Chapter

Inhibition of Bacterial Biofilm Formation

Angela Di Somma, Antonio Moretta, Carolina Canè, Arianna Cirillo and Angela Duilio

Abstract

Biofilm is a complex matrix consisting of extracellular polysaccharides, DNA, and proteins that protect bacteria from a variety of physical, chemical, and biological stresses allowing them to survive in hostile environments. Biofilm formation requires three different stages: cell attachment to a solid substrate, adhesion, and growth. The inhibition of one of these steps by small molecules, such as antimicrobial peptides, or their action on specific targets will leave pathogens armless against classical antibiotics. Any drug impairing crucial processes for bacterial life will inevitably lead to the development of drug-resistant strains, whereas the inhibition of biofilm formation might prevent the onset of bacterial resistance. In this section, we will focus on proteins involved in biofilm formation as useful targets for the development of new drugs that can effectively and specifically impair biofilm formation with slight effects on cell survival, thus avoiding the generation of drug-resistant strains.

Keywords: bacterial biofilms, biofilm inhibition, antimicrobial peptides, protein target, mechanism of action

1. Introduction

Microorganisms have the extraordinary ability to live in almost all environments and to protect themselves from external agents through sophisticated survival mechanisms. Bacteria can be found in planktonic form or in specific conditions, as sessile aggregates on both biotic and abiotic surfaces originating complex structures known as biofilm.

Biofilms are an ensemble of microbial cells irreversibly associated with a surface and enclosed in an essentially self-produced matrix. The biofilm matrix consists of polysaccharides, proteins, and DNA and constitutes a stubborn source that protects bacteria from a variety of physical, chemical, and biological stresses. One of its characteristics is the capability to impair antimicrobial molecules to spread through the polymer matrix or the ability of the matrix material to inactivate antibacterial molecules. Today, the increase and spread of antibiotic resistance among microorganisms (bacteria, fungi, viruses, and parasites) represent one of the greatest emergencies for human health worldwide [1]. Based on these characteristics, biofilm plays crucial roles in humans and nonhuman infections and represents the most important adaptive mechanism closely related to pathogenicity.

An antibiofilm agent must display several specific characteristics to target the biofilm lifestyle. First, due to the temporal biofilm heterogeneity, it must show a rapid killing ability to face a changing entity and to target cells before their entry into the biofilm community; it must be able to act in different environmental niches and to target different growth rate cells. The cells located in the periphery of biofilm are directly in contact with nutrients and oxygen, while those placed deepest in the biofilm layers may undergo lack of nutrients, anoxia, and acidic conditions. In this way, a metabolic and spatial heterogeneity is generated including both rapidly and slowly growing cells. In particular, due to environmental conditions, inside the biofilm, it is possible to find the so-called persister, dormant, quiescent cells characterized by a low rate of cell division that are believed to play an essential role in the biofilm resistance to antibiotics [2]. Other important characteristics for a good antimicrobial candidate are the ability to interfere with the production of the extracellular matrix and the possibility to penetrate the biofilm architecture. This matrix consists for 90% of EPS, whose principal components are proteins, polysaccharides, lipids, and extracellular DNA, and it is involved into the biofilm architecture maintenance. An antibiofilm agent should also be able to interfere with bacterial cell communication machinery.

This chapter aims to investigate and clarify in detail the inhibition of biofilm formation by different approaches.

Other additional aspects to consider the identification of potential antimicrobial agents are the ability to recruit immune cells and/or modulate the host immune response and the synergy with other conventional and unconventional antimicrobial compounds [3, 4].

Biofilms are very dynamic and spatially heterogeneous structures originating gradients of oxygen, nutrients, and pH, and their formation occurs through three phases: adhesion, maturation, and dispersal phase as described earlier.

2. Small molecules capable to inhibit biofilm formation

The inhibition or prevention of biofilm formation has been a subject of study for a long time. The first important action against biofilm formation is to prevent bacterial adhesion to surfaces and host tissues to reduce infection [5]. Preventing bacterial adhesion is an attractive target [6] for hampering bacterial infection, and several different strategies have been proposed including hindering cellular receptors from recognizing adhesion surfaces or inhibiting the process of bacterial adhesion. Blocking the primary colonizers can prevent initial biofilm colonization and the subsequent infection produced by planktonic cells released from the biofilm itself.

The adhesion process consists of various distinct steps. In the first step, bacterial cell establishes reversible adhesion interactions on host surfaces [7], while in the second step, a stronger type of adhesion is carried out, which involves specific molecules that bind in a complementary manner [5]. In particular, in Gram-negative bacteria, adhesion is mediated by special proteins known as adhesins associated with cell surface structures such as fimbriae or pili [8, 9]. Initial adhesion is then followed by a complex colonization process that offers a number of advantages to bacteria, including increased protection against dislocation by hydrokinetic forces from fluid surfaces or better access to nutrients released by the host cells [10]. Finally, in these favorable conditions, the development of the elaborate biofilm structures can take place.

For a long time, the first strategies used to inhibit the adhesion process were focused on the use of adhesin analogues that bind to the receptor and competitively

block bacterial adhesion [5]. However, this strategy resulted unpractical because adhesin proteins are not readily available, and they become toxic at the relatively high concentrations that had to be used. An attempt to overcome this problem consisted in the design and use of synthetic peptides mimicking the sequence of cell surface adhesins. For example, the small peptide p1025 inhibits *Streptococcus mutans* binding to dental surfaces [11]. Analogously, a fragment of the fimbrillin adhesin was found to inhibit the adhesion of *Porphyromonas gingivalis* to hydroxyapatite [12]. However, this approach showed several drawbacks as different adhesins usually mediate the adhesion process and the expression of carbohydrates or cell surface ligands may vary depending on environmental conditions, originating a large number of variables and making this approach more difficult and not very applicable.

A novel and interesting approach to inhibit bacterial adhesion consists in the use of cell coatings with antimicrobial peptides that alter the chemical properties of the surface [13, 14], thus interfering with bacterial adhesion and preventing surface binding. Although "passive," this method is rather attractive and may serve as a novel approach to address the biofilm problem on artificial medical devices. However, limited successes have been achieved so far due to attachment variability among different bacterial strains. Recently, many active polymeric coatings were designed to bind the surface and release a variety of antimicrobial molecules such as antibiotics, bacteriocins, and metal ions [15–18]. A significant reduction in biofilm formation of *Staphylococcus epidermidis* on hydrogel-coated and serum/hydrogel-coated silicone catheters was observed following the release of bacteriophagic factors from the polymer with and without supplemental divalent cations [19]. Similarly, treatment of piperacillin-tazobactam coated tympanostomy tubes reduces biofilm infection of ciprofloxacin-resistant *Pseudomonas aeruginosa* (CRPA) [20]. The negative aspect of this approach might be the continuous release of high concentration of antimicrobials in a short time by the active polymer often higher than the MIC values without a specific target. However, target release polymer can be foreseen as the new era of biofilm treatments in industrial food safety and packaging [21].

Recently, great attention was paid to a different approach addressed to killing planktonic cells for prevention and treatment of biofilms. The new catheter lock solution C/MB/P (citrate, methylene blue, and parabens) was able to act against planktonic and sessile bacteria within a biofilm preventing bacterial colonization of hemodialysis catheters [22]. Killing planktonic cells might represent a good approach, but this strategy cannot be carried out on long term because any drug targeting crucial processes for bacterial life will unavoidably lead to the development of resistant strains.

An effective and positive control of biofilm formation might be obtained by interfering with specific cellular process crucial for biofilm formation. Biofilm formation is often associated with the phenomenon of quorum sensing (QS), in which bacterial cells communicate with each other by small diffusible signal molecules [23]. Moreover, bacterial gene expression has to be synchronized to form biofilms, and to achieve this goal, the quorum-sensing (QS) mechanism is used by bacteria, producing and responding to a several intra and intercellular signals called autoinducers [24]. At low-cell densities, the autoinducer is present in the extracellular media in a small amount that is too dilute to be detected. When the cell density increases, the autoinducer concentration reaches a threshold, and the autoinducer-receptor complex (the regulatory protein) acts to induce or repress the expression of target genes. The QS controls some physiological processes such as secretion of virulence factors, biofilm formation, and antibiotic resistance in several bacterial species [25, 26]. Investigation and elucidation of

the molecular mechanisms underlying the QS effects on biofilms including the production of virulence factors may help to control bacterial infection. More than 70 species of Gram-negative bacteria communicate and control their population density and mobility via N-acyl homoserine lactones (AHLs) mediated QS and represented one of the primary scaffolds studied for the design of potential biofilm inhibitors [27]. N-butanoylhomoserine lactone 1 (C4-AHL, for the rhl system) and 3-oxo-C12-AHL 5 (for the las system) are among the most important AHLs involved in QS (REF Small molecule control of bacterial biofilms). In *P. aeruginosa*, one of the most important bacteria involved in human infections, different antibiofilm molecules focused on AHL analogues were designed to develop new strategies to impair biofilm formation. The Blackwell et al. identified, designed, and synthesized several different AHLs capable to significantly reduce biofilm formation and virulence factor production in *P. aeruginosa* [28, 29].

A different approach consisted in the use of the synthetic halogenated furanone produced by secondary metabolism of the Australian macroalga *Delisea pulchra*, which is able to penetrate the biofilm matrix and to alter its architecture in flow chambers [30, 31]. Furthermore, T315, an integrin-linked kinase inhibitor previously identified as a potential therapeutic agent against chronic lymphocytic leukemia [32], was shown to selectively inhibit biofilm formation in both *Salmonella typhi* and *Salmonella Typhimurium* at early stages of biofilm development without affecting bacterial viability. T315 was also demonstrated to reduce biofilm formation in *Acinetobacter baumannii* but had no effect on *P. aeruginosa* suggesting a bacterial specificity [33].

3. Biofilm inhibition by antimicrobial peptides

Antimicrobial peptides (AMPs) are small molecules (10–100 amino acids) widespread in nature that play an essential role in the innate immunity. Recently, much attention has been paid to AMPs as they exert a broad spectrum of action, exploiting different activities as antibacterial, antifungal, antiparasites, anticancer, and antibiofilm factors [34]. This paragraph will focus on the ability of some antimicrobial peptides to inhibit biofilm formation.

The use of antimicrobial peptides to impair biofilm formation is attracting great interest, and many peptides have already been tested on different bacterial biofilms. In particular, the molecular mechanism of biofilm inhibition by AMPs is very much under investigation. The AMPs tested on biofilms so far derive from different natural sources, such as humans, mammals, bacteria, plants, and amphibians, but many synthetic peptides have also been studied. For example, it was demonstrated that the human cathelicidin LL-37 and indolicidin peptides could prevent biofilm formation of *P. aeruginosa* by downregulating the transcription of Las and Rhl, two quorum-sensing systems [35]. Moreover, AMPs could inhibit biofilm formation by increasing twitching motility in *P. aeruginosa* through the stimulation of the expression of genes needed for type IV pilli biosynthesis and function. Type IV pilli has the main function to increase bacteria movement on surfaces, which could facilitate cell removal [35]. The synthetic antimicrobial peptide meta-phenylene ethynylene (mPE), based on magainin, was active against biofilms of *Streptococcus mutans*, both as an intracellular antibiotic by binding to DNA and as a membrane-active molecule inhibiting lipopolysaccharides (LPSs), similar to magainin action [36].

In addition, the LL-37 peptide can also inhibit initial biofilm attachment. In *Pseudomonas aeruginosa*, this peptide downregulates the expression of genes associated with the assembly of flagella involved in the process of initial adherence [37]. Antiadhesion could be one of the major AMPs antibiofilm properties leading

to their potential use as an effective pretreatment strategy. For example, the nisin peptide, which interferes with cell wall synthesis and is capable to form membrane pores, delays biofilm formation, but it does not inhibit the *Staphylococcus aureus* growth when it is immobilized in multiwalled carbon nanotubes [38].

AMPs can also cause biofilm matrix disruption. The human liver-derived hepcidin 20 peptide can reduce the mass of extracellular matrix and can alter the *S. epidermidis* biofilm architecture by targeting polysaccharide intercellular adhesin (PIA). Being endowed with nucleosidase activity, the fish-derived piscidin-3 peptide can degrade *P. aeruginosa* extracellular DNA by coordinating with Cu^{2+} through its N-terminus [39, 40].

Although several antimicrobial peptides have nowadays been studied for the inhibition of biofilm formation, a further aspect needs to be considered. Several biofilms have developed defense mechanisms to protect themselves from antimicrobial agents. The interaction with EPS is thought to be the principal reason of biofilm resistance to AMPs even if the exact mechanism is not well understood. Gram-negative bacteria, such as *P. aeruginosa*, can secrete alginate, an anionic extracellular polysaccharide consisting of uronic acid D-mannuronate and C-5 epimer-L guluronate. Alginate can interact with cationic AMPs and protect *P. aeruginosa* biofilm from the effect of the antimicrobial peptides [41]. Moreover, the peptide sensing system known as aps, first recognized in *S. epidermidis*, can protect Gram-positive bacteria from AMP action. This system upregulates the D-alanylation of teichoic acid and increases the expression of putative AMP efflux pumps. It was demonstrated that *Enterococcus faecalis* D-alanine deficient mutant is more resistant to AMPs than the wild type even if they produce less biofilm [42].

4. Biofilm inhibition by protein targets

Planktonic bacteria can adhere to different cells or tissues starting biofilm formation via production of a multitude of proteins, which act at different stages of biofilm formation. Some proteins contribute to biofilm accumulation, while others are involved into the mediation of primary attachment to surfaces [43, 44]. For this reason, the formation and the development of bacterial biofilm can be associated with the production of specific proteins, which play essential roles in the bacterial biofilm formation and development. Strategies leading to the identification of these proteins are fundamental as they could represent interesting targets to inhibit biofilm formation, allowing the development of new antibiofilm agents and procedures [45]. In this paragraph, we will focus on some target proteins involved in the production of biofilms in different bacteria: the N-acetylneuraminate lyase (NanA) in *Escherichia coli*, the bifunctional enzyme N-acetyl-D-glucosamine-1-phosphate acetyltransferase (GlmU) in *Mycobacterium smegmatis*, and the surface protein G (SasG) in *S. aureus*.

The NanA protein of *E. coli* is an enzyme able to recognize the sialic acid, a molecule essential to a number of critical biological processes, such as cell recognition, adhesion, and immune system evasion. NanA catalyzes the transformation of sialic acid into pyruvate and N-acetyl-D-mannosammine [46, 47], favoring cell-cell adhesion. Therefore, NanA plays a fundamental role in the adhesion development of host cells a process of great importance in the formation of biofilm. This enzyme is then considered an important target for developing molecules able to reduce biofilm accumulation. Recently, a relationship between methylation stress in *E. coli* and the reduction of bacterial adhesion properties thus decreasing its ability to form biofilm was reported. This phenomenon was associated with a drastic reduction in the expression levels of the NanA protein, suggesting a possible role of NanA in

biofilm formation and bacteria host interactions. Using a null NanA mutant and DANA, a substrate analog acting as competitive inhibitor, it was demonstrated that the downregulation of NanA or inhibition of its enzymatic activity affects biofilm formation and adhesion properties of *E. coli* [48, 49].

Another important protein target is GlmU, a bifunctional enzyme with acetyltransferase activity involved in the biosynthesis of Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), a key precursor of β-1,6-N-acetyl-D-glucosamine polysaccharide adhesin required for biofilm formation [50, 51]. GlmU is a possible factor involved in biofilm production in *M. smegmatis*, a nonpathogenic bacterium homologous to the pathogenic *M. tuberculosis*. The response of *M. smegmatis* to alkylating stress is different from *E. coli*, resulting in an increase in biofilm formation possibly due to a very strong defense mechanism. In this contest, GlmU has an important role in the process of biofilm production in *M. smegmatis*, being its expression highly upregulated when the bacterium needs to activate defense mechanisms [52]. Experiments with both conditional deletion and overexpressing glmU mutants demonstrated that the downregulation of GlmU decreased *M. smegmatis* capabilities to produce biofilm, whereas the overexpression of enzyme increased biofilm formation. These results were supported by inhibition of GlmU acetyltransferase activity with two different inhibitors, suggesting the involvement of this enzyme in the *M. smegmatis* defense mechanisms. Focusing on the inhibition of GlmU might then be an efficient method to disable the bacterium defense mechanism.

S. aureus is a common pathogen responsible for nosocomial and community infections being able to colonize the squamous epithelium of the anterior nares. One of the adhesins likely to be responsible for this ability is the *S. aureus* surface protein G (SasG), which promotes cellular aggregation leading to biofilm formation [53, 54]. SasG comprises an N-terminal A domain and repeated B domains with only the B domain required for the accumulation of biofilm. Expression of SasG does not increase the adherence of bacteria, and it is not involved in primary attachment but plays a role in the accumulation phase of biofilm formation [55]. For different aspects and playing different roles, NanA, GlmU, and SasG may all represent interesting targets to address the inhibition of biofilm production.

5. Conclusions

Currently, biofilm infections constitute a serious medical problem, and their treatment is far from being satisfactory. Biofilm formation inhibitors have several potential therapeutic applications as coatings in medical devices or in the prophylaxis of implanted surgery. In this respect, the identification of new strategies to counteract biofilm formation is a broad subject of study. The antibiofilm activity of many molecules such as proteins, peptides, and small organic molecules is currently under investigation. Each of these molecules is endowed with specific characteristics and can exert its ability to inhibit bacterial biofilm formation with different mechanisms. Antibiofilm agents are able to act both at the initial stages of biofilm formation, such as bacterial adhesion to the host surface, and on preformed biofilm, leading to the disruption of the EPS architecture. Many small organic molecules are able to interfere with the bacterial QS system, but their lack of activity in *in vivo* models and the high toxicity make these molecules of limited use in clinical applications.

As antimicrobial peptides show a broad spectrum of action, exploiting different activities including antibiofilm capabilities, these molecules might be considered as new promising factors to impair biofilm formation that exploit different mechanisms to hamper biofilms at different stages.

The administration of a single antibiotic is often not enough to eradicate bacterial invasions, and a high concentration of the antibiotic can be extremely toxic. A possible solution might be the coadministration of antibiotics with antibiofilm peptides that allow the use of low antibiotic concentrations. This strategy can be tuned to affect biofilms without killing bacteria, thus avoiding the emergence of drug-resistant populations through synergy with existing antibiotics.

Acknowledgements

This work was supported in part by MIUR grants ARS01_00597 Project "NAOCON" and PRIN 2017 "Identification and characterization of novel anti-tumoral/antimicrobial insect-derived peptides: a multidisciplinary, integrated approach from in silico to *in vivo*."

Conflict of interest

The authors declare no conflict of interest.

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