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Abstract

Biofilms formed by pathogenic bacteria and fungi are associated with a wide range of diseases, from device-related infections (such as catheters or prosthetic joints) to chronic infections occurring on native tissues (such as lung infections in cystic fibrosis patients). Biofilms are therefore responsible for an important medical and economic burden. Currently-used antibiotics have mostly been developed to target exponentially growing microorganisms and are poorly effective against biofilms. In particular, even high concentrations of bactericidal antibiotics are inactive against a subset of persistent biofilm bacteria, which can cause infection recurrence despite prolonged treatments. While the search for a magic bullet antibiotic effective against both planktonic and biofilm bacteria is still active, alternative preventive and curative approaches are currently being developed either limiting adhesion or biofilm formation or targeting biofilm tolerance by killing persister bacteria. Most of these approaches are adjunctive using new molecules in combination with antibiotics. This review presents promising approaches or strategies that could improve our ability to prevent or eradicate bacterial biofilms in medical settings.

Highlights

- Currently-used antibiotics were developed to target planktonic bacteria
- Recent discoveries on biofilm properties led to promising anti-biofilm strategies
- Biofilm inhibition should integrate biocidal and non-biocidal approaches
- Jamming bacterial communication and regulation can prevent biofilm formation
- Major anti-biofilm approaches rely on matrix dissolution and potentiation of existing antibiotics against persisters

Introduction

Since the first observation of a direct link between development of biofilm and persistent infections [1-3], modern medicine is facing a double challenge: getting around the increasing concern of multidrug antibiotic resistance and tackling sources of biofilm-related infections. There is probably little hope to witness the rapid development of novel antibiotic molecules that would not only overcome multidrug resistance but also be more efficient than current antibiotics against medical biofilms. Indeed, most of the currently-used drugs have been developed and optimized to kill planktonic microorganisms.

The identification of novel molecules designed to specifically target mechanisms involved in biofilm formation or biofilm tolerance towards antibiotics could lead to novel therapies specifically designed to be combined with antibiotics against bacterial biofilm-associated infections. This review presents recent therapeutic approaches developed to specifically target biofilm-associated bacterial infections.

Strategies targeting specific mechanisms involved in biofilm formation

Anti-adhesion strategies

One of the crucial steps in biofilm development is the initial interaction of bacteria to abiotic or biotic surfaces that can ultimately lead to colonization and infection by pathogenic bacteria. Reducing adhesion is therefore a strategy of choice to prevent biofilm formation and related infections. Among the different strategies recently developed to reduce bacterial adhesion, one can distinguish strategies that non-specifically inhibit adhesion *versus* strategies that are rather targeting specific adhesins (**Figure**).

Non-specific inhibition of adhesion

Non-specific inhibition of adhesion is generally obtained by surface modification using polymers. The type of polymers can be chosen on the basis of its anti-adhesive properties. For example, Hook *et al.* assessed hundreds of polymeric materials using an high throughput microarray assay for their anti-adhesive properties and identified materials comprising ester and cyclic hydrocarbon moieties displaying anti-adhesive activity *in vitro* against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and *in vivo*, against *S. aureus*, when grafted to silicone in a mice model of subcutaneously implanted device [4]. An efficient anti-adhesive molecule should not only limit bacterial proteins but also host proteins interaction to surfaces, therefore avoiding the formation of a conditioning film subsequently favoring bacterial colonization. Such molecules, for instance non-leaching polymeric sulfobetaine (polySB) that works as a wetting agent, have been demonstrated to reduce protein, host cells and microbial adhesion, but also thrombus formation *in vitro* and *in vivo* [5••]. More recently, a biomimetic strategy using a glycocalyx-

like molecule, methyl-cellulose, displaying anti-adhesive property for both cells and bacteria, has been used to coat totally implanted venous access ports (TIVAP). Coated TIVAP implanted in rats for several days displayed important resistance toward adhesion, strongly reduced biofilm formation by *P. aeruginosa* and *S. aureu*s as well as infective thrombus [6••].

Beyond sole anti-adhesive strategies, surfaces combining different activities such as tissue integration, biocide property and anti-adhesive activity are currently developed [7•-9•]. A recent example of such a strategy displaying promising *in vitro* activity are anti-adhesive polymer brushes composed of block copolymer Pluronic F-127 (PF127) functionalized with antimicrobial peptides (AMP), able to kill bacteria on contact, and arginine−glycine−aspartate (RGD) peptides promoting the adhesion and spread of host tissue cells [10•].

Specific targeting of adhesins

Anti-biofilm approaches targeting specific adhesins have been shown to display strong anti-adhesive and anti-infective potential. Some molecules can impede the biogenesis of adhesins such as the one developed to block different fimbrial adhesins including the well-known type 1 fimbriae involved in bladder colonization by uropathogenic *E. coli* [11-14]. Type 1 fimbriae have also been the target of sugar analogues competing with eukaryotic receptors interacting with the tip-lectin, FimH. Among several molecules, some FimH inhibitors have been shown in mice to successfully prevent catheter-associated urinary tract infections by drug sensitive uropathogenic *E. coli* (UPEC) or to treat chronic cystitis in mice infected by the multidrug-resistant UPEC clone ST131 [15••,16•]. Carbohydrate inhibitors have also been developed against *P. aeruginosa* lectins, some of which prevented lung colonization in mouse models [17,18]. Interestingly, anti-biofilm action was

also achieved by using molecules combining several activities such as maltose derivatives with bulky hydro-carbon groups that presented both a surfactant/biofilm dispersion activity and an inhibition of adhesins/receptors mediated binding of *P. aeruginosa* [19•].

Targeting biofilm maturation

Biofilm-related infection can also be reduced by blocking the biofilm maturation process (**Figure**). In most cases, strategies targeting biofilm maturation should also include treatment with an antimicrobial for an *in vivo* use to avoid the release of a massive quantity of biofilm bacteria into the bloodstream.

Major signaling pathways as antibiofilm targets

Among the major mechanisms that are governing biofilm maturation are quorum-sensing (QS) signals. We will not develop these aspects of anti-biofilm arsenal since several excellent reviews were recently written on the various strategies used to interfer with quorum-sensing including the use of analogues of homoserine lactones or AI-2 and enzymes degrading QS molecules for Gram-negative bacteria, and auto-inducing peptides or RNA-III inhibiting peptides for Gram-positive bacteria [20-22].

Discovery of the importance of small messenger molecule c-di-GMP in the physiological switch between planktonic to biofilm lifestyle is more recent and c-di-GMP is now considered as a valuable target to fight biofilm-related infections. Screening of chemical libraries led to the identification of direct or indirect inhibitors of diguanylate cyclases (the enzymes producing c-di-GMP), reducing biofilm formation such as sulfathiazole or azathioprine, an immunosuppressive drug [23-26]. Alternatively, a molecule impacting biofilm formation produced by *P. aeruginosa*, nitric oxide (NO), has been demonstrated to induce dispersal via the reduction of c-di-GMP concentrations through increased activity of

phospho-diesterases (PDE) [27], demonstrating the potential of compounds naturally produced by micro-organisms (see **Table**). In *P. aeruginosa*, NO-induced dispersal has been recently linked to a specific PDE, NdbA, whose mRNA transcription is induced by NO [28]. Interestingly, NO seems to be involved also in the dispersal of biofilms formed by other micro-organisms, however through a different mechanism involving H-NOX proteins. Therefore, development of surfaces releasing NO might be promising to control biofilm formation as demonstrated by the use of NO donor-coated urinary catheters and nanoparticles [29,30].

Direct action on matrix components to weaken biofilms

Two factors regulated by quorum-sensing and c-di-GMP play a major role in the architecture of biofilms: polysaccharides and extracellular DNA [31-34]. Thus, direct targeting of these factors instead of their signaling pathways can also be envisaged to reduce biofilm formation. Strategies using enzymatic degradation of these matrix components such as the use of DNaseI or Dispersin B, an hexosaminidase naturally produced by *Aggregatibacter actinomycetemcomitans* and hydrolyzing poly-N-acetylglucosamine (a frequent component of *E. coli*, *S. aureus* or *Staphylococcus epidermidis* exopolysaccharides), have been identified as efficient ways to disperse biofilms *in vitro* and *in vivo* (see for example, [35-37]). However, enzyme-based approaches are associated with two limitations: i) their restricted spectrum of action; and ii) the risk of immunization against these molecules. The association of chelators of divalent cations such as citrate or EDTA and biocides has also been proposed, based on their ability to destabilize biofilm matrix [38,39]. These chelators could find their interest in the case of local infection or restricted colonization such as device-related infection and have been used as preventive agents in clinical trials [40]. Additionally, EDTA was proven an efficient adjuvant to gentamicin to eradicate *E. coli*, *P. aeruginosa S. aureus* and *S.*

157 *epidermidis* biofilms including persister cells (see below) in a rat model of catheter-related
158 infection [41•].

159

160 Strategies targeting mechanisms governing biofilm tolerance
161 towards antibiotics: fighting persisters

162

163 One major problem caused by biofilms is their increased tolerance towards antimicrobial
164 agents that impairs the treatment of biofilm-related infections in clinical settings [42]. While
165 increased tolerance of biofilms is multifactorial, the main mechanism currently proposed to
166 explain such tolerance is the presence of persisters, bacteria that enter in a specific
167 phenotype state allowing them to survive in the presence of 1000 fold the minimum
168 inhibitory concentration of bactericidal antibiotics [43,44]. Persister cells have recently
169 been subjected to an intense hunt in order to limit biofilm-associated antibiotic tolerance.

170

171 Reducing persisters formation

172 There are now growing evidences that one of the main factors leading to persisters
173 formation is nutritional stress, with a major effector molecule, ppGpp, the mediator of
174 stringent response ([45], for a comprehensive review see [46••]). Regarding the central role
175 for ppGpp in persistence, it is tempting to hypothesize that strategies leading to reduced
176 level of ppGpp could help fighting persisters. Relacin, a synthetic ppGpp analog inhibiting
177 the *Bacillus subtilis* RelA synthetase activity and biofilm formation [47], and relacin
178 derivatives displayed an inhibitory activity against different Rel proteins [48]. These
179 different compounds still need to be assessed for their capacity to reduce persisters
180 formation. Another stringent response inhibitor has been identified, the peptide 1018

(VRLIVAV- RIWRR-NH_2). While a direct evidence of its activity on persister cells is missing, this peptide displayed a specific antibiofilm activity against *in vitro* biofilms formed by several species including *P. aeruginosa* or *S. aureus* by inducing ppGpp degradation [49••] and a synergistic action together with ciprofloxacin on *in vitro* biofilms of various pathogens [50•]. Interestingly, this latter study demonstrates how adjuvant therapies can allow reducing the concentration of antibiotic required to inhibit biofilm formation.

Killing persisters

Once a biofilm is mature, the last resort option for biofilm eradication is to identify compound that would increase antibiotic activity against persisters (**Figure**). Silver has been shown to potentiate the activity of several antibiotics against biofilm and persisters of Gram-negative and Gram-positive bacteria in a mouse biofilm model with subcutaneous catheter by increasing ROS production and bacterial permeability to antibiotics [51••]. Sugar metabolism was also used to obtain antibiotic potentiation against persisters through an increased aminoglycosides penetration powered by the proton motive force [52,53]. Alcalinisation by basic amino-acids such as L-arginine was also recently demonstrated to enhance aminoglycoside action *in vitro* and *in vivo* against biofilms and persisters [54•]. Anti-QS molecules such as brominated furanones have the potential to revert antibiotic tolerance of *P. aeruginosa* or *E. coli* persister cells [55,56]. Persisters tolerance could also be reduced by exploiting their weaknesses related to their slow metabolism, such as a high sensitivity to proteolysis induced by the acyldepsipeptide ADEP4 that activates the ClpP protease in Gram-positive pathogens. ADEP4 is active in combination with rifampicin in a neutropenic mouse biofilm model [57••]. Whereas the efficacy of these anti-persister approaches remains to be further validated, on-going

persister studies are likely to reveal other potential therapeutic strategies, such as the modulation of bacterial cell death [58•].

Future perspectives

Much ado for almost nothing in clinic… Why?

The intense fundamental research on biofilms led to the emergence of numerous promising antibiofilm approaches. However, despite these long-lasting efforts, one should acknowledge that the translation of *in vitro* and *in vivo* data into clinical settings is slow and somewhat disappointing. Beyond the simple explanation of the massive costs necessary for drug development toward medical usage, one can identify potential reasons explaining this delay. Not only preventive strategies are difficult to translate into the clinic, but non-biocidal preventive anti-adhesive or anti-virulence strategies face the diversity of bacterial phenotypes and may only be active against a subpopulation of bacteria encountered in clinical settings, therefore limiting their overall efficacy. Then, even if *in vitro* biofilm susceptibility testing is a mandatory first step and much efforts have been made to develop such *in vitro* testing [59], molecules identified *in vitro* should be validated using relevant *in vivo* models for their antibiofilm activity but also absence of toxicity and pharmacokinetics.

The limitation of biofilm models

Despite the diversity of both the *in vitro* and the *in vivo* models currently available to identify or test antibiofilm molecules, *in vitro* models only partially reflect *in vivo* situations, because *in vitro* biofilms are probably structurally different and respond differently as

compared with *in vivo* biofilms [60••]. In particular, antibiotic tolerance in biofilms has been obtained *in vitro* with starvation models [61•] but other stress factors could also play a role in physiopathological conditions (flow, local pH, anoxia, inflammation…). At the present time, the diversity of persister phenotypes is not known, possibly due to the diversity of the *in vitro* conditions leading to persistence and the complexity of biofilm clinical situations. Efforts should therefore be made to enrich *in vitro* models with flow conditions, type of medium used, presence or absence of blood components or even specific eukaryotic cells within the device. These issues should also apply for *in vivo* models. Beyond the question of the relevance of using rodent models, some *in vivo* models may not properly reproduce real clinical situations. One can, for example, wonder about the relevance of the rat agar beads model to faithfully reproduce chronic infection in the lungs or subcutaneous model of catheters that are not connected to the bloodstream. Furthermore, as for clinical trials, rigorous statistical analysis and experimental set-up are mandatory in order to avoid any false positive interpretation. One can however foresee that increasing use of new guidelines for reporting animal research will also improve quality of experimental *in vivo* models [62,63].

What could be the near future?

Biofilm research will certainly benefit from the development of high throughput screenings evaluating compounds in combination with antibiotics using models better mimicking *in vivo* physiological conditions and new readouts benefiting from the increased knowledge on biofilm related signaling, such as reporter genes for pathways related to persistence (ppGpp, cyclic-di-GMP for Gram-negative bacteria, persisters metabolism). It is also expected that new formulations based on polymer microparticles could also emerge and

improve the use of otherwise topic compounds for local delivery at the site of biofilm infection [64,65•]. To become more translational, biofilm research needs biomarkers and more global analyses performed directly on biofilms in clinical settings, which are currently essentially applied to *in vitro* or animal models. These omics analyses could provide new and unexpected targets. Lastly, the increasing awareness of the polymicrobial nature of biofilms should lead to the development of dedicated approaches to study bacteria-bacteria or bacteria-fungi interactions and their consequences on biofilm pathogenesis or tolerance towards antibiotics [66].

Conflicts of interest

All authors: no conflicts of interest.

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Table 1. Recent studies illustrating some promising non-pharmaceutical anti-biofilm strategies. This list is not comprehensive and is only meant to illustrate each approach.

Mode of action	*In vitro*	*In vivo*	References
Early detection			
Detection of biofilm formation in a central venous catheter (CVC) using impedimetric biosensor	Detection of *S. epidermidis* biofilm formation within the chamber of a CVC	-	[67]
Vaccination			
Immunization against Biofilm Matrix Exoproteins from *S. aureus*	-	Reduction of *S. aureus* biofilm formation in a mesh biofilm model in mice	[68]
Passive protection with a monoclonal antibody against *Enterococcus faecalis* major pili protein EbpC	Prevention of *E. faecalis* biofilm formation	Significant passive protection against *E. faecalis* endocarditis in a rat model	[69]
Inhibition of microbial adhesion			
Modification of physical architecture of the surface (Sharklet micropattern)	Reduction of *E. coli*, *P. aeruginosa*, *A. baumannii* and *K. pneumonia* adhesion	-	[70]
Bio-inspired strategies			
Quorum-sensing quencher			
New quorum-sensing quencher (F5, LasR inhibitor) from	Reduction of *P. aeruginosa* biofilm	-	[71]

T7 engineered lytic phage producing a lactonase	Inhibition of mixed *P. aeruginosa* and *E. coli* biofilm	-	[72]
Lytic enzymes from predators			
Chimeric phage endolysin degrading peptidoglycan	Disruption of *S. aureus* preformed biofilm	Attenuation of *S. aureus* mediated endophtalmitis in mice	[73]
Bdellovibrio bacteriovorus proteases and DNAse	Prevention of *S. aureus* biofilm formation and disruption of *S. aureus* preformed biofilm	-	[74]
Other activity			
Chitosan coupling with antibiotic or nitric oxide	Disruption of and inhibition of *Listeria*, *E. faecalis* and *S. aureus* biofilm by chitosan-streptomycine conjugate ; disruption of *P. aeruginosa* biofilms by chitosan-NO conjugate	-	[75,76]

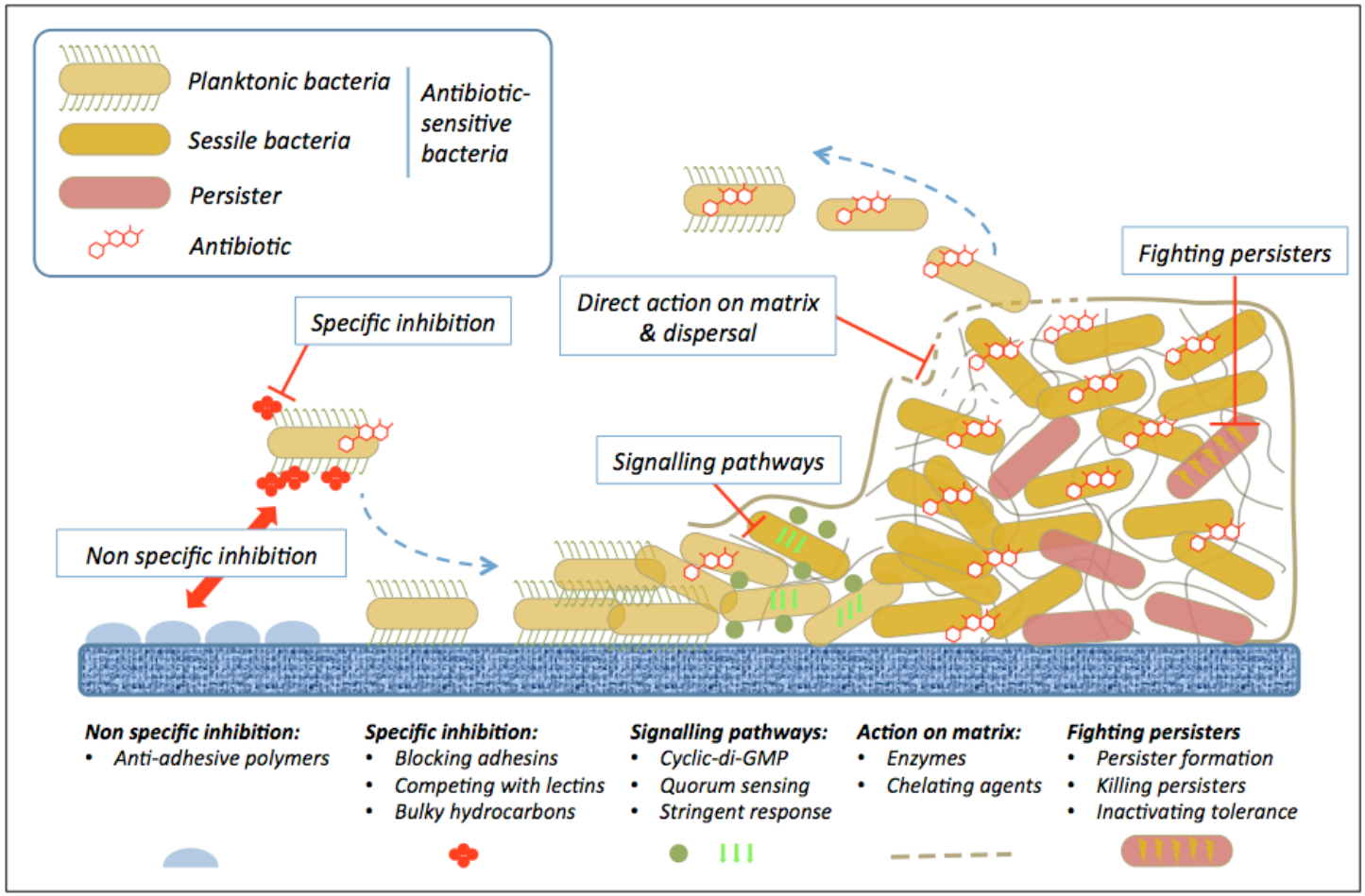

Figure 1. Biofilms: bacterial phenotypes and therapeutic targets
Schematic drawing of the successive steps of biofilm formation and maturation highlighting the different bacterial phenotypes encountered and their susceptibility to antibiotics. The five major approaches to combat biofilms are represented with their impact on biofilm formation or integrity and their possible combination with antibiotics.