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REVIEW

The role of bacterial biofilm in persistent infections and control strategies

Li Chen*, Yu-mei Wen

Key Laboratory of Medical Molecular Virology of Ministry of Education and Ministry of Public Health, Shanghai Medical School of Fudan University, Shanghai 200032, China

Bacterial biofilms can be viewed as a specific type of persistent bacterial infection. After initial invasion, microbes can attach to living and non-living surfaces, such as prosthetics and indwelling medical devices, and form a biofilm composed of extracellular polysaccharides, proteins, and other components. In hosts, biofilm formation may trigger drug resistance and inflammation, resulting in persistent infections. The clinical aspects of biofilm formation and leading strategies for biofilm inhibitors will be discussed in this mini-review.

***Keywords*:** biofilm; persistent infection; 3A remedies

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Introduction

Persistent infections are a global challenge for human beings, claiming millions of lives every year and demanding huge medical and social resources. The development of persistent infections has been exemplified as a game of "Cat & Mouse" in which the host tries to eliminate a pathogen while the pathogen tries to survive in the host. One common survival strategy employed by bacteria pathogens is to form a biofilm, an amorphous and dynamic structure that is not only resistant to antibiotics, but also resistant to host immune clearance. In bacterial infections affecting internal organs, such as *Pseudomonas aeruginosa* (*P. aeruginosa*) in cystic fibrosis pneumonia [1], *Escherichia coli* (*E. coli*) in urinary tract infections [2], and *Mycobacterium tuberculosis* (*M. tuberculosis*) in human tuberculosis [3] biofilms provide an important reservoir of cells that can repopulate colonized sites. Biofilms are also responsible for persistent *Streptococcus mutans* (*S. mutans*) infections on tooth surfaces. The *S. mutans* level rebounds in days, even after a combination of professional mechanical tooth cleaning and topical antimicrobial treatments. In addition, most nosocomial infections are persistent biofilm infections [4-5]. It is estimated that, in developed countries, over 60% of treated infectious conditions are caused by biofilm formation.

As a correlation between biofilm formation and bacterial persistence has been established [6], the possibility of using drugs targeting biofilm formation in combination with the current antibiotics is emerging as a potential therapeutic approach for this type of bacterial persistent infection. Several anti-biofilm and/or biofilm control strategies, such as anti-adhesion, quorum sensing disruption and selective targeted anti-microbial peptides, have been recently developed. These strategies will be discussed. The readers interested in the structure and function of biofilms and biofilm diseases are recommended to read the reviews by Dr. Costerton and his colleagues [4-5].

Molecular regulation of biofilm formation

Biofilm formation is a two-stage process

Biofilms are surface bacterial aggregates encased in a synthesized hydrated matrix [5]. In general, biofilm forma-

*Correspondence: Li Chen
Tel: 86 21 54237236; Fax: 86 21 64174578
E-mail: lichen_bk@fudan.edu.cn
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tion is a two-stage process that begins with the adherence of bacteria to a substrate surface (Adhesion Stage), and continues by proliferation and differentiation of the attached cells (Maturation Stage). From a molecular biology point of view, these two stages are mainly controlled by surface adhesins and cell-to-cell communication signaling pathways respectively.

Adhesins are key regulators of the adhesion stage

Pathogens colonize different sites in the human body because they express multiple adhesins. These are usually proteins that recognize specific receptors expressed at various sites of the host. Bacteria also produce different types of polysaccharides that are specifically designed to form the structural components of the biofilm. The expression of adhesins seems to be regulated by a variety of inputs. For *Staphylococcus epidermidis* (*S. epidermidis*), initial adhesion to the naked or coated polymer surface is mediated by polysaccharide adhesin (PS/A) [7-8]. The expression of PS/A is controlled by the intercellular adhesion operon (Ica). In *S. mutans*, the adhesin SpaP (PAc) is critical for *S. mutans*'s adhesion to tooth surfaces and the process is further enhanced by sucrose or pre-existing biofilms [9].

Although *S. mutans* is normally known as an oral bacterium, and the etiological agent for dental caries, it is also implicated in bacteremia and infective endocarditis. Apparently, different adhesins may participate in the adhesion of *S. mutans* on different tissues. Based on the chemical composition of serotype-specific polysaccharides, *S. mutans* can be classified into serotypes c, e, f and k with approximately 70%–80% of strains found in the oral cavity classified as serotype c, followed by e (~20%), and f and k (less than 5% each). Serotype k is more dominant in *S. mutans* identified from endocarditis samples. It has a defect at the glucose side chain in serotype-specific rhamnose-glucose polymers, which may be related to a higher incidence of detection in cardiovascular specimens, owing to reduced phagocytosis. These findings suggest that *S. mutans* is capable of surface interactions with different tissues and serotype k *S. mutans* possibly has a higher level of virulence for systemic diseases [10].

Many surface proteins are attached to the bacterial cell wall by membrane-associated transpeptidases of the sortase family [11]. These enzymes cut the target proteins at a C-terminal cell wall sorting signal (CWSS), which is typically characterized by an LPxTG motif. This forms an acyl enzyme intermediate that covalently attaches the proteins to amino groups on peptidoglycan, resulting in incorporation into the cell envelope. Substrate proteins of sortases are initially expressed in a precursor with an N-terminal signal peptide and the C-terminal CWSS. The signal sequence directs the protein for translocation across the plasma membrane *via* the Sec secretion system, until the CWSS is reached. At this point, the protein is held in the membrane by a stretch of hydrophobic amino acids immediately downstream of the CWSS. The CWSS is then available for cleavage by the membrane-bound sortase, resulting in a protein that is exposed on the bacterial surface while securely embedded within the cell envelope.

Biofilm maturation stage is controlled by quorum sensing systems

Quorum sensing (QS) is a microbial cell-to-cell communication system designated for cell-density and/or population based gene regulation. Using a QS system, individual cells can produce and release small QS signaling molecules and detect the signal in the surrounding environment at same time. Several major types of QS systems have been identified and characterized, including N-acyl-homoserine lactone (AHL) systems (from Gram negatives), 4-quinolone systems (from Gram negatives, hydrophobic signal), AgrD peptide systems (from Gram positives), and AI2/LuxS systems (both Gram negatives and Gram positives). Quorum sensing systems play critical roles in the maturing stage of biofilm formation and regulate cell differentiation and development of biofilm structures.

A breakdown of the cell-to-cell communication may keep cells in the planktonic state. It has been shown that a well-developed biofilm is more resistant to drug treatment than defective biofilms. Davies and colleagues demonstrated that a thin biofilm formed by the AHL quorum-sensing mutant *lasl* is more sensitive to treatment by antibiotics and sterilization solutions. This phenotype can be completely complemented by introducing a functional *lasl* or by adding the appropriate AHL [12].

Clinical aspects of biofilms

Detection of biofilms is a practical concern

From a clinical point of view, the first step towards a solution for biofilm-related infection is an early positive detection. The biofilm hypothesis predicts that biofilm cells are less sensitive to both nutritional stimulation and hostile attacks compared to planktonic cells. Additionally, biofilm fragments could be difficult to detect using traditional agar and culture based detection methods [3, 13]. Thus, evidence generated from direct microscopic examination and from molecular detections is preferred to verify a biofilm related condition. Although DNA and RNA based, species-specific, high-throughput detection

methods are now recommended to screen clinical samples for the presence of pathogens, these technologies are neither affordable nor available in developing countries.

Currently, biofilm specific antigens and antibodies are being pursued for both diagnostic and therapeutic purposes. In 2002, Selan and her colleagues prepared surface slime polysaccharide adhesin (SSPA) that is highly expressed in biofilm cells, and established a SSPA-ELISA assay for anti-SSPA antibodies in clinical samples. The assay was used to monitor *Staphylococcus* biofilm formation in patients receiving synthetic vascular grafts. The particular clinical challenge is that Staphylococccal biofilms on the sutures would cause around 4% of the patient grafts to simply fail and rupture without any obvious warning symptoms. The SSPA-ELISA test would help clinicians to predict the occurrence of biofilm by comparing the test results obtained at different time points. The same technology was applied to periprosthetic joint infections [14].

Antigens from the methicillin-resistant *Staphylococcus aureus* (MRSA) cell wall have been shown to be immunogenic *in vivo* and up-regulated during *in vitro* biofilm growth. Purified and recombinant forms of biofilm-up-regulated, cell wall-associated proteins were used for polyclonal immunoglobulin G (IgG) antibodies. These IgGs were utilized as imaging tools to localize areas of specific protein production within a biofilm [15]. These biofilm-specific or biofilm-enhanced antigens were applied as a therapeutic vaccine to treat MRSA caused conditions in a combination approach with vancomycin [16].

The Parsek-Singh biofilm criterion has been adopted by clinical microbiologists to track clinical biofilms. According to this criterion, a biofilm condition needs to match four conditions. (1) The pathogenic bacteria are surface associated or adherent to a substratum; (2) direct examination reveals bacteria in clusters are encased in a matrix of bacterial or host constituents; (3) the infection is localized; and, (4) the infection is resistant to antibiotic treatment despite the demonstrated susceptibility of planktonic bacteria. The guideline is a reflection of several key features of biofilms. With both DNA/RNA-based high-throughput detections and biofilm-specific antigens/antibodies as our new tools, we are expecting simple and minimally invasive detection of biofilms in the clinical setting. A positive detection of a clinical biofilm would lead clinicians to employ therapeutic approaches appropriate for persistent infections associated with biofilms.

It should be pointed out that a detected biofilm is not always disastrous if it is not causing damage to surrounding tissues. As sessile bacteria provide a protected or isolated slow mode of growth, many biofilms are well-tolerated in hosts. Dasgupta and colleagues examined more than 80 Tenchhoff catheters worn by continuous ambulatory peritoneal dialysis (CAPD) patients. Although thick biofilms were identified, the major clinical concern was acute peritonitis caused by the planktonic cells released from biofilms. The key clinical determining factor for peritonitis is host immune status and not the extent or species content of the biofilm [17].

What is our first clinical option for a detected problem? A recent review by Black and Costerton on chronic wound biofilms reasoned that as commercial topical agents and wound dressings currently in use are ineffective against the biofilm matrix, mechanical debridement appears to be essential in the eradication of a wound biofilm. Topical antimicrobial agents and antibiotics may be effective in the treatment of the wound bed after debridement in the prevention of biofilm reformation [18]. This removal/replacement plus medicine strategy is also applied for the treatment of periodontitis and most nosocomial infections.

New tests are needed for drug selections for biofilms

Biofilm formation is a main virulence determinant in many bacterial infections. It significantly increases bacterial resistance to antibiotics and innate host defenses. The drug-resistance strategy employed by sessile biofilm cells are different from the ones adopted by planktonic cells, such as activation of efflux pumps, acquisition of new enzymes and mutations of the drug targets. In general, the specific physiology of sessile bacteria and the physical and chemical barrier function of the extracellular biofilm matrix determine resistance to antimicrobials. As the sessile bacteria adopt a slow mode of growth, biofilms are relatively insensitive to bactericidal antibiotics. The effective drug concentration could be negatively affected by interactions of the biofilm matrix with antibiotics and antimicrobial peptides. These polymers may work by electrostatic repulsion and/or sequestration of antibacterial substances. Results indicated that biofilm cells may have a minimum inhibition concentration (MIC) that is 1 000 times higher than planktonic cells.

How to select the best antibiotics to treat biofilm-associated conditions is a critical clinical challenge. Planktonic bacteria have been used in standard susceptibility tests to select the most appropriate antibiotic combinations to treat biofilm conditions, such as cystic fibrosis airway infections [19]. However, this approach has a key weak point: it does not consider the drug resistance impact posed by biofilms. Recently, biofilm susceptibility methods have been developed to address this concern [20-21]. Biofilm inhibitory concentrations or minimum biofilm eradication concentrations are higher

than the corresponding MICs determined by standard planktonic methods. However, biofilm inhibitory concentrations and minimum biofilm eradication concentrations values vary greatly among the different biofilm susceptibility test methods, suggesting that the characteristics of the *in vitro* biofilms are conditional and strongly related to the laboratory system used to grow them [21]. This underscores the difficulty of selecting and developing biofilm inhibitors as well as compounds that potentiate the activity of antibiotics against biofilms. Clearly, it is important to test bacterial biofilms with clinically relevant antibiotic susceptibilities. Collective efforts from both clinicians and researchers are required to build standard biofilm inhibition and standard biofilm eradication assays. Biofilm-based strategies are needed to manage the patient's care and to develop new drug leads.

Biofilms may cause inflammation

Biofilms are protected from antibiotics and the body's immune system. For years, it was believed that leukocytes are not able to penetrate the biofilm. Leid and colleagues demonstrated that under simulated physiological conditions, leukocytes attach, penetrate, and produce cytokines in response to maturing and fully matured *S. aureus* biofilms [22]. Similar results were also observed from *P. aeruginosa* biofilms. Neutrophils that settle on biofilms appear to be unable to migrate away from the point of contact even though they are still capable of phagocytosis [23]. Neutrophil accumulation within biofilms may result in self-injury of the neutrophil by released oxidants which in turn compromises host defense mechanisms. Necrotic neutrophils can also serve as a biological matrix to facilitate *P. aeruginosa* biofilm formation [24].

Accumulated clinical evidence has indicated that biofilms associated with persistent infections can also cause chronic inflammation. Biofilm associated inflammations were identified from chronic wounds [25], cystic fibrosis [26], otitis media [27], osteomyelitis [28], prostatitis [29-30], and nosocomial infections. Anti-inflammation therapy is usually recommended for biofilms caused by systemic tissue infections.

As biofilm diseases are associated with both persistent infections and chronic inflammation, we anticipate future treatments to contain three active components, namely, antibiotics for both biofilm and planktonic cells, anti-inflammatories for neutralizing the inflammation reactions generated from biofilms, and anti-biofilm compounds for the clearance of biofilms (3A remedies for biofilms). The next section of this review will discuss the current progress of anti-biofilm research.

Research and development of anti-biofilm activities

The road from biofilm hypothesis to biofilm therapy is promising, but long. After two decades of relentless effort and a quarter of a million publications, we are still waiting for the launch of the first anti-biofilm-based product. At this point, we are optimistic that the collective efforts in the field of anti-adhesion, quorum sensing disruption and species-specific killing will deliver new and practical biofilm based medicines and/or remedies.

Sortase is a leading target for anti-adhesions

At the adhesion stage of biofilm formation, adhesins expressed on the planktonic cell and/or biofilm fragments initiate surface/tissue specific attachment. As these surface interactions are critical for biofilm formation, hundreds of surface proteins identified from different microbial species are being studied as candidate adhesins for anti-adhesion compounds. The growing information on adhesins is creating opportunities and causing confusion at the same time. The surface protein repertoire enables multiple interactions with different host components and allows versatility when it comes to occupying adherence sites. The multiple adhesin-receptor interactions with various affinities represent some of the reasons for the difficulties that have been encountered in the characterization of adhesins. For example, single-gene knockouts may reveal very little about adherence mechanisms, and antibodies generated to specific surface proteins may have little or undetectable effects on adherence.

To make the best use of limited resources, researchers are trying to focus on the reactions/steps that are shared by most of the surface proteins, instead of working on individual adhesins. The sortase of Gram positives is such a candidate. For Gram positives, peptidoglycan inside the 20–100 nm thick cell wall is covalently and non-covalently decorated with teichoic acids, polysaccharides, and proteins. This dynamic surface structure is essential for different types of interactions between bacteria and their environments and for biofilm formation. Sortases are membrane enzymes catalyzing covalent anchoring of surface proteins to peptidoglycan [11, 31]. Bacterial genome projects indicated that sortase is a common enzyme found in Gram positives with broad substrates. In *S. aureus*, around 20 candidate sortase substrates were identified from each sequenced strain, including protein A (Spa), fibronectin binding proteins (FnbpA, FnbpB), clumping factors (ClfA, ClfB), collagen adhesin (Can), heme binding proteins (IsdC, IsdB, IsdA) and other surface proteins [11]. The same is true for other Gram positives, such as *S. epidermidis* and *S. mutans*. Sortase A (*srtA*) mutants failed to display all

surface proteins, while this phenotype could be rescued by a plasmid carrying the wild type gene. In animal disease models for sepsis, abscess, septic arthritis, and endocarditis, results demonstrated that the *srtA* mutant was less virulent than wild type strains [32-34]. Interestingly, two *S. mutans* clinical isolates contain sortase mutations in the *srtA* gene. *S. mutans* Ingbritt contains an 11-base-pair deletion in the *srtA* ORF that generates a premature stop codon [35], and *S. mutans* NG5 carries a missense mutation in the *srtA* gene that results in the production of a truncated, nonfunctional enzyme [36]. Both strains secrete PAc and are unable to adhere to hydroxyapatite and to aggregate in the presence of saliva. Based upon these studies, researchers are hopeful that sortase might serve as a good drug-target for anti-adhesion activities. As sortase is a universal virulence factor for Gram positives, sortase inhibitors could have broad clinical applications.

Even before the identification of the enzyme, several compounds were recognized as sortase inhibitors, including methane-thiosulfonate and mercurial p-hydroxymercuribenzoic acid. These compounds function by blocking the Cys184 in the active pocket of sortase A. Although these compounds are useful for illustrating the action model of the enzyme, they are of limited therapeutic value due to their general toxicities. Natural compound libraries were screened for candidate inhibitors. Compounds isolated from *Cocullus trilobus* and *Coptis chinensis* exhibited lower MICs than p-hydroxymercuriabenzoic acid and were able to block the adherence of bacteria to fibronectin coated surfaces [37-38]. Libraries of small compounds have also been screened, and (Z)-diarylacrylonitriles were identified as potential leading compounds. Another strategy was to identify irreversible sortase inhibitors by screening modified versions of LPXTG, the common sortase binding site on substrates. Candidate inhibitors, such as diazoketone LPAT (LPAT-$COCHN_2$), chloromethyl ketone LPAT (LPAT-$COCH_2Cl$), were isolated for further studies [38]. The key technical issues for sortase-based anti-adhesions, such as assay design and animal models, are discussed in a review by Maresso and Schneewind.

Biofilm formation can be blocked by quorum sensing inhibitors (QSI)

At the maturation stage of biofilm formation, bacteria use cell-to-cell communication systems to regulate the expressions of the genes required and/or actively involved in the biofilm formation. A breakdown of the cell-to-cell communication may keep cells in the planktonic state. QS is a microbial cell-to-cell communication system designated for cell-density and/or population based gene regulation. Using a QS system, individual cells can produce and release small QS signaling molecules and detect signals in the surrounding environment at same time. Since the discovery of QS in the 1960s [39], several major types of QS systems have been identified and characterized, including N-acyl-homoserine lactone (AHL) systems (from Gram negatives), 4-quinolone systems (from Gram negatives, hydrophobic signal), AgrD peptide systems (from Gram positives), AI2/LuxS systems (both Gram negatives and Gram positives), and farnesol systems (from fungi). As accumulated publications imply that QS systems are actively involved in controlling biofilm formation and infections, researchers are focusing their efforts on quorum sensing inhibitors (QSI) for potential new therapeutics [40].

Among a large number of reported QSIs, furanones and RNA Ⅲ inhibiting peptide (RIP) are two classes of leading candidates. Furanones have been extensively studied for their activities and for QS inhibitory mechanisms. Several lines of evidence indicate that the furanones may act on both AHL systems and AI2 systems. Some furanones devoid of growth inhibitory effects can increase the susceptibility of biofilms to antibiotics and detergents [41]. In animal disease models, furanones could facilitate the host immune systems to clear infections [42]. Antibodies against AHLs have also been generated after conjugating AHL with carrier proteins [43]. The antibodies might have promising applications in disease control.

A linear Agr QS inhibitor known as RNA Ⅲ inhibiting peptide in its amide form (RIP, YSPWTNF-NH_2) has been shown to repress virulence, biofilm formation, and antibiotic resistance in staphylococci [44-45]. A simple mechanism of RIP function is proposed. As the cells multiply, the QS signal RAP accumulates in the supernatant, binds to its receptor, and induces the phosphorylation of TRAP. *agr* is then activated at the mid exponential phase of growth and RNA Ⅱ is produced and AgrA-D are made. This leads to the production of RNA Ⅲ, which up regulates the production of toxins at the post-exponential phase of growth. In the presence of RIP, TRAP is not phosphorylated, *agr* is not activated, and toxins are not produced. Despite promising results obtained from several animal models, the clinical application of RIP and its synthetic analogues is still in its early stage. The *in vivo* product stability and toxicity of the peptide drug are two potential concerns.

Selectively targeting antimicrobial peptides (STAMPs) is a new option for species-specific control of biofilms

Treatment of persistent infections caused by pathogens sitting in a functional biofilm community, such as

dental caries, is a clinical challenge. Conventional broad-spectrum antimicrobial treatment attacks a subpopulation of the biofilm, and may cause new problems by eliminating commensals. To build a pathogen-free biofilm, species-specific vaccines [46], anti-adhesins, (discussed above) and replacement therapies [47] have been explored. Recently, a new targeted approach named selectively targeted antimicrobial peptides (STAMPs) is emerging as a promising new strategy for species-specific biofilm control.

In short, STAMP is a modified recombinant version (or third generation) of antimicrobial peptides (AMPs). AMPs are widespread in nature and play an important role as part of innate immunity. In general, AMPs are fairly large molecules that carry a net positive charge and contain around 50% hydrophobic residues. Their mode of action involves binding to negatively-charged structural molecules on the microbial membrane. AMPs have a broad spectrum of antimicrobial activity and development of resistance is rare. Unfortunately, AMPs are difficult and expensive to produce in large quantities and are usually sensitive to protease digestion. Modifications of AMPs have resulted in the development of synthetic antimicrobial peptides, also called SAMPs (second generation of AMPs). SAMPs mimic the effect of AMPs, but have improved pharmacokinetic properties. Despite these improvements, SAMPs are still broad spectrum. This problem was solved by STAMPs.

STAMPs represent a novel strategy initially developed by Shi and his colleagues to remove pathogens from multi-species communities and/or specific clinical settings [48]. A typical STAMP contains a species-specific binding domain to facilitate the delivery of a conjoined antimicrobial peptide. The targeting peptide can be identified *via* screening peptide libraries or rational design. Targeting peptides specifically binding to *S. mutans* is derived from bacterial pheromones such as CSP (SGSLSTFFRLFNRSFTQALGK), and a targeting peptide specific for *P. aeruginosa* is derived from KKHRKHRKHRKH. A peptide of 2 to 20 amino acids is used to connect a targeting peptide to an antimicrobial peptide without interfering or reducing the activity. The technology has been applied to remove both Gram-positive strains [48-50] and Gram-negative strains [48-49]. Recently, a STAMP with two species-targeting domains was used to treat both *S. mutans* and *Pseudomonas* in a multi-species culture [49]. In an *in vitro* dynamic biofilm model, Li and colleagues demonstrated that after selective elimination of *S. mutans* from the existing biofilm with a STAMP, the treated biofilms are resistant to the recolonization of newly added *S. mutans* [50]. Although the STAMP technology may face the same challenges as RI, AMPs and other peptide based new drug candidates, STAMPs have a strong potential for novel therapeutics that may selectively eliminate pathogens, while preserving the benefits associated with a healthy normal flora.

Summary

Biofim formation is a two-stage biological process controlled by surface adhesins and cell-to-cell communication pathways. Aggregated bacterial cells protected and/or coated by extracellular matrix are insensitive to both nutritional stimulation and hostile attacks. In the human body, biofilms may trigger persistent infections with chronic inflammation. After a positive detection of biofilm related medical conditions, both surgical removal/replacement and medicinal treatment should be considered. Ideally, an effective remedy for biofilm associated conditions should contain antibiotics, anti-inflammatories, and anti-biofilm activities (3A remedies).

The road from molecular mechanisms of biofilm formation to anti-biofilm products is promising, but long. Non-invasive and/or minimally invasive detection methods and standard biofilm assays that mimic clinical conditions are opening the door for new, biofilm-oriented solutions. A large number of biofilm inhibitors are currently under comprehensive investigation. If clinicians are made more aware of the importance of bacterial biofilm formation and their associated diseases, more translational research will be designed and new therapeutic approaches may be developed.

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