Drug delivery strategies for antibiofilm therapy

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Abstract

Although new antibiofilm agents have been developed to prevent and eliminate pathogenic biofilms, their widespread clinical use is hindered by poor biocompatibility and bioavailability, unspecific interactions and insufficient local concentrations. The development of innovative drug-delivery strategies can facilitate penetration of antimicrobials through biofilms, promote drug dispersal and synergistic bactericidal effects, and provide novel paradigms for clinical application. In this Review, we discuss the potential benefits of such emerging techniques for improving the clinical efficacy of antibiofilm agents, as well as highlighting the existing limitations and future prospects for these therapies in the clinic.

[H1] Introduction

Antimicrobial resistance (AMR) is associated with ~4.95 million deaths globally,[1] and a global economic burden of over $300 billion.[2,3] Although global strategy has focussed primarily on the discovery of new antibiotic agents to circumvent drug resistance **[G]** , there have been increasingly diminishing returns due to perceived poor profitability, with no new class of antibiotic having received regulatory approval since the late 1980s.[4,5] Fundamental scientific and translational challenges such as poor penetration, efflux and rapid development of resistance have compounded the inadequacy of antimicrobial pipelines.

Approximately 80% of bacteria in chronic and nosocomial clinical infections are recognized to live within mono- or multispecies microbial communities known as biofilms **[G]** (biofilm infections) [6] Biofilms can broadly be defined as dynamic self-constructed accumulations of microorganisms that produce a matrix of extracellular biopolymers (that is; extracellular polysaccharides (EPS)). The collective behavior of bacteria within biofilms promotes communication and interaction to ensure propagation and survival. As biofilm-dwelling bacteria show markedly different behaviour from the planktonic (free-floating) bacteria that are typically used in the testing of traditional antimicrobial agents, many antimicrobials show minimal efficacy against biofilms at conventional dosages. Specifically, traditional antimicrobial therapy is often ineffective against chronic and localized infections, with biofilm-related infections conferring up to 1000x more resistance than infections caused by planktonic organisms.[7] Of particular importance clinically is the growth of biofilms on surfaces such as indwelling medical devices and mucosal tissues, and also free-floating biofilm-like aggregates[8] **(Figure 1)**.

Treatment of chronic infections has focused on early and aggressive high-dose and/or long-term antimicrobial chemotherapy, despite limited clinical evidence for biofilm eradication.[9] Therefore, there is an urgent need for innovative antibiofilm therapy strategies to address this critical challenge and to improve clinical outcomes.

Although there has been substantial growth in antibiofilm therapy research in recent years, developments in multi-omic and imaging technologies have merely scratched the surface of the remarkable complexity and spatial organization of polymicrobial biofilm infections.[10] Indeed, although preclinical studies of antibiofilm agents have shown statistically significant biomass reductions and changes in biofilm structure across common bacterial isolates, few studies have proved longitudinal biocidal effects *in vivo,* and no systemic therapy has progressed beyond Phase I clinical trials. An important factor is the lack of biocompatible antimicrobial drug delivery vehicles with drug combinations capable of inducing both biofilm dispersion and overall bactericidal effects. Many drugs either fail to accumulate efficiently beyond the biofilm matrix (for example, aminoglycosides or penicillins) or exhibit poor retention inside it (for example, fluoroquinolones or macrolides).[11,12] Hence, drug concentration within a biofilm is often sub-therapeutic, which results in a drastic reduction in effectiveness and simultaneous promotion of AMR. The demand for a robust, selective and efficacious therapy cannot be addressed in the face of such a basic delivery constraint. Motivated by this limitation, we aim to provide a critical overview of the drug delivery strategies that have been explored in antibiofilm therapy to improve clinical care. We discuss the potential benefits of such techniques in improving the efficacy of antibiofilm agents, as well as their existing limitations and prospects.

[H1] Antibiofilm agents

Treatment outcomes for biofilm-associated infections are highly variable due to increased levels of innate antimicrobial tolerance **[G]** [13] and resistance within these communities **(Figure 2).** Tolerance is defined here as the ability to survive, but not grow, in the presence of otherwise bactericidal antimicrobial agents through, for example, a reduction in growth rate or a subpopulation of non-metabolic persister **[G]** cells. By contrast, antimicrobial resistance describes acquired or intrinsic genetic mutations that permit growth of microorganisms in the presence of otherwise bactericidal or bacteriostatic antimicrobial agents (minimum inhibitory concentration (MIC) above breakpoint) through mechanisms such as efflux pumps, enzymatic drug inactivation, or modifications in drug targets.) In biofilms specifically, resistance is known to develop and propagate due to spontaneous mutations or horizontal gene transfer. Pathogenic bacteria in biofilms use both tolerance and resistance mechanisms to withstand antimicrobial challenges, although biofilm-facilitated tolerance does dematerialize when the biofilm is dispersed[14] Therefore, traditional antimicrobial chemotherapies cannot completely eliminate cells within a biofilm, which results in further development of resistant phenotypes and recurrence of persistent clinical infections. Novel antimicrobials and delivery systems tailored to biofilm infections have thus been investigated.

[H2] Dispersants.

A primary research focus for eradicating clinical biofilm infections has been the dispersal and sloughing of biofilms to remove cells from the protective EPS matrix. This approach assumes that dispersed bacteria return to a planktonic state, losing the protection conferred by the structured biofilm community, and rendering them susceptible to conventional antibiotics and host innate immunity **(Figure 3)**. As the

biofilm life cycle, bacterial survival and biofilm dispersal are interdependent, it is postulated that dispersants are less vulnerable to intrinsic resistance mechanisms.[15,16] However, natural biofilm dispersal is a complex, highly differentiated process involving a range of enzymes, environmental cues, effectors and signal transduction pathways **(Table 1).** Treatment is therefore difficult owing to the sheer diversity of biofilm modulation systems, with no single mechanism adapted by all microorganisms.[17] Moreover, the administration of many dispersants is greatly hampered by their poor solubility and rapid host immune clearance. Thus, promoting biofilm dispersion endogenously presents a substantial hurdle in drug delivery for which novel biomaterials are needed.

[H3] Matrix-degrading enzymes.

The production of matrix-degrading enzymes (MDEs) to degrade cohesive components can facilitate the transition of sessile biofilm organisms to free-floating bacteria. The nuclease-mediated degradation of extracellular DNA (eDNA) by deoxyribonucleases (DNases) demonstrated the potential of MDEs to eliminate a crucial structural component of the biofilm matrix.[17,18] Although natural DNases have received substantial attention due to their prevalence in the endogenous biofilm dispersal process, recent mechanistic work suggested that in mature bacterial biofilms, eDNA exists in a nuclease-recalcitrant Z-configuration.[19,20,21] Co-administration of MDEs with B-DNA intercalators, such as chloroquine or ethidium bromide, to drive biofilm eDNA to its native B-form G-quadraplex structure does hold promise, but DNases suffer from high environmental sensitivity, low biofilm penetration and sequence specificity.[22,23] Attempts to stabilise MDEs like glycoside hydrolases[24] through lipid-based liquid crystal nanoparticles have shown a 10-fold enhancement of the antimicrobial effect when co-delivered with tobramycin due to the targeting and degradation of the Psl polysaccharide in non-mucoid *Pseudomonas aeruginosa* biofilms.[25,26] Similar nanoformulations functionalized with different MDEs have also been developed to enhance antibiofilm effects *in vitro* and *in vivo* when co-loaded with conventional antimicrobial agents.[27-29]

DNase-functionalized nanoparticles present an exciting opportunity to enhance drug penetration. For example, DNase treatment (Pulmozyme) is already clinically used in patients with cystic fibrosis to reduce mucus viscosity in the lungs. Indeed, functionalization with DNase I improved alginate—chitosan nanoparticle delivery by almost 15% across clinical cystic fibrosis sputum samples despite its comparatively larger size (457 ± 12 nm vs 100-200 nm in similar nanoformulations).[30] Other studies examining DNase I functionalized antimicrobial-loaded nanoparticles also demonstrated encouraging outcomes, with one showing eradication of more than 99.8% of a 48 h *P. aeruginosa* biofilm *in vitro* (which is considered mature) compared to 70% eradication with free drug.[31] Similarly promising results have been reported with other enzyme-immobilization techniques, including chewing gum-based delivery,[32] magnetoreceptors[33] and covalent coating of medical devices.[34,35] However, it is important to recognize that no single enzyme or enzyme combination can completely degrade all polymers in a biofilm matrix, and no structural component exists in identical quantities across even closely related biofilm species.[36] Polymers themselves can also develop protection against enzymatic activity as the biofilm matures through polymer-vesicle interactions.[37] Further investigation is therefore needed to assess the synergistic efficacy of these MDE combination therapies to better understand their clinical potential.

[H3] Quorum-sensing inhibitors.

Many bacterial species communicate using secreted chemical signalling molecules (that is; autoinducers) to coordinate and execute colony behaviour upon reaching a critical population density (that is; quorate).[38,39] By selectively interfering with these processes, quorum-sensing inhibitors (QSIs) have been proposed as an antibiofilm strategy to hinder the initial adhesion and subsequent formation of biofilm communities. As these compounds do not exert a selective pressure on bacterial growth, it has been suggested that they should not become susceptible to AMR mechanisms.[40] Despite this, natural and synthetic QSIs have yet to show clinical efficacy as a monotherapy.[41] Although this may be attributable to a number of factors, including the polydiversity of quorum sensing systems and the inability of QSIs to efficiently permeate the biofilm matrix, loading of tobramycin and lipophilic QSIs on squalenyl hydrogen sulfate nanoparticles yielded 3-fold higher permeation and complete eradication of *P. aeruginosa* biofilms at circa 8-fold lower tobramycin concentration than free drug and QSIs alone.[42] Similarly promising results were observed in an *ex vivo* 3D skin infection model using ciprofloxacin in combination with a QSI encapsulated within alginate nanoparticles, demonstrating complete clearance of 24 h (that is; not fully mature) *P. aeruginosa* biofilm infections.[43]

Unfortunately, the diversity of quorum sensing systems regulating biofilm growth and dispersal makes it highly unlikely that molecules regulating specific signalling pathways could be used as broad-spectrum biofilm dispersants. Furthermore, preliminary evidence suggests that human microbiota homeostasis can be disrupted[44] by therapies known to target signalling factors in multiple species. The effects of QSI on signalling factors in eukaryotic mammalian cells must also be carefully considered. Numerous *in vivo* and *in vitro* studies have identified an association between *N*-acyl-homoserine lactones (AHLs) and the induction of pro-inflammatory and pro-apoptotic responses, including a direct disruption of regeneration processes.[45-47] Nevertheless, whilst these issues represent important obstacles to the further development of QSI, they do not diminish the importance and potential that this novel strategy offers towards combatting narrow-spectrum clinical biofilm infections, particularly when coupled with a growing understanding of bacterial cell–cell signalling networks and creative drug-delivery strategies.

[H3] Reactive oxygen species and nitric oxide.

Reactive oxygen species **[G]** (ROS) and reactive nitrosyl species **[G]** (RNS) have garnered interest as highly reactive molecules capable of damaging DNA, reducing biofilm biomass and inducing biofilm disruption.[48-51] Indeed, oxidative and nitrosative stress on biofilms has been explored extensively both endogenously[52] and exogenously[53] to alter biofilm formation. However, the short-lived nature and poor metabolic half-lives of these highly toxic species mandates innovative delivery and therapeutic strategies to facilitate their use clinically.

To overcome this challenge, a range of molecules and particles has been developed capable of releasing reactive species under specific biological conditions. For example, at the acidic pH levels common for pathogenic bacteria, iron oxide nanoparticles have demonstrated high peroxidase-like activity, locally catalysing H_2O_2 to produce free radicals for simultaneous bacterial killing and EPS structure breakdown A study has reported the complete inhibition of biofilm accumulation on a human-derived *ex vivo* tooth and an *in vivo* rodent *Streptococcus mutans* biofilm model using a clinically approved iron oxide nanoparticle formulation (Ferumoxytol) with H_2O_2. Within 5 minutes of topical application, they observed a largely amorphous and scattered EPS with >99.9% biocidal activity on treated *S. mutans* biofilms *in vitro* with no discernible effects on oral microbiota composition or damage to surrounding tissue *in vivo*[54] In a follow-up randomized clinical crossover study, *S. mutans* was completely eradicated from multispecies intraoral biofilms treated with the H_2O_2/iron oxide nanoformulation with no adverse

signs in the oral cavity.[55] Pre-clinical studies using nitric oxide (NO) donors showed similar encouraging results, suggesting that local release of NO may trigger biofilm dispersal. Although conventional NO donors lack the stability and specificity needed to achieve the necessary localized delivery, recent work conjugating NO donors to various polymeric and nano-based systems has shown promise in enhancing NO donor stability and increasing local concentration of pharmaceutically active NO.[56-58]

[H2] Bacteriophages.

Bacteriophages (phages) represent an alternative to conventional antibiotics, able to treat multi-drug resistant strains and self-replicate within the infection site to maintain bactericidal concentrations. Although phages have been used to treat resistant infections since the 1920s in many countries of the former Soviet Union,[59] multiple challenges hinder their wide-spread adoption globally, including poor penetration of biofilms, narrow species-specific selectivity, high rates of anti-phage resistance, host immune stimulation, poor stability, complex regulatory requirements and insufficient large-scale purification procedures.[60-62] Detailed reviews have been published on these challenges[63,64] and therefore this section will focus on the optimization of phage delivery for clinical application.

[H3] Liposome-encapsulated phages.

Various strategies have been explored for encapsulating phages to enable deeper penetration at infection sites. In healthy mice, phage titers persisted 120 hours post-intraperitoneal administration of a liposome-encapsulated phage cocktail, versus 36 hours for free phage.[65] Although the biodistribution study failed to account for phage replication in the infected state, liposomal phages produced a one-log reduction in bacterial burden in *Klebsiella pneumoniae*-infected mice and a substantial decrease in host inflammatory markers compared with free phage. Similar outcomes have been observed after oral administration of liposome-encapsulated phages,[66,67] although these studies also raised instability concerns in acidic gastric fluids, where the titer of encapsulated phages was reported to fall by 4-5 log units. Moreover, when encapsulating both *Escherichia coli* T3 and *Staphylococcus aureus* K phages, aggregation and interaction with the liposomal bilayer, respectively, were observed.[118] The substantially larger (~300 nm) size and lower encapsulation efficiency (~50%) of liposomal phages compared with conventional liposomal formulations may also limit their utility.[68] To improve this, attempts to encapsulate the mycobacteriophage TM4 into giant unilamellar vesicles (GUVs; 1-100 μm) demonstrated an over 4-fold better cellular uptake but with poor control over particle size.[69] Microfluidic methods have refined this approach[70], but success seems to be phage-dependent, and is constrained by its low throughput and poor scalability.

[H3] Alternative encapsulation strategies.

Alternative encapsulation techniques such as niosomes,[71] transfersomes[72] and hydrogels[73] have also been explored as strategies to enhance phage stability and delivery. Hydrogels have found widespread application as commercial wound dressings by exploiting their tunable controlled release properties. It has been proposed that hydrogels encapsulating phages using either chemical or physical crosslinking could be used to both treat and prevent biofilm-related infections. Researchers have demonstrated the successful encapsulation of *E. coli* HZJ phages embedded within alginate hydrogel fibers,[74] and in vitro, ~70% *E. coli* cell death and successful prevention of biofilm formation was observed using similar encapsulation methods.[75] However, it is important to note that only 10% of phages were released from the hydrogel after 24 hours, which may explain the sub-optimal bactericidal efficacy. Loading of LM99

phages in alginate hydrogels resulted in superior antimicrobial responses, with 97% of phages released over 24 hours, killing over 99% of multi-drug resistant (MDR) *Enterococcus faecalis in vitro* and *ex vivo*.[76] Microencapsulation of phages for oral delivery with other polymers such as Eudragit[77] has also been explored[78] with success *in vitro* and *ex vivo* but *in vivo* data have so far been less promising, with phage–hydrogels showing only a one-log reduction in bacterial load compared with hydrogels alone.[79] Although this inefficiency may be due to the low titer of phages loaded within the hydrogel, it nonetheless emphasizes the need to correlate *in vitro* and *in vivo* data to determine antimicrobial and antibiofilm effects.

[H1] Supramolecular formulations

The dynamic qualities and integration capacity of supramolecular self-assembly endow extraordinary functions, providing an opportunity to intervene when conventional therapies fail[80] **(Figure 4).**Many antibiofilm supramolecular delivery systems (**Table 2**) have been explored to enhance the clinical use of existing drugs. In this section, we review how supramolecular assemblies **[G]** can improve drug delivery to the infection site, facilitate biofilm penetration, integrate dispersal and bactericidal effects, and provide innovative therapeutic strategies for clinical application.

Fundamentally, a key challenge in drug delivery is engineering systems that are capable of specifically targeting the disease site without affecting healthy cells and tissues. Conventional antibiotics generally exhibit negligible preferential accumulation in infected tissue and encounter further difficulties bypassing the biofilm matrix and diffusing into the intracellular milleu,[81,82] which leads to non-specific interactions with host cells, tissues and the resident microbiota, , low local concentrations and poor pharmacokinetic stability. Formulated supramolecular carriers should therefore remain stable and intact before reaching the infected site to prevent off-target effects; bypass the biofilm matrix and interact exclusively with the pathogenic bacteria; selectively accumulate in the infected area at bactericidal concentrations; overcome conventional antimicrobial efflux mechanisms; and prevent drug molecules from prematurely degrading both in the body and in storage.

[H2] Improving stability of conventional antimicrobials.

Antimicrobial and antibiofilm agents can be encapsulated, adsorbed or attached to supramolecular assemblies to modify their size, shape, surface chemistry and surface charge, and hence improve their pharmacokinetic and pharmacodynamic profiles. Hydrophobic drug molecules, such as antimicrobial peptides, can be encapsulated within the hydrophobic cavities of macrocyclic supramolecular systems in an aqueous solution with high binding affinities and good colloidal stability in various physiological environments. This is particularly relevant given the sigmoidal correlation between antimicrobial activity and hydrophobicity due to enhanced lipid membrane binding.[83] Supramolecular systems can also prevent enzymatic hydrolysis and proteolytic degradation in blood, liver and kidneys. For example, 92.7% of the antibiotic Mutacin 1140 bound to blood serum components, thus decreasing antimicrobial bioavailability and inhibiting activity against *S. pneumoniae*.[84] In studies evaluating encapsulation of antimicrobials in liposomes, supramolecular assembly increased elimination half-life and maintained effective therapeutic concentrations far beyond that of free drug *in vivo*.[85-87] These benefits in stability were further conserved when evaluating intratracheal administration in a rat model of pulmonary *Burkholderia cepacia* infection. Encapsulation of tobramycin in liposomes prolonged its elimination half-life significantly from 12.9 h to 19.7 h, and consequently improved the overall pulmonary uptake concentration over 8-fold.[88] Alternative strategies to use supramolecular assembly were illustrated in a study that designed a ‘trap’ to bind free

lipopolysaccharide (LPS), preventing colistin–LPS interactions, and substantially increasing the antimicrobial efficacy of the antibiotic in a pulmonary *Acinetobacter baumannii* infection mouse model.[89]

[H2] Overcoming antimicrobial resistance with supramolecular platforms.

Because most intrinsically bactericidal supramolecular platforms exert their action *via* different mechanisms from those used by conventional antimicrobials and antibiofilm agents, it has been hypothesized that these formulations may bypass resistance defence mechanisms. For example, metallic nanoparticles have been widely explored for their relatively non-toxic yet potent antibiofilm and antibacterial effects through reduction in EPS production, interruption of biofilm–substrate interactions, activation of macrophages, ROS generation and enhanced permeability of the cellular membrane.[90,91] They also seem to pose minimal risk to host cells. Loading antibiotics onto metallic nanoparticles has been shown to yield a synergistic effect, with enhanced antimicrobial activity at concentrations below the MIC of the antibiotic or the nanoparticles alone.[92] Supramolecular formulations could potentially also enhance permeabilization of bacterial membranes by promoting membrane fusion or endocytosis. This is particularly important in treating bacteria that have evolved to limit the entry of antimicrobials through mutations in genes encoding porins.[93] In penicillin-resistant *S. aureus*-infected macrophages, bioconjugation of penicillin G to geranyl nanoparticles significantly decreased intracellular bacterial counts by more than 99.9%.[94] Only supramolecular penicillin could be detected intracellularly in the host after 90 minutes of incubation, which is likely to be attributable to host and bacterial intracellular degradation of penicillin or to its excretion through efflux pumps, both of which are known *S. aureus* resistance mechanisms. It is likely that the non-biological makeup of these formulations could enable evasion of bacterial intracellular and extracellular enzymes. Whilst this ‘mix-and-match’ strategy may suffer from difficulties in regulatory approval and clinical translation, it nonetheless provides a promising framework for re-using existing antimicrobials to treat MDR infections. A summary of these strategies is presented in **Table 2**. In relation to biofilms, mannitol was seen to activate dormant persister cells and increase conventional antimicrobial activity.[95] Indeed, modelling of the heterogenous biofilm population suggested that a biofilm with plentiful nutrients is substantially more susceptible to antimicrobials, both as free drug and within a supramolecular platform, than are biofilms in low-nutrient systems.[96] However, this phenomenon may not be conserved across bacterial phenotypes and species given their substantial heterogeneity in modulating behaviour of biofilms in response to nutrient supplementation and other changes in the biofilm microenvironment. Similarly, the possibility of co- or cross-resistance (that is; resistance to two bactericidal compounds on either the same genetic element or system) to these supramolecular formulations is feasible, particularly in metallic nanoparticles, and this co-resistance is becoming increasingly prevalent in the environment. [97]

[H2] Enhancing antimicrobial delivery.

[H3] Passive delivery strategies.

Successful passive delivery to the biofilm matrix requires supramolecular formulations to have high aqueous solubility, successful encapsulation or embedding of the compound to avoid degradation and sustained drug release to maintain therapeutic concentrations.[98] To optimize the benefit afforded by supramolecular assembly, careful consideration of the formulation process is required to maximize biofilm deposition and improve selectivity towards specific bacterial strains. For non-specific biofilm interactions, the surface charge of the vehicle membrane has a critical role. Given the primarily

polyanionic biofilm matrix present in most cases, a range of cationic supramolecular assemblies has been developed to promote rapid facile biofilm and bacterial cell binding. A group has reported the rapid penetration and distribution of cationic quantum dots but not of neutral nor anionic analogs into *E. coli* biofilms.[99] Cationic nanoparticles have also been observed to aggregate on planktonic bacteria,[100] localizing on hydrophobic anionic hotspots to modify the cell surface, prevent biofilm formation and induce bacterial cell death *in vitro*, thus demonstrating the potential therapeutic benefit against both planktonic and biofilm phenotypes.[101] However, this success has not been mirrored *in vivo* following systemic administration, where positively charged carriers are easily captured by macrophages and often interact with blood components. To avoid rapid clearance by the mononuclear phagocyte system (MPS), zwitterionic particles responsive to the infection microenvironment have been developed. Typically, using pH-responsive charge-reversal lipids or polymers, supramolecular carriers can be negatively charged at pH 7.4 in circulation and positively charged in the acidic environment of the bacterial biofilm. In evaluating one such micellar formulation composed of PEG (polyethylene glycol) and pH-responsive PAE (poly(β-amino ester)), substantially higher *S. aureus* biofilm penetration and accumulation of the lipophilic dye Nile Red were observed, compared with formulations lacking the presence of a pH-responsive component, where no penetration was observed.[102] Despite this, only minor differences in bacterial cytotoxicity were seen when comparing formulations with and without a pH-responsive component *in vivo*, which suggests the need for further considerations beyond a cationic surface charge.[103] Indeed, whereas most bacteria have a polyanionic biofilm matrix due to the presence of uronic acid or metal-bound pyruvate, it has been recognized that the positively charged exopolymer polysaccharide intercellular adhesin (PIA) is also an integral and essential factor of the extracellular matrix.[104] Therefor, biofilms possessing PIA have shown considerable resistance against cationic compounds such as antimicrobial peptides. To advance this basic formulation strategy, detailed mechanistic studies evaluating the spatial heterogeneity of particle biofilm charge interactions and distribution are needed.

Researchers have studied the interaction and diffusion of particles with sizes ranging from 0.9 nm to 135 nm within *Pseudomonas fluorescens* biofilms using fluorescence correlation spectroscopy (FCS).[105] Testing a wide range of polymer, metallic and polystyrene nanoparticles, they found that self-diffusion within the biofilm decreased exponentially with the square of nanoparticle radius. Others reached similar conclusions with 40-550 nm particles on *Burkholderia multivorans* and *P. aeruginosa*, finding that smaller particles can achieve deeper biofilm infiltration.[106] Specifically, the authors observed an upper threshold of 100-130 nm for optimal penetration of both biofilms, which suggests that the mesh size of the biofilm matrix and the size of the diffusion channels between bacteria clusters may exclude larger particles. This hypothesis was further supported by evidence showing variable diffusion of nanoparticles following alteration of biofilm growth conditions, which modified both exopolymer and microbial density. Decreasing particle size also increased retention time in the body and the likelihood of bacterial intracellular endocytotic uptake. By reducing particle size to <2 nm, small molecule-modified gold nanoparticles produced a 60-fold increase in antimicrobial efficacy against Gram-positive bacteria compared with 3.5 nm diameter particles.[107] Other researchers developed a pH- and lipase-sensitive micelle for simultaneous charge reversal and size shrinkage for spatiotemporal release of azithromycin. By reducing the size 3-fold in the presence of lipase, the formulation was observed to promote extravasation and eliminate *P. aeruginosa* biofilms on pre-colonized catheters *in vivo*.[108] However, it remains to be seen whether the observed size-based phenomena are preserved in heterogenous clinical biofilm infections where pharmacokinetic parameters must be factored.

Steric stabilization via PEGylation is a well-explored approach in parenteral drug delivery to enhance circulation time. However, when the affinity of 0-9% PEGylated liposomes to *S. aureus* biofilms was evaluated, adsorption to biofilms was decreased with increasing PEG concentration[109] Interestingly, no such antagonistic effect was observed with a similar formulation against *Staphylococcus epidermidis* biofilms,[110] which suggests that the impact of PEG on surface binding of particles onto biofilms may differ considerably across strains and indeed biofilm growths. Glycosylated particles have also shown improved antimicrobial targetability and delivery because of their ability to adsorb and fuse with bacterial biofilms.[111] Building on the principle of fusing with bacteria to proximally release antimicrobials, fusogenic liposomes (Fluidosomes™) that contain tobramycin have been approved for treatment of pulmonary infections caused by both *P. aeruginosa* and *B. cepacian*. Fusogenic liposomes differ from conventional lipid formulations as they contain asymmetric lipids such as phosphatidylglycerol to induce disorder in membrane lipid packing and to facilitate spatial control of drug delivery. Although their efficacy against planktonic bacteria is well explored, with reductions in bacterial growth over 100-fold compared with free drug,[112,113] the activity of fusogenic liposomes against clinical biofilms is less well known. Only a single reported study evaluating its antibiofilm effect could be found, showing over 10-times greater inhibition of *S. aureus* biofilm viability compared to free drug at 10x MIC and no statistically significant inhibition at 1x MIC.[114] The formulation tested, however, was based on a different formulation than the one clinically approved Further insights into its antibiofilm mechanism of action and optimization of membrane fluidity and lipid packing composition is needed.

[H3] Controlled delivery strategies.

Whereas passive targeting is applicable to a wide range of clinical biofilm infections, its efficacy is hindered by its lack of specificity. Active targeting and/or stimuli-responsive release strategies may yield more biofilm- and strain-specific antimicrobial interactions. Stimuli-responsive agents can also prevent premature release of cargo to avoid damage to surrounding microbiota **(Figure 5)**. Drug delivery vehicles can be functionalised with biomarker-targeting ligands to increase drug accumulation selectivity and facilitate bacterial cell uptake. Common targets that are overexpressed or solely expressed on bacterial cell membranes include EPS adhesins, exopolysaccharides, DNABII family proteins or toxins such as phenol-soluble modulins (PSMs).

Due to their high target specificity, affinity and wide availability for many of the target antigens, antibodies have traditionally been the preferred ligand for active targeting. As the phenotypic expression of target epitopes often varies considerably among bacteria, monoclonal antibodies have been developed against specific EPS components, including polysaccharide/adhesin (PS/A) to inhibit *S. epidermidis* in a rabbit model for central venous catheter infection[115], poly-N-acetylglucosamine (PNAG)[116], protein A[117] and surface accumulation-associated protein (Aap)[118], although antibiofilm efficacy seems to be strain- and species-dependent.[119] To address this, a phenotypic screening strategy was developed to identify monoclonal antibodies capable of binding to common epitopes in patients convalescing from *P. aeruginosa* infections.[120] The authors identified the polysaccharide Psl, showing that its targeting increased opsonophagocytic killing of *P. aeruginosa*, inhibited biofilm adhesion to lung epithelial cells and offered prophylactic protection against reinfection in multiple models. Interestingly, regardless of the number of antibodies conjugated or the net liposome charge, binding affinity of immunoliposomes to *Streptococcus oralis* biofilms was shown to be higher than that of traditional anionic liposomes yet lower than that of ordinary cationic vesicles, which suggests that electrostatic passive interactions may still outweigh active targeting strategies.[121] Building upon this minor advantage in drug delivery,

antibodies targeting biofilm components have also been observed to elicit antibiofilm effects themselves; for example, antibodies targeted against eDNA-binding proteins that provide structural support (that is; DNABII). When applied *in vivo* against biofilms in several infection models, destabilisation of the biofilm matrix was confirmed, enabling swift bactericidal effects when combined with antimicrobials.[122,123]

Although antibody-mediated targeting can increase site-specific antimicrobial delivery to biofilms, incorporated ligands must be homogenously distributed on the surface of a supramolecular assembly to facilitate binding. Furthermore, antibodies may elicit an immunogenic response and/or denature *in vivo*. To overcome this, *aptamer*-targeted systems have been developed. Aptamers are short-strand oligonucleotides or peptides with high affinity and specificity to a range of target molecules, offering good physicochemical stability and economical production, making them excellent substitutes for antibodies in targeting biofilms.[124] Researchers demonstrated the conjugation of a *S. aureus* SA31-specific aptamer onto liposomes for localized delivery of vancomycin:[125] after a 1-hr incubation, all viable and culturable bacteria within *S. aureus* biofilms were eradicated with 2-fold greater penetration of aptamer-tagged liposomes. Aptamer-functionalised carbon nanotubes loaded with ciprofloxacin also showed superior bactericidal and antibiofilm activity compared with untargeted nanotubes and aptamer-functionalized ciprofloxacin in *P. aeruginosa* biofilms.[126] Despite their promise, the ability of aptamers unique to a particular bacterial strain to treat polymicrobial infections is intrinsically constrained. To expand its therapeutic potential, aptamers tailored specifically for disease-related biofilm components rather than bacteria must be developed.

Light as an external trigger for drug release and/or activation may facilitate greater spatiotemporal control compared with both passive and active targeting pathways. Photodynamic therapy (PDT) typically involves the application of a minimally toxic drug (photosensitizer) that produces ROS locally when exposed to light. Photosensitizers are typically encapsulated within supramolecular assemblies, enabling selective uptake by bacterial cells through passive accumulation whilst illumination of this area, typically with a laser, then generates oxidative stress and bacterial membrane deformation. This approach has shown promising results *in vitro*, *in vivo* and clinically, with 95.4% destruction of *S. aureus* biofilms in rats reported following implantation with photosensitizer-loaded mesoporous polydopamine nanoparticles.[127] Using a photoactivatable porphyrin–phospholipid liposome, over 90% of loaded ciprofloxacin was released in less than 30 seconds at high fluence rates (200 mW/cm^2), inhibiting growth of *B. subtilis in vitro*.[128]Interestingly, no differences in bacteriostatic effects were observed with or without activation of the drug by laser treatment , which suggests that passive accumulation of liposomes contributed substantially to the observed results. Clinically, antimicrobial PDT (aPDT) has shown significant reductions in total bacteria counts in the treatment and maintenance of chronic periodontitis after mechanical debridement.[129,130] However, low numbers of controlled and homogenously designed studies have yielded high variability of clinical outcomes, with no statistically significant difference observed between PDT and laser alone in a recent meta-analysis.[131] Whereas additional trials must be conducted to confirm the efficacy of aPDT as a therapy, the use of PDT to reduce periodontal inflammation does hold promise as a symptomatic adjunct rather than microbiological.

Unfortunately, the use of light-activated therapies in humans is inherently constrained by its poor penetration. To extend the use of stimuli-responsive materials beyond superficial infections, ultrasound has been used to deliver energy in a focused manner to tissues at depths >10 cm. Ultrasound can generate oscillating gas and/or vapour bubbles from either endogenous or exogenous nuclei (acoustic cavitation)

to enhance the delivery of antimicrobial agents. Multiple studies have co-delivered nuclei and antimicrobials, including vancomycin, oxacillin, gentamicin and antimicrobial peptides, against both biofilms of Gram-positive and Gram-negative bacteria. A detailed review of this strategy can be found in Ref. [132] Direct incorporation of antimicrobials within the nuclei as a drug delivery vehicle can further increase penetration and spatiotemporal control of release. For example, a 2-fold increase in intracellular delivery of liposomal gentamicin was observed following ultrasound exposure when liposomes were conjugated to lipid-coated gas microbubbles.[133] Loading microbubbles with antibiofilm agents such as NO has also shown success, achieving reductions in *P. aeruginosa* biofilm biomass of 94% and enhancing antimicrobial efficacy.[134]

As external triggers increase the complexity and cost of treatment, smart moiety antibiofilm delivery platforms have been designed to respond to the biofilm microenvironment. These systems release their payload in response to the altered pH or enzymes present within biofilm infections in a spatiotemporal and dosage-controlled manner. Researchers used β-lactamase (*Bla)*- and penicillin G amidase (*PGA*)-responsive polymers with multiple antimicrobials[135] and showed that methicillin-resistant *Staphylococcus aureus* (MRSA) successfully triggered release of the cargo, significantly inhibiting bacterial growth *in vitro* and enhancing wound healing *in vivo*. Similarly, pH-responsive copolymer micelles were shown to release farnesol and reduce in size in response to acidic conditions in the biofilm microenvironment.[136] Dextran-coated iron oxide nanozymes have also been formulated to show strong peroxidase-like activity at acidic pH levels.[137] When exposed to oral *S. mutans* biofilms, the nanozymes were incorporated robustly into the EPS owing to the dextran coating, which enabled localized activation of hydrogen peroxide to induce EPS breakdown and bacterial killing without adverse effects on gingival tissues or oral microbiota. However, despite these promising results it must be considered that the dynamics of the infection microenvironment is still not fully understood and is likely to depend on the infecting strain, infection site and other host factors.

[H1] Outlook

There is an urgent need for innovative strategies for the clinical treatment of biofilm-associated infections. Engineered delivery systems can substantially improve solubility, pharmacokinetics, biofilm accumulation and the bactericidal potential of antimicrobial and antibiofilm agents relative to their molecular counterparts. Nonetheless, its translational potential depends on addressing the existing limitations and knowledge gaps behind antibiofilm therapies

The terms ‘biofilm disruption’ and ‘antibiofilm therapy’ are often used interchangeably, but they do not represent the same biological outcome. Antibiofilm therapy entails the destruction of bacterial cells within a protective biofilm layer, whereas biofilm disruption refers to the sloughing of biofilms to remove cells from the EPS matrix. The latter can, in fact, promote the spread of infection and cause septic shock. Studies show that bacteria disunited from biofilms during dispersal events in mature biofilm development represent a distinct intermediate phenotype that can persist for more than two hours in the presence of dispersal agents.[138] These dispersed cells show greater virulence than planktonic bacteria and rapidly accelerate disease progression in mouse[139] and human[140] models. The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) 2014 guidelines on biofilm diagnosis and treatment consequently specify the need for ‘combinations of antibiotics with biofilm-dissolving drugs’ to facilitate antimicrobial effectiveness.[141]

‘Biofilms’ itself is a broad term to describe a highly diverse and heterogenous set of entities, including non-surface-associated aggregates. Within any given biofilm, there are multiple genotypes and phenotypes, each with unique stress responses and metabolic pathways that are regulated by microvariations in the local microenvironment, stochastic gene expression and inherent genetic variability.[142] The biofilm EPS matrix is similarly dynamic in chemistry and structure, making it highly unlikely that a single treatment will work across all types of biofilm infections, or for all clinical strains. Multiple studies have reported conflicting results regarding the efficacy of antibiofilm therapy on different bacterial strains, isolates or even in varying growth conditions. Most prominently, experimental conditions and biofilm maturity has a substantial role in impeding antimicrobial therapy either through formation of a thick, well-connected EPS layer, bacterial cell dormancy or quorum sensing. Although 24 hours is often considered the benchmark for maturity in preclinical studies, clinical infections can persist for decades and studies evaluating different antibiofilm therapies have observed a significant decline in efficacy after only 60 hours.[11] Particularly, biofilm infections may create biogenic mineral-fortified EPS matrices when matured, further inhibiting antimicrobial transport.[143] There is a need for standardization and accurate reporting of how biofilm experiments are performed to facilitate meaningful comparisons and to preserve relevance to the clinical biofilm state; for example, the minimum information about a biofilm experiment (MIABiE) criteria.[144]

As biofilms harbour dormant and viable but nonculturable **[G]** (VBNC) cells typically undetectable via routine clinical microbiological methods, there is a requirement for studies either to develop more accurate methods to confirm bacterial cell death, or to evaluate long-term treatment efficacy of new antibiofilm therapies. Common commercially available viability kits such as propidium iodide (PI) have only been validated for a very limited number of bacterial species and have been reported to erroneously stain 50-75% of culturable cells.[145]

Although the exact replication of the clinical infection state is impossible, biofilm models should recapitulate the key parameters known to influence antibiofilm therapies including interactions between drugs and the host environment; for example, the degradation of drug molecules through proteolysis or opsonization in host fluids. New therapies should also assess host toxicity and collateral damage at the site of drug-biofilm interaction as most tissue-related biofilm infections co-exist with healthy tissue and are commensal with non-pathogenic bacteria, some of which may also reside in biofilms.[146] For example, off-target disruption of healthy mucosal biofilms may elicit increased interactions between mucosal microbiota and healthy colonic epithelial cells, causing inflammation.[147] To bridge this knowledge gap, we must eliminate the pervasive false dichotomy of ‘disease equals biofilms’ and ‘non-disease equals the planktonic state’ for an appropriate understanding of antibiofilm therapy interactions.

Moreover, bacterial resistance to any given therapy must be evaluated. The growing misuse of alternative antibiotics such as biocides and metals is increasingly being observed to not only activate metal resistance genes but also promote the development of antimicrobial resistance through co-selection. Similarly, bacteria have evolved a plethora of mechanisms to target critical phases of phage proliferation, causing abortion of phage infection.[63] There have also been reports of resistance to physical stimuli such as high pressure, UV radiation, and electricity,[148] reflecting the heterogeneity and diversity of bacteria that must be considered in developing novel therapies.

The vast majority of studies targeting biofilms specifically have been conducted *in vitro* using non-clinically relevant models and treatment regimens. Very few have progressed *in vivo* and even fewer have been assessed in humans. Without proper assessment of the multifactorial parameters known to

influence antibiofilm therapies, it will be challenging to realize the clinical benefit that these therapies and delivery systems have to offer. A concerted effort of microbiologists, engineers, chemists and medical professionals combined with in-depth mechanistic, pharmacokinetic, pharmacodynamic and bactericidal studies is needed to properly assess the efficacy of these promising innovative technologies for clinical translation. However, the adoption of these novel therapies also requires substantial improvements in the diagnosis of biofilm infections, clarification from regulators on what constitutes a clinically viable treatment, and collaborations between regulatory agencies and industry partners to bring antibiofilm therapy to patients.

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Acknowledgements

The authors acknowledge their funding sources: the Engineering and Physical Sciences Research Council (EP/V026623/1).

Author Contributions

The authors contributed equally to all aspects of the article.

Competing interests

E.S. and J.L.R. are named inventors on a patent application for a microparticulate formulation of antibiotics for urinary tract infection treatment; this formulation, however, is not promoted in the review. V. C., P. S. and D. C. do not declare any competing interests.

Peer review information

Nature Reviews Microbiology thanks Oana Ciofu, Saji George and Kendra Rumbaugh for their contribution to the peer review of this work.

Table 1 Biofilm targets and pathways implicated in biofilm dispersal.

Trigger	Target	Source	Function and/or mechanism	Preclinical or clinical trial stage	Refs.
Matrix-degrading enzymes					
α-amylase	Polysaccharides	*Bacillus subtilis* and synthetic sources	Major structural biofilm matrix component	*In vitro*	149
α-methyl-galactoside	LecA and LecB lectins	*Pseudomonas aeruginosa*	Virulence factor, increases absorption of exotoxin A	*In vivo*	150
Alginate lyase	Alginate	Algae and molluscs	Major structural biofilm matrix component	*In vivo*	151
Aureolysin	Clumping factor B	*Staphylococcus aureus*	Promotion of bacterial attachment to tissue	*In vitro*	152
Cepacian lyase	Cepacian	*Bacillus* sp.	Virulence factor contributing to *Burkholderia cepacia* complex pathogenicity	*In vitro*	153
Chitosan	Chitin	*Vibrio cholerae*	Nutrient source, promotes horizontal gene transfer	*In vivo*	154
Dextranases	Dextran	Fungi and various natural sources	Major structural biofilm matrix component	*In vitro*	155
Dispersin B	PIA and PGA (PNAG)	*Aggregatibacter actinomycetemcomitans*	Major structural biofilm matrix component	Pre-Phase I	156
DNase I	Extracellular DNA	Most Gram-positive and Gram-negative bacteria	Major structural biofilm matrix component	Clinical	157
β-mannanases	Mannans	*Candida albicans*	Mediates GtfB binding for bacterial-fungal biofilms	*In vivo*	158
Endoglucanase	Cellulose and xylan	*B. subtilis*	Major structural biofilm matrix component	*In vitro*	159
Esp protease	Binding proteins (serine)	*Staphylococcus epidermidis*	Cell–cell and cell–surface interactions	*In vivo*	160
Hyaluronidase	Hyaluronic acid	*Streptococcus* sp.	Minor biofilm matrix component mediating adherence	*In vitro*	161
LapG protease	Cell surface adhesin LapA	*Pseudomonas fluorescens*	Adhesive protein necessary for biofilm attachment	*In vivo*	162
Lysozyme	GlcNAc–MurNAc bonds	Secretions	Major structural biofilm matrix component	Dietary	163
$PelA_h$ and $PslG_h$	Pel and Psl exopolysaccharides	*P. aeruginosa*	Establishment of non-mucoid biofilms	*In vivo*	25
Proteinase K	Binding proteins (serine)	*S. aureus*	Cell–cell and cell–surface interactions	*In vitro*	164
Serratiopeptidase	Binding proteins (metalloprotein	*Serratia marcescens*	Cell–cell and cell–surface interactions	Clinical	165
Subtilisins	Binding proteins (serine)	*B. subtilis*	Cell–cell and cell–surface interactions	Dietary	166
Quorum-sensing agents					
Acylases	AHL (LasI and LasR)	Various natural and synthetic sources	Inactivation of AHL by cleavage of amide side chain in ring	*In vivo*	167
B. Diffusible signal factor	Diffusible signal factor	*Burkholderia cenocepacia*	Diffusible signal factor analogue to inhibit filament formation	*In vitro*	168
cis-2-decenoic acid	Gene regulation	*P. aeruginosa*	Reverts persister cells to a metabolically active state	*In vitro*	169
Farnesol	Pseudomonas quinolone signal	Natural isoprenes and synthetic sources	Inhibition of PQS synthesis via reduced *pqsA* transcription	*In vivo*	170
Halogenated Lactones	AI-2 (LuxS)	Hydroxy acids	Inhibition of RhIR–LuxS to prevent synthesis of AI-2	*In vivo*	171
LuxO	HA–protease	*V. cholerae*	Inhibits HapR expression for reduced protease production	*In vitro*	172
Lactonases (PLLs)	AHL (LasI and LasR)	*Bacillus* sp. and others	Inactivation of AHL by homoserine lactone ring hydrolysis	*In vivo*	173

Oxidoreductases	AHL (LasI and LasR) and AI-2 (LuxS)	Various natural and synthetic sources	Hydroxylation of AHL and AI-2 to quorum sensing-inactive derivatives	*In vitro*	174
RNA-III Inhibiting peptide	*S. aureus Agr*	Synthetic peptide derivative	Competes with RAP to inhibit phosphorylation of TRAP	*In vivo*	175
Savirin	*S. aureus Agr*	Small-molecule inhibitor	Blocks transcriptional function of AgrA, inhibiting P3	*In vivo*	176
Solonamide B	*Agr*	*Photobacterium halotolerans*	Downregulation of RNAIII, AgrA-controlled virulence gene	*In vitro*	177
Microenvironment modulation					
Nutrient modulation	c-di-GMP pathway	Sugars and carbon sources	Induction of c-di-GMP pathway	*Ex vivo*	178
Glutamate	c-di-GMP pathway	Amino acid (Glutamic acid)	Induction of c-di-GMP pathway	*In vitro*	179
Lactoferrins	Iron	Milk or recombinantproteins	Iron chelation, causing starvation + disruption of membrane	*Ex vivo*	180
Nitric oxide	Phosphodiesterases	Various natural and synthetic sources	Activation of phosphodiesterases resulting in decreased c-di-GM concentration	Phase I	181
Oxygen depletion	Cellular respiration	Excess N_2, radiotherapy	Cellular apoptosis or induction of biofilm formation	Phase I	182

AHL, N-Acyl homoserine lactone

AI-2, Autoinducer-2

c-di-GMP, Cyclic di-GMP

GlcNAc, N-acetylglucosamine

GtfB, 4,6-α-glucosyltransferase enzyme MRSA, Methicillin-resistant Staphylococcus aureus

MurNAc, N-Acetylmuramic acid

PGA, poly-β-1,6-N-acetyl-D-glucosamine

PIA, Polysaccharide intercellular adhesin

PLLs, Phosphotriesterase-like lactonase

PNAG, poly-β-1,6-N-acetyl-D-glucosamine

PQS, Pseudomonas Quinolone Signal

RAP, RNAIII-activating protein

TRAP, Target of RNAIII-activating protein

Table 2: Biofilm supramolecular delivery arranged by vehicle.*

Agent	Class	Composition	Targeting moieties	Size (nm)	Tested on	Model	Refs.
Polymeric supramolecular assemblies							
AMP-cypate	AMP	Gelatin	Targeted PTT	220 ± 2	*Staphylococcus aureus; in vitro, in vivo* (mouse)	Diabetic foot ulcer	[183]
Benzalkonium Cl Sodium salicylate	Quaternary NH_4 Co-inhibitor	Mesoporous silica	Cationic and anionic dual targeting	100.0 ± 10	*Staphylococcus epidermidis*; *in vitro*	Microtiter plate assay	[184]
Chitosan	Biopolymer	Chitosan	Targeted PDT (Emodin)	35.3 ± 5.6	*Streptococcus mutans; in vitro, in situ*	Enamel slab	[185]
Metronidazole	Nitroimidazole	Hyaluronidase	Targeted PDT (Chlorin e6)	ca. 180	MRSA; *in vitro, in vivo* (mouse)	Diabetic wound	[186]
Curcumin Nisin	Phytochemical AMP	Polymeric	Targeted PDT and SDT	78.6 ± 17.9	*Acinetobacter baumannii; in vitro, in vivo* (mouse)	Third-degree burn wound	[187]
DNase I Tobramycin	Enzyme Aminoglycoside	Polymeric – Dextran SCPN	None	11.0 ± 1.0	*Pseudomonas aeruginosa; in vitro*	Flow-cell	[188]
Luteolin	Quorum sensing inhibitor	Polydopamine	pH-responsive release using $Ca_3(PO_4)_2$	ca. 280	*S. aureus*; *in vitro, in vivo* (rat)	Knee joint implant	[189]
Prangos acaulis	Medicinal plant	Chitosan	None	89.8 ± 5.8	Screen in Gram-positive and Gram-negative bacteria, in vitro	Microtiter plate assay	[190]
Tannic acid	Tannin	Polymeric	pH-responsive and salivary peptide targeting	ca. >500	*S. mutans; in vitro, in vivo* (rat)	Dental caries	[191]
Vancomycin	Glycopeptide	Mesoporous silica	Peptide UBI_{29-41} cationic interaction Peptide D_6 bone-targeting	ca. 100	*MRSA; in vitro, in vivo* (rat)	Femur implant	[192]
Lipid supramolecular assemblies							
Alpha-lipoic acid Minocycline	Anti-oxidative Tetracycline	Lipid nanoparticles	Lipase-responsive release using DSPE-PEG pH-responsive release using dendrimer shell	12.78	*S. aureus*, *Escherichia coli*; *in vitro, in vivo* (rat)	Periodontitis	[193]
DCD-1L	AMP	Lecithin nanoparticles	Anti-*E. faecalis* DNA aptamer and PDT (Emodin)	107.3	*Enterococcus faecalis; in vitro*	Microtiter plate assay	[194]
Metal supramolecular assemblies							
Chitosan Silver	Biopolymer Metal	Ag nanoparticles	N/A; coated on endotracheal tube surface	16.7 ± 4.8	*P. aeruginosa*, *S. aureus* – *in vitro* and *in vivo* (pig)	Oropharyngeal challenge	[195]
Hydrogen sulphide	Toxic gas	ZnS nanoparticles	pH-responsive using ZnS nanoparticles and PTT (ICG)	177.7 ± 4.8	MRSA; *in vitro, in vivo* (mouse)	Cutaneous wound	[196]
Lignin	Plant-derived polymer	Metal oxide	N/A; topical scaffold application	18-33	*P. aeruginosa*; *in vitro*	TCP assay	[197]
Proteinase K	Enzyme	ZIF-8 MOFs	pH-responsive; ZIF-8 and PDT (Rose Bengal)	ca. 142	*S. aureus; in vitro, in vivo* (mouse)	Cutaneous wound	[198]
Rose Bengal	Xanthene	Metal oxide	N/A; topical application on dental caries	ca. 40	*S. mutans*; *in vitro, in vivo* (rat)	Dental caries	[199]
Other supramolecular assemblies							
Cefixime Rifampicin	Cephalosporin Rifamycin	Hydrogel	N/A; implanted on urological devices	N/A	*S. aureus*, *E. coli*, *P. aeruginosa; in vitro*	Microtiter plate assay	[200]
Cellulose	Polysaccharide	Nanocrystal	No	93 x 10	*Pseudomonas savastanoi; in vitro*	Microtiter plate assay	[201]

Citric acid	Cationic acid	Nanodots	Electrostatic cationic interactions	105 ± 19	Screen in Gram-positive and Gram-negative bacteria; *in vitro*	Microtiter plate assay	[202]
Epigallocatechin Silver	Catechin Metal	Nanowires	N/A; topical application on chronic wounds	148 ± 11.2	Resistant *S. aureus*, *E. coli; in vitro, in vivo* (mouse)	Cutaneous wound	[203]
Fucoidan	Polysaccharide	Nanodots	No	7.15 ± 1.5	*E. faecalis*; *in vitro, in situ*	Dentin block	[204]
Glutathione-silver	Metal	Nanoclusters	No	7.9 ± 0.2	*S. aureus, E. coli*; *in vitro*	Microtiter plate assay	[205]
Imipenem	β-lactam	Niosomes	No	192.3 ± 5.8	MRSE; *in vitro*	Microtiter plate assay	[206]
Graphene oxide	Photosensitizer	Graphene	Anti-*P. gingivalis* DNA aptamer and PDT	21.3 ± 3.2	*Porphyromonas gingivalis; in vitro*	Microtiter plate assay	[207]
$Ti_3C_2T_x$	MXene	Nanosheets	Targeted PTT; topical administration	ca. 200	MRSA; *in vitro, in vivo* (mouse)	Cutaneous wound	[208]
Triclosan	Biocide	Nanozyme	*Lactobacillus* cell envelope	132.8 ± 9.1	*S. mutans; in vitro, in vivo* (rat)	Dental caries	[209]
Zinc oxide	Metal	Nanoflowers	No	NR	*S. aureus*; *in vitro*	Microtiter plate assay	[210]
Zinc iron oxide	Metal	Nanoclusters	Targeted PTT	130	Resistant *Helicobacter pylori*; *in vitro*	Microtiter plate assay	[211]

AMP, Antimicrobial peptide
DSPE-PEG, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)
ICG, Indocyanine green
MOFs, Metal-organic framework
MRSA, Methicillin-resistant Staphylococcus aureus
N/A, Not applicable
NR, Not reported
PDT, Photodynamic therapy
PTT, Photothermal therapy
SCPN, Single-chain polymer nanoparticle
SDT, Sonodynamic therapy
TCP, Tissue culture plate
ZIF, Zeolitic imidazolate framework
*A subset of recent papers are listed.

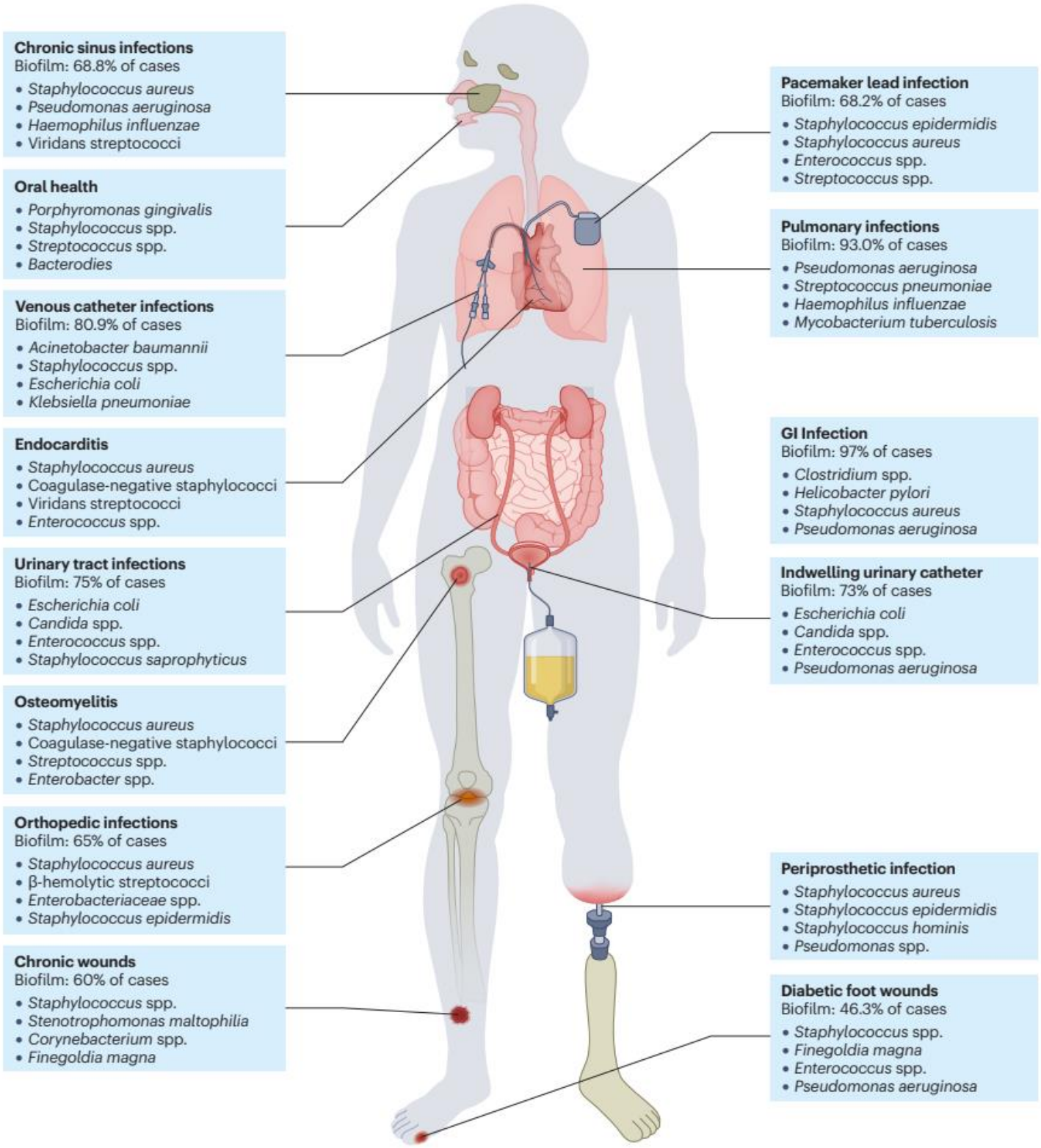

Figure 1. Sites of common clinical biofilm-associated infections and the most frequent pathogens involved in those infections

Almost all types of indwelling devices, many mucosal surfaces and diverse free-floating or embedded bacterial aggregates have been associated with the occurrence of microbial biofilms, such as chronic urinary tract infections and chronic wounds. Due to their high tolerance and resistance against conventional antimicrobials, biofilms result in recalcitrant and often chronic infections, exposing the patient to recurring symptoms and increasing the likelihood of selection of further resistance mechanisms. Statistics of biofilm cases for each infection from data from Refs. [3,5,212-219], if available.

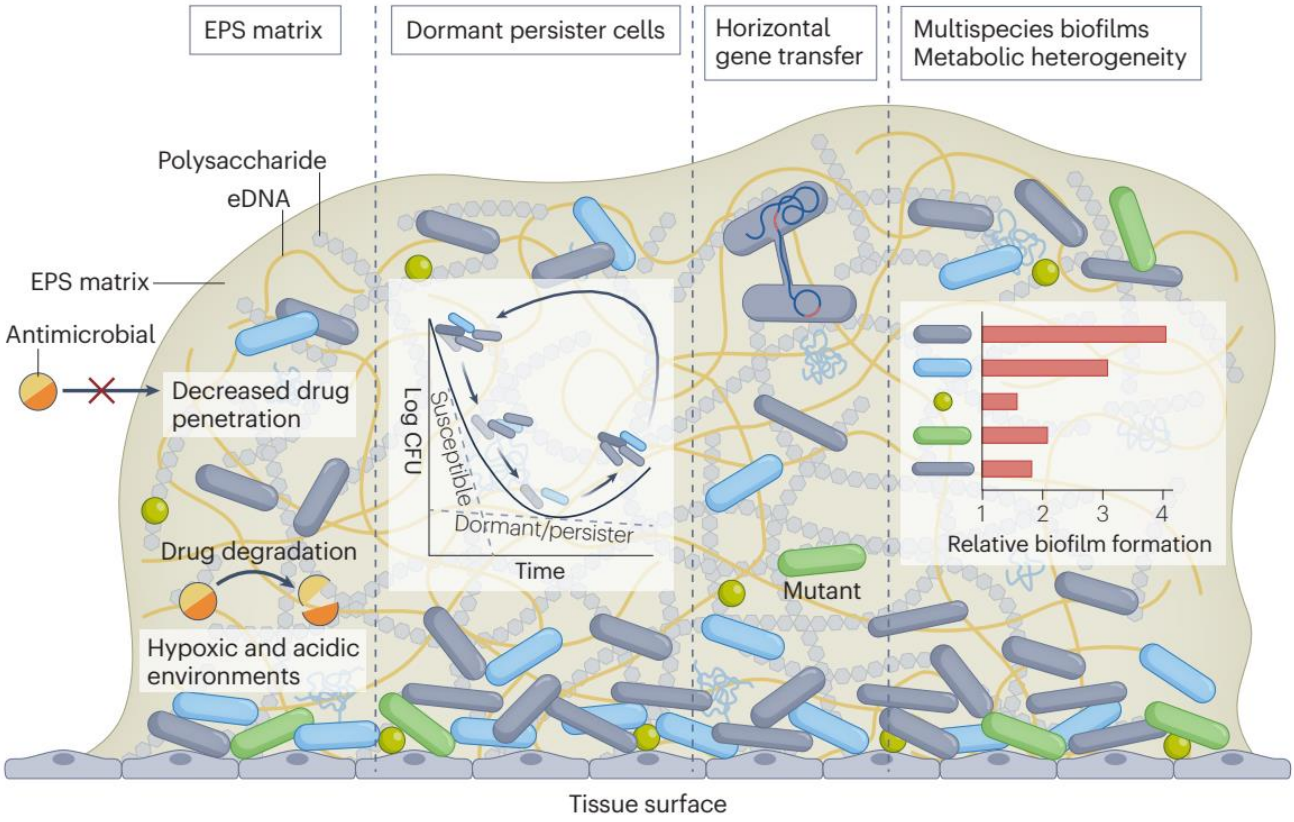

Figure 2: Challenges associated with treating biofilm-associated infections. To develop effective strategies to combat clinical biofilm-related infections requires understanding of the antimicrobial resistance and tolerance mechanisms exhibited by the bacterial communities within a biofilm. There are four primary mechanisms of resistance and tolerance: production of an extracellular polysaccharide (EPS) matrix; altered metabolism of biofilm cells, horizontal gene transfer and enhanced spontaneous mutations and a community of multispecies populations. The presence of an EPS hypoxic and acidic compartmentalised microenvironment boosts antimicrobial degradation mechanisms through interactions with diverse EPS components and also diminishes biofilm susceptibility by quenching antibiotic penetration. Cells within the biofilm can exist in a reversible metabolically stationary (dormant) phase either as 'viable-but-nonculturable' (VBNC) or 'persister' subpopulations. Antibiotics are unable to interfere with their metabolic function, enabling over 99% tolerance to conventional antimicrobials despite successful killing of susceptible populations. Within the community of biofilm-resident bacterial cells, cell–cell signalling (quorum sensing) increases the opportunity for plasmid exchange between neighbouring species (horizontal gene transfer), and increases the likelihood of spontaneous mutations, to further promote antibiotic resistance development. Multispecies biofilms (represented here by different colours and shapes) are now recognized as being omnipresent in natural environments, eliciting unique structural and functional dynamics including metabolic cross-talk, and crucially becoming more resilient to antimicrobial therapy than their single-species counterparts. Although in this representation a surface-associated biofilm is shown, similar challenges exist for treatment of non-surface attached biofilms and aggregates.

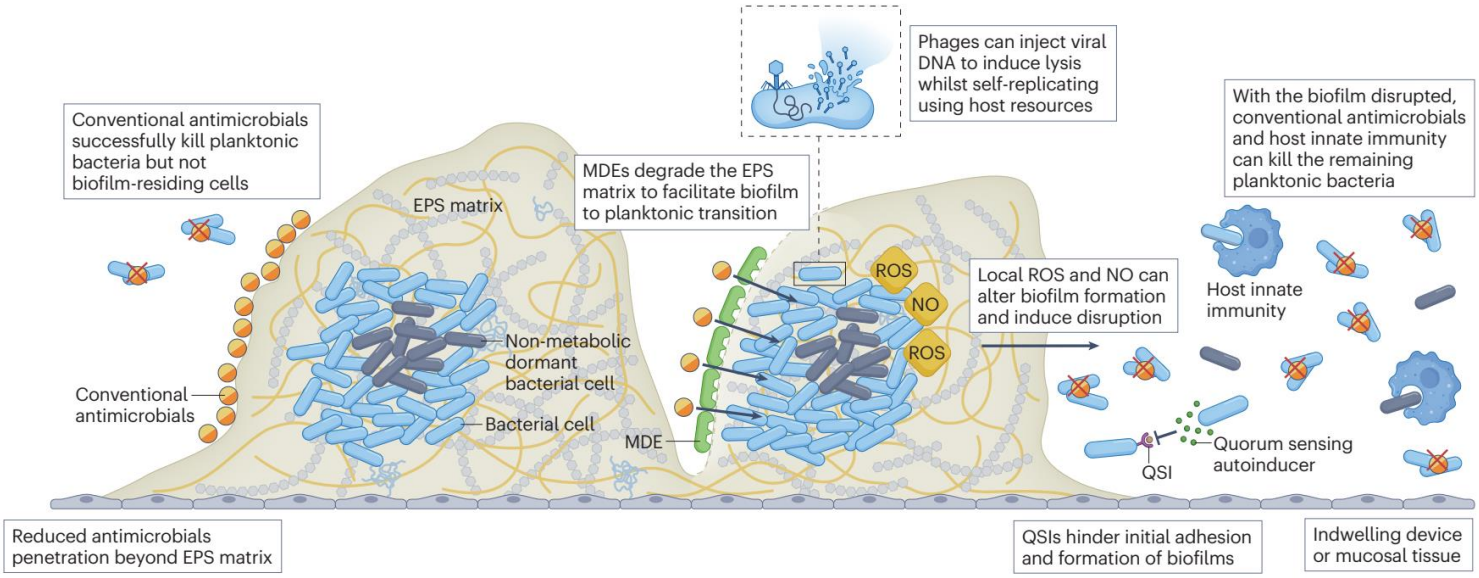

Figure 3: Mechanisms of action for antibiofilm agents. Multiple different approaches exist for disrupting the protective extracellular polysaccharide (EPS) matrix of bacterial biofilms to expose the resident bacteria, preventing biofilm formation and/or and increasing the susceptibility of bacteria to antimicrobial drugs. These include matrix-degrading enzymes (MDEs), reactive oxygen species (ROS) and nitric oxide (NO) that can induce biofilm disruption, quorum-sensing inhibitors (QSIs) that interfere with cell–cell signalling between bacteria within the biofilm to prevent them from forming a community upon reaching a critical population density, and bacteriophages (phages) that can induce cell lysis. Although in this representation a surface-associated biofilm is shown, similar approaches are used for non-surface attached biofilms and aggregates.

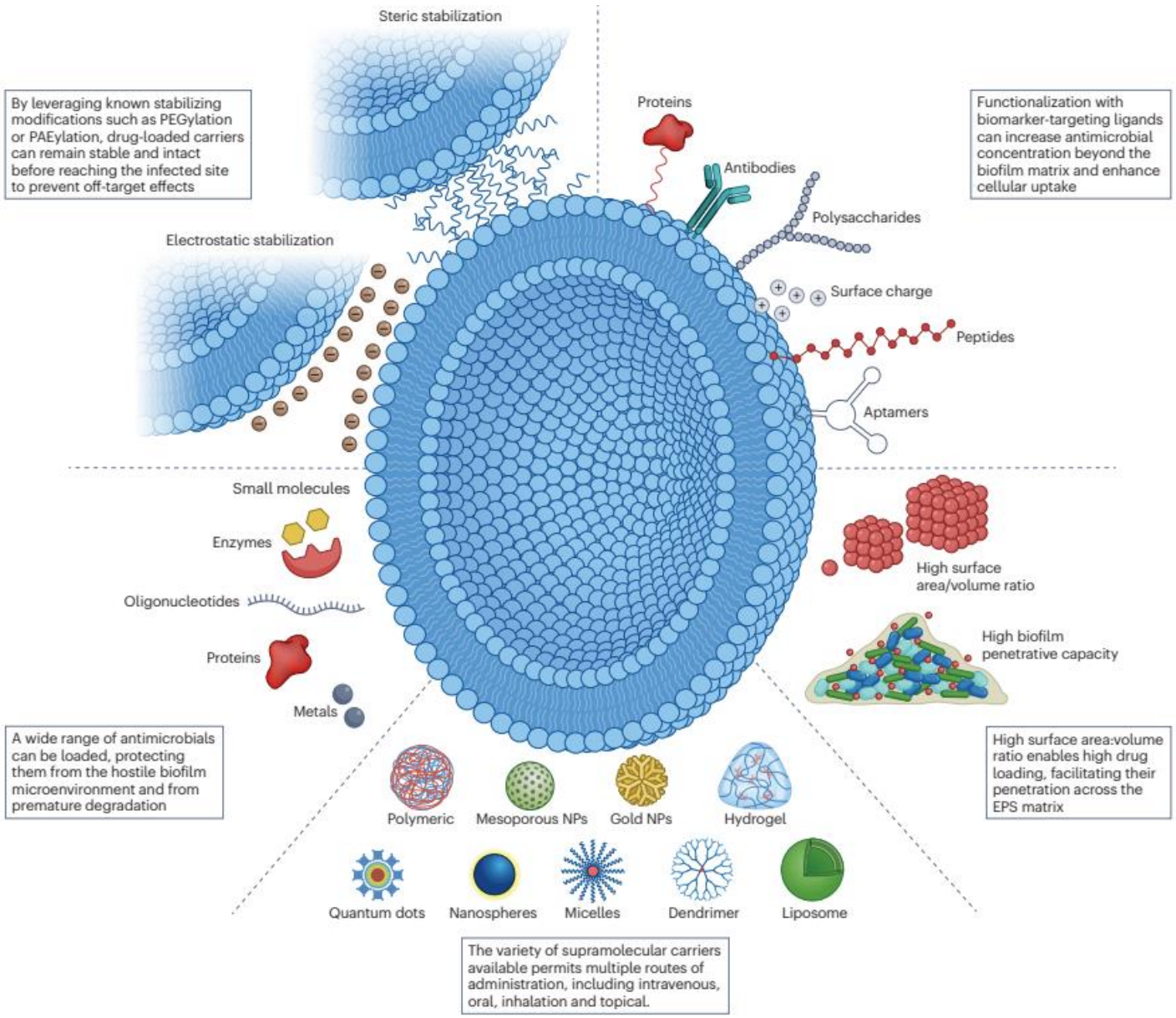

Figure 4: Unique properties and advantages of supramolecular assemblies in treating biofilm-related infections.

Functionalization of supramolecular drug-delivery vehicles with biomarker-targeting ligands, such as antibodies, or adjustments in structure and/or physiochemical properties can increase antimicrobial concentration beyond the biofilm matrix and enhance cellular uptake to elicit bactericidal effects. The high surface area:volume ratio of nanometric supramolecular structures enables high loading of otherwise hydrophobic or insoluble drugs, facilitating their penetration into the biofilm extracellular polysaccharide (EPS) matrix. The variety of supramolecular carriers available (polymeric, lipid, metallic etc.) permits multiple routes of administration, including oral, inhalation and topical. A wide range of antimicrobials, including degradable hydrophilic and hydrophobic substances, enzymes, and oligonucleotides can be loaded within supramolecular drug carriers, protecting them from the hostile biofilm microenvironment and from premature degradation. Carriers exhibit high stability both in storage and *in vivo* despite their high surface energy due to steric and electrostatic stabilization. By leveraging known stabilizing modifications such as PEGylation or PAEylation from analogous fields such as cancer therapy and RNA delivery, supramolecular carriers can improve biofilm agent stability and crucially, remain stable and intact before reaching the infected site to prevent off-target effects.

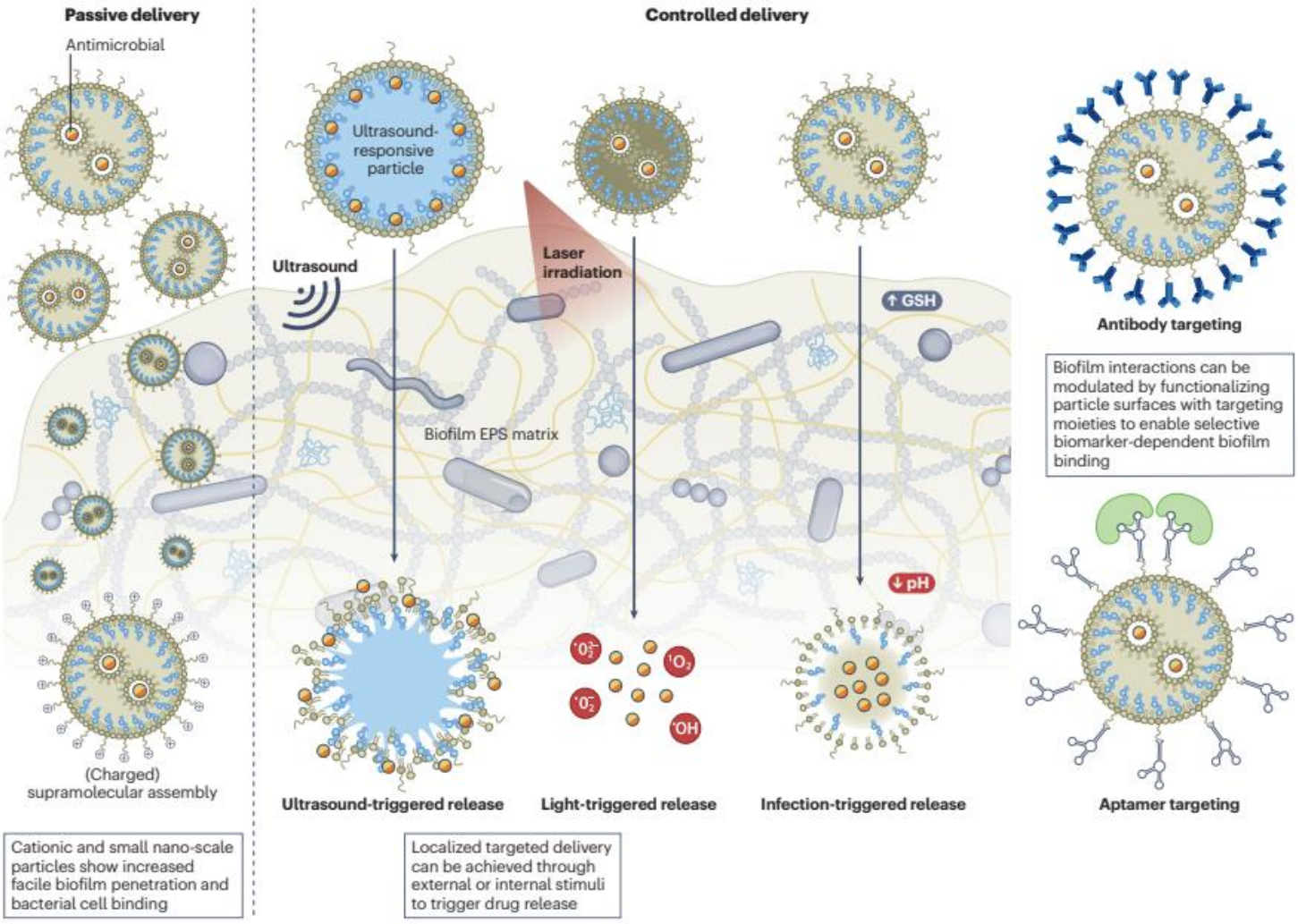

Figure 5: Supramolecular assembly delivery strategies to enhance antimicrobial delivery through the biofilm matrix.

The physicochemical properties of supramolecular structures, such as size, shape, surface charge and surface chemistry, are unique and can be directly engineered to target clinical biofilm infections (passive targeting). Given the primarily polyanionic biofilm matrix present in most cases, cationic supramolecular assemblies have been shown to promote rapid facile biofilm and bacterial cell binding, localizing on anionic hotspots within the extracellular polysaccharide (EPS) and bacterial cell surface. Similarly, small nano-scale particles have shown increased penetration of the biofilm matrix, passing through diffusion channels between bacterial clusters. To overcome the rapid clearance systemically associated with such cationic and ultra-small particles, pH and lipase-responsive components have been developed such that drug delivery vehicles exhibit charge reversal and shrink only in the presence of the acidic environment of the bacterial biofilm. Biofilm interactions can be further modulated by functionalizing the surfaces of these drug delivery systems with targeting moieties such as aptamers, antibodies, or peptides, to enable selective binding to biofilms expressing the biomarker of interest (controlled delivery). Localized targeted delivery can also be achieved through external or internal stimuli such as ultrasound, light, electricity, or exploiting the inherent infection microenvironment to trigger drug release.

Glossary

Resistance

Acquired or intrinsic genetic mutations permitting growth of microorganisms in the presence of bactericidal (or bacteriostatic) agents (minimum inhibitory concentration above breakpoint) through mechanisms such as efflux pumps, enzymatic drug inactivation, or modifications in drug targets.

Biofilms

Dynamic self-constructed accumulations of microorganisms producing a matrix of extracellular biopolymers (extracellular polysaccharides).

Tolerance

The ability to survive, but not grow, in the presence of bactericidal agents; for example, via reduced growth rate or survival of dormant persister cells.

Persister

A phenotypical survival strategy used by small populations of cells within the larger population that enter a state of dormancy and are thus protected from antibiotics functioning by disrupting metabolic activity or other growth processes. Persister cells can form in response to conditions of extreme stress, or even under optimal growth and nutrient conditions. Persister cells are thought to resuscitate *in vivo* or upon culture in laboratory conditions when the antimicrobial is removed , differentiating them from VBNCs, although there is still debate on the definitions of these phenotypes.

Reactive Oxygen Species

Derivative radicals formed by the reduction of molecular oxygen. Examples include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and hydroxyl radicals (-HO).

Reactive Nitrosyl Species

Derivative radicals formed by the reduction of molecular nitrogen. Examples include nitric oxide (NO), peroxynitrite ($ONOO^-$), and nitrous acid (HNO_2).

Supramolecular Assemblies

A complex of molecules held together by usually non-covalent bonds, usually through stoichiometrically interacting particles or in large complexes. This can include quaternary protein structures such as DNA, biological membranes, and synthetic compounds such as most drug or peptide-loaded nanomaterials.

Viable-but-nonculturable (VBNC)

Cells that survive and grow *in vivo* but are not capable of growing or dividing by conventional laboratory methods. This can be due to reduced metabolic activity as a survival strategy in response to conditions of extreme stress or inappropriate culture conditions not reflecting essential growth requirements of the in vivo environment. VBNCs have been reported as being

antibiotic, heavy metal, temperature, pH, and biocidal tolerant. In this case, some VBNCs are thought to resuscitate under specific conditions and/or with time once the stressor is removed.

Table of content:

In this Review, Stride and colleagues discuss emerging drug delivery strategies that are explored in antibiofilm therapy to improve the clinical efficacy of antibiofilm agents, highlighting their current limitations and future prospects.