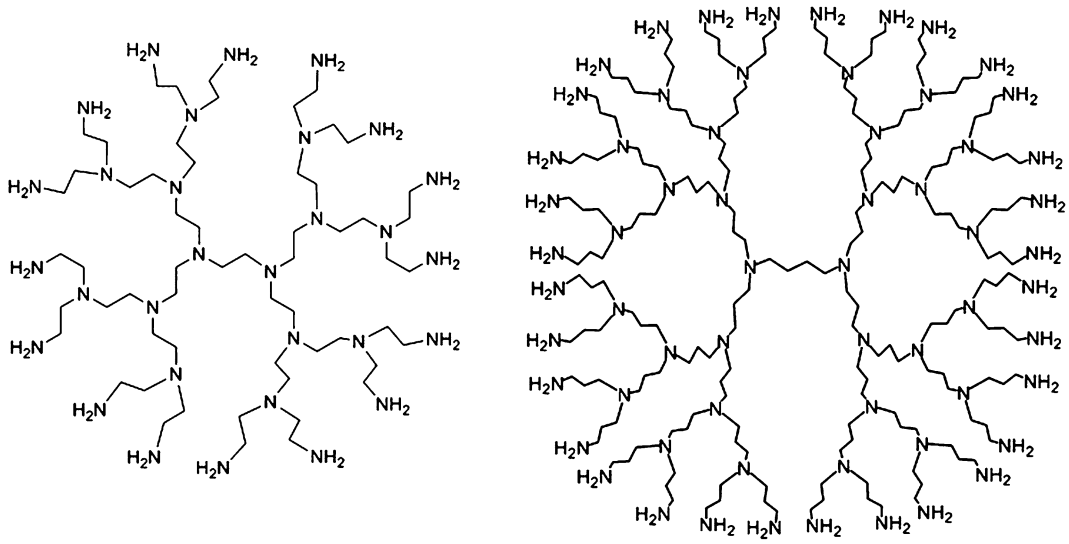

Fig. 7.4 Chemical structures of generic PEI (*left*) and PPI (*right*) dendrimers

A new methacrylamide monomer containing a pyridine moiety was synthesized by reacting methacrylic anhydride and 3-(aminomethyl) pyridine (Dizman et al. 2006). The monomer was homopolymerized in 1,4-dioxane and copolymerized with N-isopropyl acrylamide. Then, the pyridine groups of both the homopolymer and copolymers were reacted with various bromoalkanes containing 12, 14, and 16 carbon alkyl chains to obtain the polymers with pendant pyridinium groups. The quaternized water-soluble copolymers showed excellent antibacterial activities against *S. aureus* and *E. coli*, whereas the neutral polymers and quaternized water-insoluble homopolymers and copolymers were not active.

5.1.3 Hyperbranched and Dendritic Cationic Polymers

Hyperbranched and dendritic polymers, such as polyethyleneimine (PEI) and polypropyleneimine (PPI) (Fig. 7.4), possess very high positive charge density. Helander et al. (1997) found that PEI possessed a permeabilizing effect rather than a bactericidal effect against Gram-negative bacteria. A synergistic antibacterial effect of such a polymer with different antibiotics have been shown by Khalil et al. (2008). To improve PEI antibacterial activity, several modifications regarding cationic and hydrophobic groups present in the main chain have been performed (Pasquier et al. 2008; Gao et al. 2007).

Various polyethyleneglycol diacrylate (PEGDA)-based dendrimers were synthesized by Zainul Abid et al. (2010) by reaction of PEGDA with ethylene diamine and diethyl amine. Synthesized dendrimers were copolymerized with ethyleneglycol dimethylacrylate and then quaternized with hydrochloric acid. Quaternary dendritic copolymers showed antimicrobial properties depending on the concentration of quaternary ammonium groups and surface porosity.

Chen et al. (2000) studied the structure-activity relationship of quaternary ammonium functionalized polypropyleneimine (PPI) dendrimers showing that the antibacterial properties depended on the size of the dendrimer, the length of hydrophobic chains in the quaternary ammonium groups, and the counteranion.

5.2 Biocidal Polymers Mimicking Cationic Host Defense Peptides

Many different organisms produce antimicrobial peptides as part of their first line of defense (host defense antimicrobial peptides). Generally, antimicrobial peptides (AMPs) possess a small

molecular size (from 10 to 50 amino acids), amphiphilic features, with cationic and hydrophobic groups in side chain. Some of these peptides, such as defensins and magainins, are potent antimicrobials with poor or no susceptibility to bacterial resistance mechanism.

At this regard, the increase in antibiotic resistance and emergence of new pathogens has led to an urgent need for alternative approaches to infection management. Polymers mimicking natural cationic host defence peptides have emergent as promising therapeutical approaches to overcome infectious diseases (Mookherjee and Hancock 2007). The action mechanism of AMPs is different with respect to conventional antibiotics. Indeed, antibiotics generally exert their action inhibiting enzymes and DNA replication, while AMPs attack bacterial cell membranes, making them more permeable with further leakage of cellular components and cell death. In addition, cationic AMPs are selective to bacteria over human cells due to their high binding affinity with bacterial surfaces which possess a great negative charge density.

Although the marked antimicrobial activity exhibited by AMPs, their application as antibiotic substitutes has been hampered by the high cost of manufacturing, and susceptibility to enzyme action. For these reasons, in recent years, an intense research activity has been addressed to develop both small molecules and polymers mimicking antimicrobial peptides.

5.2.1 Synthetic Mimics of Antimicrobial Peptides (SMAPs)

In the last two decades, many synthetic peptides composed of L-α-amino acids (especially L-lysine) or including structures such as D-α-amino acids, β-peptides or peptoids have been developed. Indeed, chain length of 10–30 amino acid residues by using solution-coupling or solid phase synthesis can be obtained (Halevy et al. 2003). In both cases it is possible to control precisely the aminoacidic sequence.

To better resemble AMPs, the first synthesized peptides were designed in such a way that a segregation of the hydrophobic and hydrophilic groups of amino acid sequence would occur, thus inducing α-helix formation (Beven et al. 2003; Halevy et al. 2003; Tamaki et al. 2009). All of these peptide-based oligomers showed selectivity and good antimicrobial activity.

Then, also β-amino acid-based SMAPs were obtained (Liu and DeGrado 2001; Schmitt et al. 2004) that, even if formed a different type of helix, resulted active and selective. Later, many investigations have demonstrated that the helix structure of the molecule is not critical for having antimicrobial activity. Instead, it is of paramount importance that the molecule possess a local amphiphilicity and is able to self-organize into hydrophobic and hydrophilic domains upon interaction with the cell membrane.

5.2.2 SMAPs Based on Synthetic Polymers

The main advantage in using polymer systems over synthetic peptides for developing SMAPs is the possibility to produce materials on large-scale by few synthetic steps. This makes polymeric SMAPs promising candidates for therapeutical applications.

Understanding the influence of some parameters, such as spatial relationship between hydrophobic side chains and cationic groups, charge density, structure of cationic groups and so on, on the biological properties of synthetic polymers have triggered an intense research activity addressed to the design and developing of new synthetic amphiphilic cationic polymers mimicking natural host-defense peptides.

Different amphiphilic copolymers, based on a polymethacrylate or polymethacrylamide backbone having hydrophobic and cationic side chains, have been synthesized (Kuroda et al. 2009; Palermo and Kuroda 2010) (Fig. 7.5).

The hydrophobic groups and the length of the two parts have been changed to obtain non-hemolytic antimicrobial polymers. Primary, tertiary amino groups, or quaternary ammonium salts have been used as sources of cationic moieties. In these studies it has been evidenced as the antimicrobial and hemolytic activities of the synthesized polymers depend on the properties of amino side chains as much as the hydrophobic nature of the counterpart. In addition, reversible protonation of

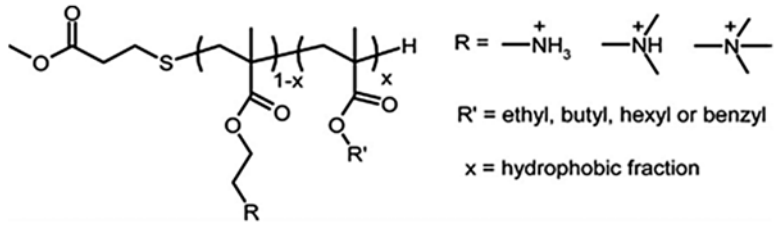

Fig. 7.5 Chemical structure of amphiphilic copolymers with different amine and hydrophobic side chains

the amino groups is more appropriate to obtain non-toxic antimicrobial polymers.

6 Future Perspectives

The field of antimicrobial polymers has increasingly grown over the past 10 years, and is expected to have a further rapid expansion in the next few years. There are numerous approaches to be applied to develop antimicrobial polymers depending on the pathogens and the final application of the polymer. Accordingly, a broad variety of new classes of active compounds is now available.

Drug-releasing polymers can be considered the first milestone in the prevention of medical device-related infections. Indeed, their use to develop anti-biofilm medical devices has largely contributed in decreasing the risk of infection development and morbidity of involved patients. However, in the last 10–15 years, the application of drug-releasing polymers have elicited concerns about the local emergence of organisms resistant to the used antimicrobial. Even if to date no clinical emergence of resistant bacteria has been reported using the so far developed coated medical devices, the drug resistance of bacteria isolated from explanted devices should be always monitored. In addition, alternative strategies to the use of antibiotic or antiseptic combinations are encouraged.

At this regard, biocidal polymers have emerged as novel promising therapeutic tools since they do not release low molecular weight products in the environment, possess long-term antimicrobial activity and not promote the development of bacterial resistance (Mizerska et al. 2009). Among biocidal polymers, cationic polymers are the most promising since they are versatile and possess a broad spectrum of antimicrobial activity. Particularly, synthetic mimics of antimicrobial host defence peptides have been shown to be potently antimicrobial, and in the same time nontoxic to mammalian cells.

Alongside the development of novel antimicrobial matrices, the application of nano- or micro-technology could give a significant contribution to the fight against biofilms. Indeed, nano- or micro-particles could protect the drug from degradation, penetrate better into the biofilm matrix and release the drug in a controlled manner thus increasing drug residence time in the biofilm and efficacy. In addition, these systems could reduce drug toxicity and permit the use of drugs with unfavorable physico-chemical properties, such as poor water solubility. Drug-loaded nano- or micro-particles could be incorporated in a polymer matrix as a possible strategy to develop antimicrobial coatings.

In conclusion, the prevention of biofilm-based infections related to medical devices is still an area of active and ongoing research. The key for the success of antimicrobial polymeric materials relies on the tight collaboration of experts in the field of macromolecular science, microbiology and medicine, in projects involving Research Institutions and Industries.

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Antimicrobial Photodynamic Therapy for Treatment of Biofilm-Based Infections

8

Merrill A. Biel

1 Overview

Photodynamic therapy (PDT) is the combination of light-absorbing chemicals, light and oxygen leading to the production of reactive oxygen species resulting in the destruction or catastrophic damage to adjacent cells. Antimicrobial PDT (aPDT) is the use of PDT to eradicate pathogenic bacteria, fungus, virus or protozoa. aPDT can be deployed for water purification, air purification and human antimicrobial applications. This chapter will discuss the status of clinical applications of aPDT in human medical treatment of microbial diseases.

2 History of aPDT

The possibility to kill microorganisms by the combination of visible light and a photosensitizing dye was first described by Raab in 1900, when he observed that paramecia in the presence of sunlight or artificially produced visible light and visible light absorbing compounds, such as acridine orange or eosin, resulted in the death of the paramecia (Raab 1900). The specific aPDT action of methylene blue against *Staphylococcus aureus* bacteriophage was demonstrated by Schultz in 1928 (Schultz and Krueger 1928). Clifton and Lawler described the same aPDT effect on *S. aureus* bacteriophage by toluidine blue in 1930 (Clifton and Lawler 1930). In 1935, T'ung described the photodynamic action of methylene blue on bacteria (T'ung 1935). During this same period Fleming described antimicrobial effects of penicillin in 1928 and Domagk described the antimicrobial effect of sulfonimides in 1935. A hiatus in photodynamic research therefore occurred through the mid-twentieth century until the 1960s when a number of studies were published on the inactivation of animal viruses by PDT, including in photodisinfection of blood products (Wallis and Melnick 1965). Since that time, a significant body of preclinical and clinical work has been published supporting the effectiveness of aPDT in the eradication of bacterial and fungal biofilm, virus and protozoan infections.

3 aPDT for Acne Vulgaris – Antibacterial PDT

Acne vulgaris is the most common dermatologic disorder with up to 33 % of all dermatology visits being for the treatment of acne vulgaris (Leyden 1997). ALA PDT (DUSA Pharmaceuticals, Wilmington, MA) and MAL PDT (Galderma, Fort Worth, TX) is recognized as a useful therapy

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for treatment of non-melanoma skin cancers and actinic keratosis (Goldman et al. 2002). These photosensitizers have been shown to accumulate readily in non-melanoma skin cancer cells, actinically damaged skin cells and in the pilosebaceous unit, where acne vulgaris lesions arise. The first reported clinical trial utilizing a photosensitizer, ALA, in the treatment of acne vulgaris was described by Hongharu et al. (2000). Twenty two patients were treated with ALA and a 550–570 nm broadband light source. Significant clearance of the acne was noted after 4 weekly ALA light treatments. Since that time, numerous clinical investigations in the use of ALA and MAL PDT to successfully treat acne vulgaris using slightly different photosensitizer application times, light applications or frequency of treatment have been reported (Itoh et al. 2001; Gold 2003b; Goldman and Boyce 2003; Taub 2004; Santos et al. 2005; Rojanamatin and Choawawanich 2006; Oh et al. 2009; Alexiades-Armenakas 2006; Wiegell and Wulf 2006; Horfelt et al. 2006; Miller and Van Camp 2006). With the success of these investigator initiated studies, two multi-institutional US FDA clinical trials were performed sponsored by DUSA Pharmaceuticals using ALA for the treatment of inflammatory acne. Unfortunately, the trials did not show a statistical clinical improvement of ALA PDT for the treatment of inflammatory acne versus light treatment alone. Therefore, ALA PDT was not approved by the FDA for the treatment of inflammatory acne, although it continues to be used by clinicians in the US as an off label treatment.

4 aPDT for Condylomata Acuminata and Molluscum Contagiosum – Antiviral PDT

Condylomata acuminata (CA) are anogenital verrucous lesions caused by the HPV virus, a member of the papova viral family, which are double stranded DNA viruses. The verrucous lesions have been related to genital carcinomas and so treatment is recommended to prevent the potential conversion of these lesions into a carcinoma. Several single investigator clinical investigations have been published on the effectiveness of ALA aPDT in the treatment of condylomata acuminata. Stefanaki reported on the use of ALA aPDT in the treatment of 12 male patients with CA. The overall cure rate was 72.9 % at 12 month followup (Stefanaki et al. 2003). Wang reported a series of 164 patients with urethral CA treated with ALA aPDT. Complete response rates at 6–25 months were 95.12 % with recurrence rates of 5.13 % (Wang et al. 2004). Chen compared ALA aPDT to CO_2 laser therapy in the treatment of 86 patients with CA. Sixty five patients received aPDT and 21 patients received CO_2 laser therapy. The complete response rate for the ALA PDT group after one treatment was 95 % with a recurrence rate of 6.3 % at 12 weeks. This was better than the CO_2 laser group with less adverse events in the aPDT group (Chen et al. 2007). Liang similarly treated a group of 67 patients with ALA aPDT and 23 patients with CO_2 laser therapy. Complete clearance was noted in 95.93 % of the ALA aPDT group and 100 % of the CO_2 laser therapy group 1 week after the last treatment but recurrence rates and adverse events were much less in the ALA aPDT treatment group (Liang et al. 2009). No multi-institutional controlled clinical trials have been performed for this indication of aPDT and therefore there are no regulatory approvals for this treatment indication to date. ALA aPDT continues to be used as an off label indication for the treatment of CA.

Molluscum contagiosum (MC) are skin and mucus membrane lesions that present with a discrete skin colored smooth papule with a umbilicated center. They are caused by large DNA pox viruses. MC is commonly seen in children and in HIV and immunocompromised individuals. The incidence of MC is reported to be as high as 5 % in children and 5–18 % in the HIV and immunocompromised population. ALA aPDT has been described by several investigators to be successful in the treatment of recalcitrant MC. Gold reported on the treatment of an HIV positive patient with a dramatic resolution of the MC lesions (Gold 2003a) Moiin treated 40 patients with MC using ALA

aPDT, of which six patients were HIV positive. After receiving 3–5 aPDT treatments, there was a 75–80 % reduction in the number of MC lesions. All patients expressed a preference of ALA aPDT compared to previous therapies for their recurrent MC (Moiin and Gold 2007). ALA aPDT remains an off label indication for treatment of MC.

5 aPDT for Chronic Periodontitis – Antibacterial Biofilm PDT

Chronic periodontitis is the most common form of periodontitis and is the major cause of tooth loss in the adult population. The disease is caused by the microbiota forming the plaque biofilm on the tooth surface which elicits a host immune inflammatory response resulting in further tissue destruction. The biofilm present in the gingival crevice and the periodontal pocket is extremely diverse with up to 100 culturable species present (Haffajee and Socransky 1994). The three microbial species thought to most contribute to the development of periodontitis are *Actinoba-cillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* (Ready et al. 2008). Standard treatment regimens are based on mechanical removal of the bacterial biofilms via scaling and root planning (SRP).

Several studies have been reported of aPDT alone or in combination with SRP compared to SRP alone in the treatment of chronic periodontitis. Anderson reported the results of a randomized controlled trial of 33 patients with chronic periodontal disease treated with Periowave aPDT (Ondine Biomedical, Vancouver, BC, Canada) using methylene blue and 670 nm light. Subjects were randomized to SRP alone, Periowave aPDT alone or SRP and Periowave aPDT. Results demonstrated that the SRP with aPDT group had a significant improvement in gingival attachment level at 6 ad 12 weeks as well as improvements in probing depth compared to the SRP and aPDT groups alone (Andersen et al. 2007). A study by Chondros of 24 patients treated with SRP and aPDT also demonstrated significant improvement in bleeding on probing in the SRP and aPDT group versus the SRP group alone. There was no statistical difference in pocket depth and attachment level between the study groups (Chondros et al. 2007). Sgolastra published a systematic review and meta-analysis of the efficacy of aPDT adjunctive to SRP in patients with chronic periodontitis. Published studies using Periowave aPDT (methylene blue) and Helbo aPDT (Helbo Photodynamic Systems, Walldorf, Germany) which uses phenothiazine chloride and 660 nm light, were evaluated in this meta-analysis review. This evidence-based assessment of the literature suggested that the adjunctive use of aPDT could provide short term benefits to SRP alone. However, these beneficial effects seemed to be modest and not stable over time (Sgolastra et al. 2013). Periowave aPDT has regulatory approval for use in Canada and Helbo aPDT has regulatory approval for use in the EU and Canada.

Peri-implantitis is an inflammatory process around a dental implant, characterized by soft tissue inflammation and loss of supporting marginal bone. aPDT has been used to treat this condition in several small clinical studies. Deppe reported the results of 16 patients with peri-implantitis treated with Helbo aPDT. The study demonstrated that aPDT could stop bone resorption in moderate peri-implant defects at 6 month follow up, but not in severe defects (Deppe et al. 2013). Bombeccari reported on a randomized controlled study of 40 patients with peri-implantitis, 20 of which were treated with toluidine blue aPDT and 20 received standard surgical therapy. The aPDT treatment group was associated with a significant decrease in peri-implant bleeding and inflammatory exudate as compared to the surgical therapy group (Bombeccari et al. 2013). Esposito reported on a multi-institutional trial of 80 patients with peri-implantitis randomized to treatment with FotoSan aPDT with 630 nm light (CMS Dental, Copenhagen, Denmark) and surgery versus surgical therapy alone. The study demonstrated that adjunctive use of FotoSan aPDT with mechanical cleaning of implants did not improve any clinical outcomes when compared to mechanical cleaning alone up to 1 year after treatment (Esposito et al. 2013) aPDT remains an off label indication for treatment of peri-implantitis.

6 aPDT for the Reduction of Ventilator Associated Pneumonia – Antibacterial Biofilm PDT

The leading cause of patient death from hospital-acquired infections is pneumonia (Baughman 1999). It is reported that 15–25 % of hospital patients who require mechanical ventilation to assist with breathing develop what is known as ventilator-associated pneumonia (VAP) (Morehead and Pinto 2000). Patients who require mechanical ventilation are typically in the Intensive Care Unit (ICU) and need the assistance of a respirator for an average of 4–5 days. VAP is defined as occurring in these patients within 48 h after the start of ventilation. Once developed, VAP has significant morbidity. Based on a clinical outcome study of over 9,000 patients who developed VAP, patients with VAP had a significantly longer need for mechanical ventilation (14 days vs. 5 days), a significantly longer stay in the ICU (11 days vs. 6 days), and a significantly longer stay in the hospital (25 days vs. 14 days) (Rello et al. 2002). Furthermore, each case of VAP resulted in an increase of $40,000 dollars in mean hospital charges per patient. According to the U. S. Department of Health and Human Services, the cost of treating VAP is conservatively estimated to be $1.2 billion dollars annually (Guidelines CDC 1997). Of most concern is the associated mortality rate of 24–71 % in patients who contract VAP (Papazian et al. 1996; Fagon et al. 1996; Timsit et al. 1996; Craven et al. 1986; Kollef 1993; Torres et al. 1990).

The endotracheal tube has long been recognized as a risk factor for the development of VAP, allowing bacteria direct access to the lungs (Adair et al. 1999; Feldman et al. 1999). These tubes are commonly made of polyvinyl chloride, a surface on which local bacteria colonize rapidly to form an adhesive polysaccharide glycocalyx layer known as a biofilm (Sheth et al. 1983). The biofilm protects bacterial colonies from both natural and pharmacologic antibacterial agents, in effect increasing the virulence of the bacterial species in the intubated host (Costerton et al. 1981). This phenomenon of biofilm formation has been demonstrated to occur on the interior surface of endotracheal tubes and the subsequent dislodgement of biofilm-protected bacteria into the lungs is considered to be a significant factor in the pathogenesis of VAP (Sottile et al. 1986; Inglis et al. 1989; O'Neill et al. 1991). These life-threatening infections are perpetuated by continuous microbiological seeding from the endotracheal tube biofilms and become difficult to treat due to the propensity of the biofilm microorganisms to develop antibiotic resistance (Feldman et al. 1999).

S. aureus and aerobic gram-negative bacilli are the major pathogens responsible for VAP, accounting for 50–70 % of all cases (Fagon et al. 1996; Craven et al. 1991) Importantly, a clinical study of endotracheal tubes removed at the time of extubation demonstrated extensive secretions lining the interior of the distal third of the endotracheal tubes that were shown on scanning electron microscopy to be a biofilm. Although the biofilms were polymicrobial, gram negative bacteria were isolated from these secretions in 87 % of the cases. VAP occurred in 13 of 21 patients. Eight of the 13 patients with VAP had identical organisms cultured from secretions in the lower respiratory tract and from the secretions of the interior of the endotracheal tube (Feldman et al. 1999). Furthermore, a recent clinical study by Shah and Kollef demonstrated that significant biofilm secretions build up on the interior of the endotracheal tube over the course of intubation resulting in considerable narrowing and partial obstruction of the airway. This build-up is significant enough to increase airway pressures and lengthen the time to extubation (Shah and Kollef 2004).

Recently, a non-invasive aPDT treatment method of eradicating biofilms/microorganisms from endotracheal tubes without removing the tube from the patient has been developed (Advanced Photodynamic Technologies, Minneapolis, MN) (Fig. 8.1). The photosensitizer used for this aPDT application is methylene blue. The photodynamic mechanism of bacterial and fungal cell destruction is by perforation of the cell wall and membrane with PDT induced singlet oxygen and oxygen radicals thereby allowing the dye to be further translocated into

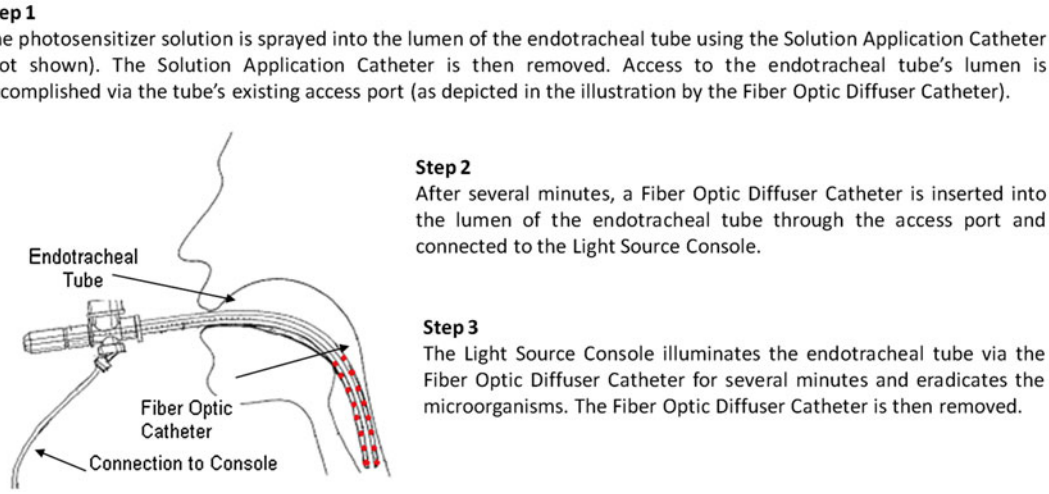

Fig. 8.1 Illustration of the PDT treatment system

the cell. Subsequently, the photodynamic photosensitizer in its new sites photodamages inner organelles such as the nucleus and induces cell death. Importantly, the PDT mechanism of microbial cell death is completely different from that of oral and systemic antimicrobial agents. Therefore, it is effective against antimicrobial-agent resistant bacteria and fungi, as well as help to prevent the development of resistant microorganisms by providing for another means of microbial eradication.

6.1 Ex vivo Endotracheal Tube Studies

In order to evaluate the effectiveness of using the combination of methylene blue and benzalkonium chloride based PDT on naturally occurring biofilms in humans, an ex vivo study was performed on endotracheal tubes with biofilms. The source material studied were endotracheal tubes coated with biofilm that were removed from patients that had received mechanical ventilation.

6.1.1 Materials and Methods

Endotracheal tubes were removed from patients and immediately cultured prior to PDT treatment using cotton tipped applicators onto TSA Blood agar (Remel, Inc., Lenexa, KS) for qualitative growth. Next 5 mL of solution in 0.45 % saline containing 200 μg/mL of methylene blue (Sigma-Aldrich, St. Louis, MO) and 0.0075 or 0.01 % benzalkonium chloride (Sigma-Aldrich, St. Louis, MO) was applied by pouring into the endotracheal tube. After a 5-min incubation time, light activation was performed using a 664 nm diode laser light (DD4, Miravant, Inc., Santa Barbara, CA) with a 2 cm cylindrical light diffuser at 70 J/cm fiber length at 300 mW for a time of 233 s. The light diffuser was inserted into the endotracheal tube so that the diffuser was at an equal distance from the wall of the tube and emitted light from the tip opening and back 2 cm. After light activation, the endotracheal tube was cultured for qualitative growth onto TSA Blood agar plates. Following a 10-min waiting period, the endotracheal tube underwent a second PDT treatment using identical parameters. Upon competition of the second treatment the tube was again cultured for qualitative growth onto TSA Blood agar plates. All TSA Blood plates were grown at 37 °C for 48 h. Results were visually scored using the following qualitative scoring system: 0 = no colonies, 1 = 1–5 colonies, 2 = 6–100 colonies, 3 = 101–300 colonies, 4 = 301+ colonies.

Table 8.1 PDT of endotracheal tubes using methylene blue and benzalkonium chloride

Patient	Intubation (days)	Pneumonia	Antimicrobial	MB (μg/mL)	BAC (%)	Treatment stage		
						M-L-	M+L+	2L+
MZ	16	Yes	Amphotericin	200	0.0075	4	4	4
			Clindamycin					
			Imipenem					
			Tequin					
			Vancomycin					
PS	1	No	Zinacef	"	"	4	2	2
GH	1	No	Zinacef	"	"	4	0	0
AR	11	No	Ciprofloxacin	"	"	4	2	2
			Tequin					
EB	2	No	Vancomycin	"	"	4	4	4
HE	6	No	Tequin	"	"	4	0	0
NB	6	No	Zinacef	"	"	4	0	0
BG	2	No	Zinacef	"	"	3	2	0
PH	1	No	Zinacef	"	"	4	4	4
AW	2	No	N/a	"	"	4	3	2
RC	x < 1	No	Zinacef	"	0.01	4	0	0
SD	1	No	Zinacef	"	"	4	0	0
MJ	1	No	Zosyn	"	"	4	0	0
SR	3	Yes	Tequin	"	"	4	2	0
MK	4	Yes	Ceftriaxone	"	"	4	0	0
MS	11	Yes	Rocephin	"	"	4	0	0
MB	1	No	N/a	"	"	0	0	0
NV	3	Yes	Ampicillin	"	"	2	0	0
JF	3	Yes	Primaxin	"	"	2	0	0
EK	x < 1	No	Zinacef	"	"	3	0	0

Growth scoring system: 0 = no colonies, 1 = 1–5 colonies, 2 = 6–100 colonies, 3 = 101–300 colonies, 4 = 301+ colonies
M-L- denotes NO MB and NO light treatment
M+L+ denotes MB and one light treatment
2L denotes MB with two light treatments

6.1.2 Results

These ex vivo studies demonstrated that methylene blue/benzalkonium chloride based aPDT treatment of endotracheal tube biofilms results in a significant reduction of the biofilm after one treatment. Sixty five percent of the endotracheal tubes treated obtained complete eradication of the pathogenic organisms after one PDT light treatment ($p < 0.05$) and another 15 % obtained a significant reduction in the pathogenic organisms after one PDT treatment. Only 15 % of the aPDT treated endotracheal tubes obtained no response (Table 8.1).

The microorganisms cultured from the ex vivo endotracheal tubes prior to PDT treatment (Table 8.2) demonstrated a predominance of Streptococcus and Staphylococcus species as well as *Candida albicans* and *Pseudomonas aeruginosa*.

A further ex vivo endotracheal tube study was performed to determine the rate of pathogenic bacterial growth in endotracheal tubes over time. Endotracheal tubes that had been in patients for various lengths of time were cut into 2 cm segments from the distal tip and microbial colony forming units (CFU) counts were obtained using culture swabs. This study demonstrated that pathogenic bacterial growth of an average of 2 log base 10 occurred by 24 h of intubation and increased with increasing days of intubation up to 6 log base 10. In addition, the maximal growth occurred at the distal tip of the endotracheal tube

Table 8.2 Microorganisms cultured from endotracheal tubes prior to PDT treatment

Microorganism	No.	Microorganism	No.
α *Streptococcus* not *Enterococcus*	16	Yeast not *Cryptococcus*	1
Coagulase negative *Staphylococcus*	15	*Streptococcus pneumoniae*	1
Candida albicans	15	*Streptococcus* Group B	1
Gram positive *bacillus*	14	*Stomatococcus* probable	1
β Streptococcus	11	*Stenotrophomonas malthophilia*	1
Gram positive *cocci*	10	*Staphylococcus*	1
Staphylococcus aureus	9	*Serratia marcescens*	1
γ Streptococcus not *Enterococcus*	8	*Proteus mirabilis*	1
Gram positive *bacillus*, res. *Corynebacterium*	7	Mold	1
Haemophilus	5	*Klebsiella pneumoniae*	1
Pseudomonas aeruginosa	4	*Klebsiella oxytoca*	1
γ Streptococcus	4	*Haemophilus* not *H. Influenzae* β-lactamase negative	1
Enterobacter aerogenes	4	Gram positive bacillus res. *Lactobacillus*	1
Neisseria	3	Gram negative *bacillus*	1
Enterococcus	3	*Enterobacter cloacae*	1
Yeast	2	*Escherchia coli*	1
Saprophytic *Neisseria*	2	*Citrobacter koseri*	1
Micrococcus	2		

and rapidly diminished over the distal 8 cm of the tube. These findings were consistent with those found in the literature (Feldman et al. 1999). Further In vitro preclinical studies of the methylene blue based photosensitizer to photoablate polymicrobial antibiotic resistant gram-negative and gram-positive bacterial biofilms in an endotracheal tube/ventilator model demonstrated a the more than 3 $\log_{10}$ reduction from baseline ($p < 0.005$) (Biel et al. 2011b). Based on these preclinical studies, a multi-institutional FDA IDE approved randomized controlled clinical trial is presently in progress to demonstrate the efficacy of aPDT in the reduction of ventilator associated pneumonia as compared to standard of care therapy.

7 aPDT of Chronic Wound Infections – Antimicrobial Biofilm PDT

Approximately 18 million people in the U.S. develop chronic wounds annually (Heffner 2003). The definition of a chronic wound is simply any condition in which the wound does not heal in a timely fashion. Chronic wounds are most frequently seen in individuals who have diabetes, spinal cord injuries, cutaneous burns, suppressed immune systems, vascular disease, and the elderly. Chronic ulceration of the skin, regardless of etiology, causes a prolonged breach of one of the primary host defenses against bacterial infection. As a result, wounds are prone to bacterial contamination and subsequent infection. In aggregate, skin ulcers represent 70 % of all chronic wounds. Skin ulcers are classified according to type and underlining cause that include venous, decubitus, and diabetic.

7.1 Venous Ulcers

Chronic leg ulceration is a very common clinical problem in the elderly. Venous ulcers constitute the majority (65–70 %) of all leg ulcers. Venous hypertension is thought to be the underlying cause of leg ulcers. Venous hypertension is the failure of ambulatory venous pressure to fall and

is well documented in patients with venous ulcers. Why venous hypertension produces leg ulcers is unclear. Current hypotheses that are considered to contribute to venous ulcer development include: (1) a blockage of oxygen or nutrients by fibrin build-up or perivascular fibrin cuffs in the leg (fibrin cuff hypothesis), (2) entrapment of growth factors by macromolecules leaking into the perivascular tissues (trap hypothesis), and (3) blockage of the capillaries by white blood cells that subsequently damage the capillaries (white blood cell hypothesis). The effects of oral drug therapy for the treatment of the underlying venous disease and subsequent prevention of leg ulceration have been disappointing (Goodfield 1997). The Wound Healing Society's standard of care for venous ulcers are compression therapy (bandaging of the leg or use of hosiery), maintaining a moist wound environment, debridement of necrotic tissue, and bacterial control (Ehrlich and Dingleman 1999).

7.2 Decubitus Ulcers

Decubitus ulcers, also known as pressure ulcers or bedsores, are the most common of all the chronic wounds. These ulcers are particularly problematic in people who are paralyzed, debilitated, or unconscious and plague the nursing home community with a 23 % incidence rate per year (Findlays 1996). In addition over one million persons are afflicted with bedsores in U.S. hospitals each year. It is reported that the cost of care for decubitus ulcers is conservatively estimated to be $55 billion dollars per year in the U.S. (Cost Savings 2003). In addition, significant morbidity and mortality is reported to be associated with these lesions. The National Decubitus Foundation reports that "hospital patients over the age of 70 with decubitus ulcers have a fourfold increase in the death rate" and "the mortality rate in hospitals for afflicted patients is between 23% and 37 %" (Cost Savings 2003). The U.S. Department of Health and Human Services Agency for Health Care Policy and Research has published clinical practice guidelines for the prevention and treatment of decubitus ulcers (Bergstrom 1994). The major preventive measures recommended by the Agency include (1) identification of high-risk patients, (2) frequent assessment, and (3) preventive measures such as regular repositioning, pressure relief bedding, moisture barriers, and adequate diet. The principal therapeutic measures recommended by the Agency include (1) pressure relief, (2) moist wound care, and (3) bacterial control. Similarly, the Wound Healing Society's standards of care for pressure ulcers include pressure relief i.e. regular repositioning and pressure relief bedding, maintaining a moist wound environment, debridement of necrotic tissue, and bacterial control (Ehrlich and Dingelman 1999).

7.3 Diabetic Ulcers

More than 15 % of the 12 million people in the U.S. with diabetes (1,800,000 people) will develop foot ulcers in their lifetime (Kahn 1999). Chronic foot ulcers account for approximately 20 % of all hospitalizations in diabetic patients (Kahn 1999). In addition, 14–24 % of people with foot ulcers will require an amputation (National Diabetes Fact Sheet 2003). The American Diabetes Association reports that approximately 82,000 lower-limb amputations are performed each year as a result of chronic diabetic foot ulcers. The most common location for foot ulcers is the plantar surface of the forefoot. These ulcers are often caused by repetitive mechanical stress that is not recognized by the patient because of peripheral neuropathy and subsequent failure to sense and relieve pressure. In addition, the presence of peripheral vascular disease with diminished blood flow and infection can lead to poor healing of foot wounds and to the development of gangrene. Nearly one half of all lower extremity amputations in diabetic patients occur as a result of uncontrolled infection, even in the presence of an adequate blood supply (Joseph and Axler 1990). The principles of good foot ulcer care include use of proper fitting foot wear, non-weight bearing limb support, debridement, control of serum glucose levels, aggressive revascularization, and infection control. Despite

much effort directed toward ulcer/amputation prevention in the last decade the incidence of foot ulcers and lower extremity amputation in people with diabetes continues to rise. The Wound Healing Society's standards of care for diabetic foot ulcers are off-loading to relieve pressure, i.e. avoidance of all mechanical stress, maintaining a moist wound environment, debridement of necrotic tissue, and bacterial control (Ehrlich and Dingleman 1999).

7.4 Chronic Wound Infections

Although there are many underlying causes, conditions, and predispositions for developing a chronic skin ulcer, the most common contributing factor for a delay in wound healing amongst *all* wound types is infection. According to the Wound Healing Society's standards of care, the U.S. Department of Health and Human Services Clinical Practice Guidelines, the American Burn Association, and the American Diabetes Association bacterial control is absolutely critical to effective wound care (Ehrlich and Dingleman 1999; Bergstrom 1994; National Diabetes Fact Sheet 2003). Uncontrolled infections can lead to significant morbidity (including sepsis, amputation) and mortality.

All wounds are colonized with potentially pathogenic organisms. Therefore, diagnosis of infection of a chronic wound is generally based on clinical rather than microbiological criteria. The presence of purulent secretions (pus) or two or more signs of inflammation (e.g., erythema, warmth, tenderness, induration) should be regarded as evidence of infection. Wound infection has also been defined as a bacterial concentration of greater than 10^5 organisms per ml of wound exudate (Noyes et al. 1967; Heggers and Robson 1969). Clinical studies have reported that only when the bacterial bioburden rises above 10^5 CFU/g of tissue is there a significant impediment to wound healing (Robson 1997; Robson et al. 1993). Additional human studies have confirmed that significant healing of decubiti only occurred when bacterial counts were less than 10^5 per ml of wound exudates (Bendy et al. 1964). Interestingly, there was no correlation between wound healing and the presence or absence of diabetes mellitus, anemia, renal insufficiency or concurrent systemic antibiotics. There was only a high correlation between healing and the presence of fewer than 10^5 gram-negative or coagulase-positive staphylococci per ml of exudate in the ulcer. Similarly, in burn victims, the presence of 10^5 or greater bacterial wound counts results in the failure of split thickness skin grafts to survive and heal (Krizek et al. 1967).

Baseline cultures of normal non-clinically infected skin contain 10^2–10^3 bacteria per gram of tissue (Heggers and Robson 1969). Based on the literature and the Wound Healing Society's Clinical Practice Guidelines, it is generally accepted that reducing the wound bacteria concentration to normal skin concentrations of 10^2–10^3 bacteria per ml of exudates, significantly promotes wound healing (Ehrlich and Dingleman 1999; Bergstrom 1994; Joseph and Axler 1990). Therefore, methods of wound treatment that do not reduce the bacterial load to a level equal to or less than normal tissue do not substantially result in the promotion of wound healing in acutely or chronically infected wounds (Noyes et al. 1967). Consequently, any clinically effective antimicrobial treatment would at least need to maintain the bacterial load of the wound to at least that of normal colonized skin.

7.5 Current Antimicrobial Wound Infection Treatment

Current clinical guidelines typically recommend antimicrobial treatment of wounds to prevent an infection, to treat an existing infection, or if an ulcer has not healed within 2 weeks. The clinical goals of antimicrobial treatment may also include improving the patient's quality of life by reducing odor, exudates, inflammation, pain and tenderness. Although antimicrobial therapy is often the first treatment of choice for controlling bacterial colonization, morbidity and limitations are reported in the literature regarding their use. In the last several years, there has been a rapid

Table 8.3 Antibiotic resistant bacteria commonly found in wound infections

Multidrug-resistant *Staphylococcus aureus*
Methicillin-resistant *Staphylococcus aureus* (MRSA)
Vancomycin-resistant *Staphylococcus aureus* (VRSA)
Vancomycin-resistant *Enterococci* (VRE)
Multidrug-resistant *Escherichia coli*
Multidrug-resistant *Pseudomonas aeruginosa*
Multidrug-resistant *Acinetobacter*

increase in the number of multi-drug antibiotic resistant strains of bacteria including both gram negative and positive types. Antibiotic resistant bacterial infections are reported to be the cause of increased morbidity, increased incidence of hospital admissions, prolonged length of hospital stays, more costly treatments, and increased mortality (Niederman 2001). A list of the most common types of resistant bacteria found in wound infections is found in Table 8.3.

The prevalence of antibiotic resistant bacteria has diminished the effectiveness of standard antibiotic therapy and has become a significant worldwide health problem that is causing the medical community to search for alternatives to antibiotic treatment. As a result, public health organizations including the Centers for Disease Control and Prevention and the World Health Organization have made the search for alternative antimicrobial treatment modalities a major priority.

Numerous investigators have reported on the effectiveness of aPDT in the reduction of antibiotic resistant bacteria in wounds in vivo (Biel 2010; Hamblin et al. 2002). Clayton reported the first patient treated with aPDT for a chronic recalcitrant venous ulceration of the lower leg that persisted for more than 12 months despite aggressive medical therapy. ALA aPDT was delivered twice weekly for 4 weeks with significant improvement in the leg ulcer (Clayton and Harrison 2007). Morley described a Phase IIa randomized, placebo-controlled study of aPDT n chronic leg ulcers and diabetic foot ulcers. 32 patients were treated with a single treatment of aPDT with PPA904 (Photopharmica Ltd., Leeds, England) and 570–670 nm broad band light. Immediately post aPDT treatment a 1 log reduction in bacterial load was noted ($p<0.001$). After 3 months, 50 % of patients treated with aPDT demonstrated complete healing compared with 12 % of patients on placebo treatment (Morley et al. 2013). Brown described preliminary results of a Phase II b study using aPDT with PPA904 in which repeat aPDT treatments to the chronic wound. Interim data on 24 patients in this trial demonstrated that 4 of 12 ulcers in the aPDT arm closed completely compared with 0 of 12 in the placebo arm. Final results of the clinical trial are pending (Brown 2012). Mannucci reported on a randomized, double blind, placebo controlled trial on patients with diabetic foot ulcers treated with aPDT using RLP068 (Molteni Therapeutics, Italy) and 689 nm light. The study was performed with aPDT as an adjuvant to oral amoxillin and clavulanic acid treatment. Results demonstrated a dose dependent reduction in total microbial load of greater than 2 log 10 compared to the control systemic antibiotic alone group (Mannucci et al. 2013). No difference in ulceration size was noted during the 15 days of followup. Further clinical trials are pending and there are no regulatory approvals for aPDT treatment for chronic wounds to date.

8 aPDT of Cutaneous Leishmaniasis – Antiprotozoan PDT

Leishmania is an intracellular protozoan parasite of the family Trypanosomatidae which is transmitted by the sandfly (Huaman and Castillo 1990). The Leishmania species parasitize tissue macrophages and clinically manifest as skin lesion, mucocutaneous ulceration or hepatosplenomegaly. The annual global incidence of cutaneous leishmaniasis (CL) is 1–1.5 million cases per year (Desjeux 2004). Most treatment options involve physical removal of the lesions, which results in disfigurement and scarring. aPDT has been reported for the treatment of CL in six clinical studies on 39 patients with a total of 77 lesions (van der Snoek et al. 2008) These studies

evaluated Metvix or ALA aPDT with repeated aPDT treatments over the course of 3 months. Clinically the CL lesions resolved after up to 20 treatment sessions (Gardlo et al. 2003; Gardlo et al. 2004; Asilian and Davami 2006). For CL, aPDT acts via a combination of direct cytotoxicity and immunomodulatory effects. Ongoing clinical studies using different photosensitizers are presently underway to improve the effectiveness of aPDT for CL treatment. Presently, there are no regulatory approvals for aPDT treatment of CL.

9 aPDT for Oral Candidiasis – Antifungal Biofilm PDT

Oropharyngeal candidiasis is an opportunistic mucosal infection caused by Candida albicans in over 85 % of cases. The four main types of oropharyngeal candidiasis are: (1) pseudomembranous (thrush), comprising white discrete plaques on an erythematous background, located on the buccal mucosa, throat, tongue or gingivae; (2) erythematous, comprising smooth red patches on the hard or soft palate, dorsum of tongue or buccal mucosa; (3) hyperplastic, comprising white firmly adherent patches or plaques, usually bilateral on the buccal mucosa; (4) denture induced stomatitis, presenting as either a smooth or granular erythema confined to the denture bearing area of the hard palate. Symptoms vary, ranging from none to a sore, painful mouth with a burning tongue and altered taste, which can impare speech, nutritional intake and quality of life.

Candida species are commensals in the gastrointestinal tract. Transmission occurs directly between infected people or on fomites. Candidia is found in the mouth of 31–60 % or healthy people (Webb et al. 1998). Oropharyngeal candidiasis affects 15–60 % of people with hematological or oncological malignancies during periods of immunosuppression (Ninane 1994). Oropharyngeal candidiasis occurs in 7–48 % of people with HIV infection and in over 90 % of those with advanced disease. In severely immunosuppressed people, relapse rates are high (30–50 %) and usually occur within 14 days of treatment cessation (Philips et al. 1996).

Mucocutaneous candidiasis can be treated either topically or with systemic antifungal agents (Sangeorzan et al. 1994; Stevens et al. 1991), but such therapy does not eradicate colonization (Sangeorzan et al. 1994). Clinical cure rates are 34–72 %. Chronic use of these agents have resulted in development of candidal resistance.

Refractory oropharyngeal candidiasis has been increasingly reported since 1990 (Heinic et al. 1993; Maenza et al. 1997; Sanguineti et al. 1993). Refractory disease tends to occur in persons with advanced HIV disease, i.e. CD4+ cell counts < 50 cells/mm^3, who have been exposed to antifungal therapy on a chronic basis (Maenza et al. 1996). A study by Johnson confirmed these results with up to 81 % of AIDS patients on chronic azole drug therapy having azole resistant *C. albicans* (Johnson et al 1995).

Teichert demonstrated the effectiveness of methylene blue based aPDT in the eradication of oral candidiasis in an immunodeficient murine in vivo model (Teichert et al. 2002). Two randomized clinical trials have demonstrated the effectiveness of aPDT in the treatment of oral candidiasis and denture stomatitis. Scwingel reported on a randomized clinical study of 21 HIV positive patients with oral candidiasis. Patients were randomized to a control group treated with standard fluconazole daily for 14 days, laser light only group at 660 nm, and an aPDT group treated with topical methylene blue and 660 nm light. Oral fluconazole was effective in the short term eradication of the oral candidiasis, but 72 % recurred within 30 days of treatment. Laser light alone was not effective as a treatment. Methylene blue aPDT resulted in 100 % clinical resolution of the oral candidiasis without clinical recurrence at 30 days after treatment (Scwingel et al. 2012) Mima described a randomized clinical trial of 40 patients with denture stomatitis treated with oral nystatin or Photogem (Moscow, Russia) aPDT and 455 nm light. Both groups demonstrated a significant reduction of culturable oral candida at the end of treatment and on 30 day followup. aPDT was found to be as effective as topical nystatin in the treatment of denture stomatitis (Mima et al. 2012). There have been no regulatory approvals to date for the treatment of oral candidiasis with aPDT.

10 aPDT for Nasal Decolonization of Staphylococcus Species to Reduce the Rate of Surgical Site Infections – Antibacterial PDT

Surgical site infections (SSI) represent a significant cost burden and resource diversion for hospitals with increased patient length of stay, morbidity and mortality (Anderson 2011; Cosgrove et al. 2005). To complicate matters, infection with an antibiotic resistant strain of bacteria such as Methicillin-resistant *S. aureus* (MRSA) can increase hospital costs up to threefold compared to infections with susceptible strains (Abramson and Sexton 1999; Engemann et al. 2003; Ben-David et al. 2009). Nasal colonization with *S. aureus* has been demonstrated to increase the risk of a SSI (Yano et al. 2000; Kluytmans et al. 1997; Perl et al. 2002; Kalmeijer et al. 2000). Pre-surgical bundles that employ mupirocin ointment and chlorhexidine body washes to decrease *S. aureus* colonization can reduce this risk (Perl et al. 2002; Bode et al. 2010; van Rijen et al. 2008; Wenzel 2010). However, mupirocin-based nasal decolonization strategies are disadvantaged by poor compliance (Caffrey et al. 2011), lengthy treatment time (twice daily for 5 days), and increasing potential for antibiotic resistance. While some studies have failed to demonstrate benefit to nasal decolonization, expert consensus is to employ decolonization strategies for high risk surgical procedures (Anderson 2011; Garcia et al. 2003; Kalmeijer et al. 2002; Konvalinka et al. 2006; Suzuki et al. 2003).

Bryce performed a single arm clinical study of the effectiveness of aPDT nasal decolonization with the MRSAid device (methylene blue with 670 nm light, Ondine Biomedical, Vancouver, BC, Canada) and chlorhexidene body wipes performed just before surgery. During a 16.5-month period, patients undergoing cardiac, orthopaedic, spine, thoracic, vascular, breast, and neurosurgical procedures were treated with intranasal aPDT in the pre-operative waiting area. Nasal swabs to assess colonization with both Methicillin-sensitive (MSSA) and MRSA were performed immediately prior to and after PDT; growth was graded as no growth, scant, moderate or heavy. 2 % chlorhexidine body washes were performed within 24 h prior to surgery. 5,691 patients were treated with aPDT. Compliance with LPN-administered PDT was 93.4 % (5691/6090). Pre-PDT colonization rates for MSSA and MRSA were 23.4 % (1315/5627) and 1.00 % (56/5627), respectively. Microbiologic bioburden reduction was successful in 81.7 % of cases. The adverse event rate was 0.12 % (7/5691). The surgical site infection (SSI) rate in the study was 1.73 % compared with a historical SSI rate of 2.7 % (Bryce et al. 2012). Further studies are ongoing in Canada. MRSAid nasal decolonization has regulatory approval in Canada for human clinical use.

11 aPDT Treatment for Chronic Rhinosinusitis – Antibacterial Biofilm PDT

Chronic rhinosinusitis (CRS) is an inflammatory disease of the facial sinuses and nasal passages that is defined as lasting longer than 12 weeks or occurring more than four times per year with symptoms usually lasting more than 20 days (Marple et al. 2009). The National Institute for Health Statistics estimates that CRS is one of the most common chronic conditions in the United States affecting an estimated 37 million Americans (National Center for Health Stats 2002). It is also estimated that CRS results in 18–22 million office visits per year and over 500,000 emergency visits per year resulting in an estimated 73 million restricted activity days with an aggregated cost of six billion dollars annually (Murphy et al. 2002; Gliklich and Metson 1995). The potential etiologies of CRS include bacteria, viruses, allergies, fungi, superantigens, exotoxins and microbial biofilms. Importantly, CRS is also considered to be a significant factor that can exacerbate asthma, chronic lung diseases, eczema, otitis media and chronic fatigue (Gliklich and Metson 1995; Hamilos 2000; Somerville 2001; Chester 1999).

Failure to effectively treat CRS not only results in prolonged illness but can also result in significant complications including osteomyelitis of the facial bones, meningitis and brain abscesses (Gliklich and Metson 1995; Chester 1999).

In clinical practice there is a significant subpopulation of patients with CRS who remain resistant to cure despite rigorous treatment regimens including surgery, allergy therapy and prolonged antibiotic therapy (Marple et al. 2009; Desrosiers and Kilty 2008; Cohen et al. 2009; Hunsaker and Leid 2008; Palmer 2005; Harvey and Lund 2007). The reason for treatment failure is thought to be related to the destruction of the sinus mucociliary defense by the chronic sinus infection resulting in the development of secondary antibiotic resistant microbial colonization of the sinuses and biofilm formation (Marple et al. 2009; Desrosiers and Kilty 2008; Cohen et al. 2009; Hunsaker and Leid 2008). Gram-negative and Gram-positive bacteria, including *Hemophilus influenzae* and *Streptococcus pneumoniae* account for 50 % of clinically sampled isolates found in CRS patients (Palmer 2005; Harvey and Lund 2007). It is increasingly reported that MRSA and multidrug resistant *P. aeruginosa* are found in the clinical isolates of CRS patients and are a cause of antibiotic treatment failures (Marple et al. 2009; Desrosiers and Kilty 2008; Cohen et al. 2009; Hunsaker and Leid 2008). Numerous investigators have reported the presence of biofilms in the sinuses of patients with CRS and consider biofilm as a cause for the recalcitrant nature of persistent CRS (Marple et al. 2009; Desrosiers and Kilty 2008; Cohen et al. 2009; Hunsaker and Leid 2008; Sanderson et al. 2006; Palmer 2005; Harvey and Lund 2007). The presence of *H. influenzae*, *S. pneumoniae* and *S. aureus* biofilms have been reported to be present in patients with an unfavorable treatment outcome after aggressive antibiotic therapy and surgery for CRS (Cohen et al. 2009; Sanderson et al. 2006; Sanclement et al. 2005). Antibiotic resistant strains of these bacteria also significantly contribute to poor clinical results with the presence of antibiotic resistant bacteria in clinical isolates as high as 30 % (Stewart and Costerton 2001). CRS with its chronic indolent course, resistance to antibiotics and acute exacerbations has a clinical course that parallels that of other persistent biofilm related inflammatory diseases (Stewart and Costerton 2001).

Significant preclinical studies have been performed demonstrating the safety and effectiveness of methylene blue and 670 nm aPDT (Sinuwave Inc., Bothell, WA) for treatment of CRS (Biel et al. 2011a; Biel et al. 2012; Biel et al. 2013). Osaki described the regression of polypoid rhinosinusitis in a dog after benzporphyrin derivative PDT with control of disease at 10 months (Osaki et al. 2012).

Desrosiers described the results of a case series of patients with recurrent CRS who failed medical and surgical therapy treated with methylene blue and 670 nm aPDT using the Sinuwave System (Sinuwave, Inc., Bothell, WA). Twenty nine sinuses (13 frontal, 6 ehtmoid and 10 maxillary) were treated in nine patients. Four patients underwent two treatment sessions. Results of treatment demonstrated that 7 of 9 patients had good or excellent clinical improvements as well as improvement in well being. Three patients had a sinus that did not respond to aPDT treatment. At 6 month follow up, one patient developed recurrent polyps at 5 months post aPDT treatment with persistent resolution of disease in the remaining patients who initially responded. The treatment was performed with topical anesthesia in an ENT office setting and was well tolerated with no evidence of mucosal irritation or pain. One patient developed transitory numbness to the cheek that lasted less than 24 h and resolved spontaneously (Desrosiers et al. 2013). Controlled multi-institutional clinical trials are presently underway. CRS aPDT using the Sinuwave System has regulatory approval for human treatment of CRS in Canada.

12 Conclusion

Antimicrobial PDT has been demonstrated in multiple human case series to be a promising clinical treatment of various bacterial, fungal, viral and protozoan diseases. Although there are to date relatively few regulatory approvals for aPDT treatment of human diseases, a number of

controlled multi-institutional clinical trials are underway to assess the effectiveness of aPDT as compared to standard of care therapies of various bacterial biofilm related diseases.

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Anti-biofilm Agents in Control of Device-Related Infections

9

Salman Ahmed and Rabih O. Darouiche

1 Introduction

> For every complex problem there is a simple solution… and it is wrong.
>
> H. L. Mencken

Biofilms pose a remarkable challenge to physicians for their diverse biochemical properties and varied presentation. Treatment strategies for biofilms are based on understanding these properties and must be tailored to the patient, implanted medical device, and organism(s) of concern (Table 9.1).

Biofilm formation occurs in a sequence beginning with adhesion of a planktonic, or free-floating, bacterium, to a favorable surface. It is there that the bacterium replicates, and along the way, undergoes important genetic and proteomic changes while the bacterium elaborates an extracellular matrix (Hoiby et al. 2010). Importantly, biofilms host an awesome diversity of phenotypic variants of the same bacterium. They may also host a variety of different bacterial species, making targeted antibiofilm therapy difficult (Donlan 2001).

The number of implanted medical devices has continued to rise, as have the number and severity of device-related infections. A decade ago, it was estimated that over 30,000,000 bladder catheters; 5,000,000 central venous catheters; and 2,000,000 fracture fixation devices were inserted annually in the United States alone (Darouiche 2001). Though less frequently implanted, the number of cardiovascular implanted devices is also large, and it is infections of these devices that cause the highest mortality: it is estimated that 450,000 vascular grafts; 300,000 pacemakers; 85,000 mechanical heart valves; and 700 heart assist devices are implanted in the United states, with prosthetic valve endocarditis carrying the highest risk of mortality of any implanted device infection. A more recent review states that over 150,000,000 intravascular devices are implanted in the United States yearly (Mermel et al. 2009).

Four major strategies are employed by healthcare facilities to reduce the risk, morbidity, and mortality associated with biofilm-associated infections (Aslam 2008). First, sterile precautions and aseptic technique during device placement are aimed at preventing infection. Second, anti-microbial or antibiotic-eluting devices may be used to prevent initial attachment of organisms to the implanted device. Third, agents may be infused to disrupt an established biofilm and eliminate the organism therein. Finally, removal of the infected device remains the definitive treatment strategy, though it comes with significant physical and psychological burden to the patient in the case of more complex implanted devices, such as fracture fixation hardware or mechanical heart valves.

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Table 9.1 Mechanisms of action of antibiofilm agents

Agent	Mechanism
Traditional antibiotics	
Minocycline	Inhibition of bacterial ribosome by binding to 30S subunit; blocks tRNA translocation
Tigecycline	Inhibition of bacterial ribosome by binding to 30S subunit; blocks tRNA translocation. Has glycylamido- substituent to reduce bacterial resistance
Daptomycin	Alteration of bacterial cell membrane
Linezolid	Prevents formation of initiation complex at bacterial ribosome by binding to 50S subunit
Vancomycin	D-alanine-D-alanine binding prevents synthesis of polymers of N-acetylmuramic acid and N-acetylglucosamine in bacterial cell wall
Rifampin	Chain termination by blocking DNA-dependent RNA Polymerase
Non-traditional chemical inhibitors	
Dispersin B	Lysis of polysaccharide intracellular adhesin (PIA)
2-aminoimidazole	Zinc chelation
Furanone	Quorum sensing inhibition
N-acetylcysteine	Repletion of glutathione
Deoxyribonuclease I	Lysis of extracellular DNA
Ethylenediaminetetraacetate (EDTA)	Chelation of calcium, magnesium, and iron
Bacteriophages	
S. epidermidis bacteriophage 456	Lysis of bacterial cells
LYSMP	Increase dispersal of bacterial cells

Unique anti-biofilm agents are being evaluated for the prevention and treatment of infections of each of the device categories mentioned above. These agents can be divided into traditional antibiotics, non-antibiotic chemical inhibitors of biofilm formation, and bacteriophages.

2 Traditional Antibiotics

Antibiotics have defined the treatment of infectious diseases since the surreptitious discovery of penicillin by Fleming in 1928. In acute infections, proper diagnosis of the suspected organism can lead to rapid and remarkable results if the correct antibiotic is administered and maintained at therapeutic levels in the affected tissue. The twentieth century has seen dramatic advances in antibiotic discovery and design, with many previously common infections now easily treated. With advancements in public health, including sanitation, vaccines, and antibiotics, the life expectancy in many industrialized nations has doubled from the beginning of the twentieth century to the beginning of the twenty-first century. The next challenge to the designers and discoverers of antibiotics are biofilm-related infections, including biofilm-associated device infections.

Antibiotic treatment of device-related infections relies on traditional mechanisms of bacterial cell death, but also requires adequate delivery of the antibiotic to adherent, biofilm-producing bacteria. Currently, several established antibiotics are used for prolonged courses of treatment for various implanted device infections. For example, cefazolin, oxacillin, and vancomycin are routinely used for up to 6 weeks for the treatment of staphylococcal prosthetic valve endocarditis. For the purposes of this discussion, we will present several antibiotics that have shown promise for the treatment of infected devices in both in vitro and in vivo studies.

2.1 Minocycline

Minocycline is a bacteriostatic, second generation tetracycline possessing a dimethylamino-group that imparts favorable pharmacokinetic properties to the drug as well as greater ability to elude bacterial resistance mechanisms. Minocycline has better oral absorption and a longer half life than other members of the tetracycline class of antibiotics (Jonas and Cunha 1982). In addition, its enhanced lipophilicity allows for improved tissue penetration (Smilack 1999). Like the other tetracyclines, minocycline results in bacterial ribosome inhibition, specifically by binding to the ribosomal 30S subunit (Schnappinger and Hillen 1996).

In a recent study, the authors used an in vitro silicone disk model to compare the efficacy of minocycline with other anti-staphylococcal agents including vancomycin, daptomycin, tigecycline and linezolid (Raad et al. 2007). After a single day of exposure, minocycline produced the greatest reduction in colony-forming units per milliliter (cfu/ml) of methicillin-resistant *S. aureus* (MRSA) isolates. Similarly, when the same model was used to simulate antibiotic lock therapy with four-hour daily treatments for five days, minocycline ranked second only to daptomycin in the reduction of cfu/ml.

In vivo evidence of the efficacy of minocycline comes from a study of orthopedic joint implants in a mouse model of MRSA infection (Bernthal et al. 2010). Bioluminescence imaging after inoculation of affected knees containing an implanted joint showed reduction in bacterial burden in those mice with antibiotic-coated implants as opposed to those with control implants. Furthermore, the mice with coated joint implants had clinical evidence of less inflammation, with less guarding and greater functional status of the affected leg.

Clinical studies of the efficacy of an antibacterial envelope that contains the combination of minocycline and rifampin for implantation of cardiac implantable electric devices (CIED) are ongoing (Bloom et al. 2011). An initial study of 624 eligible procedures performed at 10 centers across the United States suggests that this antibiotic-eluting coating may reduce the risk of implanted device infection. However, the key limitation of this study was its relatively short follow-up period of 1.9+/−2.4 months.

2.2 Tigecycline

Tigecycline is the first glycylcycline antibiotic available for clinical use (Peterson 2008). It was specifically designed with a glycylamido- substituent that prevents bacterial efflux pump action. This substituent mitigates the efflux pump resistance mechanism common to many gram-positive organisms (Projan 2000).

Tigecycline has demonstrated in vitro and in vivo activity against Staphylococcal species (Aybar et al. 2012). In one study, 75 strains of biofilm-producing *S. epidermidis* were collected from patients in a Polish hospital and treated in vitro with either tigecycline alone or tigecycline and rifampin together (Sczuka 2014). The authors performed in vitro testing of 16 of these strains and found that the combination of tigecycline and rifampin resulted in two to four fold reduction in minimum inhibitory concentration and minimum bactericidal concentrations compared to tigecycline alone. Both tigecycline and rifampin are relatively well tolerated drugs and are therefore potential therapies for biofilms formed by both coagulase-producing and non-coagulase producing staphylococci.

In vitro analysis has also shown the ability of tigecycline to interfere in biofilm formation of *Enterococcus faecalis*, even at sub-inhibitory concentrations (Maestre et al. 2012). Similarly, tigecycline has been shown to be effective in vivo in one study that analyzed three groups of rats implanted with ureteral stents (Minardi et al. 2012). Those rats implanted with rifampin-coated stents and that subsequently received intraperitoneal tigecycline had the lowest bacterial burden on quantitative urine cultures following treatment.

In another study, in vivo analysis of prosthetic graft infections treated with tigecycline, with and without the quorum sensing inhibitor FS8, were studied in Wistar rats (Simonetti et al. 2013). FS8

is a unique derivative of the RNA-III inhibiting peptide, which is known to interfere with staphylococcal quorum sensing functions (Balaban et al. 2007). Rats underwent implantation of Dacron grafts inoculated with S. aureus, strain Smith diffuse, into the back subcutaneous tissue. Rats in the contaminated group were divided into three subgroups. Group 1 consisted of rats that were implanted with untreated grafts and treated with intraperitoneal tigecycline. Group 2 consisted of rats that were implanted with FS8-soaked grafts but did not receive treatment with intraperitoneal tigecycline. The third group consisted of rats implanted with FS8-soaked grafts and that also received intraperitoneal tigecycline. Infection burden was measured by sonication and quantitative agar techniques. In vitro studies showed that the addition of FS8-pre-treatment to tigecycline reduced MIC and MBC of tigecycline four-fold. While quantitative graft cultures showed that intraperitoneal tigecycline treatment resulted in a greater reduction in bacterial burden than FS8-pretreatment alone, the group that demonstrated the greatest reduction in bacterial burden was the FS8-pretreatment group that also received treatment with tigecycline. Studies like these suggest a potential role for quorum-sensing inhibitors as antibiotic-sensitizers for treatment of implanted device infections.

2.3 Daptomycin

Daptomycin is a naturally occurring bactericidal lipopeptide antibiotic derived from the organism *Streptomyces roseoporus*. It is currently used for treatment of systemic staphylococcal infection, and requires at least once-weekly monitoring of creatine kinase as the antibiotic has been associated with myositis. Its mechanism of action is thought to be due to calcium-dependent binding to bacterial cytoplasmic membranes, resulting in cell death (Silverman et al. 2003). It has been shown to have effective MIC's at peak serum concentration for the reduction in colony forming units present in in vitro cultures (Leite et al. 2011). However, daptomycin administration did not result in a reduction in biomass, unlike the effect of rifampicin, which caused a significant reduction in biomass despite the fact that it did not induce much greater reduction in colony forming units than either daptomycin or linezolid.

In vivo studies have also shown efficacy of daptomycin as a prophylactic antibiotic for prevention of implanted orthopedic hardware infection, even at low doses (Niska et al. 2012). It has been shown elsewhere that daptomycin is effective as antibiotic lock therapy in vascular catheters (Meije et al. 2014). This study examined one isolate each of methicillin-sensitive S. aureus (MSSA) and MRSA and found that high concentration daptomycin antibiotic-lock therapy (50 mg/L) achieved negative catheter tip cultures in central venous catheters implanted in rabbits.

Importantly, daptomycin has so far largely eluded *S. aureus* resistance mechanisms (Schaad et al. 2006). This fact enables clinicians to use daptomycin judiciously in treatment of severe staphylococcal infections, and especially in those cases where vancomycin susceptibility may be compromised. Even in cases where vancomycin susceptibility is present, daptomycin may have improved penetration into biofilm matrices for treatment of device related infections. In one model of difficult-to-treat rat tissue-cage infection, twice daily dosing of daptomycin resulted in reduced colony forming units of *S. aureus* strain I20 (a known strain associated with catheter-related sepsis), presumably secondary to an enhanced pharmacokinetic profile compared to the other antibiotics studied (oxacillin and vancomycin).

2.4 Linezolid

Linezolid is the only member of the oxazolidinone class approved for clinical use (Moellering 2003). This class of antibiotics inhibits ribosome function by disrupting the assembly of ribosomal subunits, thereby blocking protein synthesis (Swaney et al. 1998). Linezolid has demonstrated in vitro and in vivo activity against staphylococcal species in a variety of settings. In an experimental model of endocarditis in white male New Zealand rabbits, both linezolid and vancomycin

were noted to reduce bacterial load in valve vegetations, with linezolid exhibiting bactericidal effect (Tsaganos et al. 2008). This bactericidal effect of linezolid was seen in kidney, liver, and spleen segments that became secondarily infected in the setting of endocarditis; with the addition of rifampicin, the bactericidal spectrum of linezolid extended to include lung tissue as well. Though linezolid has known bacteriostatic effect, bactericidal action was achieved in this study as trough serum concentrations were approximately 2–2.5 times higher than in human serum, and the trough serum concentration remained above the MIC for the duration of treatment.

In a prospective cohort study of 161 patients with documented prosthetic joint infection, administration of linezolid and rifampicin cleared infection of prosthetic hip and knee implant joint infections at a rate of nearly 70 % in 49 patients, thereby preventing implant removal (Gomez et al. 2011). These patients received the combination of linezolid and rifampicin largely because of failure of previous antibiotic regimens, and a small proportion of patients had adverse events from previous antibiotics. The study echoed a finding common in treatment of orthopedic hardware infections: often, a lengthy duration of therapy is needed in order to achieve therapeutic success. The mean (standard deviation) duration of treatment was 80.2 (29.7) days. Adverse events noted were thrombocytopenia and anemia, two well known complications of linezolid; though in this study, neither adverse event warranted cessation of linezolid therapy.

2.5 Vancomycin

The antibiotic concentration required for elimination of bacteria in sessile form, as opposed to planktonic form, can be greater than 1,000-fold (Mah and O'Toole 2001). In order to enable concentrations to approach these levels in bone, meticulous surgical debridement and lengthy antibiotic courses are required for treatment of prosthetic hardware infections. One study recently examined the concept of impregnating bone cement with polymerizable derivatives of the traditional glycopeptides antibiotic vancomycin (Lawson et al. 2010). The authors followed a strategy that has been used frequently and with good effect in many orthopedic settings (Anguita-Alonso et al. 2006). In fact, much work is being done on binding of vancomycin binding to titanium alloy in order to further reduce the risk of infection offered by titanium materials (Antoci et al. 2007). The authors found that use of this substance could reduce adherent bacteria numbers, but at the expense of several crucial mechanical properties, including compressive modulus, yield strength, resilience, and fracture strength.

2.6 Rifampin/Rifampicin

Rifampicin has bactericidal activity as it inhibits RNA synthesis by blocking the bacterial polymerase, engaging in chain termination (Campbell et al. 2001). Rifampicin has been shown to be effective in the treatment of Gram-positive and Gram-negative bacteria, as well as mycobacteria. Rifampin, for as yet-unknown reasons, has been observed to have excellent biofilm penetration and diverse tissue sites (Zavasky and Sande 1998). Indeed it has been shown to have the unique property of being able to kill staphylococci adherent to foreign material (Archer et al. 1983). However, resistance to rifampicin is a common phenomenon due to rapid mutations in the rpoB gene allowing decreased affinity of the rifampicin binding site on RNA polymerase, preventing rifampicin use as monotherapy (Aubrey-Damon et al. 1998). Therefore, rifampicin is nearly always used in combination with other antibiotics for the treatment of infection of a variety of implanted devices, most notably in the treatment of prosthetic valve endocarditis (Karchmer et al. 1983). In fact, treatment of prosthetic valve endocarditis may usually be accomplished with the same antimicrobial agent that would be used to treat an episode of native valve endocarditis due to the same organism, except in the case of staphylococcal prosthetic valve endocarditis. In this case, it is recommended that a dual or triple drug therapy be employed, at least one of which is rifampicin (Drinkovic et al.

2003). Overall, rifampin has been shown in multiple studies to be an excellent adjuvant treatment or pre-treatment that may be valuable as an antibiotic-sensitizer. Rifampin's utility extends across multiple biofilm-forming organisms and in a variety of device related infections.

3 Non-traditional Chemical Inhibitors

It is theorized that non-microbicidal mechanisms will most likely act synergistically with conventional antibiotics (Wu et al. 1998). In this vein, several agents with the capability to reduce biofilm biomass by other methods are under evaluation.

3.1 Dispersin B

One of the key elements of eliminating biofilms is to disrupt the polysaccharide matrix in which the biofilm-producing bacteria reside. Dispersin B is a B-hexosaminidase that has been shown to have activity against polysaccharide-based staphylococcal biofilms (Chaignon et al. 2007). Specifically, it lyses the polysaccharide intracellular adhesin (PIA) that is key to biofilm aggregation (Arciola et al. 2011). Dispersin B has been shown to reduce the incidence of S. epidermidis-induced biofilm formation in coated polyurethane surfaces (Marti et al. 2010). A combination of dispersin B and chlorhexidine produced a broad-spectrum antibiofilm agent which has shown in-vivo efficacy (Darouiche et al. 2009).

3.2 Aminoimidazole-Derived Antibiofilm Agents

A new class of non-microbicidal inhibitor has been shown, in vitro, to synergize with conventional antibiotics to remediate biofilm colonization and to re-sensitize multidrug resistant bacteria to the effects of conventional antibiotics (Rogers et al. 2010). It is thought that the 2-aminoimidazole compounds chelate zinc (Rogers et al. 2009). Specifically, the authors found that the addition of a derivative of 2-aminoimidazole, known as compound 1, significantly increased the potency of several commonly used drugs aimed at various biofilm-producing pathogens. For instance, the EC50 of novobiocin was reduced from 325+/−26 nM to 0.67 +/− 0.11nM with the addition of compound 1, suggesting an improvement in dispersal enhancement. And in the case of one particularly treatment resistant clinical isolate of multi-drug resistant *Acinetobacter baumannii* (MDRAB 3340), the addition of 75 uM of compound 1 reduced the imipenem MIC from 64 to 8 ug/ml.

3.3 Furanone

The furanone class of drugs is being evaluated as quorum-sensing inhibition is thought to be essential to the eradication of pseudomonal biofilms. Tympanostomy tube placement is the most common implanted medical device in the pediatric population. *Pseudomonas aeruginosa* infections of the device pose a source of potentially long-term morbidity, including hearing loss, in many children. In one in vitro study, furanone treatment alone did not result in reduction in colony counts from infected tympanostomy tubes. However, it had a synergistic effect with ciprofloxacin for treatment of tympanostomy tubes (Kim et al. 2012).

3.4 N-Acetylcysteine

Administration of N-acetylcysteine (NAC) restores glutathione stores in the body, allowing for a variety of anti-oxidant and anti-inflammatory effects. The compound has received attention as a potential adjuvant form of treatment for intravascular catheter-related infections. In a pilot study that enrolled 18 hemodynamically stable patients with catheter-associated infections, a catheter-lock solution containing NAC, tigecycline, and heparin was employed and resulted in an 83 % absence of bacteremia at ninety days (Aslam et al. 2007). Treatment of bacterial clinical isolates with a concentration of NAC 80 mg/ml resulted in bactericidal activity against clinical isolates of methicillin-sensitive and methicillin-resistant

S. aureus, *S. epidermidis*, vancomycin-resistant *Enterococcus faecalis* (VRE), *P. aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Candida albicans* and *C. krusei* as assessed by confocal microscopy measurements of biofilm thickness, number of non-viable cells, and fluorescent intensity (Aslam and Darouiche 2011).

3.5 Deoxyribonuclease I

Deoxyribonuclease I (DNase I) degrades extracellular DNA (eDNA), thereby weakening the structure and firmness of the stability of the polysaccharide biofilm (Kaplan 2009). Notably, DNase I is unable to disrupt established biofilms, but is able to inhibit biofilm formation in vitro if present in solution at the time of bacterial seeding. It is therefore thought to offer a means by which to sensitize biofilms to antibiotics, and also a preventative measure at the time of device implantation.

3.6 Ethylenediaminetetraacetate (EDTA)

Chelator therapy for treatment and prevention of biofilm associated devices is based on the findings that metallic cations are essential to bacterial cell wall growth as well as biofilm adherence (Sarkisova et al. 2005). Magnesium, for example, has been shown to increase adhesion and matrix formation by *S. epidermidis* (Dunne and Burd 1992). Iron plays an important role in cellular respiration and is also an important signal in biofilm formation; limiting its availability is therefore shown to restrict biofilm formation (Weinberg 2004). Chelators, including EDTA, are thought to prevent cell growth of planktonic organisms, prevent adherence to fibrin, and disrupt biofilm formation (Raad et al. 2008). EDTA offers the additional advantage of being a well known anti-thrombotic agent, a useful property for any implanted device (Percival et al. 2005).

In vitro efficacy of EDTA has been shown with regards to several organisms, including *P. aeruginosa*, *S. aureus*, and *S. epidermidis* (Raad et al. 2002). As an indicator of the potential use of chelator therapy for salvaging catheters infected with resistant organisms, a recent study indicated the efficacy of the combination of minocycline, EDTA, and 25 % ethanol solution as antibiotic lock therapy in eradicating several resistant organisms. The in vitro study included VRE, multi-drug-resistant *P. aeruginosa*, and MRSA (Raad 2008).

4 Bacteriophages

It is hypothesized that bacteriophages may be of great utility in the treatment of biofilm-related infections because of their ability to produce depolymerases and other catalytic enzymes that interrupt bacterial polysaccharides (Richards and Melander 2009). Some of these enzymes have been observed to have in vitro activity against gram-negative bacteria (Kimura and Itoh 2004). One of the greatest advantages of bacteriophages is that, unlike antibiotics which must be delivered through the resistant biofilm matrix, bacteriophage replicates most actively at the site of infection (Smith and Huggins 1982). In fact, a single dose of bacteriophage could potentially eradicate an entire biofilm as progeny phage replicate through the biofilm organisms (Doolittle et al. 1996).

The *S. epidermidis* bacteriophage 456 was used to pre-treat the internal lumen of hydrogel-coated silicone catheters in an in vitro model system. The authors showed "significantly reduced viable biofilm formation by *S. epidermidis* over a 24-h exposure period," on the order of log-CFU/cm2 reductions of 4.47 ($P < 0.0001$) and 2.34 ($P = 0.001$), with and without divalent cation supplementation, respectively (Curtin and Donlan 2006).

LySMP is the putative lysin enzyme of phage SMP, a dsDNA phage. It was shown to increase dispersal of *Streptococcus suis* strains SS2-4 and SS2-H in conjunction with antibiotic treatment; the combination of LySMP and antibiotic treatment resulted in synergistic killing of *S. suis* (Meng et al. 2011).

Bacteriophage cocktails, or admixtures of multiple bacteriophages targeted against specific organisms, also provide promising results

(Fu et al. 2010). In one study, phage M4 pre-treatment reduced colony forming units of *P. aeruginosa* but several strains of phage-resistant *P. aeruginosa* were found. These isolates were used to create a five-phage cocktail from a library of *Pseudomonas* phages; application of this phage cocktail resulted in elimination of >99 % of bacteria in the in vitro model biofilm.

5 Future Directions

The need for antibiofilm agents for the prevention, treatment, and control of device related infections has never been so pressing. The mortality, morbidity, and cost associated with these infections affect a large and increasing proportion of the population. Several agents are under evaluation, both among conventional antibiotics and agents with non-microbicidal modes of action. Synergy between multiple conventional antibiotics or between antibiotics and other agents will likely come to define the treatment approach towards device-related infections. Though several in vitro studies have been performed, in vivo studies remain scarce, and human studies even more scarce. Some of the challenges faced include the difficulty in creating a model biofilm for in vivo use and the propensity to opt for removal of infected devices in ill patients in whom experimental treatment may not be a viable or desirable option.

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Lipid- and Polymer-Based Drug Delivery Carriers for Eradicating Microbial Biofilms Causing Medical Device-Related Infections

10

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1 Introduction

1.1 Medical Devices

According to US Food and Drug Administration (USFDA), a medical device is an instrument, apparatus, implant, in vitro reagent, or similar or related article that is used to diagnose, prevent, or treat disease or other conditions, and does not achieve its purposes through chemical action within or on the body (which would make it a drug) (Extracted from the FDA's definition as per http://www.fda.gov/medicaldevices/deviceregulationandguidance/overview/classifyyourdevice/ucm051512.htm, accessed on 16, April 2014). Whereas medicinal products (also called *pharmaceuticals*) achieve their principal action by pharmacological, metabolic or immunological means, medical devices act generally by other means like physical, mechanical, or thermal means. Between 2004 and 2009, sales of medical devices increased by 56 %, while pharmaceutical sales rose by 38 % during the same period (O'Keeffe 2011). Medical devices vary greatly in complexity and application. Examples range from simple devices such as tongue depressors, medical thermometers, disposable gloves and bedpans to advanced devices such as complex programmable pacemakers with micro-chip technology, laser surgical devices, computers which assist in the conduct of medical testing, implants and prostheses. The medical device industry, which includes product areas such as cardiovascular and orthopedic devices, wound care products, disposable supplies, and durable equipment, is a US$200 billion business worldwide, with projected sales of US$95 billion in 2010 in the US alone (O'Keeffe 2011). Thus, medical devices are important health care innovations, enabling effective treatment using less invasive techniques, and improving health care delivery and patient outcomes, but they can also be harmful to patients. An audit conducted by the UK National Patient Safety Agency reported that device-related incidents are caused by device failure (43.8 %), inappropriate use (29.3 %), lack of training (12.3 %), and poor maintenance (1.5 %) (Vincent et al. 2001).

1.2 Classification of Medical Devices

The medical devices when they implanted or inserted into human body become the sites of competition between host cell integration and microbial adhesion while exerting the intended purpose like salvage of normal functions of

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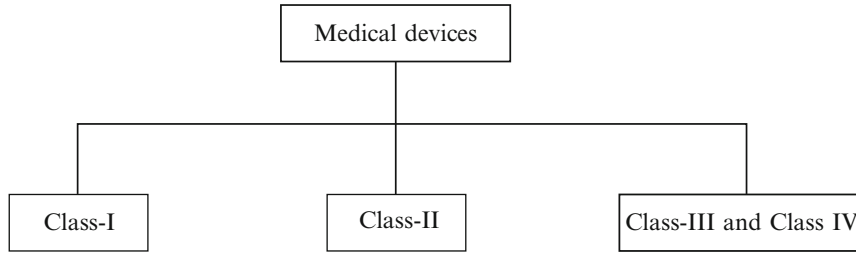

Fig. 10.1 Classification of medical devices

Table 10.1 Risk levels associated with classes of medical devices

Device class	Risk level	Examples
Class I	Lowest	Reusable surgical scalpel, bandages, culture media
Class II	Low	Contact lenses, epidural catheters, pregnancy test kits, surgical gloves
Class III	Moderate	Orthopaedic implants, glucose monitors, dental implants, haemodialysis systems, diagnostic ultrasound systems
Class IV	High	HIV test kits, pacemakers, angioplasty catheters

Extracted from: http://www.oag-bvg.gc.ca/internet/English/parl_oag_201106_06_e_35374.html. Accessed on March 15, 2014

vital organs. The regulatory authorities, therefore, recognize different classes of medical devices, based on their design complexity, their use characteristics, and their potential for harm if misused. Each country or region defines these categories in different ways. Figure 10.1 and Table 10.1 show classification of medical devices and risk levels associated with classes of medical devices, respectively. In general, class I devices present the lowest potential risk and do not require a license (non-critical type), class II devices require the manufacturer's declaration of device safety and effectiveness (semi-critical type), class III and class IV devices present a greater potential risk and are subject to in-depth scrutiny (critical type).

In addition, the regulatory authorities also recognize that some devices are provided in combination with drugs, and regulation of these combination products takes this factor into consideration. By definition, all antimicrobial-coated devices are indeed/therefore considered as combination product. Thus, if a catheter is coated with silver – probably the most common type of coating today, particularly for urinary catheters – that's considered to be a combination product. It combines an antimicrobial and an underlying device.

For the most part, device-antimicrobial combinations are used to prevent infections that are associated with the underlying use of the device. The idea behind a device-antimicrobial combination is often to either inhibit microorganisms from attaching to the device or to inactivate or kill microbes if they came into contact with the device.

1.3 Microbial Biofilms Causing Medical Device-Related Infections

Microbial biofilms are microcosms attaching irreversibly to abiotic or biotic surfaces and are promulgated as congregates of single or multiple microbial population (Tamilvanan et al. 2008).

More specifically, the medically-relevant biofilms consisted of a discrete association between a manmade device and bacteria or, less frequently, fungi. Although microbial colonization and

subsequent biofilm formation can also form on living tissues as in the case of endocarditis, dental caries, periodontitis, otitis media, biliary tract infections, and bacterial prostatitis, these established infections could be termed as nondevice-related chronic infections. Moreover, it has also been estimated that over 60 % of bacterial infections currently treated in hospitals are caused by bacterial biofilms (Costerton et al. 1999). However, this book chapter explores the potential of lipid-and polymer-based drug delivery carriers to eradicate microbial biofilms causing medical device-related nosocomial infections.

According to a patent (Liliana et al. 2008), the (substrate) materials used to manufacture medical devices can be made with a variety of polymeric, ceramic and metallic materials, as well as combinations of two or more of the same (e.g. hybrid materials). Since the medical devices are routinely being made up of many different types of surfaces, the physicochemical characteristics of the materials play a major role to act as a substrate for microbial attachment and colonization. Hence, the key to preventing device-related infections is to prevent bacterial attachment and subsequent biofilm formation. Table 10.2 shows the comprehensive lists of biomaterials used to make medical devices, and common causative organisms of biofilm to produce device related nosocomial infections.

Microbial adherence and biofilm production proceed in two steps: first, an attachment to a surface and, second, a cell-to-cell adhesion, with pluristratification of microorganisms onto the biomaterial surfaces like medical devices. Though development of device-related nosocomial infections begins with colonization of the medical device material, followed by a complex metamorphosis by the microorganisms with resultant biofilm formation, the microbial source for these infections may be acquired either from indigenous microbiota in vivo or from those micros present in exogenous sources in vitro (Rutala and Weber 1997). It has also been suggested that hospital water distribution systems are one of the most overlooked, important and

Table 10.2 Comprehensive but not exhaustive lists of substrate materials taken for comparative evaluation of microbial attachment and common causative organisms of biofilm responsible to produce device-related nosocomial infections

Biostable & biodegradable polymers	Names of pathogenic organisms including bacteria, fungi and yeast
Glass	*Staphylococcus aureus*
Ceramics	*Staphylococcus epidermidis*
Mild steel	*Staphylococcus haemolyticus*
Stainless steel	*Streptococcus mutans*
Copper	Coagulase-negative staphylococci (CoNS)
Silver	*Enterococcus faecalis* and *E. faecium*
Natural rubber latex	*Burkholderia cepacia*
Silicone	*Pseudomonas aeruginosa*
Plastics	Corynebacterium spp.
Polybutylene	Propionibacterium spp.
Polyethylene	Bacillus spp.
Polypropylene	Micrococcus spp.
Polyurethane	Enterobacter spp.
Ethylene-propylene	Serratia spp.
Polyvinyl chloride (PVC)	*Bacteroides fragilis*
Chlorinated PVC	*Mycobacterium fortuitum* and *M. chelonei*
Unplasticized PVC	*Escherichia coli*
	Proteus mirabilis
	Klebsiella pneumoniae
	Acinetobacter baumannii
	Stenotrophomonas maltophilia
	Candida albicans
	Non-albicans Candida spp.
	C. parapsilosis
	C. glabrata
	C. krusei
	C. tropicalis
	C. guillermondii
	C. dubliniensis
	C. lusitaniae
	HACEK group of organisms
	Haemophilus aphrophilus
	H. paraphrophilus
	Actinobacillus actinomycetemcomitans
	Eikenella corrodens
	Rhodotorula spp. *Hansenula anomala*, Aspergillus spp.

controllable sources of hospital-acquired infections (HAI) (Anaissie et al. 2002). According to Cozad and Jones (2003), environmental surfaces could harbor pathogenic organisms that are able to cause infectious diseases. The Centers for Disease Control and Prevention (CDC) in their "Guidelines for Environmental Infection Control in Health-Care Facilities" pinpoints the evidence of nosocomial infections associated with fomites (Sehulster and Chinn 2003). Brady et al. (2003) emphasized the increase of the role of contaminated surfaces as a potential source for infectious diseases. The majority of device-related infections, thus, occur in the early post-operative period and are likely due to contamination at the time implantation or insertion. Even though the chances of obtaining tailor-made medical devices in a sterile package are highly possible, the maintenance of the sterility of medical devices especially after opening from their package during implantation or insertion particularly in hospital premises is somehow always speculative. Because of the eventual dissemination of blood, saliva, urine, and other secretions due to the routine health care practices, the microorganisms could be spread throughout the environment even after the adaptation of some essential precautionary/preliminary disinfection and sterilization procedures within the hospital premises. Examples of principal medical devices which could be affected by biofilm associated infections include: central venous catheters, heart valves, ventricular assist devices, coronary stents, neurosurgical ventricular shunts, urological medical devices, implantable neurological stimulators, arthro-prostheses, fracture-fixation devices, inflatable penile implants, breast implants, cochlear implants, intraocular lenses and dental implants (Costerton et al. 2005).

Clinical manifestations of device-related infections are very vulnerable which include life-threatening systemic infections and device malfunction that may require device removal, urological medical devices-crystalline encrustation and pain, increased morbidity and mortality, additional hospital cost to patient, blocking encrustation and mechanical failure or fracture, etc. Furthermore, a sequence of events that leads to the foreign body reaction following implantation of a medical device, prosthesis, or biomaterial is also presented in a review (Anderson et al. 2008). It should be emphasized that the application of new microscopic and molecular techniques has revolutionized our understanding of biofilm structure, composition, organization and activities. This results in the development of novel preventive strategies for the treatment of biofilm-related diseases. A very elegant approach comprises the inhibition of regulatory systems that govern the expression of a series of bacterial virulence factors: for example, antiadhesion therapy [passive antibody therapy (Martinez and Casadevall 2005; Casadevall et al. 2004)], and synthetic peptide vaccine and antibody therapy (Cachia and Hodges 2003), inhibiting or negating cell-cell signaling (Otto 2004), negating biofilm formation by disrupting iron metabolism (Kaneko et al. 2007), and up-regulation of biofilm detachment promoters (rhamnolipids) (Boles et al. 2005). A patent describes some new molecules that are capable of inhibiting rhamnolipids and especially epithelial infiltration by gram-negative bacteria (Zulianello and Meda 2009). In particular, the invention provides proteins useful in the prevention and/or treatment of gram-negative bacteria induced infections or disorders, including nosocomial infections. Due to space constraints, I urge the reader to explore the many excellent literature reviews of medical biofilms that are directed on topics like overall biofilm formation and infection (Reisner et al. 2005a, b), biofilm diagnosis and treatment (Fux et al. 2003), general survival strategies of infectious biofilms (Foster 2005; Fux et al. 2005), biofilm antibiotic/antimicrobial resistance (Stewart 2002), quorum sensing control of biofilm formation (Bjarnsholt and Givskov 2007), and biofilm issues related to specific infections, such as: cystic fibrosis (Høiby 2002), osteomyelitis (Brady et al. 2008), otitis media (Bakaletz 2007; Post et al. 2007), and dental caries and oral infections (Offenbacher et al. 2007).

To control microbial colonization of- and subsequent biofilm formation onto- the medical devices, different approaches either to

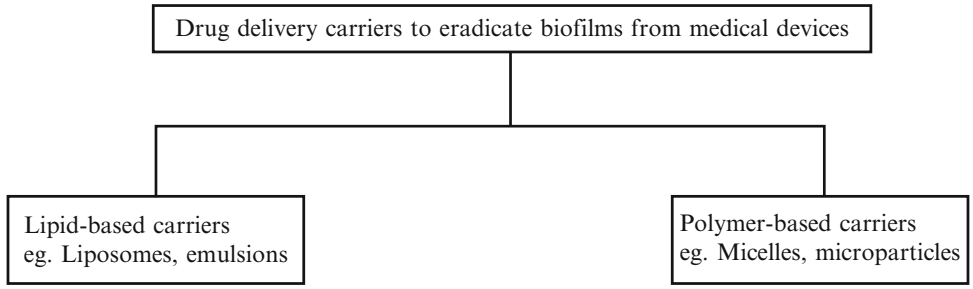

Fig. 10.2 Various drug delivery carriers

enhance the efficiency of certain antimicrobial agents or to disrupt the basic physiology of the pathogenic microorganisms including novel small molecules and antipathogenic drugs are being explored. In addition, the various lipid- and polymer-based drug delivery carriers (Fig. 10.2) are also investigated for applying antibiofilm coating onto the medical devices especially over catheters. The intention of this book chapter is to summarize the major and/breakthrough inventions disclosed in patent literature as well as in research papers related to microbial colonization of medical devices and novel preventive strategies. Therefore, both published patents and lab-based research papers are taken into consideration for the organization of this manuscript. In order to access the full-text and claim language of the respective patents, the readers should do an online search using the patent numbers provided in the reference list.

1.4 Objectives

The major objectives of this chapter are to emphasize the dearth of available information concerning the lipid-and polymer-based drug delivery carriers in eradicating the microbial biofilm development over the inserted or implanted medical devices, to describe the application potentials of these carriers in developing such microbial-resistant medical devices upon real clinical phase experiments and to suggest future research on this very important and under-researched topic.

2 Lipid-and Polymer-Based Drug Delivery Carriers

The introduction of lipid-and polymer-based drug delivery carriers in pharmaceutical sciences or other allied disciplines helps the physician to achieve the required therapeutic concentration of the drug at the diseased region of the body while minimizing drug exposure to nondiseased normal organs. To extent the pharmaceutical knowledge gained over the decades on these drug delivery carriers to medical biofilm prevention and eradication, it has become necessary first to review/focus the already developed strategies for prevention of device-related nosocomial infections. Recommended technological and nontechnological strategies in conjunction with electrical (Pickering et al. 2003; Stewart et al. 1999; Raad et al. 1996b, c; Costerton et al. 1994), ultrasound (Carmen et al. 2004a, b; Pitt and Ross 2003; Rediske et al. 2000) and photodynamic (Wainwright and Crossley 2004; O'Neill et al. 2002; Gad et al. 2000; Soukos et al. 2000) stimulation either to enhance the efficiency of certain antimicrobial agents or to disrupt the basic physiology of the pathogenic microorganisms, including novel small molecules and antipathogenic drugs, are already being explored or under exploration process. In addition, assimilation of antimicrobials in targeted drug delivery machinery has also been utilized to counter biofilms-related medical infections. Precisely, the potential of lipid-and polymer-based drug delivery carriers to eradicate biofilms from device-related nosocomial infections are therefore considered in three main categories (as shown in Fig. 10.3).

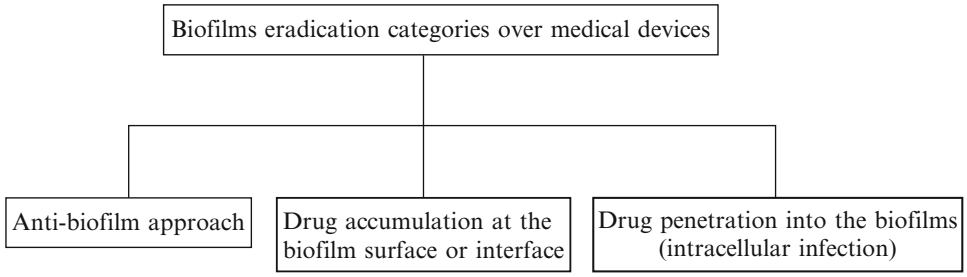

Fig. 10.3 Different categories to eradicate biofilms over medical devices

Table 10.3 illustrates a non-exhaustive and selected list of lipid-and polymer-based drug delivery carriers investigated for medical device- and non-device-related nosocomial infections. Uni-and multi-lamellar liposomes are covered in this book chapter as lipid-based systems. The selected examples for polymer-based drug delivery carriers include: implantable matrices, microparticles, fibrous scaffolds, micells and thermoreversible gels, and surface modified polymeric materials having antimicrobial/antiseptic/silver-coatings onto them. It should be added that most of these selected drug delivery carriers are prepared from biodegradable polymers like poly(lactide) (PLA) and its co-polymers with glycolide (PLGA). Some of the delivery systems such as micells and thermoreversible gels are obtained from poloxamer 188 and poloxamer 407 (also known as Pluronics® or Lutrols®). The poloxamers are a well-studied series of commercially available, non-ionic, triblock copolymers with a central block composed of the relatively hydrophobic poly(propylene oxide) flanked on both sides by blocks of the relatively hydrophilic poly(ethylene oxide) and possess an impressive safety profile and are FDA approved selectively for pharmaceutical and medical applications, including parenteral administration (Kabanov et al. 2002; Nace 1996). Another concept for the prevention of device-related infections involves the impregnation/coating of devices with various substances such as antibacterials, antiseptics and/or metals Surface-modified polymeric devices with impregnation/coating of various substances such as antibacterials, antiseptics and/or metals are also covered briefly in this chapter.

2.1 Liposomes as Drug Delivery Carriers to Biofilms

Liposomes are attractive as drug delivery/targeting vehicles by virtue of their compatibility with biological constituents and the range and extent of pay loads that they can carry. Liposomes have the potential to carry hydrophobic and hydrophilic drugs over long periods of time and also to decrease drug side effects by protecting the environment from direct contact with the drugs. It is illustrated in the liposomal literatures that liposomes need to be stable when used as drug delivery tools in vivo. There are three forms of liposome stability to consider in relation to drug delivery: chemical, physical and biological stabilities. Stability can be controlled by manipulating factors, such as pH, size distribution and ionic strength, or by using the alternative method of coating liposomes with inert hydrophilic polymers (Stealth® liposomes).

A new approach for achieving chemical and physical liposome stabilisation was developed by adsorbing them on solid surfaces, like zinc citrate (Catuogno and Jones 2003). When liposomes are adsorbed on solid surfaces, adsorption is irreversible. Liposomes can either disrupt on adsorption or adsorb intact or a combination of the two processes can occur. However liposomes adsorbed predominantly intact on solid particulates of zinc citrate.

Apart from the above-described modifications on native liposomes, surface (charge)-modified liposomes (cationic or anionic liposomes), have been recognized as an interesting and promising delivery vehicle for active and passive drug targeting purposes even with or without ligand/antibody attachment onto their surfaces. In the

Table 10.3 A non-exhaustive and selected list of lipid-and polymer-based drug delivery carriers investigated for medical device- and non-device-related nosocomial infections

Drug delivery carriers	Indication	Reference
Liposomal ciprofloxacin hydrogel (LCH)	Coating onto silicone foley catheters (in vitro and in vivo)	DiTizio et al. (1998), Finelli et al. (2002), Pugach et al. (1999)
Cationic liposomes	In vitro against immobilized biofilm formed by microorganisms	Ahmed et al. (2002)
Solid supported vesicles (SSV) containing either triclosan or penicillin-G	Zinc citrate supported liposomes against oral biofilm microorganisms (in vitro)	Catuogno and Jones (2003)
Stealth liposomes containing ciprofloxacin	In vitro and in vivo against pneumonial biofilm forming microorganisms	Bakker-Woudenberg et al. (2002)
Ambiosome®	Against candida infections (systemic candidasis)	Ambiosome fact sheet, http://www.astellas.us/docs/ambisome.pdf accessed on 11-01-2014
Antibiotic-eluting medical devices	Bone cements, fillers and coatings for orthopedic applications, wound dressings based on synthetic and natural polymers, intravascular devices, vascular grafts and periodontal devices	Zilberman and Elsner (2008)
Palacos bone cement with gentamycin and Cobalt™ G-HV (Biomet), Palacos® G (Biomet), DePuy 1 (DePuy Orthpedics), Cemex® Genta (Exactech), VersaBond™ AB (Smith and Nephew) which contain gentamicin and Simplex® P (Stryker Orthpedics) which contains tobramycin	Against biofilm microorganisms in joint infections	Buchholz and Engelbrecht (1970), Wahlig and Buchholz (1972), Buchholz and Gartmann (1972), Hanssen (2004)
Eptacin™, a biodegradable polyanhydride implant in the form of linked beads containing gentamicin	For local delivery of the antibiotic to infected bone	Li et al. (2002)
An injectable biodegradable synthetic polymeric device made of poly(sebacic-co-ricinoleic-esteranhydride) having gentamycin	For treatment of established Osteomyelitis	Krasko et al. (2007)
"Biomimetic" coating technology (hydroxyapetite-coated implant) with tobramycin	For treatment of Bone-implant-related infections	Chai et al. (2007), Alt et al. (2006)
Gentamicin poly-(DLlactic)-coated tibial nails	Internal fixation of open tibial fractures	Raschke and Schmidmaier (2004), Schmidmaier et al. (2006)
Polyhydroxyalkanoates incorporated with Sulperazone® (cefoperazone) and Duocid® (ampicillin) in the form of rods	Implant-related osteomyletis in rabbits	Gürsel et al. (2001)
"Structured films" Poly(L-lactic acid) (PLLA) and poly(DL-lactic-co-glycolic acid) (PDLGA) films containing gentamicin	To be applied on the surface of any metallic or polymeric fracture fixation device, and can therefore make a significant contribution to the field of orthopedic implants	Aviv et al. (2007), Zilberman et al. (2009), Zilberman and Malka (2009)

(continued)

Table 10.3 (continued)

Drug delivery carriers	Indication	Reference
Cylindrical composite pellets (1.0×0.9 mm) from bioabsorbable poly(d/l-lactide) matrix and ciprofloxacin	To be applied as a coating onto implant surfaces	Koort et al. (2006)
Pluronic F-127 thermoreversible gels for antibiotic delivery	To be applied as a coating onto implant surfaces and for treatment of methicillin-resistant *S. aureus* otitis media	Lee et al. (2004)
Polyvinylpyrrolidone-coated polyurethane catheter (called as Hydrocath®)	A catheter with reduced microbial adhesion, and to be used for various medical applications	Tebbs et al. (1994)
Cook Spectrum™ catheter (Cook Critical Care, Bloomington, IN, USA) and is coated on the inner and outer surface with minocycline and rifampicin	Broad-spectrum activity against gram-negative and -positive organisms and *C. Albicans*	Raad et al. (1995, 1996a, b, c, 1997a, b), Johnson et al. (1999)
Chlorhexidine and silver sulfadiazine (CHSS) catheter	A significant reduction in catheter colonisation	Quesnel et al. (1978), Brun-Buisson et al. (2004)
PET (polyethyleneterephtalate, Dacron™) and ePTFE (expanded polytetrafluorethylene) vascular prostheses soaked in an antibiotic solution or in cyclodextrins	Reduction in microbial adherence on the vascular grafts	Richardson et al. (1970), Galdbart et al. (1996), Blanchemain et al. (2007)
Erlanger' silver and Oligon' catheters (silver-coated catheters)	Significantly lower rate of catheter-related bloodstream infection (CRBI)	Böswald et al. (1999), Stoiser et al. (2002)
MgF_2-coated catheter surfaces	To inhibit bacterial colonization	Lellouche et al. (2009)

context of antibiotics and treatment of infection, liposomes have been studied for their ability to act against colonising microorganisms (DiTizio et al. 1998), to concentrate agents at biofilm interfaces (Kim et al. 1999; Jones et al. 1997) and also to be taken up into cells harbouring intracellular pathogens (Pinto-Alphandary et al. 2000; Nightingale et al. 1993; Majumdar et al. 1992; Eduardo et al. 1987; Fountain et al. 1985).

2.2 Liposomes to Reduce Microbial Adhesion/ Colonisation onto Medical Devices

Taking into consideration of the point that many catheter-related infections are due to skin organisms acquired at the time of catheter insertion, anti-colonisation strategies are still worth exploring. There is evidence that the intrinsic properties of a material might be of advantage regarding resistance to infection. Thus, improvement of the surface texture, tailoring the protein adsorption characteristics and improving the antithrombogenicity of a given material would be key factors in the development of innovative, infection resistant materials. However, this goal has to be achieved even after insertion of the devices into the bloodstream and despite the ever-occurring interactions of the device surface with host factors such as proteins and cells.

The surfaces of the medical devices are simply modified with the application of external coating substances onto them. For example, surfaces containing immobilized long-chain N-alklyated polyvinylpyridines and structurally

unrelated N-alklyated polyethylenimines were lethal to *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli*. The structure-activity analysis revealed that for surfaces to be bactericidal, the immobilized long polymeric chains have to be hydrophobic, but not excessively so, and positively charged (Lin et al. 2002).

Alternatively, there are many instances where plain broad-spectrum antimicrobials (without any carriers) have been incorporated into the device (Donelli et al. 2002; Bach et al. 1999; Schierholz et al. 1998; Sheretz et al. 1993; Tebbs and Elliott 1993). These are then eluted with the aim of preventing biofilm formation by killing early colonizing bacteria. For example, rifampin and amoxicillin have been incorporated on a polyurethane surface through introduction of polymer side-chain functional groups. This results in bacterial inhibition that can persist for several months, especially from rifampin-coated polymer (An et al. 2000a). It may be noted that antimicrobial efficacy is dependent on surface type. Incorporation of antimicrobials on an unstable surface may cause rapid release of drug. To address this, triggered release in the presence of infection has been developed. For example, neutrophil-derived factors can achieve triggered ciprofloxacin release. Similarly, macrophage-derived enzyme cholesterol esterase recognizes hydrophobic moieties and achieves drug release in polyurethanes surfaces synthesized with 1,1 diisocyanatododecane with long hydrophobic monomers (Piozzi et al. 2004). Despite enhanced bacterial killing, incorporation of antimicrobials on surfaces is limited by encapsulation efficiencies (Woo et al. 2002). Ideally, high levels of antimicrobial incorporation on modified surfaces should not affect material properties of the surface. However, a failure can cause contrasting outcomes. Sufficient antibiotic must be incorporated for the "user-lifetime" of the device and such incorporation must not damage the properties of the material, for example, lubrication, lifetime and host compatibility. There is also the nagging concern that low levels of antimicrobials could favour acquisition of antibiotic resistant organisms (Danese 2002). Worryingly, in staphylococcal species commonly associated with device-related infections, sub-inhibitory concentrations of tetracycline and quinupristin-dalfopristin enhanced biofilm development by increasing expression of the intercellular adhesion *ica* locus (Rachid et al. 2000). Furthermore, antibiotic pressure can induce the viable but non-culturable (VBNC) state in *S. aureus* growing in biofilms (Pasquaroli et al. 2013). Similarly, sub-inhibitory concentrations of tetracycline and quinupristin-dalfopristin may favor Staphylococcus biofilm formation, as well as development of antibiotic resistant organisms (Donelli and Francolini 2001). One approach of addressing this is the sequestration of biological agents on the surfaces. For example, usnic acid a naturally occurring dibenzofuran derivative and a secondary lichen metabolite, has been sequestered into modified polyurethane to achieve comparatively superior antimicrobial activity against *S. aureus* and *P. aeruginosa* (Francolini et al. 2004). This compound was able to affect the morphology (thickness and roughness) of *P. aeruginosa* biofilm without inhibiting bacterial growth, and this phenomenon is presumably indicating its interference with bacterial signaling pathways (Francolini et al. 2004). Although this technology is in preclinical stages, and requires more investigation, preliminary data are very promising.

Among the other alternative drug delivery strategies that have been developed for anti-colonisation or anti-biofilm approach, a liposomal hydrogel that reduces bacterial adhesion to silicone catheter material is most promising. Liposomes containing ciprofloxacin are sequestered within a poly(ethylene glycol)-gelatin hydrogel. Bacterial adhesion was completely inhibited on catheter surfaces throughout a 7-day adhesion assay (DiTizio et al. 1998). Using peritoneum of male Sprague-Dawley rats, a new model of persistent *P. aeruginosa* peritonitis was developed. The ability of liposomal ciprofloxacin hydrogel (LCH)-coated silicone versus plain silicone to prevent bacterial colonization at optimal conditions was compared (Finelli et al. 2002). While Plain silicone coupons in all tested rats were colonized and peritoneal washings were

consistently culture-positive, LCH coupons removed after 7 days from the tested rats were sterile, as were the peritoneal washings, and there was no evidence of peritonitis. This indicates that the LCH-coated silicone resists colonization in this rat model of persistent *P. aeruginosa* peritonitis (Finelli et al. 2002).

Pugach and co-workers (1999) developed an antibiotic liposome (ciprofloxacin-loaded liposome) containing hydrogel for external coating of silicone foley catheters and evaluated its efficacy in a rabbit model. Their goal was to create a catheter that would hinder the development of catheter associated nosocomial urinary tract infections. They inserted either an untreated, liposomal hydrogel coated or a liposome hydrogel with ciprofloxacin coated 10 F silicone foley catheter into New Zealand white rabbits and challenged the system with 5×10^6 virulent *E. coli* at the urethral meatus twice daily for 3 days. Urine cultures were evaluated twice daily for 7 days. When urine cultures became positive, the rabbits were sacrificed and urine, urethral catheter and urethral tissue were cultured. The time to bacteriuria detection in 50 % of the specimens was double for hydrogel with ciprofloxacin coated catheters versus untreated and hydrogel coated catheters. A significant ($p = 0.04$) improvement in average time to positive urine culture from 3.5 to 5.3 days and a 30 % decrease in the bacteriuria rate for hydrogel with ciprofloxacin coated catheters were noted compared to untreated catheters. A significant benefit was realized by coating the extraluminal catheter surface with a ciprofloxacin liposome impregnated hydrogel. Therefore, this procedure will provide a significant clinical advantage, while reducing health care costs substantially.

For readers' interest, four antimicrobial urinary catheters are currently marketed in the United States. They are coated with silver alloy (3 latex- or silicone-base catheters) or nitrofurazone, a nitrofurantoin-like drug (1 silicone-base catheter). Johnson and colleagues (2006) have assessed, through randomized and quasi-randomized clinical trials, the currently marketed antimicrobial urinary catheters for preventing catheter-associated urinary tract infection. According to fair-quality evidence, antimicrobial urinary catheters can prevent bacteriuria in hospitalized patients during short-term catheterization, depending on antimicrobial coating and several other variables. A similar type of clinical trials was also conducted and evaluated to find out the efficacy of silicone-based, silver ion-impregnated urinary catheters in the prevention of nosocomial urinary tract infections (Srinivasan et al. 2006). Unlike previous trials of latex-based, silver ion-impregnated foley catheters and silicone-based, silver-impregnated foley catheters were not effective in preventing the nosocomial urinary tract infections. However, this study was affected by differences in the study groups. Prospective trials remain important in assessing the efficacy and cost-effectiveness of new silver-coated products. It has to be added that from the above-described two different clinical experiments, an identical clinical trial should also be conducted for antibiotics containing-liposomal hydrogel-coated medical devices in future.

2.3 Liposomes as Drug Delivery Carriers to Biofilm Interfaces

Jones and colleagues have studied extensively on the interaction between liposomes and bacterial biofilms (Jones 2005; Kim and Jones 2004; Ahmed and Jones 2003; Catuogno and Jones 2003; Ahmed et al. 2001, 2002; Robinson et al. 2001; Kim et al. 1999; Hill et al. 1997; Jones et al. 1997). Confocal laser scanning microscopy (CLSM) has been used to visualize the adsorption of fluorescently labelled liposomes on immobilised biofilms of the bacterium *S. aureus* (Ahmed et al. 2002). The liposomes were prepared with a wide range of compositions with phosphatidylcholines as the predominant lipids using the extrusion technique. They had weight average diameters of 125 ± 5 nm and were prepared with encapsulated carboxyfluorescein. Cationic liposomes were prepared by incorporating dimethyldioctadecylammonium bromide (DDAB) or 3, beta [N-(N1,N1 dimethylammonium ethane)-carbamoyl] cholesterol (DC-chol) and anionic liposomes were prepared by incorporation of

phosphatidylinositol (PI). Pegylated cationic liposomes were prepared by incorporation of DDAB and 1,2-dipalmitoylphosphatidylethanolamine-N-[polyethylene glycol)-2000]. Confocal laser scanned images showed the preferential adsorption of the fluorescent cationic liposomes at the biofilm-bulk phase interface which on quantitation gave fluorescent peaks at the interface when scanned perpendicular (z-direction) to the biofilm surface (x-y plane). The biofilm fluorescence enhancement (BFE) at the interface was examined as a function of liposomal lipid concentration and liposome composition. Studies of the extent of pegylation of the cationic liposomes incorporating DDAB, on adsorption at the biofilm-bulk phase interface were made. The results demonstrated that pegylation inhibited adsorption to the bacterial biofilms as seen by the decline in the peak of fluorescence as the mole% DPPE-PEG-2000 was increased in a range from 0 to 9 mol%. The results indicate that confocal laser scanning microscopy is a useful technique for the study of liposome adsorption to bacterial biofilms and complements the method based on the use of radiolabelled liposomes.

Using cationic liposomes prepared from dimyristoylphosphatidylcholine (DMPC), cholesterol and DDAB or anionic liposomes substituting DMPC with PI, Robinson et al. (2001) noted that each bacterium in the biofilm adsorbed independently and that the extent of adsorption of anionic liposomes was smaller. Interestingly, when targeting mixed biofilms of *Streptococcus sanguis* and *Streptococcus salivarius* by liposomes loaded with the bactericide triclosan, anionic liposomes were most effective against *S. sanguis*, but relatively ineffective against *S. salivarius* (Robinson et al. 2001). An additional approach has been to load antibacterials into liposomes adsorbed on the surface of zinc citrate particles, used in toothpaste formulations, to produce solid supported vesicles (SSV) containing either triclosan or aqueous-soluble penicillin-G. Anionic liposomes were prepared by incorporation of PI into DMPC liposomes and cationic liposomes were prepared by incorporation of DDAB and cholesterol into DMPC. Whilst zinc citrate is itself antibacterial, it was noted that particles and empty liposomes had an additional or synergistic effect, whereas particles and liposomally encapsulated antimicrobials had an inhibitory effect on each other against *Streptococcus oralis* biofilms (Catuogno and Jones 2003). Other oral hygiene approaches have included liposomal encapsulation of the enzyme glucose oxidase and horse radish peroxidase that generate hydrogen peroxide and oxyacids in the presence of their substrates. They were effective against *Streptococcus gordonii* biofilms in a manner dependent upon liposome-biofilm and substrate-biofilm incubation times (Hill et al. 1997).

Another work by Jones (2005) has described methods for using of liposomes to deliver bactericides to bacterial biofilms. Anionic liposomes, cationic liposomes and proteoliposomes with covalently linked lectins or antibodies are designed by the extrusion technique (vesicles by extrusion, VET). The liposomes are prepared from the phospholipid dipalmitoylphosphatidylcholine (DPPC), together with the anionic lipid PI or the cationic amphiphile DDAB together with the reactive lipid DPPE-MBS, the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) derivative of dipalmitoylphosphatidylethanolamine (DPPE). Proteins (lectin or antibody), after derivatization with *N*-succinimidyl-*S*-acetylthioacetate (SATA), can be covalently linked to the surface of the liposomes by reaction with the reactive lipid, DPPE-MBS. The physical and chemical characterization of the liposomes and proteoliposomes by photon correlation spectroscopy (PCS) and protein analysis, to determine the number of chemically linked protein molecules (lectin or antibody) per liposome, are described. The liposomes can be used for carrying oil-soluble bactericides (e.g., Triclosan) or water-soluble antibiotics (e.g., vancomycin or benzylpenicillin) and targeted to immobilized bacterial biofilms of oral or skin-associated bacteria adsorbed on microtiter plates. Techniques for the preparation of immobilized bacterial biofilms, applicable to a wide range of bacterial suspensions, and for the analysis of the adsorption (targeting) of the liposomes to the bacterial biofilms are given. The mode of delivery and assessment of antibacterial activity of

liposomes encapsulating bactericides and antibiotics, when targeted to the bacterial biofilms, by use of an automated microtiter plate reader, are illustrated, with specific reference to the delivery of the antibiotic benzylpenicillin encapsulated in anionic liposomes to biofilms of *S. aureus*. The methods have potential application for the delivery of oil-soluble or water-soluble bactericidal compounds to a wide range of adsorbed bacteria responsible for infections in implanted devices such as catheters, heart valves, and artificial joints.

2.4 Liposomes as Drug Delivery Carriers in Intracellular Infections

Infectious diseases caused by intracellular bacteria present a significant challenge to antibiotic therapy. Antibiotic treatment of these types of infections has been associated with high failure and/or relapse rates (Donowitz 1994; al-Orainey et al. 1990). Intracellular pathogens, whether obligate or facultative, can hide, reside and multiply within the phagocytic cells of the reticuloendothelial system (RES), and by virtue to their intracellular location, are protected from the actions of the immunological defence cells and of antimicrobial agents (Raoult 1996; Donowitz 1994; Holmes et al. 1966; Rous and Jones 1916). The ineffectiveness of conventional antibiotics against intracellular infections may also be attributable to poor drug penetration, limited drug accumulation in subcellular compartments and/or drug inactivation by acidity in subcellular compartments (Raoult 1996; Holmes et al. 1966; Rous and Jones 1916). These factors may explain why some antibiotics are bactericidal against extracellular bacteria in vitro, but are ineffective in killing intracellular forms of the bacteria (Raoult 1996; van den Broek 1989; Rous and Jones 1916). Since the current book chapter describes mainly the potential of liposomes for eradicating biofilm consortia on device-related nosocomial infections, the potentiality of liposomes on the treatment of biofilm-mediated intracellular infections are not elaborated and only a short outline is presented. Interested readers may find further details in this particular area through review articles (Gupta and Haq 2005; Salem et al. 2005).

Ciprofloxacin, a fluoroquinolone, is a potent and broad-spectrum antibiotic, and has good antibacterial activity against most gram-negative bacteria and gram-positive cocci. Ciprofloxacin has been shown to have a superior ability to penetrate most tissues compared to other antibiotics (Kuhlmann et al. 1998; Nix et al. 1991; Dalhoff 1989; Bergan et al. 1988), accumulates in macrophages (Easmon and Crane 1985a) and neutrophils (Easmon and Crane 1985b) and is bactericidal in low pH environment (Rastogi and Blom Potar 1990). These attributes contribute partly to ciprofloxacin being the drug of choice for the treatment of infectious diseases caused by intracellular pathogens. Furthermore, ciprofloxacin, when orally or intravenously administered, is known to reach such organs as liver, spleen, lungs and lymph nodes (Hanan et al. 2000), which are important infection sites for intracellular bacteria. However, ciprofloxacin does not preferentially accumulate well at these tissues and may therefore not reach high sustain therapeutic levels at these sites.

The spontaneous uptake of liposomes by cells of the RES following parenteral administration has been exploited to target antibiotics to those intracellular sites where parasitic bacteria reside, and by virtue of sustained release properties, extend the half-life of the drug in the body. Ciprofloxacin has been incorporated with high efficiency into DSPC/cholesterol liposomes and examined in a mouse model of *Francisella tularensis* (Wong et al. 2003). Intravenous injection of liposome-encapsulated ciprofloxacin resulted in increased drug retention in the lungs, liver and spleen compared with that of free drug. Aerosolized liposomal ciprofloxacin gave complete protection against a lethal pulmonary infection of *F. tularensis*, whereas free ciprofloxacin was ineffective (Wong et al. 2003). Caution should be exercised in extrapolating data as it is clear that liposomal efficacy is dependent on the infecting organism. Liposome-encapsulated ciprofloxacin, delivered intravenously, has been compared with free drug in a rat model of

S. pneumoniae pneumonia and, whilst serum and lung lavage levels were higher (peak and area under curve), survival rates were similar (Ellbogen et al. 2003).

An interesting development of the liposomal concept has been the use of pH-sensitive liposomes in a murine salmonellosis model (Cordeiro et al. 2000). Here, gentamicin encapsulated in liposomes including a pH-sensitive lipid fusion between unsaturated phosphatidylethanolamine (PE) and N-succinyldioleyl-PE gave 153- and 437-fold greater drug levels in the liver and spleen, respectively, compared with free drug. Overall, liposomal delivery was associated with 10,000-fold greater activity than that of free drug.

2.5 Stealth® Liposomes

There is an increasing interest in developing injectable liposomes that is not cleared quickly from the circulation when liposomes are designed to reach non-RES tissues in the vascular system, extravascular sites of action, or to act as circulating drug reservoirs. Because, it is well established that when colloidal drug delivery carriers like liposomes are mixed with blood, many plasma proteins, mainly the apolipoproteins, associate with the surface of these carriers. A number of factors have been reported to influence plasma protein-liposome interactions and clearance rates including surface charge, surface coatings and lipid doses (Semple et al. 1998). It has been shown that cationic liposomes exhibit extensive interactions with plasma, resulting in immediate clot formation at charge concentrations higher than 0.5 mmol/ml (Senior et al. 1991). The circulation time for these liposomes was in the order of minutes. These findings were further confirmed for other cationic liposome formulations showing significant serum turbidity and protein binding (Oku et al. 1996). These results were expected since the majority of plasma proteins carry a net negative charge at physiological pH. The ability of anionic liposomes to interact with blood proteins depends on the nature of the anionic lipid, mainly the composition of the acyl chain (Hernandez-Caselles et al. 1993). In addition, it was found that liposomes composed of neutral saturated lipids with acyl chains lengths greater than 16 carbons bound large quantities of blood proteins and were rapidly cleared from the circulation (Chonn et al. 1994). This phenomenon was attributed to the occurrence of hydrophobic domains at the surface of the vesicles. These vesicle-blood protein interactions also depend on the lipid dose administered. Increased lipid doses result in decreased protein levels on the surface of the liposomes and longer circulation time suggesting the occurrence of a saturable protein binding mechanism (Oja et al. 1996). Finally, the most widely used approach for enhancing the circulation time of liposomes is the inclusion of amphipathic poly(ethyleneglycols) (PEGs), with a typical molecular weight of 2,000–5,000, in the vesicle bilayers which decrease sterically the adsorption of plasma proteins onto the liposome surfaces (Allen et al. 1991; Klibanov et al. 1990). To honour this particular advanced property of PEG, the terms 'Pegylation and/or Pegnology' is coined in pharmaceutical and medical literatures (Harris et al. 2001; Bhadra et al. 2002).

Somewhat counter-intuitively, stealth approaches have been adopted for delivering antibiotics. Pegylated long-circulating liposomes loaded with gentamicin were superior to free gentamicin in a rat model of *Klebsiella pneumoniae* unilateral pneumonia/septicaemic (Bakker-Woudenberg et al. 2000). Studies in vitro have also confirmed that pegylation of liposomes reduced their affinity for *S. aureus* biofilms (Ahmed et al. 2001). Here, they found that liposomes prepared from the phospholipids DMPC, dipalmitoyl PC and distearoyl PC containing DDAB (cationic) or PI (anionic) and variable amounts of dipalmitoylphosphatidylethanolamine bonded to PEG of molecular mass 2000 (DPPE-PEG-2000), exhibited decreasing electrophoretic mobilities and zeta potentials with increasing DPPE-PEG-2000 incorporation. The adsorption of liposomes to *S. aureus* biofilms followed the Langmuir isotherm and both surface coverage and the magnitude of the Gibbs energy of adsorption decreased with the extent of pegylation (Ahmed et al. 2001). A study by Bakker-Woudenberg et al.

(2001) in an experimental *K. pneumoniae* pneumonia, the therapeutic potential of ciprofloxacin was significantly improved by encapsulation in polyethylene glycol-coated ("pegylated") long-circulating (STEALTH) liposomes. Pegylated liposomal ciprofloxacin in high doses was non-toxic and resulted in relatively high and sustained ciprofloxacin concentrations in blood and tissues, and hence an increase in the area under the plasma concentration-time curve (AUC). These data correspond to data from animal and clinical studies showing that for fluoroquinolones, the AUC/MIC (minimum inhibitory concentration) ratio is associated with favourable outcome in serious infections. Clinical failures and the development of resistance are observed for marginally susceptible organisms like *P. aeruginosa* and for which sufficient AUC/MIC ratios cannot be achieved.

In the next study, the therapeutic efficacy of pegylated liposomal ciprofloxacin was investigated in two rat models of *P. aeruginosa* pneumonia (Bakker-Woudenberg et al. 2002). In the acute model, pneumonia developed progressively, resulting in a rapid onset of septicemia and a high mortality rate. Ciprofloxacin twice daily for 7 days was not effective at doses at or below the maximum tolerated dose (MTD). However, pegylated liposomal ciprofloxacin either at high dosage or given at low dosage in combination with free ciprofloxacin on the first day of treatment was fully effective (100 % survival). Obviously, prolonged concentrations of ciprofloxacin in blood prevented death of the animals due to early-stage septicemia in this acute infection. However, bacterial eradication from the left lung was not effected. In the chronic model, pneumonia was characterized by bacterial persistence in the lung without bacteremia, and no signs of morbidity or mortality were observed. Ciprofloxacin administered for 7 days at the MTD twice daily resulted in killing of more than 99 % of bacteria in the lung and this result can also be achieved with pegylated liposomal ciprofloxacin given once daily, although complete bacterial eradication is never observed (Bakker-Woudenberg et al. 2002).

Urinary tract infections (UTIs), the majority of which (80 %) are caused by uropathogenic *E. coli* (UPEC) and 25 % of all UTIs recur within 6 months. UTIs occur as a continuum of steps by ascension of UPEC from the perineum through the urethra to the bladder, passing though the ureters to the kidneys. Clinically, the symptoms of cystitis, dysuria, and frequency often precede those of upper tract disease such as flank pain and chills. UPEC have also been shown to persist and reemerge in the bladder despite antibiotic therapy. Superficial facet cells of urinary bladder express integral membrane proteins called uroplakins (UP), which can serve as the receptors for UPEC. Upon entry into the superficial facet cells, UPEC are able to rapidly replicate and form intracellular bacterial communities (IBCs), characterized by a defined differentiation program and enhanced resistance to antibiotics. Their intracellular proliferation results in communal formations with biofilm-like properties-the IBCs. The bacteria thrive tightly enmeshed in the protective matrix of the IBC within the host epithelium. Ultimately, they need to be released and dispersed in order to exit the infected cell and find new naive cells for residence. The dispersion and the exiting of the host cell are, therefore, central steps in the UPEC life cycle in the bladder (termed the IBC pathway). Although the vast majority of UPEC are cleared by host defenses within a few days, small clusters of intracellular bacteria have occasionally been observed to persist for months in an antibiotic-insensitive state. The long-term persistence in the face of antibiotic therapy suggests that these bacteria are within a protected location within bladder epithelial cells. In addition to the superficial facet epithelial cell barrier, invading bacteria also face a chemical barrier: the complex network of proteoglycans/glycosaminoglycans (GAG layer) that is woven into the urothelium and is known to act as an antimicrobial adherence factor. Parsons and colleagues (1990) have shown that protamine sulfate (PS), a highly cationic protein (pI ~12) can lead to both exfoliation of the superficial facet cell barrier and biochemical inactivation of GAGs. Furthermore, PS treatment also increases urothelial ionic permeability and facilitates bacterial entry. Thus, Hultgren and co-workers (Mysorekar and Hultgren 2006; Wright et al.

2005) reasoned that PS could be used as both a chemical exfoliant of infected superficial facet cells and as an adjuvant to facilitate bacterial entry into non-exfoliating transitional cells underlying the superficial facet cell layer. Their findings raise possible therapeutic avenues for the treatment of recurrent UTIs. They also show that inducing epithelial exfoliation by using cationic proteins (e.g., PS) can, in some cases, expel bacteria from their intracellular locations. Therefore, protamine sulfate or other similar cationic compounds could act as potential therapeutic adjuvants in conjunction with antibiotic treatment to induce stripping of the urothelial lining containing cryptic bacterial quiescent intracellular reservoirs (QIRs), thus eliminating a potential source of chronic same-strain recurrent UTI episodes.

It is well-known that cationic liposomes have already re-emerged as a promising new vaccine adjuvant technology and these lipid-bilayer vesicles have positive surface charge (Christensen et al. 2007). It has also been shown that cationic liposomes have the ability to incorporate adequate quantity of number antimicrobial agents (Sapra and Allen 2003). Hence, it would be reasonable to speculate that antimicrobial agents-laden cationic liposomes should possibly pave a new way of treating QIRs, in principle but no experimental proof, to eliminate a potential source of chronic same-strain recurrent UTI episodes.

Chronic granulomatous disease (CGD) is a genetically-determined primary immunodeficiency disease in which phagocytic cells are unable to reduce molecular oxygen and create the reactive oxygen metabolites and thus are unable to kill ingested catalase-positive microorganisms (Tauber et al. 1963). The ingested organisms will remain viable within the phagocytes where they are protected from antibiotics. This leads to recurrent life-threatening bacterial and fungal infections resulting in marked inflammation, abscess and granuloma formation. CGD is now known to be caused by a defect in the nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) oxidase enzyme of phagocytes (accessed on March 21, 2014 from http://www.emedicine.com/ped/topic1590.htm). Most cases of chronic granulomatous disease are transmitted as a mutation on the x-chromosomes and are thus called an 'x-linked trait or x-linked recessive'. A less common mode of inheritance is by autosomal recessive pattern. In this form of inheritance the disease is less severe and tends to occur at an older age (Winkelstein et al. 2000).

People with CGD are sometimes infected with unique organisms that usually do not cause disease in people with normal immune systems. The microorganisms that can cause disease in CGD patients include *S. aureus*, *E. coli*, *Klebsiella* species, *Aspergillus* species and *candida* species. Manifestations of CGD include recurrent infections of the lungs, lymph nodes and skin. Bones, liver and gastrointestinal tract are less commonly involved. The great majority of the infective episodes are caused by *S. aureus* followed by *Aspergillus* (Winkelstein et al. 2000; Gallin 1983). Obstructive lesions of the gastrointestinal and urinary tract occur in CGD especially in the x-linked form. In CGD the persistence of viable bacteria within the phagocyte in the colonic mucosa may cause excessive stimulation of the inflammatory process and subsequent mucosa damage (Sloan et al. 1996; McDermott 1994).

Antimicrobial prophylaxis, early and aggressive treatment of infections, and interferon-γ (IFN-gamma) are the cornerstones of current therapy for CGD (Al-Mobaireek 2001). Although hematopoietic stem cell transplantation (HSCT) from a human leukocyte antigen (HLA) – compatible donor can cure CGD, this approach is fraught with clinically significant morbidity and a finite risk of death. HSCT remains a controversial therapeutic modality in this disease, even when stem cells from a matched sibling donor are available. Therefore, only daily prophylaxis of infections with more potent antibacterial and antifungal antibiotics (trimethoprim-sulfamethoxazole or cephalosporin and ketoconazole or itraconazole) in conventional dosage forms is indicated in CGD. On the other hand, patients with established superficial or deep infections (vs. those with obstructing granulomas) should receive aggressive intravenous antibiotics for several weeks. It should be emphasized that it might not be

convenient for the CGD patients to take the conventional antibiotic medication for several weeks to contain their infections. Moreover, liposomes incorporating the antimicrobial agents are already available in the market in order to diminish the injection frequencies and possible side effects of the non-liposomal, conventional dosage forms of antimicrobial agent. Therefore, it should be added that significant investigations should be directed for the judicious use of antimicrobial agent-laden liposomes in CGD patients when infections occur. Whilst some debate continues amongst the scientific community as to whether improved delivery of antimicrobials to the biofilm actually represents a viable approach to eradication, given the altered metabolic state of the microorganisms in the biofilm matrix, the current status of the field is further comprehensively reviewed under polymer-based drug delivery carriers.

2.6 Polymer-Based Drug Delivery Carriers

Many reviews have highlighted the use of biodegradable polyesters as effective drug carriers including nano- or micro-particles, hydrogels, micelles and fibrous scaffolds (Rabinow 2004; Freiberg and Zhu 2004; Varde and Pack 2004; Moses et al. 2003; Sinha and Trehan 2003; Zhao et al. 2003). Inevitably, there are advantages and disadvantages associated with each delivery system, however these experimental approaches have been investigated in a number of infections, including periodontitis and osteomyelitis, as well as intracellular infections such as tuberculosis and brucellosis. Small biodegradable microspheres are useful alternatives to liposomes for targeting drugs to the monocyte-macrophage system. They tend not to suffer from the same difficulties of low encapsulation efficiency and stability on storage typically exhibited by liposomal formulations. Moreover, biodegradable microspheres prepared from PLA and PLGA can release encapsulated drugs in a controlled way, depending on the method of microencapsulation and the physico-chemical properties of the polymer and drug.

2.7 Implantable Matrices, Beads, Strut, Microparticles, Fibrous Scaffolds, Thermoreversible Gels, etc.

Polymeric materials from both natural and synthetic origins are widely recognized as carriers for effective delivery of antimicrobial agents to the infections associated with implants. There is now an enormous amount of literature in this area and no single book chapter could give comprehensive coverage. Therefore, only the main areas of research with some selected examples were indicated. I apologize in advance to colleagues whose work has been omitted through lack of space.

Several resorbable materials such as collagen (Stemberger et al. 1997), gelatin (Keogh et al. 1989), polymers in different chemistries such as polylactides (Kanellakopoulou et al. 1999; Mader et al. 1997; Zhang et al. 1994; Wei et al. 1991), copolymers of lactide and glycolide (Ambrose et al. 2003; Mader et al. 1997; Overbeck et al. 1995; Nie et al. 1995; Garvin et al. 1994), polyanhydrides (Jacob et al. 1991), and polycaprolactone (Le Rey et al. 2003; Burd et al. 2001; Hendricks et al. 2001), biodegradable bone cements (Solberg et al. 1999; Gerhart et al. 1988), hydroxyapatite and glass ceramics (Mäkinen et al. 2005; Shirtliff et al. 2002; Saito et al. 2002; Nolan et al. 1993), calcium sulfate (Nelson et al. 2002), and fibrin sealant implants (Mader et al. 2002) have been investigated for use of drug delivery systems of various antibiotics. Limited clinical reports are available from collagen-gentamicin sponge (Stemberger et al. 1997), and antibiotic impregnated calcium phosphate (McKee et al. 2002). However, FDA has not yet approved any of these materials for antibiotic therapy (Holtom and Patzakis 2003).

Prevention and treatment of osteomyelitis, particularly associated with orthopaedic implant surgery, have been the focus of many studies. Systems implanted at the same time as the prosthesis may be either non-biodegradable or biodegradable. Few selected examples are discussed below in each category. Antibiotic-polymethylmethacrylate (PMMA) cement and beads constitute an effective

system of local drug delivery of antibiotic agents in patients with bone and soft-tissue infections. Debridement followed by implantation of antibiotic-PMMA beads and systemic administration of antibiotic agents has achieved a 100 % success rate in treating chronic osteomyelitis. Recently an antibiotic-PMMA strut was used by Chen and Lee (2006) for treating spinal pyogenic spondylitis in a case report of a 57-year-old woman with C5-C6 pyogenic spondylitis, progressive kyphotic deformity, and neurological deficits. The patient underwent anterior C-5 and C-6 corpectomy and spinal reconstruction in which the antibiotic-PMMA strut was used. The strut was 14 mm in diameter and contained PMMA and vancomycin powder. The operation was technically successful, and no complication related to anesthesia or the surgical procedure occurred. At the 12-month follow-up examination, dynamic radiographs revealed cervical spine stabilization. The patient's neck pain subsided and she recovered neurologically with no residual infection. No antibiotic-PMMA strut dislodgment or failure was identified although a 9.8 % subsidence of the strut into the vertebrae was observed (Chen and Lee 2006).

Antibiotics are given systemically prior to surgery in orthopedic and trauma surgery to prevent implant-related infection. However, due to the disturbed bony structure and the local vascularity of trauma patients, an appropriate local antibiotic level might not be achieved by circulating antibiotics. In addition, the dose required for systemic administration of antibiotics is relatively high in comparison to the dose required for local administration at the implant-bone interface. In most surgical procedures that include the incorporation of implants, the tissue-implant interface is especially prone to microbial contamination. Aiming for high protective tissue levels of the antibiotic agent at the interface by local application of prophylaxis appears to be a reasonable approach. Systemic side effects of the antibiotic can be avoided and higher local drug levels can be achieved without risking systemic toxicity. Impaired local blood supply due to surgical trauma, hematoma, and edema may affect the delivery of the antibiotic when administered systemically. Therefore, several strategies for local antibiotic prophylaxis have been attempted, such as antibiotic-loaded bone cements, antibiotic-impregnated collagen sponges and polymethylmethacrylate beads (Nelson et al. 1994; Hettfleisch and Schottle 1993; Letsch et al. 1993). However, certain aspects need to be considered if local prophylaxis is to be performed: The technique of delivery must guarantee a rapid release of the antibiotic from the carrier and local drug levels well above the MIC of current microorganisms need to be achieved. The drug release must be restricted to a limited period of time to prevent development of resistant bacterial strains. Bactericidal antibiotics should be favoured over bacteriostatic. The use of self-dissolving (ie, biodegradable) drug carriers is of advantage as secondary surgery for removal is not necessary. Considering these points, a local drug delivery system for gentamicin application was developed by Schmidmaier and co-workers (2006). Gentamicin was chosen as the antibiotic as it has been used successfully as a locally applied antibiotic in orthopedic surgery (Bauer and Schils 1999; Taylor et al. 1990). Its broad antimicrobial spectrum, covering most bacteria commonly involved in osteomyelitis, and its bactericidal effect, even on non-proliferating microorganisms (Eron 1985), make it favourable for local application. In an animal experiment, the efficacy of local prophylaxis of gentamicin was compared to a systemic single shot of gentamicin and to a combination of both administrations (Schmidmaier et al. 2006). The medullary cavities of rat tibiae were contaminated with *S. aureus* and titanium K-wires were implanted into the medullary canals. For local antibiotic therapy, the implants were coated with biodegradable poly(D,L-Lactide) (PDLLA)-loaded with gentamicin. All the animals not treated with local and systemic application of the antibiotic developed osteomyelitis and all cultures of the implants tested positive for *S. aureus*. Onset of infection was prevented in 80–90 % of animals treated with gentamicin-coated K-wires, with and without systemic prophylaxis. For readers' interest, gentamicin-coated intramedullary tibial nails are CE (Conformité Européene, European Conformity)-certified for Europe and Canada and

several patients have already been treated for implant-related infection. Up to now, eight patients with open tibia fractures have been treated with an unreamed tibial nail (UTN) coated with PDLLA and gentamicin. In the 1-year follow up, none of the patients developed an infection. So far, the results suggest that a local application of gentamicin from PDLLA-coated implants might support systemic antibiotic prophylaxis in preventing implant-associated osteomyelitis (Schmidmaier et al. 2006).

Koort et al. (2006) have designed a cylindrical composite pellets (1.0×0.9 mm) from bioabsorbable poly(d/l-lactide) matrix and ciprofloxacin (7.4 wt%). In vitro studies were carried out to delineate the release profile of the antibiotic and to verify its antimicrobial activity by means of MIC testing. A long-term study in rabbits was performed to validate the release of ciprofloxacin from the composite in vivo. Therapeutic level of ciprofloxacin (>2 μg/ml) was maintained between 60 and 300 days and the concentration remained below the potentially detrimental level of 20 μg/ml in vitro. The released ciprofloxacin had retained its antimicrobial properties against common pathogens. In an exploratory long-term in vivo study with three rabbits, ciprofloxacin could not be detected from the serum after moderate filling (160 mg) of the tibia (follow-up 168 days), whereas after high dosing (a total dose of 1,000 mg in both tibias) ciprofloxacin was found temporarily at low serum concentrations (14–34 ng/ml) during the follow-up of 300 days. The bone concentrations of ciprofloxacin could be measured in all samples at 168 and 300 days. The tested copolylactide matrix seems to be a promising option in selection of resorbable carriers for sustained release of antibiotics, but the composite needs modifications to promote ciprofloxacin release during the first 60 days of implantation.

Although polymethylmethacrylate beads impregnated with gentamicin have been available for about 20 years, they have to be removed usually about 4 weeks after insertion since they are non-biodegradable (Kanellalopoulou and Giamarellos-Bourboulis 2000). A number of osteoconductive and biodegradable alternatives have been studied, including calcium phosphates such as hydroxyapatite whose chemical composition is similar to the bone mineral phase. Studies with ciprofloxacin incorporated into hydroxyapatite and poly(d,l-lactide) formulations implanted in the femur of rabbits, indicated that therapeutic bone levels were achieved over 6 weeks with release enhanced by erosion-disintegration and bone ingrowth into the implant (Castro et al. 2003). Other studies using the glycopeptide antibiotic teicoplanin, effective against *S. aureus*, indicate that it too can be effective over several weeks when incorporated into microspheres prepared from PLGA (75:25) (mol.wt. 136,000) polymer (Yenice et al. 2002, 2003). Other materials studied against implant-related osteomyelitis include poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). The release behaviour of sulbactam: cefoperazone from rods comprising 7 %, 14 % or 22 % (mol) 3-hydroxyvalerate were representative of typical monolithic devices where a rapid early release phase is followed by a slower and prolonged phase. With PHBV 22 rods, this extended phase lasted for up to 2 months, making it a promising controlled release vehicle since treatment of device-related infections is typically up to 6 weeks (Gursel et al. 2002). Release studies of antibiotics adsorbed onto the surface of hydroxyapatite cylinders having a bimodal pore size distribution also have a prolonged duration of release, attributed to the small pores, combined with favourable osteoconduction properties into the large pores (Hasegawa et al. 2004). A number of matrices have been formed using PLGA, including discs (Yoo et al. 2004) and electrospun nanofibrous scaffolds (Kim et al. 2004). In an effort to achieve the ideal drug release pattern of no lag time and zero-order release, lactide monomer or glycolide monomer have been incorporated into PLGA discs loaded with gentamicin. The idea here is that channels will form in the disc following dissolution of the monomer to aid the release of gentamicin. Discs containing 10 % monomers showed nearly zero-order release kinetics for more than 1 month (Yoo et al. 2004). Evidence for the channel-forming properties of the monomers came from the water uptake by the discs. After 7 days, the amount of water

absorbed by the control discs was 20 %, compared with 60 % in monomer-containing discs.

Fibrous scaffolds are currently receiving attention both as a means to prevent post-surgery adhesions and also to release drugs in a site-specific manner. Whilst surgical implantation is required, they could be used where surgery is already indicated and the drug release profile can be controlled by varying the scaffold's morphology, porosity and composition. Cefoxitin was incorporated into PLGA scaffolds by Kim and co-workers (2004). Here, PLGA controls the rate of degradation whilst high molecular weight PLA confers mechanical strength to the scaffold. An amphiphilic diblock co-polymer comprising poly(ethylene glycol)-b-poly(lactide) was added to the polymer solution to encapsulate the hydrophilic cefoxitin sodium, which otherwise has poor solubility in the PLGA solvent N,N-dimethyl formamide. The drug/polymer solution was electrospun in a spinneret at 23–27 kV. As the concentration of cefoxitin increased, the scaffolds changed from a bead and string morphology, attributed to insufficient stretching of the polymer jet, to a fine fibrous structure of diameter 260 ± 90 nm. The spinning process did not affect the cefoxitin and following an initial burst, prolonged release was measured for up to 1 week.

Other applications of PLGA have included incorporation of antibiotic-loaded microparticles into an injectable collagen sponge, resulting in local drug delivery combined with the tissue regeneration properties of collagen (Schlapp and Friess 2003). Several other matrices have been used, including the aluminosilcate material halloysite. Whilst chemically similar to kaolin, halloysite has a tubular structure that can be loaded with drug. Moreover, surface charge neutralisation using cationic polymers can place an additional level of control on drug release.

Periodontitis is an inflammatory response in which the structural support to the tooth is destroyed. The disease results in resorption of the alveolar bone, detachment of the periodontal ligament supporting the tooth and formation of a periodontal pocket (lesions between teeth and junctional epithelium) (Heasman and Seymour 1994). The pocket provides an ideal environment for the proliferation of a variety of pathogenic bacteria. Therapeutic approaches for the treatment of periodontitis include mechanical or surgical methods and administration of systemic antibiotics. However, for systemic administration the drugs must be given in high doses to maintain an effective concentration in gingival crevicular fluid (GCF). High doses of antibiotics cause side effects such as gastrointestinal disorders, development of resistant bacteria and suprainfection. Systemic therapy has a low benefit to high risk ratio (Heasman and Seymour 1994; Gordon and Walker 1993). With advances in understanding of the etiology and pathogenesis of periodontal disease, attention has been focused on local drug delivery systems. These include both sustained and controlled release polymeric systems which when inserted into the periodontal pocket, release antimicrobial agents above the minimum inhibitory concentration for a sustained period of time. Thus intrapocket devices have a high benefit to low risk ratio (Pandit 1997).

In one study of tetracycline incorporation into halloysite for the treatment of periodontitis, an initial burst of tetracycline release was followed by a dramatic reduction in release. Coating with the cationic polymer chitosan reduced the burst release from 45 % to 30 % and the total release over 9 days from 88 % to 78 % (Kelly et al. 2004). A key concern was delivery of the halloysite to the gingival pocket and its subsequent retention. Combination therapy based on amoxycillin and metronidazole in conventional dosage forms has been widely investigated in clinical dental practice due to its activity against a wide range of anaerobes, facultative and aerobic bacteria. The combination of amoxycillin and metronidazole has synergistic action and covers a wide range of microflora, with metronidazole inhibiting the anaerobes and amoxycillin inhibiting the facultative aerobic bacteria. Both the drugs are bactericidal in nature and are administered systemically. This is important for complete elimination of subgingivally occurring periodontal pathogens (Schwach-Abdellaoni et al. 2000). A biodegradable intrapocket device containing amoxycillin and metronidazole was prepared using 69.29 mg each of amoxycillin

and metronidazole, 2.0 % diethyl phthalate plasticizer and 750 mg PLGA (Ahuja et al. 2006). The device was optimized on the basis of evaluation parameters such as weight variation, content uniformity, surface pH, and in vitro and in vivo release studies. The films showed sustained in vitro release for a period of 16 days. In vivo release studies showed that drug concentrations were maintained above the MIC value for the entire period of the release studies. The samples from this study were capable of inhibiting the growth of most test strains. The combination of amoxycillin and metronidazole in the carrier polymer PLGA not only showed an extended spectrum of antimicrobial activity but also showed a synergistic effect against *Eubacterium limosum*, a metronidazole-resistant strain.

The polyoxyethylene-polyoxypropylene copolymer, poloxamer 407, was used for its thermoreversible properties of being liquid below 20 °C and forming a hydrogel at higher temperatures if sufficiently concentrated that is also biodegradable and relatively non-toxic. The idea here is that the delivery system should be a liquid at room temperature, so avoiding the need for refrigeration, and a device-retaining gel at the temperature within the gingival crevice. In this particular study, the transition temperature range was narrowed by the addition of polyethylene glycols, however this caused the storage modulus (solidity) of the gel to drop as well (Kelly et al. 2004). This was overcome by the addition of 1 % octyl cyanoacrylate (OCA), a powerful tissue adhesive that polymerises at neutral pH. For storage, the pH of the system was held at 4 by addition of glacial acetic acid, and upon application to the gingival crevice the natural buffer capacity of the tissue slowly brought the pH of the formulation up to neutral, allowing the OCA to polymerise. Thermoreversible polymers have also been studied as delivery matrices in their own right (Veyries et al. 1999; Esposito et al. 1996; Miyazaki et al. 1995). For example, pluronic F-127 has been used to deliver vancomycin to treat methicillin-resistant *S. aureus* otitis media (Lee et al. 2004). Based on the sol-gel phase transition measurements, a 25 % w/v pluronic solution was loaded with vancomycin and injected through a 26 gauge needle. It changed to a gel at 36–37 °C that consisted of large populations of the micelles and aqueous channels from which the vancomycin was released. Bioabsorption studies followed over 50 days indicated that the gel gradually disappeared leaving open spaces that initially showed some inflammatory exudate, but by day 50 normal tissue was observed without any inflammation, fibrosis, or open spaces from the gel.

2.8 Surface Modified Polymeric Catheter Materials

Several research groups have tried to develop polymers with new surface properties that would lead to a reduction of microbial adhesion onto medical devices, especially of catheters. Reported modified polymeric catheter materials include; polystyrene modified with copolymers of poly(ethylene oxide) and poly(polypropylene oxide) (Bridgett et al. 1992; Desai et al. 1992), photochemical coating of polymers (Dunkirk et al. 1991), polyvinylpyrrolidone-coated polyurethane catheter (called as Hydrocath®) (Tebbs et al. 1994), polyvinylpyrrolidone-coated polyurethane catheter with benzalkonium chloride (Tebbs and Elliott 1994), polyurethane surfaces modified by radiation grafting of 2-hydroxymethylmethacrylate (Jansen et al. 1987), polyurethane coated with sulfonated poly(ethylene oxide), and polyurethane possessing a chain extension provided by glycerophosphorylcholine (Baumgartner et al. 1997). An overview mainly on experimental research on the surface modification of polymers and on binding macromolecules such as albumin to surfaces in order to prevent bacterial adherence can be found elsewhere (Kohnen and Jansen 2000; An et al. 2000). So far, the only such modified polymer used in clinical applications is a hydrophilic, polyvinylpyrrolidone-coated catheter (Hydrocath®) based on polyurethane. Its relatively low thrombogenicity and low in vitro bacterial adherence should also be of benefit regarding infection resistance, although this has not yet been demonstrated in a clinical trial.

A major disadvantage of all of the previously described approaches, which aim primarily at the modification of the surface properties of basic materials (e.g., catheters or other devices) is the fact that, for thermodynamic reasons, the creation of surfaces that show a "zero" adhesion is probably not feasible. In an experimental study that investigated the relationship between bacterial adhesion and the free surface enthalpy of adhesion of a large number of differently modified polymers, Jansen and Kohnen (1995) demonstrated that it is impossible to develop a polymer surface that shows an absolute bacterial "zero" adherence in vitro. In particular, adherence of *S. epidermidis* to a variety of polymers with different surface properties, generated by means of the glow discharge technique, was investigated (Jansen and Kohnen 1995). The same authors found that adhesion of the bacteria to the modified materials decreased with increasing negative free enthalpy values. A certain minimum number of adherent *S. epidermidis* cells could be proved at positive free enthalpy values at which adhesion should be thermodynamically impeded. Hence, it seems impossible to design an absolute antiadhesive material that retains its properties even in the more complex in vivo situation, in which the native surface properties are masked by adsorption of bacterial and host components.

The biomaterials community over the past 25 years has attempted to produce anti-infective devices or implants by either (1) mechanical design alternatives (liquid-air breaks; skin cuffs; antibiotic fills; all for indwelling catheters), (2) tethered anti-infective agents, bound directly to the surface of the material (Ag coatings, tethered quaternary ammonium, synthetic antibiotics), or (3) the release of soluble toxic agents (chlorhexidine, antibiotics) into the adjacent surroundings. Mechanical design alternatives have had only marginal success and are only applicable for short-term indwelling catheters. Tethered anti-infective agents are only toxic to the initial wave of incoming bacteria and provide little residual effects once layers of dead cells accumulate, which are also inflammatory. Finally, regardless of the type of "drug-release" method used (passive vs. sustained vs. responsive), release of a toxic agent from a biomaterial of a soluble anti-infective agent will inevitably stop once the entrapped agent is depleted. Nevertheless, delivery of sublethal dosages of antibiotics can also lead to accelerated biofilm formation and induced virulence factor expression. For example, Staphylococcal biofilms are among the main causes of chronic implant-associated infections. Pasquaroli et al. (2013) have recently suggested that their transformation into viable but non-culturable (VBNC) forms (i.e. forms capable of resuscitation) could be responsible for the recurrent symptoms. *S. aureus* biofilms were exposed to different concentrations of antibiotic (vancomycin or quinupristin/dalfopristin) and/or to nutrient depletion until loss of culturability. Viable subpopulations were detected in all non-culturable biofilms. However, viable cell numbers and gene expression remained constant for 150 days from loss of culturability in cells from antibiotic-exposed biofilms, but not in those that had only been starved. Resuscitation was also obtained in rich medium supplemented with 0.3 % sodium pyruvate or with 50 % filtrate of a late-log culture. These findings demonstrate that *S. aureus* can enter the VBNC state in infectious biofilms. Furthermore, the presence of vancomycin or quinupristin/dalfopristin can inadvertently induce a true VBNC state or its persistence in *S. aureus* cells embedded in biofilms, and thus supporting the role of staphylococcal biofilms in recurrent infections.

In spite of the cited deficiencies, the usefulness of the polymeric catheter materials containing antimicrobial agents, anti-infective substances and antiseptic agents, as well as the catheter materials coated with metals and metal fluoride in controlling biofilm-related nosocomial infections are discussed one by one in the following sections.

2.9 Antimicrobial-Containing Polymeric Catheter Materials

Catheters or parts of the catheter system have been coated with antimicrobial substance (e.g. an antibacterial, disinfectant or metal ion) is bound

superficially to a catheter, either directly or by means of a carrier (as shown previously) or incorporated into the interior of the polymer. As quoted previously in the introduction part of this book chapter, all antimicrobial-coated devices are combination products. Originally, most of the effort to develop combination products was directed toward developing coatings for different types of devices. These coatings, in turn, often contained antibiotics. Probably the earliest example of such a device was bone cement, which has been mixed with gentamicin and other antibiotics since the early 1960s. The standard approach involved coating devices with various kinds of antibiotics and hoping that they would provide enough activity for a long enough period of time to prevent infections. With the evolution of materials science in the intervening years, manufacturers however have learned how to fabricate antibiotic-laden combination products with longer lives. Other materials breakthroughs have resulted in the creation of polymers that incorporate antimicrobials into the backbone of the material and then release the agent under controlled conditions as the polymer degrades. Meanwhile, efforts have been made to eliminate antimicrobials altogether from medical devices and design microbe-resistant surfaces. "Such antifouling technology," usually means using steric hindrance at the molecular level to prevent microorganisms from attaching to the surface of the device or using materials with functional groups on the surface that inhibit the growth of microorganisms. In order to obtain suitable antifouling materials, Francolini et al. (2014) have synthesized the segmented polyurethanes characterized by a hard/soft domain structure, having the same hard domain but a variable soft domain. The soft domain was constituted by one of the following macrodiols: polypropylenoxide (PPO), polycaprolactide (PCL), and poly-l-lactide (PLA). The effects of the polymer hydrophilicity and the degree of hard/soft domain separation on antifouling properties of the synthesized polyurethanes were investigated. Microbial adherence assays evidenced as the polymers containing PCL or PLA were able to significantly reduce the adhesion of *Staphylococcus epidermidis* with respect to the PPO-containing polymer.

One of the main drawbacks of most available antimicrobial-coated devices is the burst release of the adsorbed antibiotics in the first few hours, followed by a long-lasting phase of slow release at very low concentrations. This behaviour can be associated with the development of antimicrobial resistance both at in vitro and in vivo conditions. The development of an innovative catheter with long-lasting antibiofilm activity depends on the ability of the catheter constitutive polymer to adsorb large amounts of antibiotic molecules and on their long-term release at relatively constant concentrations. In this regard, it would be of better idea to develop some properly functionalized polymers that are able to adsorb large amounts of antibiotic by introducing into the polymer side chains acidic or basic groups able to interact with different classes of drugs.

There are a large number of studies on the bonding of antibacterials to biomaterials. Solovskij et al. (1983) prepared polymers to which ampicillin and 6-aminopenicillanic acid were covalently bonded and which inhibited the in vitro growth of *S. aureus*. However, most studies have focused on the incorporation or superficial coating of antimicrobials rather than on covalent bonding by chemical reaction. Sherertz et al. (1993) used a rabbit model to investigate intravascular catheters coated with several antimicrobial substances (dicloxacillin, clindamycin, fusidic acid and chlorhexidine). The frequency of catheter infections was significantly reduced compared with the control group when the dicloxacillin-coated catheter was used. Similarly, the incorporation of flucloxacillin, clindamycin and ciprofloxacin into polyurethane polymers demonstrated a considerable reduction of the in vitro adherence of *S. epidermidis* (Jansen et al. 1987; Jansen and Peters 1991). An another approach involves the loading glycopeptide teicoplanin into a commercially available central venous, hydrophilic-coated polyurethane catheter (Hydrocath®) and shows the capability of this catheter to prevent colonisation with *S. epidermidis* and *S. aureus* for a period of at least 48 h (Romano et al. 1993; Jansen et al. 1992). When teicoplanin and silver were incorporated into the Hydrocath®, the catheter shows a considerable

activity against *S. epidermidis*, *E. coli* and *Candida albicans* (Jansen and Kohnen 1995).

Heparin coatings or bindings have also been shown to prevent microbial adhesion and colonization in vitro and in vivo (Appelgren et al. 1996). Heparin binding reduces fibronectin deposition on vascular catheter surfaces and makes the catheter negatively charged, thus preventing thrombosis and reducing microbial colonization (Russell et al. 1987). This antiadhesive activity of heparin results in a significant reduction of catheter-related infections, as recently confirmed by a randomized-controlled clinical trial of heparin-coated and uncoated non-tunnelled CVCs inserted in 246 patients (Abdelkefi et al. 2007) and a retrospective comparative analysis of 89 coated and 86 uncoated tunneled dialysis catheters (Jain et al. 2009). Among the strategies pursued to control the interactions between material surfaces and biological tissues, the immobilization of non-fouling polymers on biomaterial surfaces as well as the synthesis of the so-called biomimetic polymers is considered promising approaches to elicit specific cellular responses. In order to obtain materials able to prevent infectious and thrombotic complications related to the use of blood-contacting medical devices, heparin-mimetic segmented polyurethanes were synthesized and fully characterized (Francolini et al. 2012). Specifically, sulfate or sulfamate groups, known to be responsible for the biological activity of heparin, were introduced into the side chain of a carboxylated polyurethane. Due to the introduction of these groups, the obtained polymers possessed a higher hard/soft phase segregation (lower glass transition temperatures) and a greater hydrophilicity than the pristine polymer. In addition, the synthesized polymers were able to significantly delay the activated partial thromboplastin time, this increased hemocompatibility being related both to polymer hydrophilicity and to the presence of the -SO3H groups. This last feature was also responsible for the ability of these biomimetic polymers to prevent the adhesion of a strain of *S. epidermidis*.

Kamal et al. (1991) have evaluated the efficacy of a cefazolin-containing catheter (in which cefazolin was bound to benzalkonium chloride) in a prospective, randomized trial. There was a significant decrease in catheter colonisation (7-fold) as determined by the semiquantitative tip culture method (Maki et al. 1977) and no CRBSI was observed in this study. A comparative study involving before and after the routine use of cefazolin catheters in the ICU, the authors described a marked reduction in the rate of CRBSI from 11.5 to 5.1 infections per 1,000 catheter days (Kamal et al. 1998).

Raad et al. (1995, 1996a, b, c) reported on the broad-spectrum activity against gram-negative and –positive organisms and *C. albicans* of a minocycline-rifampicin catheter based on in vitro and animal data. This catheter has been marketed as the Cook Spectrum™ catheter (Cook Critical Care, Bloomington, IN, USA) and is coated on the inner and outer surface with minocycline and rifampicin, which have a synergistic or additive action in combination. In a prospective, randomized clinical trial (Raad et al. 1997a, b), the minocycline-rifampicin catheter was compared with an uncoated control catheter and demonstrated a statistically significant decrease in catheter colonisation (8 % vs. 26 % for the control catheter, $p<0.001$) and in CRBSI (0 % vs. 5 %, $p<0.01$). In a large multicenter trial, the minocycline-rifampicin catheter was compared with another commercially available catheter containing chlorhexidine and silver sulfadiazine (the CHSS catheter), which is being described further in the next paragraph (Johnson et al. 1999). It was found that the minocycline-rifampicin catheter was 3-fold less likely be colonised (7.8 % vs. 22.6 % for the CHSS catheter, $p<0.0001$) and 12-fold less likely to lead to CRBSI (0.3 % vs. 3.4 %, $p<0.002$). This difference has been explained by the fact that minocycline-rifampicin catheters are coated internally and externally (in contrast with the first-generation CHSS catheter), the combination of minocycline and rifampicin showing superior surface activity than chlorhexidine and, finally, that the minocycline-rifampicin catheter retain surface antimicrobial activity longer in situ (Crnich and Maki 2002). Although resistance against minocycline and rifampicin could not be detected in clinical trials, this remains of concern

as in vitro development of resistance has been demonstrated (Tambe et al. 2001). In their next study, Raad et al. (2007) demonstrated that linezolid and vancomycin, administered alone, were less effective in decreasing the viability of biofilm-embedded *S. aureus* than daptomycin, minocycline and tigecycline. However, when rifampicin was added to linezolid or vancomycin, an enhancement of their activity in biofilm killing was observed (Raad et al. 2007). Kim et al. (2008) showed that combined and sequential treatments with tobramycin and silver enhanced antimicrobial efficacy by > 4,200 % in *P. aeruginosa*. The authors concluded that the use of combinations of agents that have similar antimicrobial behaviours, but that are not too oxidative, i.e. silver and tobramycin, might be an effective strategy for preventing microbial adaptation and facilitating the antimicrobial action of agents. Similarly, the combination of silver/ciprofloxacin was demonstrated to have a synergestic effect in killing *S. epidermidis* growing as biofilms (Donelli et al. 2009; Francolini et al. 2010).

Additional approaches on antibacterial-containing catheters include: the adsorption of cefamandole nafate on functionalized urethane catheters that were then used to coat a commercial CVC (Donelli et al. 2002) and the use of a combination of an antibacterial substance (rifampicin) in combination with an antifungal substance (miconazole) in a polyurethane catheter (Schierholz et al. 2000). Other combinations of antibiotics and antifungal drugs exhibiting synergistic activity include: (1) aminoglycosides and fosfomycin against *P. aeruginosa* biofilm in a rat model (Cai et al. 2009); (2) anprocide and bacitracin or oxacillin against *S. aureus* and *S. epidermidis* biofilms in vitro (Pettit et al. 2009); and (3) amphotericin B, caspofungin or fluconazole in combination with a high-dose doxycycline against *C. albicans* biofilms in vitro (Miceli et al. 2009).

A disadvantage of all these approaches might result from the risk for development of resistance against the antimicrobial agents, especially if antibacterials considered as first-line drugs in the therapy of infections and are used as an active part of the modified catheters.

Donelli et al. (2006) performed a combined entrapment in functionalized polyurethanes of fluconazole and albumin, as a pore-forming agent, in order to obtain good and controlled release over time of the antifungal drug, thus inhibiting *C. albicans* growth and biofilm formation on polymeric surfaces for up to 8 days. In fact, to increase and control drug release from the polymer matrices, Ruggeri et al. (2007) experimented with two pore-forming agents at different molecular weights, PEG (molecular weight = 2,000, 10,000 and 35,000) and bovine albumin (molecular weight = 69,000), which were incorporated into the polymer bulk together with antibiotic or antifungal molecules, thus obtaining different degrees of impregnation and release. These authors demonstrated that polyurethanes containing PEG 10,000 + cefamandole + rifampicin were active against a rifampicin-resistant *S. aureus* strain for up to 23 days (Ruggeri et al. 2007).

Given the well-known decreased antibiotic susceptibility of bacteria growing in the sessile mode, experiments were carried out with *S. epidermidis* and *S. aureus* grown as biofilms on untreated or Dispersin B-treated polyurethanes. As Dispersin B is a b-N-acetylglucosaminidase able to dissolve the staphylococcal exopolysaccharide matrix (Kaplan et al. 2003; Kaplan 2009), it has been demonstrated that this enzyme is able to promote the antimicrobial and antibiofilm activity of cefamandole nafate (Donelli et al. 2007), sodium dodecyl sulphate (Izano et al. 2007) and triclosan (Darouiche et al. 2009).

2.10 Antiseptics-Containing Polymeric Catheter Materials

Antimicrobial substances that differ from antibacterials, such as antiseptics, have also been used to develop new catheter materials. The disinfectant Irgasan® was incorporated into several polymer catheters, showing a reduction of infections in rabbits (Kingston et al. 1986). Jansen et al. (1992) used the hydrophilic Hydrocath® catheter to incorporate iodine, leading to a polyvinylpyrrolidone-iodine-complex on the

inner and outer catheter surface. In vitro adherence of various microorganisms (*Staphylococcus spp*., *E. coli*, *Candida spp*., *Pseudomonas spp*.) was completely inhibited for the time of iodine release. After iodine exhaustion, re-loading of the catheter was possible. Tebbs and Elliott (1994) incorporated benzalkonium chloride into triple-lumen Hydrocath® catheters and demonstrated a long-lasting antimicrobial activity of the catheters against *staphylococci* and a somewhat lesser activity against gram-negative bacteria and *C. albicans*.

The most promising development in this field was a catheter using a combination of an antiseptic (chlorhexidine) and silver sulfadiazine (CHSS catheter). This catheter became available ~12 years ago, is polyurethane-based and impregnated with minute amounts of chlorhexidine and silver sulfadiazine (ArrowGard, Arrow International, Reading, PA, USA). A synergistic effect of chlorhexidine and sulfadiazine has been shown in vitro (Quesnel et al. 1978). This first-generation CHSS catheter is coated only on the exterior surface and exhibits antimicrobial properties for ~15 days. Since its introduction >8 million catheters have been sold worldwide and a considerable number of randomized clinical trials have been performed with this type of catheter (Sheng et al. 2000; Hannan et al. 1999; Collin 1999; Heard et al. 1998; George et al. 1997; Logghe et al. 1997; Maki et al. 1997; Tennenberg et al. 1997; Bach et al. 1996; Ciresi et al. 1996; Pemberton et al. 1996). In the study with the greatest patient numbers, which also used molecular methods for the confirmation of CRBSI, the CHSS catheter was associated with a 2-fold reduction in the incidence of catheter colonisation and a 5-fold reduction of CRBSI (RR 0.21, 95 % CI 0.03, 0.95; p = 0.03) (Maki et al. 1997). As the first-generation CHSS catheters are coated only externally, colonisation of the inner lumen as a result of hub contamination might also be of greater relevance with longer duration of placement. For these reasons, a new second-generation CHSS catheter has been developed that is coated both internally and externally, and that exhibits enhanced chlorhexidine activity (ArrowGard Plus, Arrow International, Reading, PA, USA). Clinical trials with this new type of catheter are also carried out and a significant reduction in catheter colonisation was observed (Brun-Buisson et al. 2004). Development of resistance to chlorhexidine has been demonstrated in vitro (Tattawasart et al. 1999). However, in vitro resistance to either chlorhexidine or silver sulfadiazine associated with the use of the antimicrobial catheter has not yet been reported. Anaphylactoid reactions, probably due to chlorhexidine, have been reported from Japan and UK, but have not been observed in the US so far (Oda et al. 1997).

Minocycline-rifampin-impregnated central venous catheters (M/R CVCs) have been shown to be efficacious in reducing CRBSI and inhibiting the biofilm adherence of resistant gram-positive and gram-negative pathogens, with the exception of *P. aeruginosa* and *Candida spp.* To expand the spectrum of antimicrobial activity, a novel second-generation M/R catheter was developed by adding chlorhexidine (CHX-M/R) (Raad et al. 2012). CVCs and peripherally inserted central catheters (PICCs) were impregnated with CHX-M/R and compared with first-generation M/R catheters, CHX-silver sulfadiazine-treated CVCs (CHX/SS-CVCs), chlorhexidine-treated PICCs, and uncoated catheters. A biofilm catheter colonization model was used to assess the efficacy of catheters against MRSA, vancomycin-resistant *Enterococcus faecium* (VRE), *P. aeruginosa*, *C. albicans*, and *Candida glabrata*. CHX-M/R-impregnated CVCs were the only antimicrobial catheters that completely inhibited the biofilm colonization of all resistant bacterial and fungal organisms tested at all time intervals, and they were significantly superior to uncoated catheters (all P values were ≤ 0.003). Furthermore, CHX-M/R-coated CVCs had a significantly more effective and prolonged (up to 3 weeks) antimicrobial activity against MRSA and P. aeruginosa than M/R, CHX/SS, and uncoated CVCs ($P < 0.0001$). Similarly, CHX-M/R-coated PICCs were also superior to M/R-coated and CHX-coated PICCs in preventing biofilms of MRSA, VRE, *P. aeruginosa*, and Candida species (P value = 0.003 for all). Our study shows that novel CHX-M/R-coated catheters have unique properties in completely inhibiting biofilm colonization

of MRSA, VRE, *P. aeruginosa*, and fungi in a manner superior to that of M/R- and chlorhexidine-treated catheters.

Resistant gram-negative bacteria are increasing central-line-associated bloodstream infection threats. To better combat this, chlorhexidine (CHX) was added to minocycline-rifampin (M/R) catheters (Jamal et al. 2014). The in vitro antimicrobial activity of CHX-M/R catheters against multidrug resistant, gram-negative *Acinetobacter baumannii*, *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *Stenotrophomonas maltophilia* was tested. M/R and CHX-silver sulfadiazine (CHX/SS) catheters were used as comparators. The novel CHX-M/R catheters were significantly more effective ($P < 0.0001$) than CHX/SS or M/R catheters in preventing biofilm colonization and showed better antimicrobial durability.

2.11 Metals-Coated Polymeric Catheter Materials

Among metals with antimicrobial activity, silver has raised the interest of many investigators because of its good antimicrobial action and low toxicity (Gosheger et al. 2004). Silver has also extensively been used for the development of infection-resistant urinary catheters.

Sioshansi (1994) used ion implantation to deposit silver-based coatings on a silicone rubber, which thereafter demonstrated antimicrobial activity. Silver-copper surface film, sputter-coated onto catheters materials, also showed antimicrobial activity *P. aeruginosa* biofilm formation (Mclean et al. 1993). In a relatively recent piece of research, an ion beam technique applying low implantation energy has been used for the formation of silver nanoparticles on the surface of polymers that exhibited and improved effect on bacterial adhesion (Davenas et al. 2002). Jansen and Kohnen (1995) developed an antimicrobial polymer by binding silver ions to acid modified, negatively charged polyurethane surface. Another approach is loading of a hydrophilic polyurethane catheter with silver nitrate (Gatter et al. 1998). In addition, surface-coated polyurethane catheters with a silver surface thickness of 15–20 Å have been investigated with regards to their biocompatibility and antimicrobial efficacy, showing markedly decreased adherence of gram-positive and -negative microorganisms in vitro (Jansen et al. 1994). Further interest has been raised regarding devices in which silver is distributed in form nanoparticles or in combination with other elements such as carbon and platinum. The 'Erlanger' silver catheter used a microdispersed silver technology to increase the quantity of available ionized silver (Böswald et al. 1999). The 'Oligon' catheters are composed of polyurethane in which carbon, silver and platinum particles are incorporated, which leads to an electrochemically driven release of silver ions in the outer and inner vicinity of the catheter surface. However, a peripherally implanted central catheter based on this technology (Olympicc ™, Vygon, Cirencester, UK) has been withdrawn from the market at least in Germany because of mechanical problems associated with this type of catheter. A more recent development is the Oligon Vantex® catheter (Edwards Life Science, Irvine, CA, USA) (Ranucci et al. 2003). Other approaches are catheters with the 'active iontophoresis' technology in which microorganisms are repelled by current generated from a carbon impregnated catheter (Liu et al. 1993) or where low amperage current is produced by two electrically charged parallel silver wires helically wrapped around the proximal segment of silicon catheters (Raad et al. 1996a).

Several clinical studies have been performed with silver-containing intravascular catheters. In a randomized, prospective study in haemato-oncological patients, a silver sulfate- polyurethane catheter (Fresenius AG, Bad Homburg, Germany UK) was associated with a significantly lower rate of CRBSI compared with the control group (10.2 % vs. 22.5 %, $p = 0.01$) (Goldschmidt et al. 1995). In three trials the 'Erlanger' silver catheter in which the silver is microdispersed was evaluated (Stoiser et al. 2002; Böswald et al. 1999; Carbon et al. 1999). In the adult population, a reduction in catheter colonisation and in

'catheter associated sepsis' was observed. However the authors used criteria for determining CRBSI that differed from most other studies. Furthermore, clinical investigation failed to show a statistically significant difference in the colonisation rate of the silver catheter compared with a control catheter (Stoiser et al. 2002). Ranucci et al. (2003) compared the Oligon Vantex® catheter, composed of a silver, carbon and platinum with a benzalkonium chloride-treated catheter (Multi-Med, Edwards Life Sciences, Irvine, CA, USA) in a prospective randomized trial. Use of the Oligon Ventax® catheter decreased the rate of catheter colonization by 11 %, while the rate of CRBSI did not differ significantly between the Oligon Vantex® and control group.

2.12 Metal Oxide–Fluoride Nanoparticle Coated Sterile Surfaces to Inhibit Biofilm Formation

The inherent resistance of biofilms to killing and their pervasive involvement in implant-related infections has prompted the search for surfaces–coatings that inhibit bacterial colonization. One approach comes from recent progress in nanotechnology, which offers an opportunity for the discovery of compounds with antimicrobial activity, as well as the use of "nanofunctionalization" surface techniques. Recent examples include the direct antibacterial properties of colloidal ZnO nanoparticles toward a broad range of microorganisms (Jones et al. 2008; Brayner et al. 2006) or the selective targeting of Au nanorods toward pathogenic bacteria and killing them by applying photothermal treatment (Norman et al. 2008). Other examples include the functionalization of biomaterials with antibacterial properties by coating (Roe et al. 2008), impregnation (Raad et al. 2008; Shi et al. 2006; Flemming et al. 2000), or embedding nanomaterials (Chang et al. 2008; Beyth et al. 2008).

Fluorides are well known for their antimicrobial activity (Marquis et al. 2003; Marquis 1995). This activity is mediated via three major mechanisms: (1) the formation of metal fluoride complexes, especially with Al and Be cations, which interact with F-ATPase and nitrogenase enzymes inhibiting their activities (Sturr and Marquis 1990); (2) the formation of hydrogen fluoride (HF), which disturbs the proton movement through the cell membrane (Guha-Chowdhury et al. 1997a); and finally (3) F^- or HF can directly bind and inhibit specific cellular enzymes. For example, enolase (an important enzyme in glycolysis) is known to be inhibited by a complex of F^- and Mg^{2+} at micromolar concentrations in low pH (Guha-Chowdhury et al. 1997b).

Recently, Lellouche et al. (2009), utilized a simple and fast microwave-based synthesis method to synthesize MgF_2 nanoparticles (MgF_2.Nps), and characterized their activity against two common nosocomial biofilm-forming pathogens (i.e., *E. coli* and *S. aureus*). Scanning and transmission electron microscopic techniques indicated that the MgF_2.Nps attach and penetrate into the cells. Flow cytometry analysis revealed that the Nps caused a disruption in the membrane potential. The MgF_2.Nps also induced membrane lipid peroxidation and once internalized can interact with chromosomal DNA. Based on these findings, these authors further explored the possibility of using the MgF_2.Nps to coat surfaces and inhibit biofilm formation. A microwave synthesis and coating procedure was utilized to coat glass coupons. The MgF_2 coated surfaces effectively restricted biofilm formation of the tested bacteria. The effectiveness of MgF_2 coated surfaces to inhibit bacterial colonization as a function of time was examined (Lellouche et al. 2009). As can be seen in Fig. 10.4a, b, the coated surfaces are able to restrict *S. aureus* and *E. coli* biofilm formation throughout the entire 3 days. Microscope evaluation of the surfaces clearly shows that the coated surfaces do not allow bacterial colonization and biofilm formation compared to the untreated controls. It is important to note that only on the third day do single cells begin to appear on the MgF_2 coated surfaces and many of those (~50 %) are dead (i.e., stained red) based on a live–dead staining (Fig. 10.4a). This data is also supported by viable counts obtained directly from the biofilm formed on the surfaces. Uncoated glass surfaces supported a massive biofilm formation (12.6×10^{11}

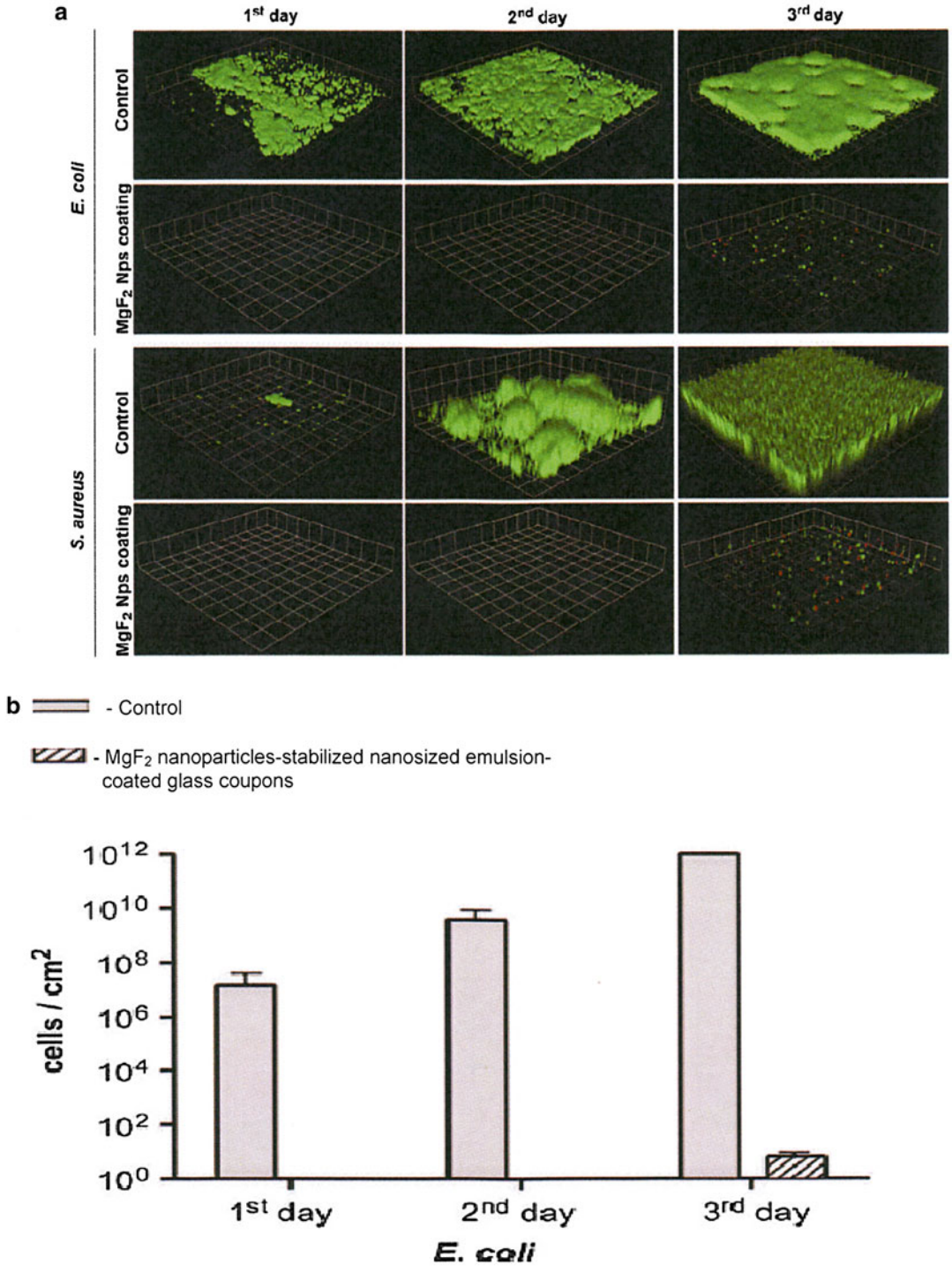

Fig. 10.4 Extended antibiofilm activity of MgF_2.Nps coatings on glass surfaces. (**a**) Confocal laser scanning microscopy (*CLSM*) images of *E. coli* and *S. aureus* following biofilms formation over the course of three consecutive days on uncoated and MgF_2.Nps coated surfaces. *Green* and *red* staining represents, respectively, live and dead bacterial cells. In all images, 1 unit equals 13.8 mm. (**b**) Viable count of the biofilm cells. (control refers to the biofilm development on uncoated surface) (Reproduced with permission Lellouche et al. 2009)

and 11.6×10^{11} CFU cm^{-2} for *E. coli* and *S. aureus*, respectively, for the 3rd day) while MgF_2 coated surfaces dramatically restricted bacterial colonization (9.3 and 8.0 CFU cm^{-2} for *E. coli* and *S. aureus*, respectively, in the last day). These results suggest that MgF_2 nanoparticles are effective in restraining bacterial colonization of the surface. Furthermore, these results also highlight the potential of using MgF_2 nanoparticles for the design of sterile surface coatings that may be useful for various medical applications including the management of device-related nosocomial infections.

Very recently, Lellouche et al. (2012a) reported one-step synthesis and coating procedure that yielded a homogenous MgF_2 nanoparticle layer on both the inside and outside of the catheter and the coated catheters were analyzed by high resolution scanning electron microscopy and energy dispersive spectroscopy. The coating thickness was found to be varied from approximately 750 to 1,000 nm on the inner walls and from approximately 450 nm to approximately 580 nm for the outer wall. The coating consisted of spherical MgF_2 nanoparticles with an average diameter of approximately 25 nm. These MgF_2 nanoparticles-modified catheters were investigated for their ability to restrict bacterial biofilm formation. Two bacterial strains most commonly associated with catheter infections, *E. coli* and *S. aureus*, were cultured in tryptic soy broth, artificial urine and human plasma on the modified catheters. The MgF_2 nanoparticles-coated catheters were able to significantly reduce bacterial colonization for a period of 1 week compared to the uncoated control. Finally, the potential cytotoxicity of MgF_2 nanoparticles was also evaluated using human and mammalian cell lines and no significant reduction in the mitochondrial metabolism was observed. Altogether, they have concluded that the surface modification of catheters with MgF(2) NPs can be effective in preventing bacterial colonization and can provide catheters with long-lasting self-sterilizing properties.

In the next report, Lellouche et al. (2012b) have shown a novel water-based synthesis of MgF_2 nanoparticles using sonochemistry. The sonochemical irradiation of an aqueous solution of [Mg(OAc)(2)·(H(2)O)(4)] containing acidic HF as the fluorine ion source afforded crystalline well-shaped spherical MgF_2 nanoparticles that showed much improved antibacterial properties against two common bacterial pathogens (*E. coli* and *S. aureus*). They have also able to demonstrate that the antimicrobial activity was dependent on the size of the nanoparticles. In addition, using the described sonochemical process, they have coated glass surfaces and demonstrated inhibition of bacterial colonization for 7 days. Finally, the antimicrobial activity of MgF_2 nanoparticles against established biofilms was also examined. Taken together they have highlighted the potential to further develop the concept of utilizing these metal fluoride nanoparticles as novel antimicrobial and antibiofilm agents.

A further novel strategy that represents a promising, but still poorly investigated tool for biofilm eradication from device surfaces and surrounding tissues is represented by the use of nanoparticles able to target antimicrobial agents, alone or possibly in combination with *quorum* sensing (QS)-interfering agents or enzymes (Chaignon et al. 2007). In fact, nanoparticles, either polymeric or inorganic, can be properly targeted to a localized area in which the drug release is planned to occur. In this regard, magnetically driven nanoparticles can be easily guided to a specific body area by the application of an appropriate magnetic field (Corchero and Villaverde 2009; Xie et al. 2009). Thus far, magnetic nanoparticles have been applied for imaging, delivery and targeting in cancer therapy (Gindy and Prud'homme 2009) and, very recently, were also demonstrated to be able to lower in vitro the OD of *S. epidermidis* broth cultures as well as to promote bacterial death for up to 48 h (Taylor and Webster 2009). Francolini and Donelli (2009) have also developed iron oxide-based magnetic nanoparticles exhibiting antimicrobial activity against both gram-positive and gram-negative bacteria after their coating with a silver-containing polymer and ciprofloxacin as a model of an antimicrobial agent.

3 Challenges Faced by the Manufacturers of Combination Devices

In the US, domestic and foreign manufacturers are required by the Good Manufacturing Practices regulations to have a quality system for the design and production of medical devices (plain devices without antimicrobial combination) that are intended to be sold in the country (Lowery et al. 1996). The primary objective of the quality system is to prevent defects in the design, manufacture, and shipment of products. The Quality System Regulation requirements for device manufacturers are as follows: various specifications and controls for the devices must be in place for devices; devices must be designed under a quality system; finished devices must meet these specifications; devices must be correctly installed, checked, and serviced; quality data must be analyzed to identify and correct quality problems; and complaints must be processed. Customer feedback on a regular basis, system audits, management reviews, and corrective and preventive actions are required for the system to remain dynamic and ensure improvements in the medical device, labeling, packaging, or quality system (Lowery et al. 1996). In Canada, the medical device industry must have a quality system certificate issued by the Canadian Medical Devices Conformity Assessment System as proof that manufacturers have complied with the appropriate regulatory system requirement. Class I medical devices and importers or distributors of medical devices do not have any regulatory quality system requirement [Quality systems ISO 13485 (webpage on the Internet). Ottawa: Health Canada; 2013. Available from: http://www.hc-sc.gc.ca/dhp-mps/md-im/qualsys/index-eng.php. Accessed on February 26, 2014)].

Post-market surveillance (PMS) represents a feasible means of reducing the risk of adverse medical device events (AMDEs) that are detectable and traceable. This approach would prospectively monitor safety and effectiveness; more rapidly identify and communicate incident data to avoid further events; guide the development of training, organizational process improvement, or other patient safety interventions; and direct decision making about funding or replacement by purchasers and policymakers. Medical device problems may surface years after they have been used or implanted in thousands of patients. As major changes to the medical device approval process are unlikely in the near future, policies intended to detect and reduce harms of medical devices must instead target the PMS space. The FDA published two reports in 2012 and 2013 on strengthening their national system for medical device PMS [fda.gov (homepage on the Internet). National medical device postmarket surveillance plan. Silver Spring, MD: US Department of Health and Human Services, Food and Drug Administration; 2014. Available from: http://www.fda.gov/aboutfda/centersoffices/officeofmedicalproductsandtobacco/ cdrh/cdrhreports/ucm301912.htm. Accessed on February 26, 2014]. The national surveillance system is intended to effectively communicate accurate information on the benefits, risks, and safety signals associated with the use of medical devices, from reliable data sources in a cost-effective manner, and simplify regulatory approval for new and current devices. The actions outlined to help achieve the desired functionality of the surveillance system are the (1) introduction of a unique device identification system; (2) establishment of national and international device registries; (3) update of existing adverse event reporting and analysis; and (4) development and use of new research methodologies for evidence generation, synthesis, and appraisal (US Food and Drug Administration. *Strengthening Our National System for Medical Device Postmarket Surveillance. Update and Next Steps*. Silver Spring, MD: US Food and Drug Administration; 2013. Available from: http://www.fda.gov/downloads/MedicalDevices/Safety/CDRHPostmarketSurveillance/UCM348845.pdf. Accessed on February 26, 2014).

Health Canada is responsible for the surveillance and reporting of device advisories, warnings, or recalls and posts this information in the Advisories, Warnings and Recall Database and

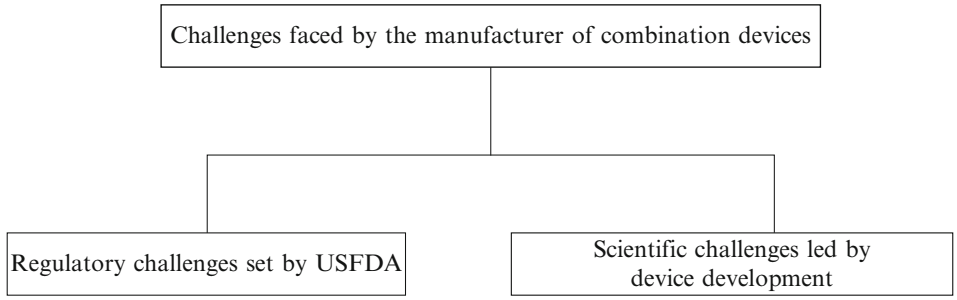

Fig. 10.5 Challenges faced by the combination devices manufacturers

Drug and Medical Device Recall Listing available on their website [Advisories, Warnings and Recalls-Drugs and Health Products (webpage on the Internet). Ontario: Health Canada; 2013. Available from: http://www.hc-sc.gc.ca/dhp-mps/advisories-avis/index-eng.php#share. Accessed on April 24, 2014]. The regulator issues device advisories, warnings or recalls by rich site summary (RSS), Twitter, email, and web postings, but if clinicians are not aware of or communicating this information to patients, patients are not providing fully informed consent about potential risks to which they may be exposed. An Auditor General review, however, has recommended improved collection and analysis of PMS data and communication of safety concerns to all stakeholders [Office of the Auditor General of Canada (homepage on the Internet). Chapter 6 – Regulating medical devices – Health Canada. In: Jun 2011 status report of the Auditor General of Canada. Ottawa: Office of the Auditor General of Canada; 2011. Available from: http://www.oag-bvg.gc.ca/internet/English/parl_oag_201106_06_e_35374.html. Accessed on March 15, 2014]. Ideally, information about device safety, clinical effectiveness, and potential risks communicated to clinicians would then be shared with patients for shared decision making about device use. This is fundamental to ethical and patient-centered care (Neumann and Tunis 2010; Curfman et al. 2009; Ross et al. 2008). An article by Polisena et al. (2014) describes the various components/measurements (an adverse medical device events database, a medical device/equipment library, education and training, and an open communication and feedback strategy) to find out the effectiveness of the proposed medical device surveillance system framework after it has been implemented in a Canadian hospital facility. A suggestion was given to establish/maintain the close linkages among these components and with external medical device/equipment networks to the hospital.

As far as the medical devices with antimicrobial combination, two distinct types of challenges confront the manufacture of combination devices: regulatory and a scientific (Fig. 10.5).

On the regulatory side, FDA in the past few years has been requiring human clinical trials to support any infection-reduction claims. This requirement, however, retards the development of new antimicrobial combination devices because small companies have difficulty recruiting the large numbers of patients required to conduct trials. Moreover, the incidence of many kinds of device related infections is very low, ie., less than 1 % for many kinds of orthopedic devices, for example. Moreover, because of concerns that low-level release of antimicrobials over time could select for antimicrobial-resistant bacterial populations, FDA has been asking manufacturers to demonstrate that their antimicrobial combination devices will not result in the generation of resistant isolates. Finally, manufacturers should also demonstrate that their antimicrobial combination devices will not significantly affect host-normal flora – a suggestion that is difficult to meet from a clinical-trial standpoint.

From a science standpoint, the main challenge is ensuring that a sufficient concentration of an antimicrobial agent will be available at the device surface for an extended period of time – for example, 30 days. For long-term implantable devices, extended antimicrobial protection may provide the host tissue with sufficient time to integrate with the device, reducing the risk of infection. So the big scientific challenge in this juncture is, "How do you achieve good antimicrobial activity for 30 days?" One of the most promising technologies is a polymer-antimicrobial combination in which the polymer is part of the actual delivery system. In such designs, the antimicrobial becomes part of the device, and we can achieve drug residence as long as the device remains intact. Advances have also been made in the development of antithrombogenic surfaces that reduce blood-clot formation – a method that is also known to inhibit microbial adhesion (Francolini et al. 2012). Because several types of bacteria require blood components to be able to attach to surfaces and form biofilms, reducing thrombus formation on medical devices that make contact with the blood can minimize microbial adhesion and the incidence of infections. So the engineered surfaces that both greatly limit the amount of thrombus formation and also seem to retard microbial adhesion to surfaces. But at the molecular level, it is however ultimately material structures that will sterically inhibit the development of bacteria on device surfaces. This also includes fungi, which are also associated with device-related infections.

4 Current and Future Developments

Various preventive strategies are already developed to control the microbial colonization of medical devices. Advancement of biofilm structure analyses using different microscopical techniques even at genomic/proteomic level helps the researcher to investigate novel small molecule and antipathogenic drugs, and to develop new pathways for the enhancement of the efficiency of certain antimicrobial agents. In addition, the various lipid-and polymer-based drug delivery carriers are also investigated for applying antibiofilm coating to prevent the microbial colonization of medical devices especially over catheters. Although one cannot predict what specific technology will be in place 10 years from now, it would be reasonable to say that ultrasonic/electronic-activated drug delivery will play an ever-increasing role in enhancing the permeation of certain antimicrobial agents through the biofilm microcosm. Furthermore multiple preventive strategies combining the ultrasonic/electronic with lipid-and polymeric-drug delivery carriers are also one of the potential thrust area for future investigation. However, it should be mentioned that the nature of biomedical/pharmaceutical research affords a broad range of investigational topics at the preclinical stage, not all of which may be explored in subsequent clinical studies.

5 Conclusion

The use of surgically implanted devices is increasing as a means to improve quality of life, and in some cases, to survival rates. However, these foreign bodies, once implanted, are sites of competition between host cell integration and bacterial adhesion. If bacteria are able to adhere successfully, they will undergo biofilm formation, which alters their properties and renders them resistant to commonly used antibiotics. Considering the additional medical expenses required for the removal of already implanted but being infected medical devices, it becomes necessary to look for the alternative ways of eradicating the device-related nosocomial infections. Native and stealth (pegylated) liposomes were in fact investigated extensively for improving antiadhesive property of the implant material, for concentrating the encapsulated antimicrobial agents in an adequate amount at the infected surfaces of the medical devices, and for targeting the antimicrobial agents to biofilm-associated intracellular infections. On the other hand, biodegradable and non-biodegradable polymer-based matrices,

beads, microspheres, strut, gels, fibrous scaffolds, etc., and surface (properties) modified polymeric catheter materials such as antimicrobial, antiseptic or metallic substances-coated polymeric materials were also developed in an attempt to eradicate biofilm-associated infections especially in implant and in periodontal cavity by the local delivery of the entrapped antibiotic substances. The advantages of these novel drug delivery carriers are mirrored by a high number of high quality scientific papers, published in conventional and open-access journals. However, the potential of lipid-and polymer-based drug delivery carriers in eradicating biofilm consortia in device-related nosocomial infections is not achieved fully in terms of further clinical application and subsequent approval from health care authorities. Recent developments in microscopy imaging and surface-analytical techniques allowed the quantitative in situ investigation of cell/surface interactions at submicron scale, providing information on the strength of microbial cell attachment to solid substrata and the properties of macromolecules involved in this process [See details in a review by Beech et al. (2005)]. Gaining deeper insight into the fundamental mechanisms of biofilm-mediated deleterious interfacial processes together with understanding of the physiology of biofilm bacteria at the genomic and proteomic levels will, undoubtedly, result in the development of practices that will aid in their control especially through lipid-and polymer-based drug delivery carriers. Hence, it is necessary to establish closer collaborations between scientists working in universities or research institutes and industrial investigators to hasten achievement of the above objectives and find more advanced solutions to prevent microbial biofilm causing medical device-related infections.

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Index

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