Demolishing the Great Wall of Biofilms in Gram-negative Bacteria: To disrupt or disperse?

Running title: Biofilm dispersal and disruption

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Abstract:

Bacterial infections lead to high morbidity and mortality globally. While current therapies against bacteria often employ antibiotics, most bacterial pathogens can form biofilms and prevent effective treatment of infections. Biofilm cells can aggregate and encased themselves in a self-secreted protective exopolymeric matrix, to reduce the penetration by antibiotics. Biofilm formation is mediated by c-di-GMP signaling, the ubiquitous secondary messenger in bacteria. Synthesis of c-di-GMP by diguanylate cyclases (DGCs) leads to biofilm formation via the loss of motility, increased surface attachment, and production of biofilm matrix, whereas c-di-GMP degradation by phosphodiesterases (PDEs) causes biofilm dispersal to new sites via increased bacterial motility and matrix breakdown. The highly variable nature of biofilm development and antimicrobial tolerance imposes tremendous challenges in conventional antimicrobial therapies, indicating an imperative need to develop anti-biofilm drugs against biofilm infections. In this review, we focus on two main emergent approaches - active dispersal and disruption. While both approaches aim to demolish biofilms, we will discuss their fundamental differences and associated methods. Active dispersal of biofilms involves signaling the bacterial cells to leave the biofilm, where resident cells ditch their sessile lifestyle, gain motility and self-degrade their matrix. Biofilm disruption leads to direct matrix degradation that forcibly releases embedded biofilm cells. Without the protection of biofilm matrix, released bacterial cells are highly exposed to antimicrobials, leading to their eradication in biofilm infections. Understanding the advantages and disadvantages of both approaches will allow optimized utility with antimicrobials in clinical settings.

Keywords: Biofilms; dispersal; disruption; antimicrobial treatment; chronic infections

Main Text:

1. Introduction

1.1 Biofilm-mediated infections

Modern medicine has effectively eliminated acute infections caused by pathogenic bacteria and prevented the deaths of millions over the past century. Yet, such diseases are increasingly replaced by chronic infections caused by biofilms, which now accounts for 80% of all bacterial infections [1]. Biofilms are bacterial communities growing in their slimy exopolymeric matrix, after colonizing any biotic or abiotic surface. They colonize most surfaces in the human body and medical devices, causing persistent infections such as chronic wound infections, endocarditis, keratitis, and lung infections to implant-associated infections [2]. One biofilm-forming pathogen is *Pseudomonas aeruginosa*, an opportunistic Gram-negative pathogen that causes 90,000 deaths annually [3] by causing life-threatening infections in burn wounds and pneumonia [4,5].

1.2 Clinical approaches to develop anti-biofilm therapies

While most research into antimicrobials focuses on the treatment of acute infections caused by free-swimming planktonic bacteria, few commercial anti-biofilm drugs are available. Most anti-biofilm treatments currently employed by clinicians are mostly based on aggressive early surgical removal and delivery of sustained antimicrobial chemotherapy. Combinatorial antimicrobial treatment such as colistin or tobramycin inhalations with oral ciprofloxacin are frequently used to treat *P. aeruginosa* biofilm infections in cystic fibrosis lungs [6]. However, treatment at higher antibiotic doses for sustained treatment periods, such as 3 million units of colistin administered three times per day may pose health risks to patients [7]. Furthermore, antibiotic resistance via efflux pumps, acquired resistance plasmids, or phenazine metabolites in bacteria has significantly reduced treatment efficacy [8-10]. Hence, the discovery of novel anti-biofilm drugs with lower toxicities and suitability for combinatorial use with antibiotics can potentially eradicate biofilm infections, minimize treatment duration, and lower mortality rates.

Here, we summarize, assess, and compare anti-biofilm compounds that can disperse or disrupt biofilms. Firstly, we provide background on biofilm physiology and its role in chronic infections. Next, we analyze the anti-biofilm strategies adopted by biofilm dispersal and disruption agents. Finally, we discuss the future directions of anti-biofilm therapies based on both approaches.

2. Biofilm physiology and its roles in infections

Briefly, the life cycle of bacterial biofilms involves planktonic cells attaching to a surface and producing an exopolymeric matrix for biofilm growth and formation, with the mature biofilm eventually dispersing to new sites of infection (Figure 1). Unlike 'nomadic' free-swimming planktonic bacteria, biofilm cells survive as 'city-like' multicellular communities in self-produced exopolymeric matrix (EPS). The EPS comprises exopolysaccharides, adhesion proteins, and extracellular DNA (eDNA), primarily providing structural integrity to biofilms [11].

In chronic infections, biofilms can easily attach onto human tissues and extracellular matrix, where dead cells and blood supply contribute to nutrient availability for biofilm growth [12]. Furthermore, the EPS acts as a protective barrier to reduce antimicrobial penetration, whereas dormant persisters render biofilms extremely tolerant of antimicrobials [13,14]. These confounding factors may lead to inaccurate results in antibiotic susceptibility tests, which are typically performed on planktonic bacteria in clinical bacteriology, leading to inappropriate clinical doses and incomplete eradication of infections [15]. The exopolymeric matrix protects biofilm cells from phagocytosis and assists in immune evasion against the host immune system [16].

Understanding the mechanisms driving the biofilm life cycle can provide hints for the development of novel anti-biofilm approaches [17]. Currently, two primary bacterial mechanisms are extensively studied - quorum sensing (QS) and c-di-GMP secondary messenger system. QS allows planktonic cells to coordinate their behavior in forming biofilms via surface attachment, exopolymer production, and biosurfactant (rhamnolipids) synthesis [18,19]. QS operated in a cell density-dependent manner – bacterial populations will coordinate their gene expression once the autoinducers accumulated past a concentration threshold [20]. Coordinating bacterial populations in biofilm dispersal also requires QS [21], where *P. aeruginosa* biofilms secrete QS- controlled rhamnolipids as biosurfactants for detachment during dispersal [22].

The biofilm life cycle is mediated by c-di-GMP, an intracellular secondary messenger employed by many Gram-negative bacterial species [23]. Synthesis of c-di-GMP levels within bacterial cells by diguanylate cyclases (DGCs) often leads to biofilm formation via surface attachment, reduced motility, and production of the exopolymeric matrix. Degradation of c-di-GMP by phosphodiesterases (PDEs) to levels lower than that in planktonic cells will lead to biofilm dispersal and increased flagellar motility [23] (Figure 2). Clinical isolates from various bacterial

species, such as the rough and small colony variants (RSCVs) of *P. aeruginosa* and enteroaggregative *Escherichia coli*, possess constitutively high intracellular c-di-GMP levels [24,25].

While our review focuses on both QS and c-di-GMP signaling, there are other mechanisms mediating biofilm life cycle, such as the two-component system (TCS) [26] and iron siderophore (pyoverdine) signaling [27], which contributes to further complexity in biofilm physiology. One prominent example is the GacA/GacS TCS involved with pyoverdine synthesis and interaction with c-di-GMP signaling [28]. Hence, the difficulty of treating biofilm infections is attributed to complex and unique biofilm physiology, which warrants an urgent need to develop innovative anti-biofilm approaches and drugs against biofilm infections.

3. Development of anti-biofilm chemotherapy:

To effectively eradicate biofilms, many anti-biofilm approaches have been developed, mainly mechanical removal of biofilms, which includes electricity and sonication [29,30], and chemotherapy, which is the focus of this review. Novel drug candidates must be developed for effective anti-biofilm therapy of chronic infections in humans.

Current discovery of anti-biofilm compounds requires either high-throughput screening (HTS) or systems biology approaches. Being focused on a specific target, HTS is usually achieved via *in vitro* screening of compound libraries, such as the identification of biofilm-inhibiting patulin from thousands of natural extracts [31]. The *in-silico* structure-based virtual screening is also an alternative that employs molecular docking to identify which molecule from the compound libraries can bind and inhibit a biofilm effector. For instance, emodin identified from traditional Chinese medicine extracts could inhibit QS-mediated TraR in *E. coli* [32].

Systems biology approaches are employed in various ways to facilitate the discovery of compounds with novel or multiple mechanisms of action. Conventionally, we identify potential mechanisms of action by comparing the transcriptomic or proteomic signatures of wild-type and deletion mutants [33] or treated with an unknown chemical and antibiotics with known mechanism of action [34]. Undirected approaches with large compound libraries are gaining traction in anti-biofilm discovery. For instance, functional metagenomics from seawater samples are employed

to identify anti-biofilm compounds against *Vibrio cholerae* and *P. aeruginosa* [35,36], while transcriptomics and proteomics are used to study the anti-biofilm activity of 3-Furancarboxaldehyde On Group A Streptococcus [37].

Anti-biofilm chemotherapy can be achieved via a variety of approaches: inhibition of bacterial attachment to surfaces and biofilm formation, active dispersal of biofilms, and disruption of biofilm matrix (Figure 2). Since most biofilms are already established in chronic infections which rendered inhibitors of biofilm formation unsuitable, this review will focus on active dispersal and disruption of biofilms. Although both approaches are previously grouped as 'active dispersal,' we will differentiate them in this review for clarity.

Active dispersal and disruption seemingly have the same objective – to ensure the dissolution of EPS and release of biofilm cells, which presumably return to the planktonic phase. As released bacterial cells are wholly exposed to antimicrobials, combining either approach with antibiotics will eradicate biofilms and prevent freed pathogens from causing disseminated infections. However, both methods are fundamentally different (Figure 3). Active dispersal involves a chemical stimulus that signals bacterial cells to terminate their biofilm lifestyle and exit the EPS via the self-production of matrix-degrading enzymes and expression of motility apparatus (flagella or pili). Biofilm disruption is the direct targeting or degradation of biofilm matrix, leading to the catastrophic collapse of biofilm structure and ejection of embedded cells. It is crucial to understand their differences so that we can take advantage of their strengths and reduce their limitations for treating biofilm infections.

3.1 Inducing biofilm dispersal

While biofilm dispersal is a highly regulated natural process, we can manipulate biofilm cells into leaving their protective 'wall' of EPS by providing a specific stress stimulus and allowing antibiotics to eradicate freshly dispersed cells (Figure 4A). Active biofilm dispersal can be achieved via three approaches: QS targeting agents, DGC inhibitors, and PDE activators (Figure 4A).

3.1.1 QS-targeting agents

Exogenous QS signaling molecules or associated products are reported to induce biofilm dispersal. Although QS autoinducers can induce biofilm formation in native bacterial species, they can also act as chemorepellants. For instance, exogenous AI-2 autoinducer could induce

Helicobacter pylori biofilm dispersal [38], while AIP-1 could reduce *S. aureus* biofilms after 48 hrs [39]. Interestingly, N-(3-oxo-dodecanoyl) homoserine lactone from *P. aeruginosa* could cause biofilm dispersal in a foreign species, *Escherichia coli* [40]. Diffusible signal factor (DSF), a fatty-acid-based QS system found in *P. aeruginosa*, *Burkholderia,* and *Xanthomonas* species, was a cross-kingdom signaling molecule which caused biofilm dispersal in prokaryotic and yeast species when added exogenously [41,42].

QS-mediated products can also induce biofilm dispersal. QS-mediated rhamnolipids from *P. aeruginosa* acted as biosurfactants that disperse biofilms of other species [40,43]. Moreover, surfactin secreted from *Bacillus subtilis* could disperse biofilms from food-borne pathogens such as *Listeria monocytogenes* and *Salmonella enterica*, grown on polystyrene material [44]. While promising in dispersing biofilms, such biosurfactants are cytotoxic to host cells [45], which can limit their applications in clinical settings.

3.1.2 DGC inhibitors

Inhibition of c-di-GMP signaling is an attractive approach to target various pathogenic biofilms. DGC inhibitors identified from high-throughput screening could reduce c-di-GMP levels and decrease biofilm formation [46]. Terrein could inhibit both QS and DGC, effectively reducing virulence and biofilm formation in *P. aeruginosa,* respectively [47]. C-di-GMP analogs, such as triazole-linked analogs, could compete with c-di-GMP for active sites in DGCs, to disrupt c-di-GMP signaling [48]. However, it is essential to note that such inhibitors must penetrate biofilm matrix to reach bacterial cells. Thus poor permeability of these compounds may significantly limit their therapeutic purposes in established chronic infections. The permeability issue should be addressed by either using nanoparticles or utility in thin nascent biofilms.

3.1.3 PDE activators

PDE activators can induce biofilm dispersal via c-di-GMP degradation *in vitro* and *in vivo* [49,50]. Most notably, nitric oxide (NO) could induce dispersal in many bacterial species at sub-lethal concentrations [51], by binding NO-sensing H-NOX-domain protein [52] and activating PDEs (DipA and NbdA in *P. aeruginosa*) that degrade c-di-GMP [49,53]. Given the DGCs/PDEs redundancy in each bacterial species, it is essential to note that not all PDEs control biofilm dispersal. Instead, PDEs such as RocR controlled swarming and production of virulence factors [54]. Hence, it is crucial to ensure that activators must correctly activate PDEs with known dispersal roles.

Despite its low cost and ease of administration via inhalers, using gaseous NO is clinically challenging due to its poor stability, pleiotropic effects on the host body in systemic exposure such as vasodilation, and lack of specificity against bacterial species. Various NO donors, such as sodium nitroprusside [55], were identified with varying NO production and half-lives. To improve the specificity of NO to biofilms, cephalosporin-3'-diazeniumdoilates, made of stable NO donor diazeniumdiolate conjugated to cephalosporin, could deliver NO after being degraded by biofilm-produced beta-lactamases, to eradicate *Haemophilus influenzae* biofilms [56]. Nitroxides, such as 4-carboxy-2,2,6,6-tetramethylpiperidine 1-oxyl (carboxy-TEMPO), 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO) and 5,6-dicarboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl (DCTEIO), which were sterically hindered NO mimetic analogues, could disperse biofilms [57]. As such, nitroxides were utilized in combination with ciprofloxacin to eradicate biofilm effectively [58].

Despite its aforementioned limitations, NO had been tested in clinical trials against biofilm infections. Low-dose NO inhalation was tested in cystic fibrosis patients with pneumonia, with markedly reduced biofilm size and improved patient outcomes [59]. A phase II clinical study is currently in progress, where patients with cystic fibrosis will inhale 0.5% NO four times a day [60]. Hence, the prospect of using NO as a PDE activator remains promising in dispersing biofilms in infections.

3.2 Disruption of the biofilm matrix

Although EPS composition varies across time, location, nutrient availability, mechanical or shear stresses, and bacterial species, EPS functions are consistent – bacterial adherence, acting as a scaffold for biofilms and barrier against host effectors or antimicrobials. To nullify EPS functions, we will discuss three approaches that directly disrupt biofilm matrix: enzymatic and non-enzymatic degradation of EPS components; and antibody targeting of exopolymeric matrix (Figure 4B).

3.2.1 Enzymatic degradation of EPS components

Since the EPS contains mainly exopolysaccharides [61], matrix-degrading enzymes can degrade EPS to release biofilm cells. The earliest report of glycosyl hydrolase, which hydrolyzed poly-acetyl glucosamines in biofilms in various bacterial species, was Dispersin B, which was produced by *Aggregatibacter actinomycetemcomitans* [62]. *P. aeruginosa* self-produced glycoside hydrolases, such as PslG and PelA, were recently discovered to disrupt Psl and Pel

exopolysaccharides, thus improving antimicrobial treatment and immune clearance respectively [63,64]. Alginate, another exopolysaccharide expressed by CF-derived mucoid *P. aeruginosa* and coccoid *Helicobacter pylori*, was effectively disrupted by alginate lyase [65,66]. Further, α-amylase and cellulase could disrupt *Staphylococcus aureus* and *Pseudomonas aeruginosa* polymicrobial biofilms in wounds to some degree [67].

The DNase can also disrupt biofilms by degrading the eDNA integrated with other EPS components for structural support [68]. Recombinant human DNase I (dornase alfa) was shown in clinical trials to degrade microbial eDNA in the sputum of patients with cystic fibrosis [69] and ventilator-associated infection in preterm infants [70].

Lastly, the degradation of adhesion and extracellular proteins by proteases can disrupt biofilms efficiently. A plant protease, ficin, was recently discovered with the ability to disrupt *Staphylococcus aureus* biofilms and release biofilm cells [71]. Serine proteases from other bacterial species, such as *Bacillus pumilus*, were effective against *Serratia marcescens* biofilms [72]. As proteases are commonly used as therapeutics in treating cardiovascular diseases, it is highly probable that future clinical trials featuring anti-biofilm proteases will be conducted.

3.2.2 Non-enzymatic degradation of EPS components

A few compounds that can cause EPS degradation had been identified. The FDA-approved N-acetyl cysteine (NAC), whose mucolytic ability to dissociate disulfide bonds in extracellular mucin proteins and reduce their viscosity, was subsequently expounded into a potential anti-biofilm agent. NAC was employed in modern applications, such as combinatorial treatment with cysteamine, an antioxidant against mixed biofilms of *Streptococcus pneumoniae* and *Haemophilus influenzae* [73]. Another garlic-derived compound, Rafflinose, could inhibit biofilms by binding to lectin-A, which is an exopolysaccharide-binding protein in *P. aeruginosa* [74].

An alternate non-enzymatic approach involves the use of biological hydrogels such as mucins to trigger biofilm dispersal via the activation of flagella, promoting of native microbiota colonization, and prevention of pathogen attachment [75]. One biopolymer currently tested in clinical trials is SP1SN13, a poly-N-(acetyl, arginyl) glucosamine (PAAG) glycopolymer with anti-biofilm effects on *P .aeruginosa* in cystic fibrosis patients [76]. After a successful Phase I determining safety and tolerability in patients, SP1SN13 had received orphan designation by FDA and will undergo Phase II clinical trial in late 2019.

3.2.3 Specific targeting of the exopolymeric matrix by antibodies

Another anti-biofilm approach is to employ antibodies specific against EPS components. For instance, monoclonal antibodies specific against epitopes on *P. aeruginosa*-produced exopolysaccharide Psl were developed to target biofilms formed by clinical isolates [77]. Since secreted Psl is easily accessible by antibodies, the anti-Psl antibody was effective at promoting opsonized phagocytic killing of *P. aeruginosa* biofilms and inhibiting the pathogen from adhering to the cell surfaces. The bispecific antibody (MEDI3902) specific against both Type III Secretion System and Psl was subsequently developed with a broad coverage against *P. aeruginosa* [78]. A Phase I clinical trial with MDI3902 was reported recently with acceptable safety and tolerability in healthy volunteers [79], paving the way for Phase II clinical trial.

Antibodies are also developed to target adhesion proteins. The integration host factor (IHF) specifically targeted eDNA-binding proteins (DNABII) in biofilms in *Escherichia coli* biofilms [80] and *Porphyromonas gingivalis* biofilms [81]. It is currently co-administered with recombinant type IV pili, leading to the disruption of biofilms formed by *Haemophilus influenza* in otitis media [82].

4. Comparison between biofilm dispersal and disruption in anti-biofilm treatments:

Since both approaches are different from each other, it is critical to consider their strengths and limitations to realize their full potential in eradicating biofilm infections (Table 1).

4.1 Strengths and limitations in active dispersal

Many biofilm dispersal agents, such as NO, are quick-acting chemicals. NO can be applied on a wide range of microbial species, ranging from Gram-positive and Gram-negative bacteria to yeast, thus it is useful in treating polymicrobial biofilm infections [51]. The use of low-dose and non-bactericidal concentrations of NO or NO-deriving compounds can also induce biofilm dispersal. Using pico-nanomolar NO concentrations for biofilm dispersal can prevent undesired effects of high NO concentrations on the human body, such as vasodilation in blood vessels, damage to host cells, inhibition of lymphocyte proliferation and delays in healing. Moreover, elevated NO concentrations have a counterintuitive effect of protecting bacteria from antibiotics, leading to poor antimicrobial effects [83].

Aside from chemical-specific side effects, there are other factors to consider when using biofilm dispersal agents. Biofilm-dispersed cells possessed lower c-di-GMP levels than in planktonic and biofilm cells of multiple species [49], which gave rise to a unique transient dispersal phenotype with heightened expression of virulence factors [49,84,85]. Worse, biofilm-dispersed cells possess different antibiotic resistance via different stimuli [86], such as increased resistance to colistin in *P. aeruginosa* [87]. The increased resilience against immune and antimicrobial stresses is probably because dispersed cells have to 'arm' themselves in advance for protection against predators or chemical stimuli as they leave the biofilm's shelter [49]. Hence, caution may be required when biofilm-dispersed cells are not entirely eradicated by antimicrobials, potentially leading to complications where residual bacteria enter the bloodstream.

Lastly, the penetrability of drugs into biofilms remains a challenge to achieve in developing dispersal agents. While NO has high penetrability into biofilms [51], other dispersal agents may face difficulty in penetrating the biofilm matrix and reaching deeply embedded cells even if they fulfill the Lipinski's Rule of Five. However, the high lability of NO can shorten its duration of effective dose, so stable NO donors or sustained NO treatment are required to treat biofilm effectively. Hence, we must consider these factors in the future design of biofilm dispersal agents.

4.2 Strengths and limitations of biofilm disruption

There are several advantages of employing biofilm disruption of anti-biofilm strategy. Firstly, biofilm disruption promotes the catastrophic collapse of the biofilm structure rapidly at low concentrations, which is attributable to the highly efficient enzymatic activity of matrix-degrading enzymes. For instance, nanomolar levels of PslG (IC_{50} = 10 nM) could disrupt Psl fibers within 4 minutes and disassemble *P. aeruginosa* biofilms in 30 minutes [63].

While active dispersal can induce physiological changes to biofilm and dispersed cells, it is unclear if biofilm disruption has any immediate physiological effects on bacterial cells upon sudden release from their bounds. However, studies had shown that *P. aeruginosa* cells released from biofilms by PslG or EndA have similar antibiotic resistance to planktonic cells or biofilms cells from which they originate [63,88], implying that bacterial cells did not have time to adjust their physiologies significantly after sudden release from biofilms. These treatments are advantageous as biofilm-disrupted cells remain susceptible to antimicrobial treatment, so standard dosages of antimicrobials may be enough to eradicate both biofilm and disrupted cells.

Next, biofilm disruption can circumvent the need for penetration by biofilm disruptors into biofilms. Biofilm disruptors can demolish the biofilms from the outside at accelerated pace, without having to pass through the biofilm matrix and microbial cell walls. In contrast, depending on the chemical penetrability into biofilms, dispersal agents may require hours to days for active dispersal to occur [49,51].

The specificity of biofilm-disruptors is also an essential factor to be considered for anti-biofilm treatment. The matrix-degrading enzymes are in general specific to the species, but cross-reactivity is possible if the chemical composition of substrates is similar. For instance, PslG is specific to a few close-related species, such as *P. aeruginosa* and plant pathogen *Pseudomonas syringae*, but has no effect on *E. coli*, *S. aureus*, *S. enterica* and *Candida albicans* [63]. Yet, DNase applies to a broader multitude of bacterial species, as it does not distinguish eDNA produced by different bacterial species.

However, biofilm disruptors are not without limitations. Like most enzymes, matrix-degrading enzymes suffer from poor stability, susceptibility to host degradation, sensitivity to changes in temperature, and pH. Furthermore, complex pharmacodynamics and pharmacokinetics in hosts can compromise the efficacy of biofilm disrupting enzymes *in vivo*. Despite showing the viability and safety of using disrupting agents in animal studies [67], they can alert the host immune system if they originate from non-human species [89]. Lastly, previous concerns over expenses, low production yield and lack of genetically optimized enzymes contribute to the difficulty in applying this method clinically [90].

5. Future Outlook

Current treatment with antibiotic cocktails is inadequate for eradicating biofilm infections. It is increasingly evident that biofilm elimination requires the simultaneous degradation of the biofilm matrix and the killing of released bacterial cells. The development of combinatorial therapy of anti-biofilm agents with antibiotics or an anti-biofilm agent-antibiotic conjugate drug will realistically attain an achievable treatment outcome in clinical settings.

With rapid advances in compound discovery, it is imperative to identify novel anti-biofilm compounds that can disrupt or disperse biofilms. Natural compounds from plants are excellent sources of potential anti-biofilm compounds, where many QSIs are previously identified [91], while the recently-discovered pyocins and norspermidine displayed promising potential against biofilms [92,93].

Understanding both active dispersal and disruption will assist in designing better anti-biofilm drugs suited for various clinical purposes. Most importantly, cells released from biofilms by either approach should be contained to prevent the immediate dissemination of pathogens into the circulatory system. Next, the shortcomings of each approach listed previously should be addressed to improve the chances of developing a suitable drug. For instance, it is crucial to re-evaluate the minimal inhibitory concentration (MIC) of accompanying antimicrobial coupled with biofilm dispersal agents, which requires further toxicology and safety studies by pharmacologists and biologists. As for enzymatic-based biofilm disruption, industrial cooperation is necessary to keep manufacturing costs down and develop a practical non-immunogenic formulation.

Finally, as most studies were conducted either *in vitro* or animal infection models, the newly identified compounds must be evaluated extensively with clinical trials. Future directions should focus on the discovery of novel anti-biofilm agents, with high efficacy against biofilm infections and low toxicity, coupled with the ease of translating to cheap and practical pharmaceuticals.

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Table 1: General comparison between active biofilm dispersal and biofilm disruption.

	Biofilm Active Dispersal	Biofilm Disruption
Treatment	Use of chemical stimulus which signals bacterial cells to terminate their biofilm lifestyle and exit the EPS via the self-production of matrix-degrading enzymes and expression of motility apparatus (flagella or pili).	Direct targeting or degradation of the biofilm matrix, leading to the catastrophic collapse of biofilm structure and ejection of embedded cells
Penetrability into biofilms	Dispersal agents need to diffuse into EPS and enter biofilm cells.	Biofilm disruptors can demolish biofilms from the outside quickly, bypassing the need to pass through biofilm matrix and microbial cell walls.
Types of agents	Mostly chemicals which act as stress to stimulate biofilm dispersal	Mostly enzymes which catalyzed the degradation of EPS
Physiology of released cells	Dispersed cells have unique physiology from biofilm and planktonic cells [49]	Disrupted cells have similar physiology with biofilm cells [63]
Antimicrobial resistance of released cells	Dispersed cells may have altered resistance/sensitivity to antibiotic classes [87]	Disrupted cells have similar resistance/similarity to antibiotic classes as biofilms [63]
Virulence of released cells	Dispersed cells have higher production of virulence factors than biofilm and planktonic cells [49]	Disrupted cells have similar virulence as biofilm cells [63]

Figure legends:

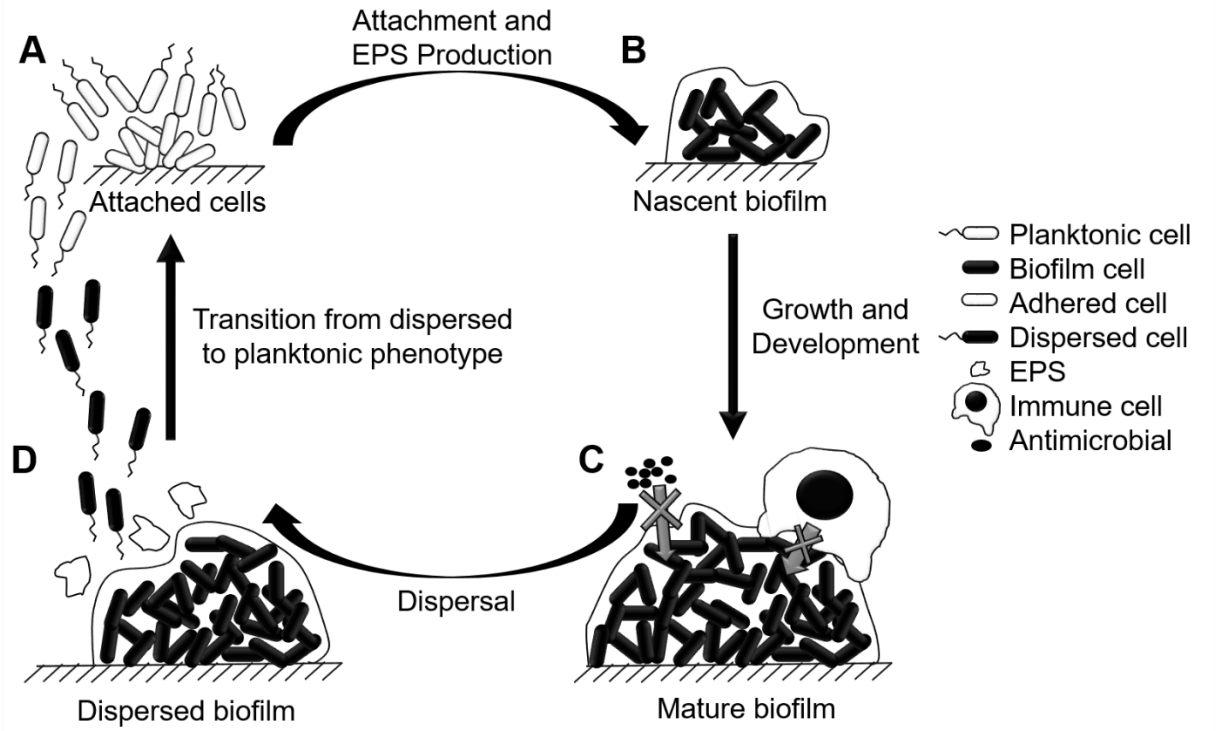
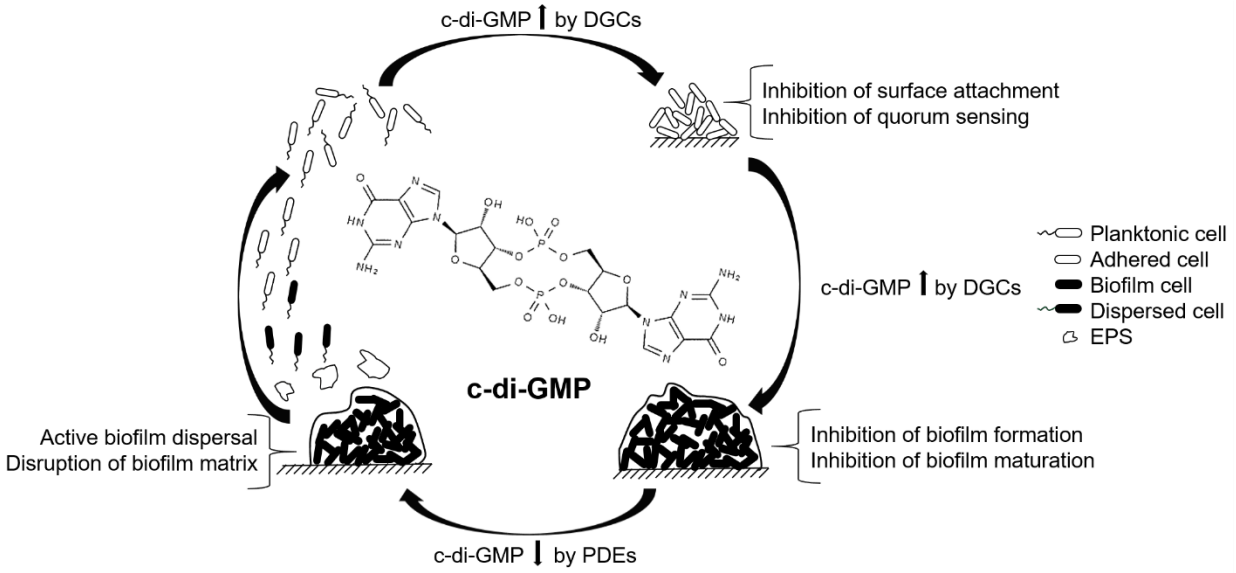


Figure 1: Typical biofilm life cycle in bacteria. (A) The planktonic cells first attach onto a surface. (B) Bacterial cells lose their motility and start to produce EPS for biofilm formation. (C) Biofilm matures and thickens where antibiotics and immune cells are unable to penetrate efficiently into the biofilm. (D) As biofilm ages, the cells disperse from the biofilms and enter a different transient dispersal phase. After a while, the dispersed cells reverted to planktonic cells, where the life cycle restarts.



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693 **Figure 2:** Role of c-di-GMP signaling in the biofilm life cycle and the potential targets against it.
694 An increase in c-di-GMP levels by DGCs, in general, leads to biofilm formation, while reduction
695 by PDEs induces biofilm dispersal. Strategies developed against biofilms include inhibition of
696 bacterial attachment to surfaces and biofilm formation, active dispersal of biofilms, and
697 disruption of the biofilm matrix.

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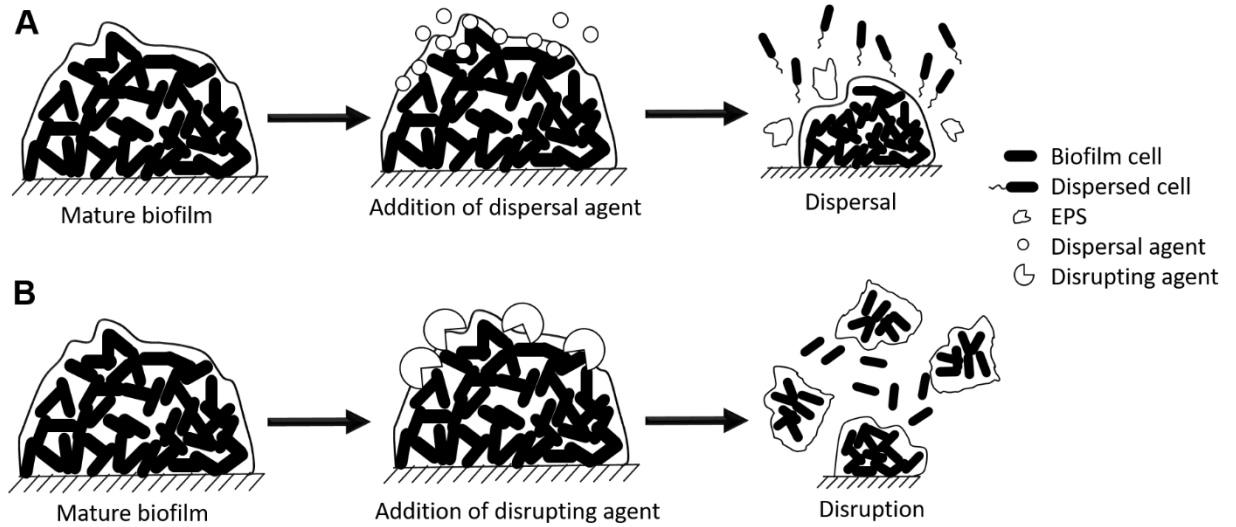

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700 **Figure 3:** Comparison of biofilm dispersal and disruption. (A) Biofilm dispersal. The dispersal
701 agent acts as a stimulus to drive biofilm cells into dispersal, leading to considerable changes in
702 physiology to dispersed cell state. (B) Biofilm disruption. The biofilm disrupting agent directly
703 degrades the biofilm matrix to eject biofilm cells with little changes to their physiological state.

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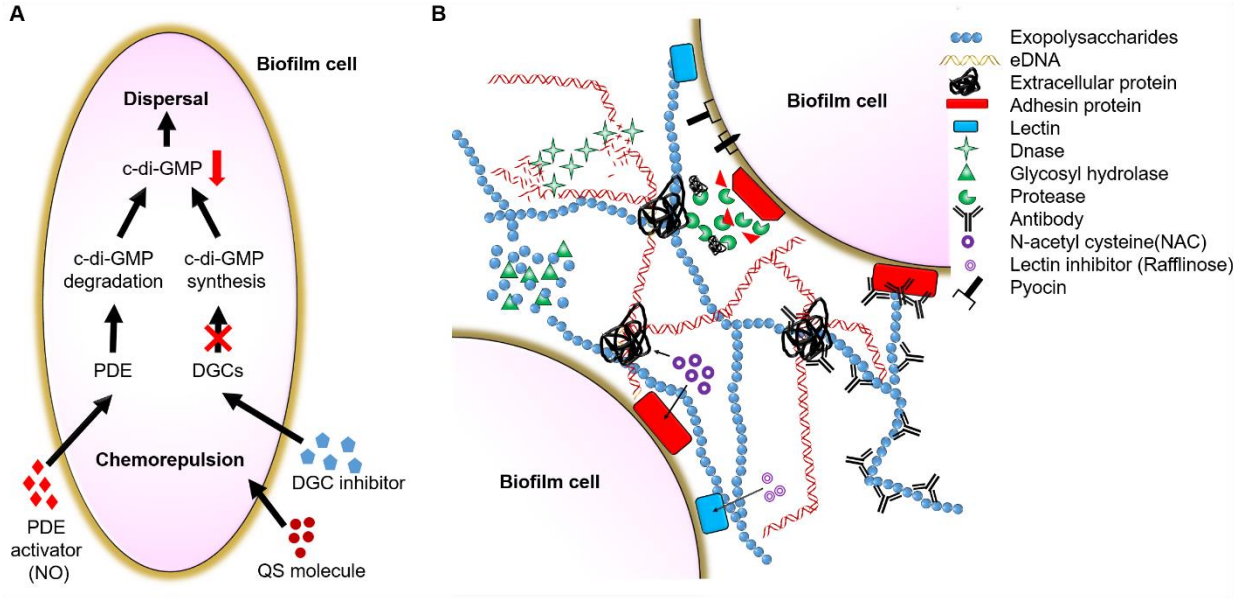

Figure 4: Development of various anti-biofilm strategies. (A) Biofilm dispersal strategies include using QS molecules as chemorepellants, DGC inhibitors (c-di-GMP analogs), and PDE activators (such as NO). (B) Biofilm disruption strategies include enzymatic degradation of EPS by glycosyl hydrolase, protease and DNase; non-enzymatic degradation of EPS components by N-acetyl cysteine; and antibody targeting of EPS.