**Title:** Targeting Biofilms: Current and Prospective Therapeutics

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**Abstract**

Biofilm formation is now recognized as a key virulence factor for a wide range of chronic microbial infections. While it has been well known for decades that bacteria and fungi in biofilms become highly tolerant of antibiotics, the development of effective therapeutics has lagged behind our growing understanding of biofilm biology. The multifactorial nature of biofilm development and drug tolerance imposes significant challenges to conventional antimicrobials, and indicates the need for multi-targeted or combinatorial therapies. In light of the discrepancy between the explosion of papers presenting multitude of methods to control biofilms and the sparsity of biofilm specific treatments available to the clinician, in this review, we focus on current therapeutic strategies and those in development for the treatment of biofilm infections, which target vital structure-function traits and drug tolerance mechanisms, including the extracellular matrix and dormant cells. We emphasize strategies that are supported by *in vivo* or *ex vivo* studies, highlight emerging anti-biofilm technologies, and provide a rationale for multi-targeted therapies aimed at disrupting the complex biofilm microenvironment.

**Introduction**

Since 1980 microbial biofilm formation has gone from being seen as an arcane behavior of bacterial populations, to being recognized as a principle virulence factor in many localised chronic infections. Biofilm infections commonly recur sometimes after long periods of clinical quiescence. This is not primarily due to genetic resistance that arises by mutation, although the increased microbial cell density may favour transfer of resistance genes. Rather microorganisms residing in biofilms may develop tolerance to traditional antibiotics or antimicrobial agents manifested by metabolic dormancy or molecular persistence programs. Moreover, the important role of the extracellular matrix in confering antimicrobial tolerance to biofilms is being recognized1. Advances in multi-omic and imaging technologies have also revealed the remarkable complexity and spatial organization of polymicrobial biofilm infections2. Accordingly our increased understanding of biofilms is rapidly changing the strategies used to treat these challenging infections (Fig.1). Nonetheless, biofilm control remains tenuous with few new therapeutic options currently available clinically. Part of the issue holding up progress in preventing and fighting these infections is that when bacteria are in biofilms they are notoriously difficult to culture using conventional clinical culture methods so that they are often completely missed.

The underlying premise of this review is that biofilm infections are not easily amenable to existing antimicrobial treatment or “single magic bullet” approaches because biofilm recalcitrance is a consequence of complex physical and biological properties with multiple microbial genetic and molecular factors, and also frequently involve multi-species interactions. Although a diverse range of microbes (Gram+ and Gram-, motile and non-motile, aerobic, anaerobic and facultative bacteria, and fungi) form biofilms, biofilms share many common features (Text Box 1). In nearly 20 years since the “universal” role of cell signalling in biofilm formation, signalling-based therapeutics have yet to be introduced for the clinical management of biofilm infections due to the extraordinary layers of complexity in cell signalling networks. Similarly the emergence of materials science and surface modifications incorporating anti-adhesion technologies and utilizing the strategy of biomimicry or surface textures and chemistries borrowed from plants and animals3 promised to prevent microbial adherence and subsequent biofilm formation. Although many studies show statistical reductions in biofilm or alterations in biofilm structures in the laboratory, few were tested or validated using *in vivo* or human cell models. Many more studies report only early time points, fail to use clinically-relevant treatment regimens or consider the presence of molecularly complex host fluids at the site of biofilm infections.More recent approaches include targeting the extracellular polymeric substance (EPS) matrix, a ‘multifunctional scaffold’ that supports and protects embedded bacteria. However, the dynamic variability of EPS composition and the interactions between various components4 add new levels of complexity, and provide challenges for developing anti-EPS therapeutics5.

Several excellent reviews discuss how microbes develop pathogenic biofilms and the protective mechanisms deployed against antibiotics, antimicrobials and innate immunity 1,6,7. Our review focusses on the challenges facing the development of biofilm-specific therapeutic strategies, how new insights into the chemistry and structure of the EPS matrix, active biofilm dispersal pathways, and a recognition of the role of dormant persister cells or slow-growing subpopulations in conferring antibiotic tolerance are being exploited to target biofilm infections.We also review developing technologies that promise to enhance the efficacy of current modalities or provide novel anti-biofilm effects, including challenges to ensure biocompatibility and therapeutic efficacy, which are both critical for clinical translatability. Where possible we focus on technologies that have shown efficacy in preclinical trials, robust animal or human cell infection models. Since there are more potential anti-biofilm therapeutic strategies than we can possibly discuss in detail in one review, we provide a comprehensive list, their developmental stage and a brief pros and cons statement in Table 1 and in Supplemental Table S1 with references.

Finally , we provide a rationale for the thesis that treating biofilm infections requires combination therapies or those that target more than one component of the complex biofilm microenvironment, similar to tumouregenesis8. Importantly, biofilm infections reflect an interplay between the host and opportunistic pathogens, each with their own reservoir of survival strategies, often within a complex microbiota. Polymicrobial biofilms pose an additional challenge, requiring antimicrobials that are effective on all microorganisms in the biofilm and limiting the efficacy of species-specific anti-biofilm strategies. All of these challenges contribute to why so few therapies have yet to be translated clinically. We hope this article will stimulate new insights and hypotheses that help develop more robust and efficacious therapeutic approaches to resolve biofilm-associated infections

**Current therapeutic approaches**

Many biofilm management strategies being devised in the clinic and used by surgeons, are largely based on an approach from cancer treatment (Box 2): early and aggressive debridement/washout and local delivery of high and sustained chemotherapy (antibiotics)9.

Given the devastating consequences of allowing a biofilm infection to persist, surgeons are undertaking earlier and more aggressive treatment, including revisiting “old” last-resort antibiotics such as colistin10. Another established approach used for intravenous catheter-related infections is lock therapy11. Following a decision to treat rather than remove certain types of catheters, the potential to leave biofilms intact (but containing dead cells) includes the potential to promote colonization by other microbes. This illustrates a crucial point regarding biofilms: killing does not necessarily eradicate the biofilm. Therefore the challenge of using antimicrobial agents, which may kill microorganisms but leave behind other biofilm components, must be addressed.

**Side Note:** Lock therapy is an approach, where a high concentration antibiotic solution is injected into the catheter lumen and sustained for an extended period to eradicate bacteria. Catheter locks have been used to combat sepsis since the 1980s, however with the understanding that infecting microbes are present as biofilms on device materials, antimicrobial treatments are now specifically tailored to improve efficacy11.

Since understanding the mechanisms of biofilm formation derives primarily from how they form on solid surfaces, most anti-biofilm clinical trials or FDA-approved therapeutics have focussed on indwelling medical devices. Current anti-biofilm technologies generally can be divided in two groups: surface coating or eluting substrates impregnated with antibiotics/antimicrobials (e.g. acrylic beads with absorbable antibiotic-loaded bone cement to prevent orthopaedic infection12) for biofilm prevention or physical-mechanical approaches (e.g. high velocity spray and jet irrigators) aimed at disruption. An advantage of local antibiotic delivery is that higher localised antibiotic concentrations can be achieved for longer periods than is possible by systemic administration. Several antimicrobial metal or inorganic coatings have also reached clinical application to prevent biofilm formation13. These include silver coating in endotracheal tubes, catheters, megaprostheses, wound dressings and copper alloys in hospital surfaces (supplemental Table S1). With respect to treating prexisiting biofilms, laboratory studies show that significant reductions in biofilm viability require extended incubation periods with high antibiotic concentrations, *in situ* release offers an important approach14,15. Although antibiotic-impregnated beads in bone cements or dental restorative materials were used before biofilm formation was recognized as a distinct etiological factor, they represent a class of technologies that are now being re-examined to gain better understanding on their effect in controlling biofilms.16

Mechanical disruption using water sprays and jets have been developed and used for pathogenic biofilm removal and for irrigation, including debridement of surgical site infections to remove necrotic tissue, exudates or dental biofilms. High speed imaging has provided important information on fluid-biofilm-surface interactions and show that while a significant amount of biofilm is removed from the impact area the biofilm becomes fluidized and spreads across the surface17. The ability of biofilms to become fluidized likely explains the tenacity of bacteria on surfaces after pulsed lavage18 and may contribute to the low success rate of irrigation and debridement alone in treating periprosthetic infections. An advantage of water-based jets is that antimicrobial agents can be readily added so that the fluid doubles as a delivery device as well as creating mechanical forces acting on the biofilm. However, inspite of advances in biofilm specific-clinical therapies, particularly in indwelling devices, most approaches still entail conventional antibiotic-based therapy or topical broad-spectrum antimicrobials.

**EPS Targeting Strategies**

The composition and structure of the EPS matrix is highly variable depending on the type of microorganism, local mechanical shear forces, substrate availability, and the host environment. EPS production is dynamic, mediating microbial adherence to a surface, cell-cell adhesion/aggregation and biofilm formation.19

**Side Note:** The EPS can contain exopolysaccharides, fibrous and globular proteins (including extracellular enzymes), lipids and nucleic acids/eDNA and can be surface-associated or secreted locally or deposited on abiotic and biotic surfaces.

In addition to promoting microbial adhesion, the EPS matrix serves as a 3D scaffold providing cohesiveness, mechanical stability, and protection against host effectors and antimicrobial therapies. The EPS matrix can modulate chemical/nutrient gradients and delineate pathogenic environments (such as acidic pH and hypoxia), which are key virulence attributes, including recalcitrance1,4. Thus, targeting the EPS represents an existential threat to biofilms20, and exogenous targeting may be an effective strategy to remove biofilm, disaggregate bacteria and disrupt the pathogenic environment. Targetting can be achieved by: (i) inhibiting EPS production, (ii) binding EPS adhesins on the microbial surfaces to block adhesion, or (iii) degrading EPS in established biofilms (Fig 2).

**Figure 2. Targeting the biofilm EPS**

Disrupting EPS synthesis/secretion and EPS adhesins binding.

Detailed characterization of pathways regulating biofilm EPS matrix gene expression has identified several extracellular and intracellular signalling networks as well as non-signalling mechanisms for inducing EPS production. In general, cyclic-di-GMP and cyclic-di-AMP21 control various EPS-producing exoenzymes, polysaccharides and adhesins that are potential anti-biofilm candidates to inhibit or disrupt biofilm EPS22,23. These nucleotide-signalling molecules regulate glucan-producing exoenzymes (e.g. glucosyltransferase) in Gram+ *Streptococcus mutans* as well as the aggregative exopolysaccharides Psl/Pel in Gram- *P. aeruginosa*. Several potential small inhibitors of di-guanylate or di-adenylyl cyclase have been identified through library screening or *in silico* drug discovery combined with bioactivity assessment using *in vitro* biofilm models,24,25 although their antibiofilm efficacy awaits further *in vivo* validation.

Likewise, inhibition of EPS glucan synthesis by glucosyltransferase using small molecule inhibitors reduced the accumulation of pathogenic biofilms on teeth, and supressed the onset of oral diseases *in vivo* without disturbing resident microbiota.26,27 These small molecule inhibitors alone are not superior than current chemical modalities for oral biofilm control (chlorhexidine) or tooth decay prevention (fluoride), however when used in combination, EPS inhibitors can significantly enhance their therapeutic effects26. Inhibitors of adhesin production and adhesin-binding antibodies or peptides have also been developed to disrupt bacterial binding to host surfaces. Small molecules (e.g. peptides, mannosides) targeting host-microbe matrix interactions have shown efficacy in prevention and treatment of both bacterial and fungal biofilm infections *in vivo*.28,29 Mannoside inhibitors of the bacterial adhesin FimH (alone or combined with trimethoprim-sulfamethoxazole) prevented catheter-associated urinary tract infection (UTI) in mice by reducing *Escherichia coli* colonization 2-log, and treated chronic cystitis by reducing the *E. coli* population 3-log28,30. A recent study has also attempted to address the low half-life and bioavailability of these *O*-mannosides by creating *C*-mannosides which have increased metabolic stability and *in vivo* efficacy, whereby prophylactic treatment reduced the *E. coli* burden 2-log and treatment of chronic infection resulted in a 4-log reduction in a UTI mouse model31. Similarly, ring-fused 2-pyridones, which inhibit curli and type-I pili biogenesis, have been shown to reduce uropathogenic *E. coli* bladder colonisation more than 10-fold and the development of intracellular bacterial communities in an *in vivo* mouse UTI model32. Several other biomolecules binding to EPS adhesins as part of anti-adhesion methods to prevent bacterial infections has been discussed in detail elsewhere33.

Targeting the EPS chemistry and structure

Exopolysaccharide-degrading enzymes such as glucanohydrolases (dextranase and mutanase) and Dispersin B can disrupt the matrix of pathogenic oral biofilms and glycoside hydrolases have been used to degrade a mixed-species *S. aureus* and *P. aeruginosa* biofilm grown in a murine model of chronic wounds 34-36, although poor retention and enzymatic stability (e.g. susceptibility to proteolysis) may compromise efficacy *in vivo*35. Nevertheless, a purified serine protease, Esp, from *S. epidermidis* inhibited *S. aureus* biofilm formation and eradicated pre-existing biofilms *in vitro*, while enhancing susceptibility to the antimicrobial β-defensin 2 and reducing *S. aureus* nasal colonization in humans37. Another approach used endolysins (bacteriophage-encoded peptidoglycan (PG) hydrolases), which enzymatically degraded the bacterial cell wall PG38. Engineered PG hydrolase constructs with distinct antimicrobial activities degraded multiple unique bonds in the PG structure specific to *S. aureus*39 increasing killing and biofilm removal in animal models. Fusion proteins derived from multiple bacteriophage endolysins may also reduce the risk of antibiotic resistance, and show sufficient specificity to avoid targeting commensal strains. Glycoside hydrolases were recently shown to both disrupt pre-existing *P. aeruginosa* biofilms and potentiate neutrophil mediated killing40.

Likewise, DNases have shown efficacy in disrupting biofilms41. Consistent with the role of eDNA in EPS and in early biofilm development, DNase I is effective in disrupting early biofilms *in vitro* and *in vivo41,42*. Notably, other biomolecules, including polysaccharides and proteins also associate with eDNA contributing to biofilm structural integrity, which may explain the efficacy of DNase in treating nascent biofilms. Few studies have used DNase to specifically target biofilms *in vivo*, however, it was shown to significantly decrease *Gardnerella vaginalis* colonization on vaginal mucosal epithelial cells in a murine model43. Therapeutic use of recombinant human DNase I (dornase alfa) degrades neutrophil and microbe-derived DNA in cystic fibrosis (CF) airways, reducing sputum viscosity44. An intervention study of dornase alfa in CF patients with early lung disease showed significantly improved lung function and lower risk of exacerbation compared to placebo groups, with a potential decrease in the rate of lung function decline in children45. A clinical trial investigating the efficacy of dornase alfa for the treatment of chronic otitis media, at the time of tympanostomy tube insertion to promote bacterial clearance from the middle ear combined with antibiotic drops is under evaluation46,47.

Matrix-degrading enzymes can help disperse bacteria in biofilms for more effective killing when combined with antimicrobial agents. Targeting EPS can also disrupt the viscoelastic properties to further weaken biofilm cohesiveness and enhance antimicrobial efficacy, including host mediated antimicrobial responses. Recent studies showed that glucano-/glycoside-hydrolases and DNases enhanced antimicrobial delivery and potentiated killing by antibiotics or antimicrobial peptides when used in combination against pre-formed biofilms *in vitro*48,49. Overall, EPS synthesis inhibitors or EPS degrading approaches, which lack intrinsic antibacterial activity, appear to be a promising adjunctive approach for biofilm control that could potentially enhance the killing efficacy of antimicrobial agents and promote biofilm removal when co-administered.

*EPS targeted antibodies and nucleic acid binding proteins*

Vaccine approaches pose several challenges as an anti-biofilm therapeutic strategy, since vaccines are microbe specific and clinical isolates from biofilm infections show considerable variability in genotype and/or the phenotypic expression of vaccine-targeted epitopes50. A more effective approach may be to use antibodies to targeted biofilm antigens. Monoclonal antibodies to *P. aeruginosa*-derived EPS selected using a human antibody phage library identified epitopes that bound to Psl, a polysaccharide widely present in *P. aeruginosa* clinical isolates51. Psl was shown to be a serotype-independent, antibody-accessible antigen, and anti-Psl antibodies increased opsonophagocytic killing of *P. aeruginosa*, inhibited attachment to lung epithelial cells *in vitro*, and showed prophylactic protection in multiple animal models of *P. aeruginosa* infection. Additionally, vaccine-elicited antibodies to *Enterococcus faecalis* pilus tip (EbpA) abrogated bacterial binding to fibrinogen and biofilm formation in a mouse model of CAUTI52. Notably, EbpA did not mediate *E. faecalis* adhesion directly to the catheter material, but rather inhibited binding to fibrinogen deposited on the catheter surface, thus preventing subsequent bacterial aggregation and biofilm formation, since fibrinogen can also be incorporated into biofilm EPS. This approach highlights why using a complex host-microbe model can reveal additional targets. In another approach, a multivalent vaccine exploiting both planktonic and biofilm-expressed polypeptides from *S. aureus* showed increased efficacy in combination with antibiotics compared to antibiotic treatment alone in a rabbit model of osteomyelitis53.

Nonetheless targeting broadly conserved antigens in microorganisms is desirable. The DNABII family of DNA-binding proteins have emerged as playing a key role in providing structural integrity to eDNA, a widely distributed constituent of the EPS matrix in many biofilms54. The high binding affinity of integration host factor (IHF) has specifically been exploited to target nucleoproteins in biofilms and widely tested in animal models. *E. coli* IHF antibodies are cross-reactive, binding to DNABII in multiple bacterial species, resulting in biofilm destabilisation and the release of individual bacteria. Anti-DNABII immunotherapy has shown efficacy *in vivo* against biofilms in numerous types of bacteria including oral bacteria55, uropathogenic *E. coli*56 and *P. aeruginosa* biofilms in a mouse lung infection model, when combined with antibiotic therapy. It has also shown efficacy with MRSA compared with antibiotic alone in murine models57,58. In a combinatorial approach without using antibiotics, DNABII antibodies were combined with a vaccine strategy. A study with nontypeable *H. influenzae* (NTHi) in an animal model of otitis media used IHF and recombinant soluble Type IV pili (rsPilA) co-administered with an adjuvant and delivered by transcutaneous immunization to achieve early NTHi eradication and prevention of disease 59. This approach also resulted in the disassembly of NTHi biofilms established prior to immunization leading to resolution of existing disease.

**Inducing biofilm dispersal**

Biofilm dispersal has been shown to be a regulated process that involves EPS matrix degradation, and the triggering of this response has provided research strategies designed to promote biofilm self-disassembly. These approaches, for the most part, assume that dispersed bacteria have returned to an active state akin to their planktonic phenotype, rendering them more susceptible to conventional antibiotics. Furthermore, liberated inactive cells will also have lost a degree of protection conferred by their association with the biofilm community and structural organization. Regardless of their dispersed state, it remains vitally important in the clinical setting that dispersive or exogenous EPS-degrading agents be administered alongside systemic antibiotics to avoid recolonization or bacteremia, and potentially septicaemia.

Targeting cyclic-di-GMP pathways

The intracellular secondary messenger nucleotide c-di-GMP plays a key role in the biofilm lifecycle of both G+ and G- bacteria whereby increased levels promote biofilm formation and reduced levels disassembly60. The enzymes governing c-di-GMP levels, diguanylate cyclases (synthesis) and phosphodiesterases (breakdown), possess GGDEF, EAL and HD-GYP domains that are found in numerous bacterial phyla. This signalling pathway therefore offers an attractive strategy for the treatment of multiple species, although the complexity of c-di-GMP regulation makes it challenging to control61. Few studies however showing biofilm dispersal have been tested with relevant cell models *in vitro* or *in vivo* using animal models. One study used a *P. aeruginosa* construct containing an exogenous *E. coli* phosphodiesterase. When expression was induced *in vivo* it resulted in reduced c-di-GMP and dispersal of biofilms on silicone implants in a mouse foreign body infection model62. While in principle it supports the potential use as an anti-biofilm strategy for this pathway, the authors noted limitations of the study including an increased bacterial burden in the spleen. C-di-GMP is also a potent stimulator of host immunity via interferon responses, and therefore it may be difficult to attribute effects on biofilms specifically *in vivo63.*

A well-characterized approach to modulate c-di-GMP levels is though nitric oxide (NO).

**Side Note:** Nitric oxide (NO) is an ubiquitous signalling molecule found in both prokaryotic and eukaryotic systems. In mM concentrations and greater it is cytotoxic but in pM and nM can be used to form oxidative/nitrosative reactive species that interact with proteins, DNA and metabolic enzymes, resulting in anti-biofilm effects. Since NO is so labile the optimal concentration to disperse biofilms is difficult to measure however NO microelectrodes are highly sensitive and offer excellent spatial and temporal resolution in tissues or body fluids.

NO was first shown to regulate c-di-GMP levels and mediate biofilm dispersal in *Pseudomonas aeruginosa64* at low concentrations, and has since been reproduced in several other bacterial species65. The use of gaseous NO or spontaneous NO-donors, however presents clinical challenges due to potential cytotoxicity from systemic exposure, lack of specificity in targeting biofilm infections and cost. However, a proof of concept preclinical study using low-dose gaseous NO in the pM to nM range, was recently shown to reduce *P. aeruginosa* biofilm aggregate size in sputum as a primary clinical outcome in a small number of patients with CF66. Patients did not exhibit adverse effects to NO therapy. Although biofilm (aggregate size) was significantly decreased, NO did not reduce CFU as seen in another study67, perhaps because patients continued to receive antibiotic therapy throughout the study period. However a Phase I clinical trial is ongoing to study the efficacy and safety of NO in CF patients68.

To address the cost of administering gaseous NO and potential systemic cytotoxicity issues, cephalosporin-3´-diazeniumdiolates (C3Ds), comprised of a stabilized diazeniumdiolate NO-donor attached to the 3’-position of cephalosporin, have recently been developed to selectively deliver NO to bacterial biofilms69. These pro-drug candidates aredesigned to specifically release NO upon cleavage of the cephalosporin β-lactam ring via bacterial β-lactamases and have been shown to be effective in dispersing *in vitro P. aeruginosa* biofilms69. NTHi biofilms grown on primary ciliated epithelia also showed enhanced sensitivity to azithromycin, reducing viability 2-log when a specific C3D, PYRRO-C3D, was used as an adjuvant, a response attributable to dispersal and modulation of metabolic activity70. This effect was also demonstrated in a study using primary epithelial cells from patients with primary ciliary dyskinesia (PCD), a disease that compromises mucociliary clearance. PCD airway cells showed increased susceptibility to NTHi biofilm formation compared to non-PCD epithelial cells, and PYRRO-C3D in combination with antibiotic significantly decreased NTHi viability 2-log compared to antibiotic treatment alone71. Treatment of these healthy and PCD airway co-cultures had no effect on transepithelial electrical resistance suggesting that epithelial barrier function was unaffected. Although this alone is not a sufficient assessment of toxicity, the targeted release of low NO concentrations (48 - 90 nM) should improve patient safety. One concern regarding the administration of these treatments, however, is that in delivering NO in this form biofilms are also subjected to low doses of antibiotic (the cephalosporin backbone), an undesirable driver for the development of antibiotic resistance in biofilms.

Outside of the use of inhaled NO for lung infections NO-donor instability is also an issue and this is being addressed by developing nitroxides (sterically hindered NO analogues) which exert biological responses via NO-mimetic properties72. These molecules (carboxy-TEMPO, CTMIO, DCTEIO) elicited biofilm dispersal in *P. aeruginosa* and *E. coli* similar to NO, with carboxy-TEMPO also reducing tolerance to ciprofloxacin*72,73*. Treatment with carboxy-TEMPO however, failed to disperse MRSA biofilms, indicating that this approach may be restricted to biofilms formed by certain species, an ongoing concern for those infections where polymicrobial biofilms are common.Other drugs in development include ciprofloxacin-nitroxide conjugates, which similar to C3Ds, combine antibiotic activity with a donor compound74, and fimbrolide-NO donor hybrids, which simultaneously target quorum sensing (QS) and NO pathways75.

Targeting quorum sensing

The role of QS systems in biofilm development and dispersal offers another intensely studied strategy for the development of novel therapeutics. QS requires signal binding to a corresponding transcriptional regulator, which activates the downstream transcription of select targets. Because production of so many virulence determinants in pathogenic bacteria requires cell-cell communication, QS inhibitors (QSI) targeting the AHL-QS system in G- bacteria or the QS systems in G+ bacteria have been extensively evaluated for efficacy on clinically relevant bacterial biofilms using *in vitro* and *in vivo* models. The QS autoinducer, AI-2, for example, acted as a chemorepellent in *Helicobacter pylori* regulating the proportion and spatial organisation of biofilm cells76. Treatment of *in vitro* biofilms with exogenous AI-2 resulted in both a reduction in the proportion of adherent cells and dispersal76. The autoinducing peptide type I (AIP-I) also triggered dispersal in MRSA biofilms on titanium disks rendering detached MRSA more susceptible to treatment with rifampicin and levofloxacin77. Additionally, the RNAIII-inhibiting peptide (RIP) resulted in a 7-log reduction in MRSA versus 5-log reductions seen with RIP-soaked Allevyn or teicoplanin treatments alone in a mouse wound model78. Starkey et al. (REF) used a high throughput screen to identify a benzamide-benzimidazole “M64” derivative that interferes with the Pseudomonas Quinolone Signal (PQS) quorum sensing system which regulates biofilm formation and the production of virulence factors in *P. aeruginosa*79. Interestingly, M64 reduced both the virulence and persistence of PA14 in a mouse model of burn and lung infections when used alone and reduced the bacterial load further when used in combination with ciprofloxacin. M64 also was not cytotoxic to murine macrophages and reduced the number of persister cells in the population.

Although the increased efficacy of antibiotic treatment with QSI *in vivo* is promising reduced bacterial loads are often strain and biofilm model dependent80. Furthermore, QS molecules can be washed away during biofilm initation, while EPS matrix can bind/sequester QS molecules and the effects may be limited to highly localized areas within the biofilm structure1, thereby needing access and specific targeting where active QS-signalling is occurring. These factors in addition to the complexity in cell signalling networks, make it a challenging therapeutic approach albeit such inhibitors can be used in combination with other strategies.

**Metabolic interference**

The potential of exogenous amino acids in the treatment of biofilms has garnered considerable interest, with specific amino acids shown to impact both biofilm metabolism and development. L-arginine serves as a substrate for alkali production by arginolytic bacteria (e.g. *Streptococcus gordonii*), which can neutralize acids and modulate pH homeostasis within oral biofilms clinically81. Treatment of polymicrobial biofilms comprised of *Streptococcus mutans*, *S. gordonii*, and *Actinomyces naeslundii* with L-Arg suppressed *S. mutans* growth and resulted in substantial reduction in insoluble EPS and altered biofilm architecture82. In addition to pH modulatory effects81, L-Arg was also capable of repression of genes involved in the production of insoluble EPS and bacteriocin in *S. mutans*, while increasing hydrogen peroxide production by *S. gordonii82* . L-Arg reduced biomass and altered EPS architecture in *S. gordonii* biofilms83, and also destabilized multispecies oral biofilms, reducing viability, and increasing susceptibility to cetylpyridinium chloride84. An alternative amino acid, L-methionine, was also identified as a promising adjuvant for treating *P. aeruginosa* biofilms, triggering disassembly and increasing sensitivity towards ciprofloxacin in a mouse model of chronic pneumonia, and enchancing survival85. This activity was attributed to up-regulation of four different *DNase* genes and the subsequent degradation of eDNA in the EPS, although the exact pathways regulating this response were not determined. Interestingly, L-Met appears to have been chosen for this study following screening of a selection of D- and L- amino acids for their activity against *P. aeruginosa* biofilms. Given the diversity in amino acid utilization between bacterial species it is unlikely that a single amino acid would be universally effective, however, their importance, and that of bacterial metabolism in general, should not be underestimated as prospective area for the development of future treatment strategies.

Another approach is based on evidence that iron metabolism is important in biofilm formation in several pathogens86-89. Iron acquisition is key in the ability of pathogens to establish infection and epithelial cells with the F508 CF transmembrane receptor (CFTR) mutation showed increased biofilm formation by *P.aeruginosa* linked to increased availability of iron90. In a non-antibiotic strategy to exploit this vulnerability gallium, which is chemically similar and can substitute for iron, interfered with *P. aeruginosa* growth and iron metabolism,killed planktonic bacteria in an acute mouse pneumonia model and reduced bacterial counts in established biofilm by 3 logs in a chronic biofilm lung infection model91. Gallium was administered via inhalation and uptake was dependent on pyoverdin *in vitro*, however *in vivo* it was not clear if gallium had other anti-inflammatory effects. Notably, *in vitro* gallium directly reduced bacteria compared to approaches using iron chelation, since *P. aeruginosa* posseses redundant iron receptor/uptake systems. Nonetheless, using iron chelators adjunctively with tobramycin reduced *P. aeruginosa* on a co-culture model of human bronchial epithelial cells from a CF patient with the CFTR F508 deletion resulted in a 7-log reduction in viable bacteria and also prevented biofilm formation on CF bronchial epithelial cells92. More recently the oxidation state of iron was shown to be important93. This study examined mucus from the airways of CF patients and found that ferrous iron was the primary form of bioavailable iron, which also correlated with CF lung disease, whereas ferric iron did not. This study highlights the importance of directly investigating the phenotypic state of bacteria *in situ* in human infections and its potential translational relevance in informing new therapeutic approaches.

**Targeting dormant biofilm cells**

Targeting pathways to induce processes such as dispersal requires that cells are metabolically active. However, available evidence also shows that dormant cells or persisters residing within biofilms play a key role for drug tolerance (Box 3). It is therefore attractive to consider antimicrobial approaches that physically or chemically disrupt cells rather than interfering with cellular processes. Non-discriminating oxidizing agents such as hypochlorite and hydrogen peroxide have been used as irrigants in wound94 and endodontic debridement95, however studies reveal that even strong oxidizers like sodium hypochlorite fail to eradicate biofilms96 likely because long term exposure is not possible due to cytotoxicity concerns. Broad-spectrum cationic biguanides such as chlorhexidine or quaternary ammonium adhere to cell walls and disrupts cell membranes. However, penetration was limited over the expected timescales used in *ex-vivo* dental biofilms97 with longer term exposure increasing cytotoxicity and clinically impractical.

Other exploratory avenues include antibiotics that treat infections caused by slow-growing bacteria. Rifampin, used to treat staphylococcal orthopaedic-implant infections raises concern about the development of rifampin resistance. However used in combination with other antibiotics, rifampin and fosfomycin enhanced efficacy in treating foreign body MRSA biofilm infections *in vivo*98. Likewise, disrupting a target in dormant cells can kill persisters. Acyldepsipeptide antibiotic (ADEP4) can activate the ClpP protease in dormant persister cells in Gram positive bacteria so the cells effectively “digest” themselves. Although the concept of endogenously activating cytoplasmic enzymes for proteolytic degradation in biofilms is elegant it should be noted that ClpP is not an essential enzyme and ClpP null mutants are not influenced by ADEP4. To address this a combination of ADEP4 combined with rifampin showed good efficacy in a chronic biofilm mouse deep “abcess-like” infection model99 using various *S. aureus* species as challenge organisms. However, this study illustrates that careful consideration needs to be given to antibiotic pairings, particularly in this case since rifampin resistance is common and would likely develop in a ClpP null mutant.

Antimicrobial peptides (AMP) represent another approach in treating biofilms that is independent of microbial activity.

**Side Note:** Antimicrobial peptides are a subset of host defense peptides with antibiotic activity. Peptides such as LL-37 (cathelicidin) and human -defensins are rapidly-acting, small molecule effectors in the innate immune arsenal of the host.

An important advantage of AMPs is that they are widely conserved and therefore attractive as broad-acting antimicrobial agents that may be useful against both bacterial and fungal biofilms100,101. Conversely, species-specific targeting is also possible with synthetic AMPs consisting of dual functionally independent moieties (a broad-spectrum AMP with a killing moiety, and a species-specific binding peptide with target specificity). This approach may remove specific pathogens such as *S. mutans* from oral multispecies biofilm communities to promote a ‘healthy-like microbiome’ as demonstrated *in vitro*102. Another advantage is that the AMP pore-forming activity targets respiring cells as well as persister and dormant populations, reducing the potential for bacteria to develop AMP resistance. AMPs thus have potential as anti-biofilm therapeutics. Synthetic peptides that modify specific AMPs sequences were designed that showed both inhibitory activity and, with antibiotics enhanced killing of *P. aeruginosa* biofilms in invertebrate infection models103. Specific peptides also triggered degradation of ppGpp, preventing the accumulation of this secondary messenger and abrogating biofilm formation of several G+ and G- pathogens101. However, AMPs can bind to EPS matrix components and to other host molecules, reducing their effectiveness and microbial proteases may further diminish AMP potency104 requiring more pre-clinical efficacy studies. Additionally, the high cost of AMPs is a barrier for clinical development and commercialization, although using chloroplast-based technology for large-scale production in automated greenhouses may mitigate costs49. Nevertheless, AMPs can be immobilized onto solid surfaces to enhance anti-biofilm efficacy or specificity. This was particularly effective as a polymer-based approach on catheters, since AMP-brush coatings significantly reduced *P. aeruginosa* adhesion and infection over 7 days in a mouse model of urinary tract infection105. Furthermore, a structurally nanoengineered antimicrobial peptide polymers exhibited potent killing activity against several G-, colistin-resistant and MDR pathogens, while demonstrating low toxicity and efficacy in an animal model of *Acinetobacter baumannii* infection106. The recent completion of two phase II clinical studies of brilacidin (a membrane acting AMP mimetic) as an intravenous agent for skin infections demonstrate the feasibility of AMPs for systemic therapeutics107.

AMP can also enhance conventional antimicrobial activity, while combination with anti-EPS strategies may further increase the access and permeabilizing properties of AMPs once in the biofilm49,101. Although targeting tolerant cells is a promising approach, reaching the target cells embedded within biofilm either topically or systemically and be active across a spatially and chemically heterogeneous microenvironment remain significant challenges *in vivo*. The stability and durability of AMP coatings within the body is also an issue which needs to be further addressed, particularly where wear might be expected due to shear caused by moving tissues and fluids.

**The promise of new technologies**

While our understanding biofilm microenvironments is evolving, technological advances have provided unprecedented avenues to develop multi-targeted therapeutic approaches that prevent and disrupt biofilms or enhance anti-biofilm drug efficacy (Fig. 3). Nano/chemical engineering approaches provide unparalleled flexibility to control the composition, size, shape, surface area/chemistry, and functionality of nanostructures that can be used to develop a new generation of modified materials or coat existing solid surfaces for biofilm prevention. Functionalized nanoparticles, including stimuli-triggered activation, can be designed to enhance penetration and selectively target or release drugs locally after bacterial attachment or within biofilms. As new technologies with novel antibiofilm effects are emerging at a remarkable pace, here we focus on overall concepts while providing insights for their clinical potential based on recent studies using *in vivo* models. We have provided a full list of current and prospective technologies and additional references in Supplemental Table S1.

Surface modifications

Surface-tethering or antibiotic/biocide incorporation as a chemical surface modification has long been studied to inhibit bacterial adhesion and biofilm formation108. However, sustaining anti-biofilm efficacy and therefore translation to clinical or environmental benefit has been challenging. The size of the biocidal reservoir and chemical composition is often limited by the potential for deleterious effects, exemplified by both silver nanoparticles (NPs) and tributyltin coatings which have previously demonstrated host and environmental toxicity respectively109,110. Additionally, antimicrobial reservoirs are often subject to progressive decreases in efficacy, either through the finite nature of the reservoir or nonspecific absorption of exogenous surfactants and proteins

Advances in material and surface engineering have created well-defined topographic patterns capable of controlling biofilm formation without including antimicrobial agents111.

**Side Note:** Topographic patterns include protruding squares, cone-shapes, wrinkle and ridge-like patterning or nanopores that exhibit anti-adhesion properties.

The most well-established ordered topography is the Sharklet™ surface. Inspired by shark skin and its inherent anti-biofouling properties, microscale ribs of various lengths are combined into a repeating diamond micropattern, creating a textured surface capable of retarding macro and micro biofouling112 as well as bacterial colonisation and biofilm formation when incorporated into medical device surfaces113. Surface modifications are mostly focused on nonspecific protein repulsion and the inhibition of bacterial colonization. This can be challenging due to the structural and physio-chemical diversity of the numerous proteins in biological fluids surrounding a surface in a biomedical setting 114. Hydrophilic polymer brushes/tethered polymers such as poly(ethylene glycol) (PEG) are widely used in the prevention of medical device fouling115. While early bacterial adhesion is attenuated, likely due to the inhibition of an initial protein priming layer, the multifaceted nature of bacterial colonization (often involving non-proteinaceous adhesins) can lead to eventual biofilm formation116. Further studies with “super-hydrophilic” (super-wet) or “super-hydrophobic” surfaces have decreased protein deposition and bacterial attachment of clinically relevant surfaces114,117.

**Side Note:** Super-hydrophobic surfaces maintain air at the solid-liquid interface when hydrated leading to improved functionality via water repellency or reduced drag

Incorporation of these materials into medical devices shows promising, but variable results. While recently greater sustainability of super-hydrophobic surfaces upon mechanical abrasion has been demonstrated118, the antibiofilm effects of these surfaces are often transient or subject to species bias119. Short to medium-term biofilm suppression may be sufficient however to permit effective immune and prophylactic responses and tissue integration of a foreign body.

The development of functionalized medical implant surfaces with a vast array of antimicrobial and antibiofilm properties has been intensely studied, particularly with respect to titanium implants. To employ these surfaces in biomedical applications, modern surface design has been largely driven by top-down methods such as lithography, imprinting and others120 to produce a vast array of antibacterial coatings, including but not limited to, silver, copper, titanium dioxide and chitosan. Furthermore, emerging bottom-up approaches using nanomaterials as ‘building blocks’121 and surface attachment and immobilization of biomolecules, including antimicrobial peptides/proteins and polysaccharides122,123 have also generated antibacterial surface coatings. Some capitalize on the unexpected discovery that certain bacterial polysaccharides can inhibit biofilm formation123. One of the most studied, hyaluronic acid (HA), reduced *S. aureus* adhesion to HA-coated titanium surfaces124 and poly(methyl methacrylate) intraocular lenses125.

Bottom-up surface-assemblies can also be combined with top-down surface processing126 to generate nanocoatings with anti-biofilm properties and biocompatibility127. Recent *in vivo* studies demonstrated the feasibility and efficacy of tunable multi-layer nanocoatings that released different combinations of antibiotics128 or sequential delivery of gentamicin and an osteoinductive growth factor129 in a time-staggered manner for prevention of biofilm-associated infection and bone tissue repair around implants. Both demonstrated the ability to prevent biofilm formation on the device surface, relative to uncoated controls, were able to clear infiltrating bacteria and prevent colonization of the implant while promoting bone formation and osseointegration. Importantly, long term host retention and release and biocompatibility were demonstrated providing promise for their more wide-spread application in orthopaedics.

Efforts to created surfaces with even more control over the specificity and sensitivity of their antibacterial or antibiofilm capabilities have led to “smart surfaces”.

**Side Note:** Smart surfaces elicit their effect only upon contact with certain physiological or physiochemical cues to provide targeted application, increasing therapeutic precision and reducing the risk of cytotoxity.

Known as stimuli-responsive or triggered anti-biofilm surfaces, triggers include pH, temperature, salt concentration, metabolites, electrical currents and photoactivation which induce surface area, topographical and chemical changes as well as heat generation or drug release that can kill or repel bacterial attachment (Supplemental Table S1). The design principles for controlling bacterial adhesion or biofilm removal mechanisms that may be triggered on demand are intriguing. However, the effectiveness of these approaches have been evaluated largely *in vitro.* Consequently, as with all surface modifications, whether functionality will remain *in vivo* upon binding endogenous host proteins in saliva, blood, synovial fluid, urine is unclear. Another consideration is that bacteria which come to the surface and may be killed but remain attached may also mask the underlying technology and even provide a nutrient source for subsequent bacteria. Thus it is important to not only have a killing effect but also a self-cleaning mechanism, possibly facilitated through mechanical shear of surrounding body fluids or tissues. Furthermore, challenges to enhance mechanochemical stability and overcome coating deterioration and dissolution and non-adverse host reactions to the coating itself will need to be addressed in future studies to facilitate clinical application130-132.

Nanoparticles

The versatility and bioactivity of nanoparticles (NPs) provided multiple avenues for biomedical applications, becoming an increasingly popular approach for the development of antibiofilm approaches. NPs with intrinsic antimicrobial activity, primarily inorganic materials such as silver, can act as anti-biofilm agents or as nanocoatings (as described above). Due to their flexible chemistries and morphologies, they can also serve as drug delivery vehicles (nanocarriers) with organic NPs accounting for over two-thirds of the systems approved for human use133. Furthermore, both inorganic and organic NPs can be combined or modified by adding new molecules (hybrid NPs) to enhance their biological properties or provide multifunctionality. Since excellent in-depth reviews on the principles and current applications of NPs, particularly silver, are available13,134, we focus on clinically used lipossomal NP for drug delivery and emerging technologies, including stimuli-triggered activation, that have shown efficacy *in vivo*.

**Side Note:** Nanoparticles are structures with a size range between 1-1000 nm, and classified as organic or inorganic that exhibit biological properties of their own (e.g. antibacterial) or can be used as drug delivery carriers/vehicles.

Liposomes, physiologically compatible vesicles composed of one or more phospholipid bilayers, represent one of the the most widely developed organic NPs for drug delivery with good biofilm penetration, biocompatibility and demonstrable efficacy against biofilms of a wide variety of bacterial species incorporating a diverse number of antibiotics135,136. These nanocarriers can protect the antimicrobial agent from deleterious matrix interaction or enzymatic inactivation and degradation at the infection site by other bacterial and host components. The lipid structure can also fuse with the bacterial outer membrane releasing the drug directly into the cell, thereby potentially maximizing therapeutic effects while reducing host cytotoxicity136. Furthermore, the liposomes can carry more than one drug by co-encapsulation and can be also functionalized to increase targeting specificity and triggered release. Importantly however, some studies have highlighted a reduced efficacy of liposomal-encapsulated antimicrobials dependent on the environment in which the biofilm resides, with the potential for host and microbe-derived substances such as mucus and alginate to inhibit bacteria-liposome interactions137. Nevertheless, several formulations are in preclinical studies and clinical trials with some available commercially138. Liposomal ciprofloxacin and amikacin, for example, have shown promise in the management of chronic lung infection in cystic fibrosis139,140. The potential of liposomes to act as delivery agents for other antimicrobials, such as NO, whilst yet to reach human trials, has demonstrated significantly reduced *S. aureus* biofilm mass compared to controls in a sheep model of chronic rhinosinusitis141. Whilst this study did not note any negative clinical symptoms, a transient increase in heart rate and decrease in mean arterial pressure was observed in the animals which requires further investigation.

Despite encouraging data with conventional nanoparticles, new generations, some with multi-functionality or on-demand activation upon specific stimuli similar to “smart” surfaces, represent the most widely developed NP class in current development (Supplemental Table S1). Recent studies with inorganic NPs such as iron oxide (Fe3O4) with peroxidase-like functionality and the capacity to catalyze hydrogen peroxide (H2O2), at concentrations ranging from 0.1-1% H2O2, in a dose- and pH-dependent manner showed potent effects against virulent oral biofilms *in vivo*142. Under acidic (pathological) conditions, NPs activated free-radical generation from H2O2 *in situ* that simultaneously degraded the biofilm matrix and rapidly killed embedded bacteria (>5-log reduction of viable cells vs. control within 5 min, and 5000-fold more effective than 1% H2O2 alone)143. Daily topical treatments effectively reduced the onset and severity of dental caries (tooth decay), preventing cavitation altogether in a rodent model of the disease. The pH-dependent functionality prevents catalytic reaction at physiological pH and unmitigated free-radical production, improving biocompatibility.

Stimuli-triggered mechanisms by NPs can also enhance the selectivity of drug activation or delivery to biofilm cells, protecting host tissues and microbiota while targeting infective agents within pathological microniches142,144,145. The anti-biofilm activity of farnesol (an antibacterial agent) delivered via acidic pH-triggered polymeric NPs was enhanced 4-fold (vs free drug), significantly improving the drug efficacy against an oral biofilm infection *in vivo* following topical treatment145. These water-soluble polymeric nanocarriers can encapsulate hydrophobic/apolar drugs into aqueous solution, critical in practical product development. Similarly, NPs conjugated with a pH-responsive element144 or pH-sensitive surface charge switching146 were developed to increase biofilm penetration and selective bacterial binding for targeted delivery and antibacterial activity in acidic conditions.

Another exciting area of NP development is increasing specificity by selectively targeting matrix constituents or through the introduction of bacteria-specific ligands, to improve both efficacy and biocompatibility. NPs functionalized with matrix-digesting enzymes (DNase) and loaded with ciprofloxacin eradicated established *P. aeruginosa* biofilms without cytotoxicity against macrophages48. Likewise, NPs conjugated to dornase alfa (DNase) bound to alginate targeting *P. aeruginosa* EPS to deliver tobramycin, resulted in better penetration and DNA degradation in CF sputum and increased protection against bacteria in an invertebrate infection model147. Functionality can be extended to NPs designed to release and activate NO *in situ,* demonstrating effective antifungal and antibacterial effects, biofilm inhibition and EPS matrix degradation *in vitro* and *in vivo*148,149. Furthermore, recent developments in synthetic chemistry and biotechnology used antimicrobial peptides100 or aptamers150 linked to NPs surfaces to enhance their killing efficacy, specificity or functionality.

Overall, NPs offer a promising therapeutic platform for the development of new effective antibiofilm approaches. However, whilst the development of novel NPs has continued apace, there is a continually widening gap between the number of new formulations under laboratorial investigation and those in clinical use. Further advances in this field should focus on enhancing *in vivo* efficacy (vs. current modalities) and biocompatibility, and understanding the potential toxicity and the metabolism of NPs in the body. Affordable large scale manufacturing would be also required for product development to the healthcare market. Nevertheless, the availability of previously FDA-approved NPs augurs well for clinical translation.

**Future directions**

Biofilm initiation involves complex and dynamic surface-microbe-EPS interactions, promoting microbial attachment and development of a cohesive, functional multicellular 3D structure. The EPS matrix provides an essential scaffold for microbial organization and a heterogeneous microenvironment. Upon biofilm establishment, the adhesive strength and viscoelastic properties make biofilm removal from surfaces difficult, and resident microorganisms become tolerant to antimicrobials. While tolerance is a common feature of biofilms, the mechanisms underlying tolerance as a microbial survival strategy are multifaceted. Likewise a reciprocal multifaceted approach to biofilm control is far more likely to achieve clinical success than a futile search for a “magic bullet” (Box 2). Understanding the complexity of biofilm biology highlights the role of complementary strategies that target both the microbes and the surrounding matrix to either prevent biofilm initiation or disrupt existing biofilms. The challenge of using antimicrobials alone, which may kill microorganisms, but leave behind biodegradable substrates for microbial reutilization, must be addressed. Thus, eliminating existing biofilms may require simultaneous degradation of the protective EPS-matrix, and targeting and killing both resident microbes and dispersed cells. The complexity of polymicrobial interactions (synergistic, cooperative or antagonistic), spatial organization and community behaviour with host immunity factors further reinforces the need of a combinatorial therapy. Rapid advances in drug discovery methods should accelerate the identification of EPS-inhibitors, biofilm dispersal inducers and dormant cell-targeting agents as well as combining with host modulation therapies151,152. However, further validation of proof-of-concept work using clinically relevant animal models as well as clinical trials are needed for rigorous evaluation. Specifically using primary human cells in co-culture to evaluate host-microbial response has the increased translational potential of investigating human cells with specific genetic mutations such as in CF or PCD. Opportunities to create physico-chemical and biological structures, including organ-on-chip, within microfluidic devices or using 3D printing may also help assess treatment efficacy in conditions mimicking in vivo-like environment.

Furthermore, new technologies with anti-biofilm properties have been developed in recent years, including ‘smart-release’ or “on-demand activation” of bioactive agents when triggered by pathogenic microenvironments (e.g. acidic pH or hypoxia), for enhanced selectivity and controlled *in situ* drug delivery. However, the vast majority of the studies were conducted *in vitro* using non-clinically relevant models or treatment regimens, with many failing to progress to *in vivo* studies and even fewer to clinical application. Complex microbiota, where commensals co-exist with potential pathogens, provide a significant challenge in developing antimicrobial agents against a particular microbial species. The presence of biological fluids that change surface chemistries poses yet another challenge. Drug penetrability into existing biofilms should be also considered, since these factors affect both potential cytotoxicity and antibacterial efficacy and the potential for *de novo* generation of antimicrobial resistance where bacteria are subjected to sub-lethal concentrations. Antibodies, aptamers or peptides on nanoparticles all greatly enhance specificity, although higher costs and additional chemistry may be limiting factors. A key approach may be to trigger antimicrobial activity in response to pathogenic microenvironments (e.g. acidic pH, hypoxia or pathogen-derived metabolites). Thus, the biological effects can be tuned to specifically target the biofilm microenvironment, degrade the matrix and kill resident bacteria, thereby eradicating the pathogenic niche with precision and minimal cytotoxicity to surrounding tissues. Nevertheless, we noted a significant discrepancy between the research efforts on new technologies and commercialization. A concerted effort with chemists, engineers and biomedical researchers combined with toxicology and safety studies, will help clinicians to assess the efficacy of these new technologies in clinical trials. However, a successful translation is not just dependent on efficacy of the technology, but also regulatory agencies and industry efforts to bring it to the market. Future directions should focus on achieving maximal efficacy and specificity with minimal toxicity and long-term therapeutic effects along with industry partnerships to develop low-cost and practical formulations for clinical use.

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**Competing Interests Statement**

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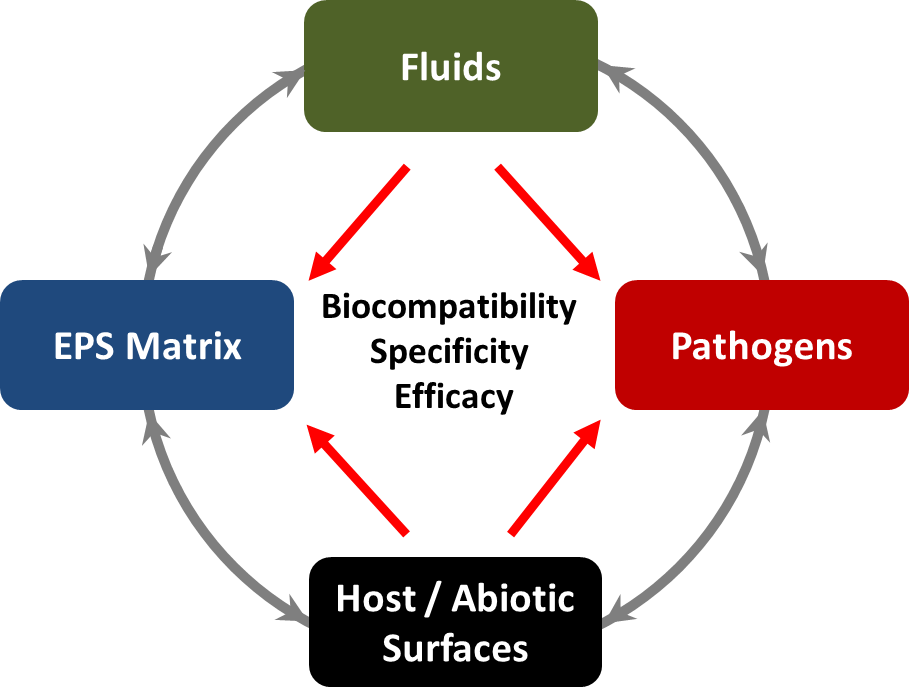
**LIST OF TEXT BOXES**

**Text Box 1.** Common features of microbial biofilms

|  |
| --- |
| *Composition and physical – chemical aspects* |
| **Adherence** to manufactured materials and biotic surfaces, using both specific and non-specific adhesion mechanisms. |
| **Extracellular polymeric substance (EPS) matrix.** Although the chemical and physical composition of EPS varies between species and growth conditions the EPS provides a scaffold for mechanical stability as well as creating compartmentalized chemical and physical microenvironments affording protection to the cells within a heterogeneous 3D structure. |
| **Architecture –** While there is some variation in the structure of *in vitro* grown biofilms there are a limited number of common forms (flat patches, mounds, mushrooms, towers, ripples, streamers) which are not generally species specific but largely dependent on biofilm maturity EPS production and growth conditions (nutrients, hydrodynamics). Biofilms seen in many clinical specimens tend to consist of aggregates of cells of varying sizes and mixed-species in polymicrobial systems 153. |
| **Viscoelasticity/Cohesiveness** facilitates remaining attached to surfaces when subject to mechanical forces5. |
| **Heterogeneity*.*** Heterogeneous and compartmentalized microenvironments can modulate microbial activity, intercellular signalling and metabolic exchange locally, orchestrating spatial localization of cells and communal behaviour for enhanced biofilm tolerance/persistence. |
| *Physiological / regulatory aspects* |
| **Developmental life cycle.** *Pseudomonas aeruginosa* as a model biofilm organism is widely adopted for biofilm formation by many different species, since the key elements, attachment, growth, maturation and dispersal are widely conserved among biofilm forming pathogens. However, there are fundamental differences between Gm+ and Gm- organisms such as the presence of a capsule, pili and flagella on surfaces that may affect surface attachment. |
| **Diffusible cell signals** co-ordinating population behaviour154 and metabolic activity |
| **Altered microenvironment formation** from the development of gradients in nutrients, pH and oxygen as a consequence of metabolic activity of the biofilm microbes and diffusion limited mass transport into and out of the biofilm EPS. |
| **Dormant** **or slow growing sub-populations** such as persisters and small colony variants (SCVs), which are tolerant to antibiotics and can be harboured in the stability of the biofilm microenvironment. |

**Text Box 2.** Lessons learned from cancer.

Over 100 years ago Paul Ehrlich, the German physician scientist used the term ‘magische kugel” to describe an ideal hypothetical therapeutic agent that specifically targets and kills disease-causing cells. In the context of cancer the target of such a magic bullet was the newly discovered receptors found on tumor cells. Another magic bullet was interferon, the media hyped cytokine discovered in 1957. However, a magic bullet for cancer therapy remains elusive in part because over the last 40 years of intense molecular research, it has been shown that “cancer is not simply a single disease that affects many parts of the body”. Rather, it is many different diseases with common themes that can cause different kinds of disorders in many of our organs”155. In addition individual tumors exhibit substantial chemical and clonal heterogeneity which combined with the ability of cancer cells to rapidly adapt, challenge both broad spectrum and targeted therapies. In tumors, the structure and composition of extracellular matrix is often altered, creating a favorable cellular niche for malignant transformation and cancer progression. Biofilms share similar common themes (Table 1) including creating their own microenvironments with unique chemical, physical, phylogenetic, genotypic and phenotypic heterogeneities. Early cancer therapy borrowed from approaches to treat acute bacterial and viral infections by targeting individual cells (with antibiotics and vaccination), with limited success. Our current understanding of biofilm biology is following a similar path to tumor biology. Rather than piled-up assemblages of clonal cells, evermore layers of complexity reveal dynamic self-constructed ecosystems within a matrix containing highly heterogeneous and compartmentalized milieu. Like cancer more effective biofilm therapies will likely need to target the complete microenvironment as well as the individual cells within 156.

**Multi-targeting Approach to Combat Biofilms.** The physical and biological complexity of biofilms and high tolerance to antimicrobials make them a formidable opponent to conventional therapeutic approaches. Biofilm targets include the microbial cells (often polymicrobial) and the EPS matrix and therapeutics can be delived from the surrounding fluid and the surfaces below.

We envision exogenous approaches (such as anti-adhesion materials and coatings, adhesin-blocking agents) to complement or synergize with endogenous activation (such as immunity modulation) to prevent microbial attachment to host or abiotic surfaces in diseased patients. Likewise, combination of approaches that degrade the protective matrix, activate dispersal, and target the resident pathogens, persisters and dispersed cells without affecting commensals may be required to eliminate existing biofilms. Long-term effects of anti-biofilm surfaces in the presence of biological fluids conditioning as well as enhanced drug penetration and targeting without toxicity or allergic reactions are required for *in vivo* efficacy. These combined with clinically relevant treatment regimen (either topical or systemic) and long-term effect assessment should help translate the many laboratorial concepts into real breakthough products with success in clinical trials.

**Text Box 3**. Persistence, resistance and tolerance.

Persistence, resistance, tolerance, persister. There is often confusion in the use of these terms when used to describe the inability of antibiotics (and antimicrobial agents) to inhibit or kill biofilm bacteria to the same extent as planktonic cultures 157. *Resistance* usually has an underlying heritable genetic basis which might be acquired through point mutation or horizontal gene transfer and is defined through standardized MIC and MBC assays. *Tolerance* is less well defined and is arguably more appropriately used when antibiotic susceptible strains (by MIC and MBC) require much higher concentrations to obtain similar log-reductions when growing in the biofilm phenotype. Importantly, tolerance can lost when biofilms are dispersed into single cells, thus dispersal strategies are normally considered as adjuvants an antimicrobial therapy. However, dispersed planktonic aggregates of cells may still retain tolerance. *Persistence* (and “Persistent biofilm”) is a term that is loosely used to describe a clinically protracted unabated biofilm infection despite treatment. However, persistence in this sense should not be confused with “persisters” 158 or sub-populations of cells with a distinct dormant phenotype affording them protection against antibiotics, which kill the metabolically active population. Persister cells can occur in both planktonic and biofilm cultures, but the stressful conditions, physical stability and protection from host phagocytes afforded by the biofilm microenvironment appear to contribute to harbouring microbial populations, which grow and repopulate once the antibiotic stress is removed. It is thought that these populations are tolerant of conventional antibiotics because there are no active cellular processes to interrupt. These subpopulations can form spontaneously or be induced from environmental stresses in the biofilm microenvironment159. We direct the reader to a review by Conlan et al. for further details of the role of persisters in biofilm infections158.

**LIST OF FIGURES**

**Figure 1.** **Opportunities for therapeutic intervention during various biofilm life-cycle.**

Blocking the mechanisms associated with initial microbial attachment and microbe-surface interactions, including targeting membrane-associated EPS. 2. Inhibition of early stages of biofilm development including EPS production and cellular division. 3. Disruption of formed biofilms, including physical removal, EPS-matrix degradation, targeting the creation of pathogenic microenvironments (low pH or hypoxia) and social interactions (in polymicrobial biofilms) as well as dormant cells. 4. Dispersing biofilms by inducing EPS matrix remodelling or activation to dispersal mechanisms.

**Figure 2. Targeting the biofilm EPS.** Disruption of EPS components, and the underlying mechanisms that are responsible for their production and secretion, represent attractive targets for the development of anti-biofilm strategies, some of which have potential efficacy across microbial species. Some of these approaches include (1) EPS degradation: Treatments have been developed that directly target the eDNA (DNAses), exopolysaccharide (dispersin B, glycoside hydrolases, monoclonal antibody vaccines), and protein (DNABII family antibodies) components of the matrix. (2) EPS adhesin-binding antibodies or inhibitors and phage-encoded peptidoglycan hydrolases have been developed to target bacterial adhesion and biofilm initiation. (3) Inhibitors of EPS synthesis and the secretion systems have also shown promise to disrupt biofilm accumulation. (4) Endogenous pathways that induce biofilm dispersal can also be targeted, including the regulation of c-di-GMP/c-di-AMP levels using exogenous NO and inhibitors, or quorum sensing using a variety of inducing peptides and messenger molecules. Importantly, all of these treatment strategies, alone or in combination, can lead to inhibition of biofilm formation, disrupt biofilm integrity and/or promote the release of individual bacterial cells that are more susceptible to conventional antibiotic treatment enhancing clinical efficacy

**Figure 3.** **Technological approaches to combat biofilms.** Recent advances in materials science and nanotechnology now provide hitherto unachievable capabilities to engineer a wide array of anti-biofilm interventional strategies. (1) The material and surface properties, such as surface charge, hydrophobicity, roughness, topography and chemistry among others, can be modified to prevent bacterial attachment and therefore attenuate or block biofilm formation. Additionally, “smart” or stimuli-triggered responsive surfaces can be constructed which elicit their anti-biofilm effect only upon the physical or chemical cues of the bacteria themselves. (2) Advancement in nanoparticle synthesis has produced a diverse array of approaches to combat biofilms should they establish themselves. Inorganic metallic and organic nanoparticles have been increasingly evaluated in order to improve, not just their anti-biofilm efficacy, but also their biocompatibility to reduce host toxicity concerns. The NPs can be used as coatings (nanocoatings), incorporated into materials (composites/fillings) or alongside conventional antimicrobials as well as other approaches designed to physically disrupt or remove the biofilm. Furthermore, new generation of AMPs and aptamers also display anti-biofilm-specific properties, which can be also used to enhance targeting specificity and efficacy of NPs (hybrid NPs). (3) New technologies for physical biofilm removal, including mechanical, energy and light-based disruption, may further improve biofilm intervention strategies. Given the multifaceted nature of biofilm formation and the complex microbial interactions with the surrounding physical and chemical environment, a combination of these approaches may be required to successfully combat biofilm-mediated disease.

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