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Novel approaches to combat bacterial biofilms

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15 **Abstract**

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

30

31

32

33 **Highlights**

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

40

41 **Introduction**

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

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

54

55

56 **Strategies targeting specific mechanisms involved in biofilm**

57 **formation**

58

59 **Anti-adhesion strategies**

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

66

67 ***Non-specific inhibition of adhesion***

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

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

86 Beyond sole anti-adhesive strategies, surfaces combining different activities such as tissue
87 integration, biocide property and anti-adhesive activity are currently developed [7-9]. A
88 recent example of such a strategy displaying promising *in vitro* activity are anti-adhesive
89 polymer brushes composed of block copolymer Pluronic F-127 (PF127) functionalized with
90 antimicrobial peptides (AMP), able to kill bacteria on contact, and arginine-
91 glycine-aspartate (RGD) peptides promoting the adhesion and spread of host tissue cells
92 [10].

93

94 ***Specific targeting of adhesins***

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

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

109

110 **Targeting biofilm maturation**

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

115

116 ***Major signaling pathways as antibiofilm targets***

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

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

131 phospho-diesterases (PDE) [27], demonstrating the potential of compounds naturally
132 produced by micro-organisms (see **Table**). In *P. aeruginosa*, NO-induced dispersal has
133 been recently linked to a specific PDE, NdbA, whose mRNA transcription is induced by NO
134 [28]. Interestingly, NO seems to be involved also in the dispersal of biofilms formed by
135 other micro-organisms, however through a different mechanism involving H-NOX proteins.
136 Therefore, development of surfaces releasing NO might be promising to control biofilm
137 formation as demonstrated by the use of NO donor-coated urinary catheters and
138 nanoparticles [29,30].

139

140 ***Direct action on matrix components to weaken biofilms***

141 Two factors regulated by quorum-sensing and c-di-GMP play a major role in the
142 architecture of biofilms: polysaccharides and extracellular DNA [31-34]. Thus, direct
143 targeting of these factors instead of their signaling pathways can also be envisaged to
144 reduce biofilm formation. Strategies using enzymatic degradation of these matrix
145 components such as the use of DNaseI or Dispersin B, an hexosaminidase naturally
146 produced by *Aggregatibacter actinomycetemcomitans* and hydrolyzing poly-N-
147 acetylglucosamine (a frequent component of *E. coli*, *S. aureus* or *Staphylococcus*
148 *epidermidis* exopolysaccharides), have been identified as efficient ways to disperse
149 biofilms *in vitro* and *in vivo* (see for example, [35-37]). However, enzyme-based
150 approaches are associated with two limitations: i) their restricted spectrum of action; and ii)
151 the risk of immunization against these molecules. The association of chelators of divalent
152 cations such as citrate or EDTA and biocides has also been proposed, based on their
153 ability to destabilize biofilm matrix [38,39]. These chelators could find their interest in the
154 case of local infection or restricted colonization such as device-related infection and have
155 been used as preventive agents in clinical trials [40]. Additionally, EDTA was proven an
156 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

181 (VRLIVAV- RIWRR-NH₂). While a direct evidence of its activity on persister cells is
182 missing, this peptide displayed a specific antibiofilm activity against *in vitro* biofilms formed
183 by several species including *P. aeruginosa* or *S. aureus* by inducing ppGpp degradation
184 [49²²] and a synergistic action together with ciprofloxacin on *in vitro* biofilms of various
185 pathogens [50²³]. Interestingly, this latter study demonstrates how adjuvant therapies can
186 allow reducing the concentration of antibiotic required to inhibit biofilm formation.

187

188 **Killing persisters**

189 Once a biofilm is mature, the last resort option for biofilm eradication is to identify
190 compound that would increase antibiotic activity against persisters (**Figure**). Silver has
191 been shown to potentiate the activity of several antibiotics against biofilm and persisters of
192 Gram-negative and Gram-positive bacteria in a mouse biofilm model with subcutaneous
193 catheter by increasing ROS production and bacterial permeability to antibiotics [51²⁴].
194 Sugar metabolism was also used to obtain antibiotic potentiation against persisters
195 through an increased aminoglycosides penetration powered by the proton motive force
196 [52,53]. Alcalinisation by basic amino-acids such as L-arginine was also recently
197 demonstrated to enhance aminoglycoside action *in vitro* and *in vivo* against biofilms and
198 persisters [54²⁵]. Anti-QS molecules such as brominated furanones have the potential to
199 revert antibiotic tolerance of *P. aeruginosa* or *E. coli* persister cells [55,56]. Persisters
200 tolerance could also be reduced by exploiting their weaknesses related to their slow
201 metabolism, such as a high sensitivity to proteolysis induced by the acyldepsipeptide
202 ADEP4 that activates the ClpP protease in Gram-positive pathogens. ADEP4 is active in
203 combination with rifampicin in a neutropenic mouse biofilm model [57²⁶]. Whereas the
204 efficacy of these anti-persister approaches remains to be further validated, on-going

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

207

208

209 **Future perspectives**

210

211 **Much ado for almost nothing in clinic... Why?**

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

224

225 **The limitation of biofilm models**

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

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

245

246 **What could be the near future?**

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

253 improve the use of otherwise topic compounds for local delivery at the site of biofilm
254 infection [64,65]. To become more translational, biofilm research needs biomarkers and
255 more global analyses performed directly on biofilms in clinical settings, which are currently
256 essentially applied to *in vitro* or animal models. These omics analyses could provide new
257 and unexpected targets. Lastly, the increasing awareness of the polymicrobial nature of
258 biofilms should lead to the development of dedicated approaches to study bacteria-
259 bacteria or bacteria-fungi interactions and their consequences on biofilm pathogenesis or
260 tolerance towards antibiotics [66].
261

262 **Conflicts of interest**

263 All authors: no conflicts of interest.

264

265

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271 We apologize to those authors whose work or publications could not be described or cited.

272

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276 as:

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

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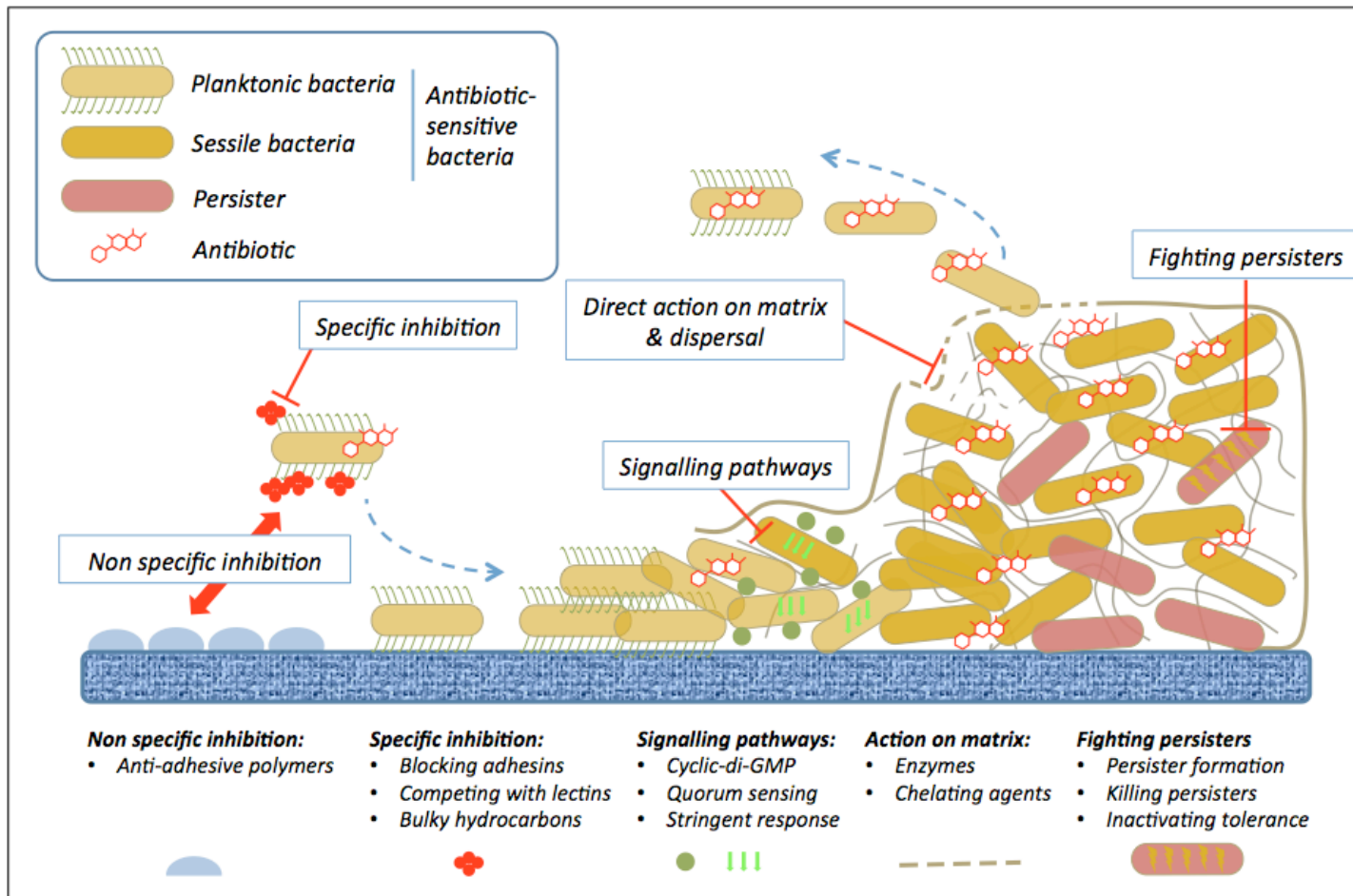


Figure 1. Biofilms: bacterial phenotypes and therapeutic targets

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.