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Current Research Approaches to Target Biofilm Infections

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Abstract
This review will focus on strategies to develop new treatments that target the biofilm mode of growth and that can be used to treat biofilm infections. These approaches aim to reduce or inhibit biofilm formation, or to increase biofilm dispersion. Many antibiofilm compounds are not bactericidal but render the cells in a planktonic growth state, which are more susceptible to antibiotics and more easily cleared by the immune system. Novel compounds are being developed with antibiofilm activity that includes antimicrobial peptides, natural products, small molecules and polymers. Bacteriophages are being considered for use in treating biofilms, as well as the use of enzymes that degrade the extracellular matrix polymers to dissolve biofilms. There is great potential in these new approaches for use in treating chronic biofilm infections.

Keywords: antibiofilm strategies, antimicrobial peptides, bacterial biofilms, dispersal, enzyme treatments, infections, matrix polymers, small molecule inhibitors

Introduction

Biofilms are aggregates of bacteria growing together in a community surrounded by a protective and adhesive extracellular matrix (ECM) of exopolysaccharides (EPS), extracellular DNA (eDNA) and proteins (1-3). The formation of a biofilm involves the following stages: attachment to a surface, formation of microcolonies, maturation and dispersal (4). Biofilms are a successful long-term survival strategy employed by bacteria in the environment and during infection due to the resistance to hostile conditions, antibiotic treatment and to immune evasion (4, 5). Biofilms have been demonstrated to be more than 1000-fold resistant to treatment with conventional antibiotics normally used to treat planktonic cells (6). Resistance to antibiotics in biofilms is multifactorial and due to poor penetration of antibiotics into the biofilm through the ECM, the presence of multidrug resistant persister cells, slow growth rates and antibiotic indifference, as well as the expression of specific resistance mechanisms of cells within biofilms (6-8).

Biofilms are often associated with human disease and are responsible for the majority of bacterial infections (9). Biofilm-related infections develop on mucosal surfaces and include lung infections of Cystic Fibrosis (CF) patients, chronic obstructive pulmonary diseases, otitis media, sinusitis, and chronic wound infections (10-14). Biofilms also commonly develop on the surfaces of medical implant devices including catheters, prosthesis, pacemakers, and intrauterine devices, to name a few, and are responsible for 50% of nosocomial infections that occur when patients have indwelling medical devices (15). Medical implants or devices such as an indwelling catheter or a respiratory apparatus are particularly susceptible to biofilm formation because the host immune response is reduced in areas of the body in contact with foreign devices (16). As a result, infections associated with medical implants and devices are a problem due to growth of the bacteria, a lowered immune response, and resistance of the bacterial biofilm to antibiotic treatment. The only solution is most often to remove the implant, which is traumatic to the patient and costly (17).

Biofilms play a major role in infectious disease and pose a significant challenge in the treatment of these infections. Since conventional antibiotics were designed to target planktonic cells, there are currently no drugs available to specifically treat biofilm-related infections (5, 18). It is imperative to develop new treatments that will be effective in eliminating these infections and reducing the costs associated with complications from the use of medical devices. This review will outline the advances made in the discovery of novel antibiofilm strategies with the potential to treat biofilm-related infections.

Some antimicrobial peptides have antibiofilm activity

Antimicrobial peptides (AMP), also known as host defense peptides (HDP), are conserved antimicrobial molecules that are produced by virtually all organisms (20, 21). These peptides are composed of 12-50 amino acids with an excess of lysine and arginine residues, which make them cationic (20, 21). They are also very hydrophobic, which enhances their antimicrobial activity as they are able to interact with bacterial membranes (22). Most AMPs have direct antimicrobial activity by disrupting bacterial membranes, and others have immune modulating activity without strong direct antimicrobial effects (8). A variety of natural and synthetic peptides have recently been shown to have a novel antibiofilm activity against both Gram-positive and Gram-negative bacteria (23-32). Synthetic antimicrobial peptides may be good candidates for treatment of biofilms as they are small, less costly to produce, demonstrate low toxicity, are relatively stable, and have specificity for biofilms in lower doses than the minimum inhibitory concentration (MIC) for planktonic cells (22).

A number of synthetic and naturally occurring peptides have been shown to have broad-spectrum antibiofilm activity (23-32). One synthetic peptide of interest, 1018, based on the amino acid sequence of a peptide named Bac2a, derived from the naturally occurring bovine HDP bactenecin, was found to be very effective against biofilms produced by a number of pathogenic bacteria (27). Although this peptide did not exhibit strong antimicrobial activity against planktonic cultures, it did demonstrate antibiofilm activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella enterica*, and *Burkholderia cenocepacia* at sub-MIC concentrations (0.8 μg/ml for dispersal and 10 μg/ml for cell death) (30). In addition it was found that this peptide targeted the stress response nucleotide ppGpp for degradation. This stress response effector normally binds to RNA polymerase in order to induce biofilm formation and maintenance (30). Given the conserved function of ppGpp in Gram-positive and Gram-negative bacteria, this may explain the broad-spectrum antibiofilm activity of peptide 1018 (33).

Our recent research has been aimed at evaluating the effectiveness of a number of synthetic AMPs for activity against biofilms using the BioFlux microfluidics system, a physiologically relevant system that employs the use of shear flow for the development of *in situ* biofilm formation. A number of synthetic peptides were developed based on the sequence of 1018 and these were tested for antibiofilm activity using this relatively high throughput screen (HTS) system that enables screening of synthetic peptides with visualization and analysis of cell viability following treatment (34).

Synthetic AMPs (274) were screened for effectiveness against biofilms in a 48-well plate format. Images of the biofilms were acquired following peptide treatment in both bright-field and fluorescence in order to visualize the integrity of the biofilm, the amount of viable cells (due to a chromosomal insertion of green fluorescent protein which is expressed in growing cells) and non-viable or membrane compromised cells (determined by propidium iodide staining), and to calculate the overall destruction of the biofilms. A number of peptides were found to demonstrate significant efficacy in eliminating biofilms and decreasing the viability of the cells, including some D-

enantiomeric peptides (35). A number of peptides were effective against *P. aeruginosa* biofilms, and we have recently identified some peptides effective against *K. pneumoniae* biofilms as well (in preparation). All of the effective peptides were found to have MIC values much higher than the concentration needed to eliminate or reduce biofilm development. The specificity of these peptides for biofilms raises questions about the structures of these particular peptides and their mechanism of action. Segev-Zarko *et al*. (32) recently found that a number of antimicrobial peptides composed of 6 lysine and 9 leucine residues in alternative sequences, had differing effects on biofilms. Some peptides degraded biofilms by killing embedded cells and some by causing bacteria to detach or disperse (32). The elucidation of mechanisms of action of AMPs on biofilms and whether they inhibit or eliminate biofilms will add valuable insights in the adoption of these peptides for the treatment of biofilm infections (32).

Bacteriophage therapy to fight biofilm infections

Bacteriophages are another approach to consider in the treatment of biofilm infections. These viruses infect and replicate within the bacterial cell and then lyse their host. Bacteriophage therapy has actually been used for over 50 years, but the emergence of multi-drug resistant bacteria and the continued development of resistance in many bacteria have prompted more studies into the use of bacteriophage as a means of treating infections (36). The advantage of using bacteriophage is that they can infect and kill both antibiotic sensitive and resistant bacteria (9).

A number of studies have been conducted that have shown the efficacy of using bacteriophages in biofilm infections (36-38). Bacteriophages have been shown to be effective against wound infections caused by *S. aureus* and multi-drug resistant *S. aureus* biofilms (39, 40). They have also been shown to clear biofilm infections caused by *P. aeruginosa* (41). Two lytic phages were recently described that were found to reduce Staphylococcal biofilms by 2 logs and the frequency of bacteriophage resistance developing in the bacteria was sufficiently low to merit these bacteriophages as potential candidates for therapy (42). Another recent study found a bacteriophage, EFDG1, to have effective lytic activity against planktonic and biofilm cultures of *Enterococcus faecalis* and *E. faecium* isolates, regardless of their antibiotic resistance profile (43). In addition, EFDG1 efficiently prevented an *ex vivo E. faecalis* root canal infection (43). There are a number of advantages in using bacteriophages to treat biofilm-related infections. Phages are specific, inexpensive, should not affect the normal microflora due to their specificity for one organism, and are synergistic with conventional antibiotics (44). Further studies into the effect of phage therapy and synergy of bacteriophages with antibiotics may prove to be a useful strategy in the treatment of biofilm infections.

The other advantage to using bacteriophages is the potential to engineer these viruses to have increased killing efficiency against biofilms. Bacteriophages frequently express enzymes to degrade bacterial cell walls and cell contents. Hughes *et al.* (45) identified the importance of the enzymatic attack of SF153b bacteriophage against *Enterobacter agglomerans* strain 53b biofilms. A depolymerase enzyme disrupts the EPS layer and allows the phage to infect and kill biofilm cells, events that lead to the disruption of the biofilm structure. Other studies have demonstrated T7 engineered phage expressing the EPS-degrading enzyme Dispersin B to be more efficient in killing *E. coli* biofilms than phages alone (46). A recent study utilizing a phage expressing a lactonase enzyme that degrades quorum sensing bacterial signaling molecules was shown to be effective in preventing biofilm formation in mixed cultures of *P. aeruginosa* and *E. coli* (47).

Small molecules with antibiofilm activity that reduce virulence

The universal first step of biofilm formation is attachment to a surface and several approaches are aimed at blocking initial adhesion. Using a rational approach, Svensson *et al*. (48) designed a new class of small molecules, derived from the saccharide binding PapG adhesin molecule from *E. coli* type 1 pili. These molecules are named pilicides and mimic the pilus protein and target periplasmic chaperones, thereby blocking pili assembly and function. Reduced pili expression decreases virulence and biofilm formation in uropathogenic *E. coli* (UPEC). Similarly, other peptidomimetic ring-fused 2-pyridones that share common chemical structures with pilicides are able to prevent UPEC biofilm formation *in vitro* and *in vivo*. These compounds prevent biofilm formation in a curly fiber- and type 1 pili-dependent matter, attenuating UPEC virulence in mice urinary tract infection model (49, 50). Considering the high degree of conservation and the importance of pili and other chaperone pathways in Gram-negative bacteria, pilicide-analogues may be useful for future therapeutic approaches in prevention of biofilm formation (49, 51, 52).

Scientists have also searched for potential active antibiofilm compounds among small molecule libraries. Regarding natural products, previous reviews have described the antibiofilm properties of plant extracts, such as garlic and cranberries, halogenated furanones isolated from the red algae *Delisea pulchra*, salicylic acid and cinnamaldehyde, among others (53, 54). The polyphenolic compound tannic acid found in tea was shown to block *S. aureus* biofilm formation, as well as limit oral colonization in a rat infection model (55).

Quorum sensing (QS) signaling systems are responsible for the coordination of gene expression at a bacterial community level, which includes controlling the expression of virulence factors, as well as influencing the formation of biofilms (19, 54). Many natural products act as quorum sensing inhibitors, and therefore have beneficial effects towards reducing biofilm formation (54). In addition, QS inhibitors also reduce the virulence of *P. aeruginosa* and *B. cenocepacia* in multiple animal models of infection, and importantly, are synergistic when combined with conventional antibiotics, leading to increasing bacterial killing (54, 55, 56).

Analogs of bromoageliferin, a natural product from marine sponges, were shown to have antibiofilm activity against *P. aeruginosa* (57). The same group later characterized an antibiofilm molecule with broad-spectrum activity. Dihydrosventrin (DHS) was identified from screening of a 50-member library of derivatives of bromoageliferin and was able to inhibit and disperse biofilm in *P. aeruginosa* (PAO1, PA14 and mucoid isolate), *A. baumannii*, and *Bordetella bronchiseptica* (58). Further derivatives of DHS were constructed as a library of 2-aminoimidazole (2-AI) analogs, and were very effective in both inhibiting biofilm formation, as well as dispersing preformed biofilms (59). Several of these compounds have antibiofilm activity at concentrations less than their bactericidal concentration, similar to some antimicrobial peptides (53, 59). A compound from the 2-AI library appeared to block biofilm formation through a zinc-chelating mechanism, as the compound could bind zinc, and excess zinc blocked its antibiofilm activity (60). Other 2-AI derivatives were also shown to act synergistically with antimicrobials to sensitize resistant bacteria without showing increased toxicity in combination with antibiotics, supporting its possible use as a therapeutic adjuvant for resistant bacterial treatments (61, 62).

HTS for identification of molecules with antibiofilm activity

Another approach used by researchers for the identification of molecules active in preventing biofilm formation is the screening of large chemical libraries. The use of HTS techniques allows the testing of a massive number of samples in a short period of time. One of the earliest HTS used a luminescence-based approach to quantitate *P. aeruginosa* biofilm biomass formed on 384-well format pin devices, as opposed to conventional crystal violet (CV) biofilm staining (63). After screening 66,095 compounds, 30 molecules were identified that

blocked biofilm attachment by greater than 50% when used at concentrations less than 20 μM (63).

Other HTS approaches were devised that targeted specific mechanisms of biofilm formation. The signaling molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) accumulates under conditions that promote EPS production and biofilm formation, and appears to be universally conserved in Gram-negative bacteria (64). Therefore, researchers have screened for antibiofilm molecules that block the synthesis of c-di-GMP or that reduce the expression of c-di-GMP-controlled promoters. In the first approach, the antibiofilm screen was to identify compounds that reduced the congo red (CR) phenotype of *E. coli* colonies on agar plates, as it is known that EPS and curli production is required for the red phenotype (64). Screening of a 1,120-member drug library allowed the identification of sulfathiazole, as an inhibitor of c-di-GMP biosynthesis.

In a second c-di-GMP targeted approach, Sambanthamoorthy *et al.* (65) screened approximately 66,000 compounds by using a transcriptional luciferase reporter to a c-di-GMP responsive promoter and searched for compounds that reduced expression and luminescence. Antibiofilm compounds that repressed this transcriptional reporter and also blocked biofilm formation in *Vibrio cholerae* were identified. The lead compound was the molecule 5-methoxy-2-[(4-methylbenzyl)sulfanyl]-1H-benzimidazole, which had broad-spectrum antibiofilm activity and blocked biofilm attachment when polystyrene surfaces were coated with the compound (65). However, this lead compound did not cause dispersion from preformed biofilms.

HTS for antibiofilm drugs have also been performed using a 3,080-member in-house pre-fractionated marine natural products library to identify inhibitors of *V. cholera*e biofilm formation (66). In this approach, biofilms were quantitated in 384-clear well bottom microplates using epifluorescence microscopy to image *gfp*-tagged *V. cholerae* biofilms in a single focal plane. This HTS lead to the further identification of a novel antibiofilm compound auromomycin (67). Recently, the same group extended this HT imaging approach to identify biofilm inhibitors, as well as inducers of dispersal in *P. aeruginosa* biofilms (68).

Although numerous effective antibiofilm molecules have been identified to date, most of them lack toxicological and pharmacological testing for a better understanding of their mechanism of action (69). Expecting to bypass this difficulty and aiming to come out with a new antifungal compound that could be easily approved for faster commercialization, Siles *et al*. (70) looked for antibiofilm agents against *Candida albicans* in a 1,200-member small molecules library constituted of Food and Drug Administration (FDA)-approved compounds. These compounds have well understood mechanisms of action, pharmacological characteristics and toxicological properties. Their screen identified 38 compounds from heterologous pharmacological classes with potent antifungal biofilm properties, reducing *Candida* biofilm formation over 50%. This significantly higher rate of “hits” (3.25%), compared to other HTS reports (<0.1%), is not unexpected when acknowledging that the library contained only drug-like molecules. From the 38 initial hits, follow up dose-dependent assays identified two polyene antifungal drugs, six antiseptics/antimicrobials and three miscellaneous drugs that were effective against formation and destruction of preformed *C. albicans* biofilms (70).

Compounds and enzymes to disperse or dissolve biofilms

Another possible approach treat bacterial biofilms is the use of compounds that cause dispersion from aggregates or enzymes that degrade the polymers of the ECM and thereby dissolve biofilms. One of the earliest dispersal agents was the discovery of cis-2-decenoic acid (C2DA), an unsaturated fatty acid produced by several types of bacteria (71). Other biofilm dispersants include D-amino acids, which are

produced by bacteria throughout growth (72), salvipisone, a diterpenoid isolated from hairy root of *Salvia sclarea* (73), are able to disperse biofilms in a range of Gram-positive and Gram-negative clinically relevant bacteria.

Biological surface-active agents, also known as biosurfactants, are a heterologous and versatile class of chemicals with amphiphilic properties, produced by microorganisms (74). Biosurfactants are another promising class of substances with possible implementations on the treatment of biofilm-related infections. In a recent review, Banat *et al*. (74) highlighted some properties of biosurfactants towards clearance or prevention of biofilms, including inhibition of initial adherence and disruption of biofilm structure, in a range of bacterial and fungal strains. Synergistic inhibition effect with conventional antimicrobials has also been described (75).

Polysaccharides (PS) are also a class of natural substances that have been recently shown to possess non-microbicidal antibiofilm properties (76). In a review by Rendueles *et al.* (76), several examples of antibiofilm PS (APS) are described, including a secreted *E. coli* group II capsular PS, that blocked biofilm formation of both Gram-positive and Gram-negative bacteria (76, 77). Interestingly, known matrix polymers that promote aggregation in *P. aeruginosa* can actually prevent biofilm formation by other species (76). APS were recovered as secreted products from planktonic, agar and biofilm cultures, but membrane-linked lipopolysaccharide also possesses antibiofilm properties. These compounds do not inhibit growth, but are generally thought to act as biosurfactants, capable of modifying cell-surface interactions (76).

The complex constitution of the biofilm matrix has been described. As there is a considerable variation among biofilm constituents within different species (78), multi-enzymatic formulations seem to be necessary for an adequate biofilm control (79) and it has been proposed already that enzymatic degradation of EPS, proteins and eDNA are involved in cell dispersal from biofilms and may be significant for the development of new therapies (80-82).

Dispersin B is a naturally occurring enzyme produced by *Aggregatibacter actinomycetemcomitans* and known to degrade EPS. This enzyme inhibits biofilm formation and disperses preformed biofilm in diverse bacterial strains. In a recent study, Gawande *et al*. (83) showed that combined therapy of Dispersin B with broad-spectrum KSL-W cationic antimicrobial peptide showed synergetic antibiofilm and antimicrobial activity in MRSA, *S. epidermidis*, Coagulase-negative Staphylococci (CoNS), *A. baumannii,* Vancomycin-resistant Enterococci, *K. pneumoniae*, and *P. aeruginosa* chronic wound infection-related bacteria.

Recombinant human DNase I, Dornase alfa (Pulmozyme®), is one of the therapies used to reduce mucus thickness and improve lung function in people with CF (69, Frederiksen et al, 2006). This recombinant enzyme also degrades eDNA of bacterial biofilms and causes a significant decrease in bacterial colonization in the lower respiratory tract of CF patients (84), Deoxyribonuclease has broad-spectrum antibiofilm activity because of the universal role of eDNA in the biofilm matrix (85). In addition to EPS and DNA degrading enzymes, proteases or chitinases are also useful to reduce biofilm formation (86, 87).

This data highlights the importance of diverse biofilm matrix polymers in the development and maintenance of the biofilm structure, and the possibility of using enzymes in prevention/dispersal of these bacterial communities. Despite the success in degrading biofilms, caution should be exercised with this approach as releasing planktonic bacteria may also pose a risk to increased dissemination and possibly increased severity of disease.

Conclusions

It has been estimated that 80% of infections are caused by biofilms. We have presented a number of strategies that have shown significant promise towards the development of antibiofilm treatments. These treatments have demonstrated either inhibition or degradation of biofilms, either alone or in synergy with

conventional antibiotics. Biofilms can be targeted by dispersal using certain small molecules or AMP or by degradation of the ECM using enzymes or engineered bacteriophage. Additionally, the cells within the biofilms may be lysed by bacteriophages and AMPs. Some compounds have been shown to inhibit biofilm formation rather than eliminate it, by inhibiting specific pathways essential to biofilm formation. Some of the treatments described in this paper may achieve more than one function, such as dispersal and killing, or antivirulence activity. Finally, HTS has facilitated the identification of many new antibiofilm candidates.

Some of the potential advantages of these strategies are that they may be less toxic and effective at concentrations lower than the concentration to inhibit planktonic cells.. However, as indicated, some of the compounds identified need to be further characterized in terms of toxicity and required dosage. Compounds or molecules that have antibiofilm activity will also need to be characterized structurally and their mechanism of action on biofilms needs to be better studied. The variety of solutions identified for the treatment of biofilm infections is very promising in light of the urgent need for alternatives to conventional antibiotics.

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