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REVIEW

Bacterial Biofilm Destruction: A Focused Review On The Recent Use of Phage-Based Strategies With Other Antibiofilm Agents

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Abstract: Biofilms are bacterial communities that live in association with biotic or abiotic surfaces and enclosed in an extracellular polymeric substance. Their formation on both biotic and abiotic surfaces, including human tissue and medical device surfaces, pose a major threat causing chronic infections. In addition, current antibiotics and antiseptic agents have shown limited ability to completely remove biofilms. In this review, the authors provide an overview on the formation of bacterial biofilms and its characteristics, burden and evolution with phages. Moreover, the most recent possible use of phages and phage-derived enzymes to combat bacteria in biofilm structures is elucidated. From the emerging results, it can be concluded that despite successful use of phages and phage-derived products in destroying biofilms, they are mostly not adequate to eradicate all bacterial cells. Nevertheless, a combined therapy with the use of phages and/or phage-derived products with other antimicrobial agents including antibiotics, nanoparticles, and antimicrobial peptides may be effective approaches to remove biofilms from medical device surfaces and to treat their associated infections in humans.

Keywords: biofilms, extracellular polymeric substance, phages, phage-derived enzymes, nanoparticles, antimicrobial peptides

Introduction

Despite being found naturally as individual cells in planktonic form, most bacteria are capable of forming multiple cell structures called biofilms by adhering to biotic and abiotic surfaces. Bacterial biofilm formation is regarded as one of the most important strategies of survival that increases the virulence to be more pathogenic and consequently contributes to further resistance to antibiotics.[1] Biofilms formed on human tissues and surfaces of medical devices, including implants, are implicated in the pathogenesis of chronic bacterial infections, such as urinary tract infections, pneumonia, orthopedic infections, oral infections, wound infections, and cystic fibrosis among others.[2,3] Bacterial biofilms, despite their beneficial use for biological stages of wastewater treatment, waste reactors, numerous food production systems, and space travel,[4] have enormous negative implications on human health.

Bacteriophages or phages, are viruses that infect bacterial cells with high specificity. They are the dominant biological entities in the planet and those phages with virulent or lytic to bacteria are also capable of infecting and destroying biofilm-forming bacteria.[5] The threat posed by increasing incidence of

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161

antimicrobial-resistant (AMR) bacteria coupled with the paucity of new antibacterial drug intervention has revived interest in using bacteriophages against AMR. Similarly, the ineffectiveness of antibiotics on biofilms has resulted in a growing scientific interest in phages as an alternative strategy in controlling and preventing biofilm formation.[6] Indeed, the interaction of phages and biofilms as a subject of research in scientific publications have risen exponentially in the last decade.[2,7–9] Reports from the majority of studies on application of phages and phage-encoded proteins as alternative approaches to control and prevent biofilms formed particularly by ESKAPEE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.* and *Escherichia coli*) are encouraging.[10–15]

In the search for effective alternative approaches to combat biofilm-forming bacteria and their associated infections, some advanced and relevant techniques for biofilm destruction have been intensely emphasized. These strategies include biofilm degradation by electrochemical method which is a combinatorial effect of applying lower dose antibiotics in a weak electric field, the use of antimicrobial lipids as biofilm inhibitors and inhibition of alarmone scheme by natural peptides in response to environmental stress.[16] Others include interruption of molecular system of biofilm formation and irrigation techniques including passive ultrasonic, subsonic, or laser-activated irrigation for removal of root canal biofilms.[17,18] Among these techniques, the use of phage-based strategies was least emphasized, whereas they are economical and potentially modifiable. It is thus essential to discuss phages and bacterial biofilms. Therefore, the authors in this review present an overview of existing literature on bacterial biofilms and give an insight on the most recent use of phages as well as phage-derived enzymes as natural armament to destroy bacterial biofilms. The authors also illustrate the synergistic use of phages or phage-derived proteins and other antimicrobial agents that the former serve as vehicles for delivering antibiofilm agents to eradicate biofilms.

The Bacterial Biofilm
The Genesis of Bacterial Biofilms

Biofilm is a complex integrated aggregate of bacterial cells associated with adherence to both biotic and abiotic surfaces. This microbial or bacterial community is encased in a matrix of hydrated extracellular polymeric substances (EPS) produced predominantly by the cells themselves within the biofilm structure.[1] The EPS matrix consists of polysaccharides (40–95%), proteins (1–60%), lipids (1–40%), nucleic acids (extracellular (eDNA) and RNA) (<1%), lipoproteins, enzymes, and inorganic components. Typically, 2–35% of the biofilm volume is composed of the microorganisms (less than 10% in bacterial biofilms) while the matrix accounts for about 90% of the total biofilm mass.[19,20] Clearly, variation exists in the physical and chemical constituents of biofilm between different species of bacteria depending on the microorganisms' type, stress level, nutrients availability, and host environment.[21] As one of the critical steps in biofilm formation, the matrix provides structural support and protection for bacterial communities. Besides offering architectural stability and forming a defense shield from antimicrobial factors, the bacterial extracellular matrix plays alternative roles such as serving as signal targets and amplifiers, mediation of migration and colonization, capturing of cations, and exchange of genes.[22] Pores and channels within the biofilm aid in the transport of nutrients, gases, water, and other molecules within the matrix and between the biofilm and the environment. It is noteworthy that the main component of the matrix is water (up to 97%), which baths the architectural and functional components of the matrix.[1] In effect, by forming biofilm, bacteria are able to adapt to the surrounding environment and also able to survive in hostile environmental conditions.

Bacterial Biofilm Formation

Biofilm formation is a complex cooperative group process, which occurs in step-by-step processes and involves chemical communication within and between cells. This cell-to-cell communication system is coordinated by crosstalk of various signaling networks including two-component systems (TCS), diguanylate cyclase (DGC) systems, and quorum sensing (QS).[2,23] The TCS, composed of histidine sensor kinase and response regulators, regulate signal transduction via phosphorylation or cyclic di-GMP (c-di-GMP), a secondary messenger, which permits DNA specific binding for modulation of gene expression. As a key signal network molecule synthesized by the DGC system, c-di-GMP level depends on membrane or cytoplasmic DGC and phosphodiesterase activities working solely or as part of TCS. Signal transduction by c-di-GMP involves allosteric modification of enzymes, interaction with transcription factor, riboswitch, and participation

in post-transcriptional and post-translational processes within the cell. These activities by c-di-GMP coordinate the transition of bacteria from planktonic to biofilm growth mode.[23]

The QS system involves two main factors, the autoinducer (AI) and the receptor, which is based on population cell density or concentration. The AIs are small diffusible signaling molecules produced by the bacteria, which are detected by the receptors once a concentration threshold is achieved.[24] Gram-negative and -positive bacteria secrete N-acyl homoserine lactones (AHL) and autoinducer oligopeptides (AIP) as signaling molecules, respectively, as well as AI-2.[24,25] The binding of the AIs to their cognate receptors activates specific gene expression including biofilm formation and antibiotic resistance among others.[23] Biofilm formation occurs within and between species of bacteria as well as interkingdom signaling among plants, fungi, and host cells, suggesting that microorganisms in a biofilm interact by self-talk, crosstalk and listen in.[26] Biofilm formation

proceeds in four stages: (i) adhesion (ii) microcolony formation (iii) maturation (iv) dispersion (Figure 1).

Adhesion

Bacterial attachment to a living or non-living surface begins the initial stage of biofilm formation with the generation of conditioning film which changes the physico-chemical nature of the surface to initiate the adhesion process. Adhesion occurs through reversible and irreversible attachment processes.[24] Reversible adhesion is a temporary attachment of the free-living bacteria to the conditioned surface, which causes a weak adhesion mediated by non-specific van der Waal's, electrostatic and Lewis's acid-based electronic forces.[27] In contrast, irreversible adhesion is a permanent adhesion, which causes strong attachment of the bacteria to any surface, mediated by bacteria specific adhesion pili (fimbriae) and flagella. Bacterial adhesion is greatly affected and
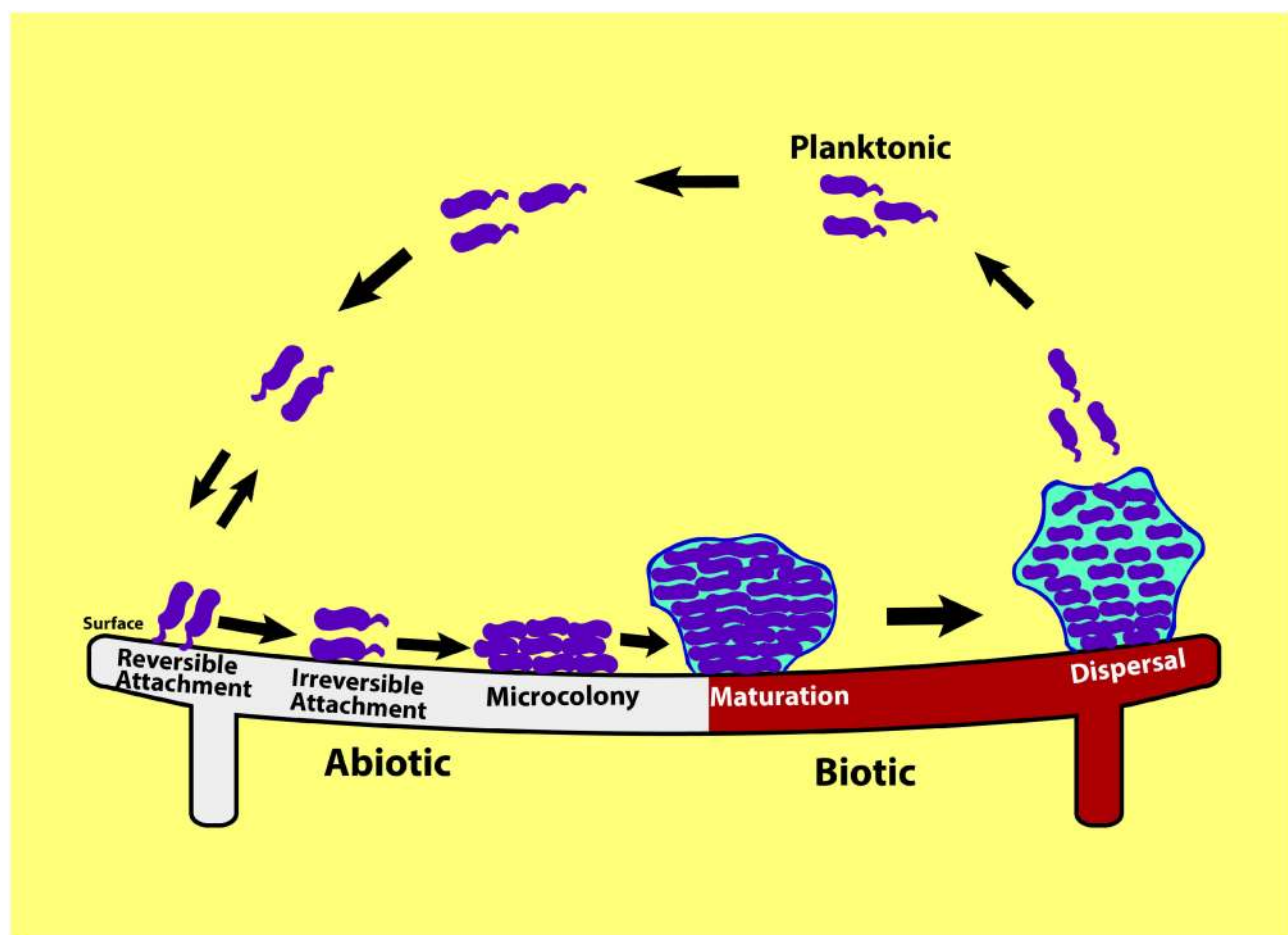

Figure 1 Schematic representation of stages of biofilm formation. Formation of biofilm begins with reversible and then with irreversible adhesion of planktonic cells to the surface. Bacteria start to multiply and form micro-colonies which develop into the mature biofilm. In the last stage, bacterial cells multiply quickly, and start to detach and disperse. This process enables the immotile bacteria to convert to motile forms that can help to spread and colonize new surfaces.

supported by EPS composition, nature of the surface, cell surface hydrophobicity, and flagella-pili-fimbriae movement. A rough surface provides a higher adhesion than a smooth surface. Likewise, due to the decreased in force of repulsion between the bacteria and the surface, hydrophobic and non-polar surfaces such as plastics and Teflon provide a higher adhesion than hydrophilic and polar surfaces such as metals and glasses.[25,28]

Microcolony Formation

Once adhesion is established, bacterial cells divide and multiply to develop three-dimensional (3D) clusters and aggregates called microcolonies. Microcolonies grow via cell proliferation to synthesize the EPS matrix for adhesion to surfaces, cohesion among cells, scaffolding cells together, maintaining the 3D architecture, and protection of the microcolonies. The EPS protects the microcolonies against various stresses such as mechanical removal, host immunity, metallic cations, oxidation, and antimicrobials with enhanced drug tolerance. EPS as a physical boundary can sequester or trap various substances to create nutritional and chemical gradient for the diffusion of oxygen, signaling molecules, inorganic ions, metabolites, and other solutes across the biofilm 3D architecture.[29] Bacterial microcolonies normally consist of many types of microcommunities that coordinate with one another for substrates exchange, metabolic products dissemination, and metabolic end-products excretion. The EPS of Gram-positive bacteria is cationic and that of Gram-negative bacteria is neutral or polyanionic.[25]

Maturation

The formation of microcolonies from layered cells and small clusters leads to the generation of a thin biofilm to begin the maturation phase, with the synthesis of EPS matrix from main structural components (polysaccharide, protein, and eDNA). Clusters develop into macrocolonies (large microcolonies) with the displacement of cells from the substratum to form channels and voids, which facilitate the exchange of nutrients and waste products by infusing fluid into the biofilm.[30] Polysaccharide forms the core of the matrix whereas eDNA is involved in horizontal gene transfer. The maturation of the biofilm through signal molecules, causes structural changes, as well as many changes in the expression of genes coding for different virulent factors. These changes include loss of cellular motility by expressing flagella-free phenotypes, reduction in protease and phospholipase C synthesis, decrease in the

synthesis and release of toxins, and production of rough and sometimes mucus-like polysaccharide to better adapt to certain conditions of the biofilm microenvironment.[23] The morphological changes in the biofilm enable metabolic adaptation under aerobic and anaerobic conditions that results in metabolically distinct microcolonies, whose presence may not only decrease or eliminate the time required to adapt to nutrients and oxygen stress but also provide an important metabolic context to resist antimicrobials present within the microenvironment.[31]

Dispersion

The final stage of biofilm formation is cell dispersion and the switch of sessile cells to the planktonic mode of growth, for colonization of new sites, to form a new cycle of biofilm. Dispersion is an active event induced or triggered by self-synthesized signaling molecules or cues such as fatty acid signaling molecules, and environmental conditions such as oxygen depletion, nutrient availability, starvation due to cessation of oxygen or nutrients, nitric oxide, and iron, which ultimately result in the overall reduction in the levels of c-di-GMP via series of post-transcriptional modifications.[32] Low c-di-GMP levels, in turn, upregulate genes involved in cell motility, such as flagella synthesis or chemotaxis, and in EPS matrix degradation, such as matrix-degrading enzymes, endonucleases such as endA, which degrades DNA present in the matrix, and glycoside hydrolases such as pslG and pelA, which degrade the matrix polysaccharides Psl and Pel. Concurrently, genes involved in EPS production, such as polysaccharide synthesis, and in attachment, such as fimbriae synthesis, are downregulated. Phenotypes related with low c-di-GMP levels include increased motility, reduced adhesiveness, reduced matrix synthesis and dispersion.[30,32]

Burden of Bacterial Biofilm Occurrence in Medical Field

Biofilm control and prevention is an enormous problem currently for the food industry, agricultural sector and the medical field. The widespread occurrence of bacterial biofilms in every habitat on earth,[33] including biofilms on medical device surfaces and in human tissues, pose a major threat causing chronic infections. According to the Center for Disease Control and Prevention (CDC), it is estimated that biofilms are responsible for over 65% of all chronic bacterial infections, while the National Institutes of Health (NIH) estimates around 80% of

microbial infections and over 60% of nosocomial infections.[32] Biofilm formation on or within medical devices, implants, and prostheses, cause device-associated infections such as urinary tract infections, orthopedic infections, endocarditis, periodontitis, gingivitis, osteomyelitis, cystic fibrosis, pneumonia, and wound infections, notably by multidrug-resistant (MDR) bacteria from the ESKAPEE group, as well as other Gram-negatives and -positives.[34]

Biofilm burden in medical field is exacerbated by antibiotics resistance of bacteria in biofilm communities contributing to persistent infections. With about 500–5000-fold increase in resistance to antibiotics compared with nomadic cells,[35] bacterial biofilms formation has rendered conventional antibiotics ineffective and insufficient at eradicating biofilm-mediated infections.[36] Furthermore, bacteria in biofilms are tolerant to antiseptic agents, germicides and the response of host immunity regardless of their location.[24,36] The tolerance of bacterial biofilms to antimicrobials depends on several factors, which stem from different intrinsic and acquired resistance mechanisms of bacteria. Notable among them is the generation of semi-dormant cells from the deepest biofilm regions with reduced metabolic activity called persister cells.[24] Biofilms contain a great population of persister cells, which are tolerant to all conventional therapeutics. As reported, resistance of bacteria in a biofilm may be due to (a) restriction of antibiotics diffusion by polymeric matrix, (b) interaction of the biofilm matrix with antibiotics that can retard and lower their activities, (c) action of the modifying enzymes such as β-lactamases or aminoglycoside adenylyltransferases, (d) alteration in metabolic activity inside the biofilm (chemical microenvironment), (e) genetic modifications of target cells or camouflaging the target sites, (f) slow growth rates of bacteria in which drugs are not effective, (g) generation of persister cells, which are tolerant to different antibiotics, (h) multiple microbial species, (i) extrusion of antibiotics using efflux pumps, and (j) the age of the biofilm. Thus, this multifactorial nature of bacterial biofilm formation and antimicrobial resistance impose great challenges for the adaptation of conventional antibiofilm therapeutic strategies.[19,24]

Bacteria–Phage Co-Interaction Within the Biofilm

Bacteriophages are considered to be the most abundant microorganisms on earth with numbers reported to be 10 times more than their bacterial hosts.[37] Two types of phages exist depending on the life cycle: lytic or virulent phages, which exhibit lytic cycle by replicating their genome and subsequent release of assembled progeny phages causing rapid cell destruction and lysis of the host cells; and temperate or lysogenic phages, which exhibit lysogenic cycle by persisting as prophages within the genome of the host bacteria to achieve a co-existing state. The later cycle may undergo the lytic cycle following induction by environmental stimulus.[38]

As natural enemies of bacteria, phages are perfectly adapted to destruct biofilms using different mechanisms such as by degrading the extracellular matrix, penetrating the biofilm and infecting the bacteria (Figure 2). One of such mechanisms is the stimulation of the host bacteria to produce EPS-degrading enzymes. These host induced enzymes breakdown the rich polysaccharides and proteins within the extracellular matrix to facilitate phage penetration, replication, and elimination of the bacteria present in various metabolic states via lytic activity.[5] In addition, phages can express enzymes with exopolysaccharide degrading activities (polysaccharide depolymerases) that degrade extracellular polymers by digesting the polysaccharide matrix and proteins in the biofilm that surrounds the bacteria as well as polysaccharide forming capsules and lipopolysaccharides. This process clears the bacterial protection barrier and then allows the entry of phage particles into the biofilm to replicate within the bacteria.[27] High rates of phage replication occur given the high densities of bacteria in the biofilm structure. Phage induced bacterial lysis results in the release of progenies as they achieve local lysis of susceptible cells and as associated enzymes weaken the bacterial cell wall and degrade EPS within the biofilm.[39] Lytic phages retain lytic activity against persister cells with reduced metabolic activity. Lysogenic phages can also integrate into the bacteria genome causing the bacteria to float naturally without adhering to surfaces to initiate the formation of mature biofilms.[27]

Biofilms defensive mechanisms can resist phage infection by affecting phage adsorption, penetration, diffusion, and proliferation within the formed biofilms. Factors such as structure and thickness of the biofilm matrix, age of the biofilm, physiological heterogeneity within the biofilm, and the bacterial species or strains that form the biofilm in multispecies state, may limit phage infection and activity of the biofilm.[40] Another interesting mechanism to prevent infection of phages is to specifically recognize the nucleic acids of the phages and destroy them.
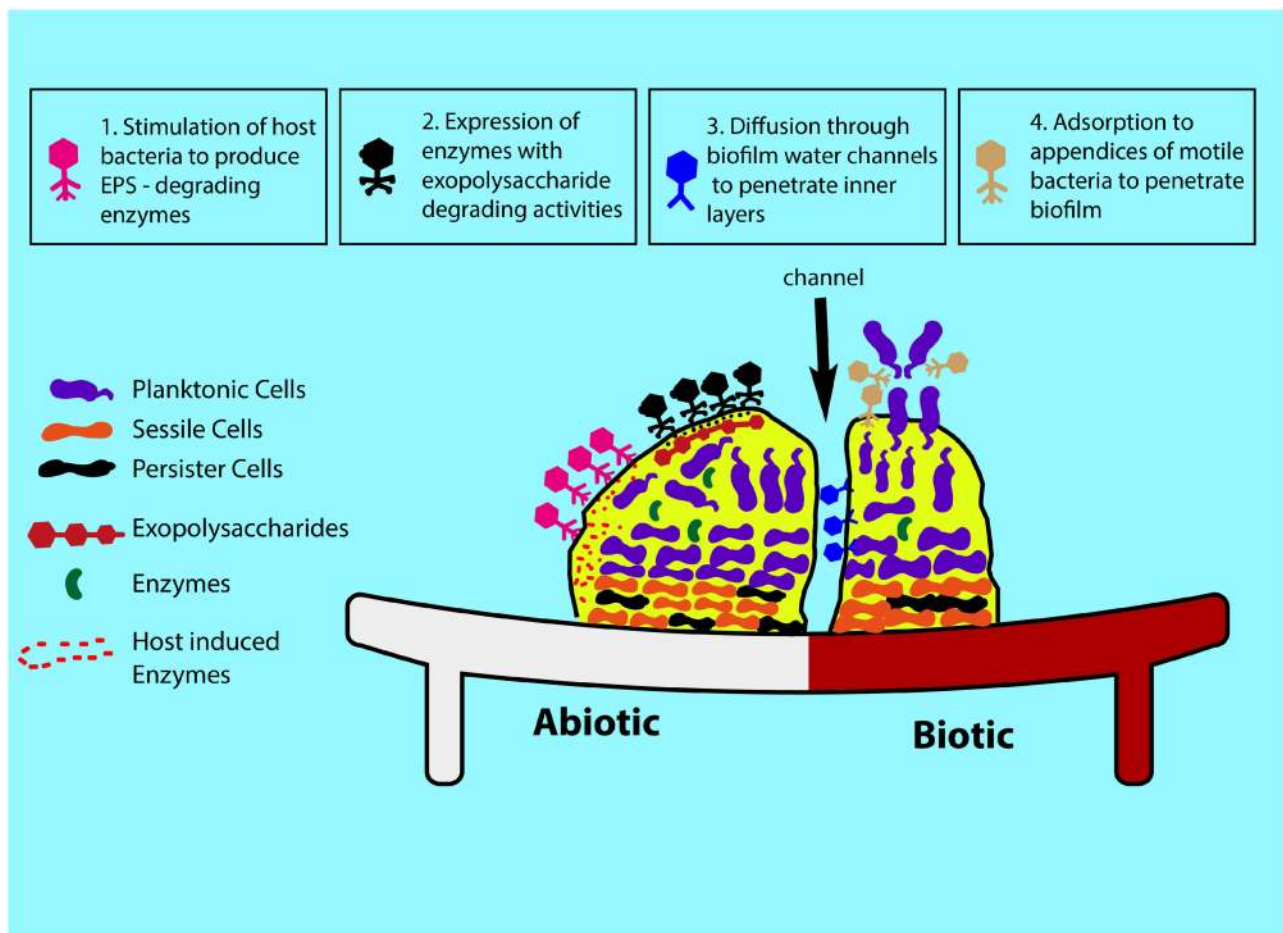
Figure 2 Schematic representation of phage mechanisms of biofilm destruction.

Bacteria use restriction-modification (R-M) system, defense island system associated with restriction modification (DISARM), prokaryote argonaute proteins (pAgos) and clustered regularly interspaced short palindromic repeats (CRISPR) – Cas9 to prevent phage infection.[41] As a final barrier to phage infection, the bacteria can use an abortive infection system that leads to the death of the infected cell, preventing the spread of phages through the community.[27]

To overcome these resistance mechanisms, phages have developed several strategies, however. Phages are equipped with specific enzymes such as hydrolases, endolysins and depolymerases to overcome the structure, thickness, composition, and age of the biofilm with associated matrix as well as the bacterial cell structural parts.[42] Phages can diffuse through biofilm water channels and penetrate the inner biofilm layers.[43] Phages can also adsorb reversibly to the appendices of motile bacteria to penetrate inside the biofilm.[44] Phages can tackle persister cells through the release of

intracellular material which triggers the metabolism of the persister cells for phage infection and replication.[2] Strategies by which phages escape the bacterial immune systems include the potential escape from R-M systems by lacking the endonuclease recognition site throughout the genome, acquisition of point mutations in the spacer sequence and production of anti-CRISPR protein which interferes with the system to escape the CRISPR/Cas9 system.[45]

The interaction of phages with bacteria is often seen as an antagonistic co-evolutionary cycle. The presence of phages may contribute to active biofilm formation as eDNA release through phage-mediated cell lysis by prophages, is responsible for horizontal gene transfer, which triggers stringent response of stabilizing the biofilm matrix.[46,47] Phage interaction with bacteria may cause changes in the biofilm matrix leading to enhanced biofilm adhesion, virulence, dispersion of biofilms, colony variation and antibiotic tolerance.[48] Owing to the co-evolution mechanism, phages are thus

seen as actively involved in biofilm formation either as promoting or destructing agents.[49]

Application of Phages in Bacterial Biofilm Destruction

The tolerance of bacterial biofilms to antibiotics and host immunity has resulted in the search for alternative methods against bacteria in biofilms as well as antibiotic-resistant strains. Phages and phage-derived products have triggered scientists as the most important alternative to antibiotics in preventing and treating biofilms and associated infections. However, development of efficient phage-based treatments requires a deeper understanding of bacteria resistance to phages and co-evolutionary mechanisms between phages and bacteria, to minimize the likelihood emergence of resistance. Phage-based treatments for bacterial biofilm destruction includes the use of mono phages, phage cocktails, genetically engineered phages, and phage-derived enzymes.[43] Some of the most recent application of phages and phage-derived products in bacterial biofilm destruction are summarized in this section (Figure 3).

Application of Mono Phages

Application of mono phage in bacterial biofilm destruction involves the use of naturally occurring strictly virulent or lytic phages that do not encode genes for virulence, toxins or AMR. In addition, the phage should not be able to mediate horizontal gene transfer or transduce infected bacterial cells. Single phages usually have narrow host range as they are generally specific for a limited set of strains of the same bacterial species. Their applications as therapeutic and biocontrol agents in clinical, veterinary, environmental and food bacterial isolates are enormous and encouraging.[50] Recently, phages PSTCR4 and PSTCR6, as part of 17 characterized novel phages, exhibited efficient reduction of well-established *P. stuartii* biofilms formed in catheter models. In addition, the phages showed killing capabilities in solid and liquid cultures in various patterns and levels of effectiveness. The phages, found to be free of virulence factors and resistance genes, suggest their potential as reliable therapeutic agents for phage therapy against *P. stuartii* biofilms in bacteriuric catheterized patients.[51] In studying oral biofilm elimination as one of the main targets for caries prevention, phage SMHBZ8, isolated from human saliva samples, showed effective prevention of biofilm and reduction of existing biofilm of *S. mutans* in biofilm cultures and cariogenic dentin model.[52] In a similar study, individual phages isolated from sewage samples prevented biofilm formation of *S. mutans* up to 97% by inhibiting the expression of genes involved in biofilm production.[53] Both studies suggest that mono phages may be possible candidates of phage therapy
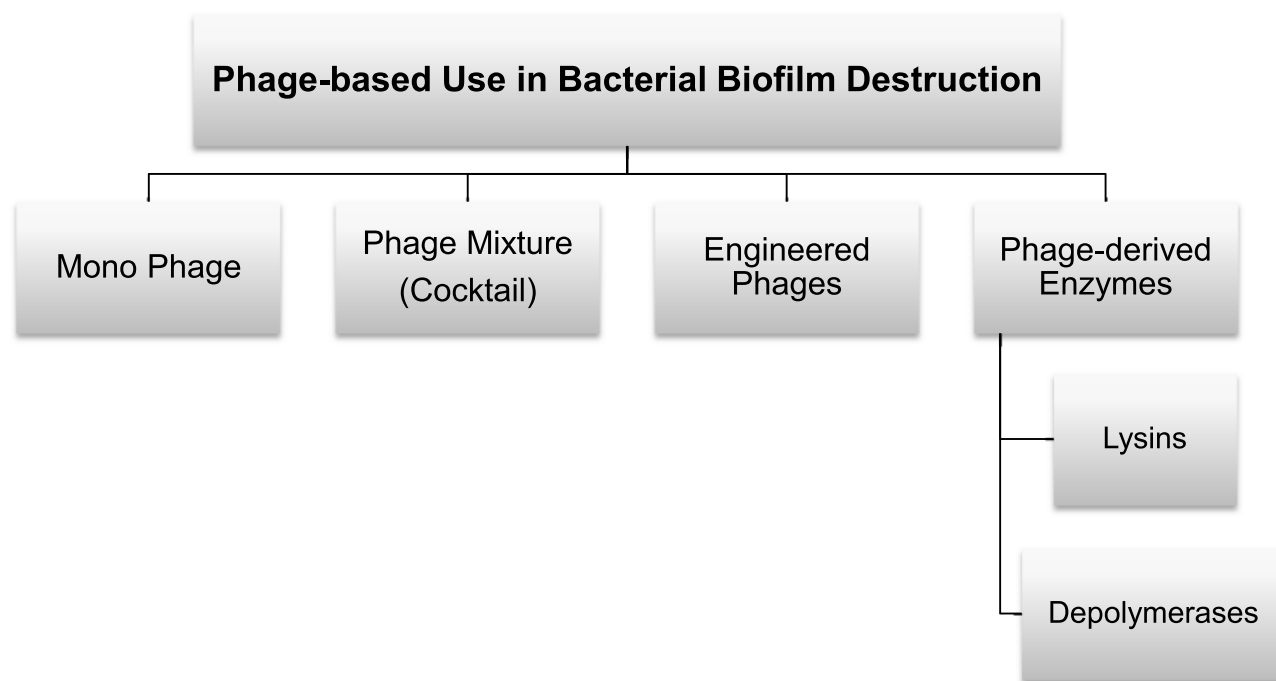

Figure 3 Phage-based treatment options in bacterial biofilm destructions.

for dental caries. To evaluate the application of phages in thermal and industrial environments, the effectiveness of single phages was assessed in biofilms of *P. aeruginosa* isolated from drinking and thermal water. The phages showed a maximum reduction of biofilms established on a stainless-steel coupon between treated and untreated surfaces.[54] Interestingly, in a study on biofilms of methicillin resistant *S. pseudintermedius*, a zoonotic pathogen isolated from canine and veterinary workers, low concentrations of phages pSp-J and pSp-S applied, inhibited biofilm formation.[55] Numerous mono phages have been applied to significantly inhibit or decrease to minimum levels of viable bacteria cells in biofilms and in liquid cultures with no considerable toxicity to mammalian cells. Notable among them are biofilms formed by *E. faecalis*,[56] *S. aureus*[57] and *E. coli*,[58] all belonging to the ESKAPEE group. As revealed in these studies, anti-biofilm effects of lytic mono phages are concentration-dependent. Too high concentration of phages can interfere with the destruction of biofilm, and too low concentration of phages may not be adequate to infect and penetrate the biofilm. Therefore, treatment time is a major factor for bacterial biofilm destruction rather than the concentration of phages and so the appropriate concentration of phages should be applied according to the usage.[55]

Application of Phage Cocktails

Phage therapy is commonly applied in the form of phage mixture or cocktail targeting either mono or several bacterial strains. The logic behind the use of phage cocktails arises from the fact that simultaneous treatment targeting a variety of bacterial receptors with diverse antibacterial pathways will more efficiently decrease the bacterial burden, expand host range coverage, lysis potential, mitigate resistance or development of lysogenic strains.[59] Simultaneous application of phages as found in several study models show greater efficacy in biofilm destruction than mono phage application.[55,58]

In a recent study, phage cocktail composed of four lytic phages, completely inhibited the growth of MDR *E. coli* and significantly prevented the development of biofilms compared with single phages. The phage mixture caused strong biomass reduction of biofilm and showed the highest biofilm inhibition up to nearly 87%.[60] To broaden lytic spectra and increase the efficiency of therapeutic phage mixture, phages with and without polysaccharide degrading enzymes should be included in cocktails preparation. A study reported that a combination of 4 phages lysed all

studied *K. pneumoniae* strains, although one of the phages lacked genes encoding polysaccharide depolymerases involved in degradation of the biofilm matrix.[61] Similarly, Phages ΦKpnM-vB1, ΦKpnP-vB2 and ΦKpnM-vB3 isolated and characterized, did not only have lytic activity on *K. pneumoniae* and *E. coli* strains, but also, were highly efficient in reduction of Klebsiella biofilms when applied as a cocktail.[62] In a *Galleria mellonella* infection model, co-incubation of monophage Sb-1 and PYO bacteriophage, two commercially available phage formulations, eradicated MRSA biofilm and prevented biofilm formation by completely abolishing heat production of MRSA.[63] Unlike mono phage applications which are mostly lytic phages, phage cocktail could be a mixture of only lytic phages, temperate phages or both. Four temperate phages Trsa205, Trsa207, Trsa220 and Trsa222 in a cocktail, was capable of removing 65% of *S. aureus* biofilms, in addition to lysing two-thirds of the isolates.[64] Due to their broad host range coverage, phage cocktails are effective on mixed-species biofilms. For example, phage cocktails AB-SA01 and AB-PA01 which target *S. aureus* and *P. aeruginosa*, respectively, when combined together, significantly reduced biofilm biomass in mixed-species biofilms, compared to the respective phage cocktail treatment.[65] A mixture of two phages, philPLA-RODI and philPLA-C1C, showed a reduction in the amount of adhered bacterial cells to about 2 log units in both mono-species and dual-species biofilms of *S. aureus* and *S. epidermidis*.[66] These studies suggest that application of phage cocktails other than individual phages in biofilm models, is highly efficient at destroying bacterial biofilms.

Application of Genetically Engineered Phages

Phages which lack enzyme-encoding genes are genetically engineered to express degradation enzymes for adsorption, penetration and diffusion of the phages through the EPS-matrix for biofilms destruction.[67] For example, a modified T7DspB *E. coli* phage has been designed to express intracellularly a hydrolase that is released during infection as well as to the extracellular matrix enhancing biofilm degradation. Testing on *E. coli* biofilms showed the efficient expression of biofilm dispersing (DspB) enzyme during phage infection, causing biofilm degradation rate of about 99.997% and a decrease in the population of viable bacterial cells in the biofilm by 4.5 orders of magnitude, around 100 times higher than the efficiency of the parent T7 phage.[68] Similarly, the construction of

bacteriophage T4 Rnl1 exerts antibiofilm activity instead of bactericidal activity against *S. mutans* with a decrease in biofilm biomass resulting from scattered microcolonies and larger channels surrounded by minor or reduced exopolysaccharides.[69] An engineered T7 bacteriophage encoded with a lactonase enzyme when constructed, expressed the AiiA lactonase to effectively degrade AHLS from many bacteria. Addition of the engineered phage to mixed-species biofilms of *E. coli* and *P. aeruginosa* inhibited biofilm formation.[70] Some temperate phages with non-lytic features are genetically engineered into lytic phages with the production of endolysins useful for biofilm destruction and removal.[71] With about half of the sequenced bacteria being lysogens, temperate phages could be easier to find than isolating lytic phages from nature.[72]

In a recent study, endolysin PM-477 of the type 1,4-beta-N-acetylmuramidase encoded on *Gardnerella* prophages when recombinantly expressed, demonstrated strong bactericidal activity against four different *Gardnerella species*. By domain shuffling, several engineered endolysins with 10-fold higher bactericidal activity than any wild-type enzyme was generated. When tested against a panel of 20 *Gardnerella* strains, the most active endolysin, PM-477, showed minimum inhibitory concentrations and had no effect on beneficial lactobacilli or other species of vaginal bacteria. Furthermore, when tested on vaginal samples of fifteen patients with either first time or recurring bacterial vaginosis, PM-477 killed the *Gardnerella* bacteria in thirteen of the cases and physically dissolved the biofilms without affecting the remaining vaginal microbiome. The high selectivity and effectiveness in eliminating *Gardnerella*, both in cultures of isolated strains as well as in clinically derived samples of natural polymicrobial biofilms, makes PM-477 a promising alternative to antibiotics for the treatment of bacterial vaginosis, especially in patients with frequent recurrence.[73] Such manipulation of phage genetic material for effective destruction of bacterial biofilms from biotic or abiotic surfaces may become a feasible option of the 21st century.

Phage-Derived Enzymes
Some enzymes encoded with phages maybe useful for treating bacterial infections and biofilms. Under current safety standards and regulations, the application of phage products is easier than use of the phage itself. In relation to this, two main types of phage degradation enzymes are

useful in the removal of biofilms: lysins and depolymerases.

Application of Lysins
Lysins are peptidoglycan hydrolases encoded by Gram-positive infecting phages and include muramidase, transglycosylase, glucosaminidase, amidase, and endopeptidase. The application of endolysins against Gram-negative pathogens is impaired by the presence of a protecting outer membrane layer, however, the combination with membrane permeabilizers turned out to significantly improve lysin efficiency.[74,75] More recently, genetic engineering has allowed the design of lysin/cationic peptide combination called Artilysins; the lysin/bacteriocin version to obtain Lysocins, and the lysin/phage receptor binding proteins to generate Innolysins, as an anticipated promising strategy.[2]

In terms of antibiofilm activity, phage lytic proteins offer interesting properties. In a recent study on investigating the combination of a phage-derived lytic protein, CHAPSH3b, and the virulent bacteriophage phiIPLA-RODI, the results showed that synergy exist between both antimicrobials for the removal of *S. aureus* biofilms, with greater reductions in viable cell counts observed when phage and lysin were applied together compared to the individual treatments. Time-kill curves and confocal microscopy revealed that the fast antibacterial action of CHAPSH3b reduces the population up to 7 hours after initial exposure, which is subsequently followed by phage predation, limiting regrowth of the bacterial population.[76] The amidase domain of phage vB_LmoS_293 was shown to inhibit biofilm formation of *L. monocytogenes* on abiotic surfaces. *S. pyogenes* biofilm matrix refractory to conventional antibiotics were readily destroyed by endolysin PlyC with minimum biofilm eradication concentration values of two and four orders of magnitude lower by mass and molarity respectively, than the conventional antibiotics.[77] Potent antibiofilm agent, lysin CF-301, removed *S. aureus* and mixed-species biofilms formed on polystyrene, glass, surgical mesh, and catheters, with an improvement in antibiofilm activity when combined with cell wall hydrolase lysostaphin.[78] Similarly, phage endolysin LysCSA13 when applied, showed high efficacy in removing staphylococcal biofilms on various surfaces, including polystyrene, glass, and stainless steel, with about 80–90% decrease in biofilm mass.[79] In a study involving Gram-negative bacteria, the endolysin of *A. baumannii* bacteriophage D2, Abtn-4, was found to have broad antimicrobial activity against MDR

S. aureus, P. aeruginosa, K. pneumoniae, Enterococcus and *Salmonella* in the absence of outer membrane permeabilizers. Abtn-4 had the ability to reduce biofilm formation and showed antimicrobial activity against phage-resistant bacterial mutants.[80] With their activities independent of the bacterial physiological state, lysins are relevant for biofilm removal especially phage-resistant bacteria.

Application of Depolymerases

Phage depolymerases are proteins encoded in the region of structural genes in a phage genome which recognize, bind, and digest the polysaccharide compounds of bacterial cell walls. EPSs are mainly responsible for the structural and functional integrity of bacterial biofilms and have an influence on their virulence. Interestingly, Gutiérrez et al[81] applied the EPS depolymerase Dpo7, derived from bacteriophage vB_SepiS-phiIPLA7, against staphylococcal biofilms. The study revealed that over 90% of biofilm-attached cells were removed by Dpo7 in all polysaccharide producer staphylococcal strains except polysaccharide-independent biofilm formed by *S. aureus* V329. Additionally, the pretreatment of polystyrene surfaces with Dpo7 resulted in the reduction of biofilm biomass by 53–85% in two-thirds of the tested strains. EPS depolymerase Dpo7 has the ability to inhibit biofilm formation and can also disperse biofilms generated by different strains of *S. epidermidis* and *S. aureus*.[81]

Recently, the enzymatic activity of a capsular polysaccharide depolymerase TSP of phage φAB6, to degrade *A. baumannii* biofilm, showed significant inhibition of biofilm formation and degradation of formed biofilms. Additionally, TSP inhibited the colonization of *A. baumannii* on the surface of Foley catheter sections, indicating that it can be used to prevent the adhesion of *A. baumannii* biofilms to medical device surfaces.[82] In another study, recombinant Dep42, a putative tail fiber protein with depolymerase activity from bacteriophage SH-KP152226, showed specific enzymatic activities in the depolymerization of the K47 capsule of *K. pneumoniae* and significantly inhibited biofilm formation and/or degrade formed biofilms. The study also showed that Dep42 could enhance polymyxin activity against *K. pneumoniae* biofilms when used in combination with antibiotics, suggesting that combination of identified novel depolymerases encoded by the phages with antibiotics may represent a promising strategy to combat infections caused by MDR and biofilm-forming bacteria.[14]

Apart from their individual activities as antibiofilm agents, effective removal of biofilms can be achieved with a combination of lysin and depolymerase. In a study on investigating the efficacy of endolysin LysK and poly-N-acetylglucosamine depolymerase DA7 against staphylococcal biofilms, in addition to LysK and DA7 removing static and dynamic biofilms from polystyrene and glass surfaces at low micromolar and nanomolar concentrations respectively, a combination of the enzymes significantly reduced viable cell counts compared to individual enzyme treatment.[83] Besides their ease of application, phage enzymes can offer to serve as potential new candidates of antibiofilm agents and antimicrobial drugs (enzybiotics) more than live phages particularly in the advent of phage-resistant bacteria.

Application of Phages in Combination with Other Alternative Antibiofilms

Sometimes, phage therapy as sole antibiofilm agents to destroy bacterial biofilms is inadequate. So far, other than phage therapy, several novel approaches against bacterial biofilms have been proposed.[84] These include surface modification or coating methods,[85] the use of anti-matrix agents such as enzymes (DNase I or dispersin B)[3,86] and chelators of divalent cations,[87] the use of naturally occurring antimicrobial peptides[88,89] or their synthetic derivatives,[90] and the use of QS inhibitors such as curcumin,[91] quercetin,[92] halogenated furanone compounds,[93] RNA-III-inhibiting peptide, ginseng extract, garlic extract, and pyrrhocoricin or microcin B17.[94] Others include nanotechnology with the use of metal-based nanoparticles (silver, gold, titanium, copper or zinc), green nanoparticles and polymer-based nanoparticles,[21,95] physical methods such as scrubbing and flushing, disinfection methods using chemical alkali-based and acid-based agents, ethanol, chlorine dioxide, or hydrogen peroxide,[94] use of monoclonal antibodies,[96,97] and antimicrobial photodynamic therapy (PDT) using light-activated photosensitizers that generate cytotoxic species such as reactive oxygen species (ROS).[98,99] To completely eradicate bacterial biofilms, a simultaneous or sequential combination approach of phage therapy with other alternative antibiofilm agents is recommended. The combination therapy is composed of phages and/or phage-derived enzymes with nanoparticles, chemical compounds, antimicrobial peptides, and disinfectants (Table 1).

Table 1 Examples of Combination of Phages or Phage-Derived Products and Antimicrobials Applications Against Bacterial Biofilm Formation

Phage/Phage-Derivative	Antimicrobial Agent Used	Biofilm Type	Results	Reference
Environmental phage-based cocktail	Antibiotics (Ciprofloxacin, sulfamethoxazole/trimethoprim, Gentamicin, Tobramycin, Meropenem, Imipenem)	*Acinetobacter baumannii* in a human urine model	Reduction of biofilm biomass and clearance of persister cells	[100]
Bacteriophage Brsv	Amikacin	*Proteus mirabilis* 3059	Eradication of biofilm	[101]
Commercially available phages Sb-1 and PYO	Ciprofloxacin	Dual-species of *S. aureus/P. aeruginosa*	Complete eradication of dual-species biofilms	[102]
Phage EFLK1	Vancomycin	Vancomycin-resistant *Enterococcus faecalis*	Reduction of biomass by 87%	[103]
Phage vB_PaeM_P6	Ciprofloxacin	*P. aeruginosa*	Inhibition of biofilm	[104]
Phage E79	Aztreonam lysine	*P. aeruginosa* PA01	Reduction in biofilm growth over 3-fold	[105]
Phage-encoded endolysin LysP108	Vancomycin	Methicillin-resistant *S. aureus* XN108	Inhibition of biofilm	[106]
Bacteriophage (Xccϕ1) - hydroxyapatite complex	Saturated long – chain fatty acids	*Xanthomonas campestris* in a flow cell system	Removal of biofilm	[107]
Phages KPO1K2	Divalent Co (II) ions	*K. pneumoniae* B5055	Significant reduction in biofilm	[108]
Mixture of RNA bacteriophages	Chorine	*P. aeruginosa* in a continuous flow system	Removal of biofilm by 97 ± 1%	[109]
Phage EC3a	Honey	*E. coli*	Removal of biofilm	[110]
phage ϕ44AHJD	Green synthesized silver nanoparticles	*S. aureus*	Rapid dispersion of biofilm	[111]
Phage-borne depolymerase	Chlorine dioxide	*Klebsiella spp*	Elimination of biofilm by 92%	[112]
T7Select phage	Antimicrobial peptide 1018	*E. coli*	Eradication of biofilm	[113]
P22 phage	EDTA and nisin	*Salmonella Typhimurium*	≥ 93.2% inhibition and 70% reduction of biofilm	[114]
Phage SA46-CTH2	Nisin	*S. aureus*	Reduction in biofilm	[115]
Phages PN05 and PN09	Carvacrol	*P. syringae* pv. *actinidiae*	Prevention of biofilm regrowth	[116]

The combination therapy is mainly based on the use of phages and/or phage-derived products with antibiotics. The synergistic actions of mono phages, phage cocktails, or phage-derived enzymes with antibiotics have revealed successful results. The development of phage-based therapy combined with antibiotics might be an advantageous weapon in the arms race between human and MDR or phage-resistant bacteria. In a study by Henriksen et al,[117] single phage treatment of *P. aeruginosa* PAO1 early biofilm stage reduced up to 98% of biofilm biovolume.

However, repeated phage treatment increased the biovolume and remained unaffected by the phage treatment, until a combination treatment of phages with ciprofloxacin caused a significant reduction in the abundance of cells in the biofilm.[117] The combination treatment of phage PEV20 with ciprofloxacin in another study enhanced biofilm eradication compared with single treatments.[118] Likewise, a combination treatment of phage Sb-1 with antibiotics exerted a synergistic effect in eradicating MRSA biofilm[119] while similar results have been reported in other studies.[120,121]

In the application of phages with antibiotics, consideration must be given to the order of therapeutic administration, as well as nature of their interactions, as these factors may affect the outcome of the therapy. However, to achieve optimum synergy, phage treatment must precede antibiotics, as demonstrated in these studies.[122,123] Papaianni and his team[107] applied a combinatorial antibiofilm strategy based on the use of fatty acids and the bacteriophage Xccϕ1-hydroxyapatite complex optimized against *Xanthomonas campestris* mature biofilm. The synergic action of these elements was demonstrated and the efficient removal of *X. campestris* mature biofilm was also proven in a flow cell system, making the proposed approach an effective solution to enhance plant survival in *X. campestris* infections.[107] Manoharadas and his associates[111] demonstrated a concerted action of green synthesized silver nanoparticles and bacteriophages in removing pre-formed *S. aureus* biofilms from an inert glass surface in a time dependent manner. The results demonstrated, for the first time, the rapid cooperative dispersion of the bacterial biofilm. In addition, the synergistic activity of the nanoparticles and bacteriophages cause the loss of viability of the biofilm entrapped bacterial cells thus preventing establishment of a new infection and subsequent colonization. This work further opens up a platform for the combinational therapeutic approach with a variety of nanoparticles and bacteriophages against mono or poly bacterial biofilm in environmental, industrial or clinical settings.[111] Using these combinational therapeutic strategies depends on the intention of preventing or treating biofilms related to device-associated nosocomial infections and biofilms formed on native host tissues. Unfortunately, not all of the alternative strategies can be used to treat infections associated with biofilms formed in a patient's body, for example, surface coating metals, disinfectants, chemical compounds, and mixtures.

Application of Phages as Vehicles to Deliver Antibiofilm Agents

Phages, in addition to their lytic potential and antibiofilm activities, can be modified to be used as delivery vehicles of cargos such as nucleic acids, nanomaterials, therapeutic drugs and diagnostic probes.[67,124] In such cases, temperate phages may be of interest for delivering programmable DNA nucleases associated with CRISPR to reverse antibiotic resistance and destroy plasmids that confer antibiotic resistance.[125] In this current view, phages can also be modified to deliver antibiofilm agents for biofilm destruction. For example, an injectable hydrogel capable of encapsulating *P. aeruginosa* phage and delivering active phage to the site of bone infections was engineered. While retaining the bacteriolytic activity after encapsulation and release from the hydrogel, phage-encapsulating hydrogels, apart from effectively killing host bacteria in both planktonic and biofilm phenotypes in vitro, achieved a 4.7-fold reduction in live *P. aeruginosa* counts at infection site of murine radial segment infected with *P. aeruginosa*, compared to phage-free hydrogels at 7 days post implantation.[126] In another study, a rare bacteriophage was used as a green route to synthesize gold nanoparticles (AuNPs). In addition to showing antibacterial activity against different bacterial pathogens, phage inspired AuNPs inhibition of about 80% of *P. aeruginosa* biofilms was reported.[127] In using magnetic phage-nanocomposite conjugates (PNCs) to target bacteria in biofilm inner layers for bottom-up eradication, phages PEB1 or PEB2 were covalently conjugated with magnetic colloidal nanoparticle clusters of different sizes. Both small and large PNCs dispersed phages evenly throughout the biofilm bottom, significantly disrupting the biofilm bottom layer and detaching the biofilm within 6 hours, with efficient biofilm removal for dual and multi-species biofilm.[128] Phages with broad host range are also conjugated with magnetic colloidal nanoparticle clusters to facilitate biofilm penetration and subsequent removal in established biofilms.[129] With the continuous evolution of bacteria, such manipulation of phages with nanoparticles, may more than ever, be needed now in the fight against biofilm forming and/or their associated human infections due to antimicrobial resistant bacteria.

Conclusions

Naturally, most bacteria live in the form of biofilms. Biofilms occurrence on medical device surfaces and human tissues, as well as their tolerance to antibiotics, disinfectants and host immunity, brought them to burden in human lives. As a niche not well covered by currently available antibiotics alone,

bacterial biofilms necessitate the search for alternative approaches to completely destruct them. Several novel approaches against bacterial biofilms such as nanotechnology, irrigation techniques, surface modification methods and use of natural peptides have been proposed. Nonetheless, phage-based treatments which include the application of mono lytic phages, phage cocktails, engineered phages, and phage-derived enzymes, appear to be effective in destroying bacterial biofilms. Higher efficacy was, however, perceived with a combination therapy of phages or phage-proteins and other alternative antibiofilm agents, as well as phages conjugated with nanoparticles, as delivery vehicles of these antibiofilm agents. To completely eliminate bacterial biofilms, it is recommended that;

(I) more knowledge on mechanisms of phage application is still needed to increase world acceptance of phage-based therapies.

(II) future research should aim to broaden the scope of application of phages by promoting engineered phages as well as their enzymes with other alternative agents such as, nanoparticles and antimicrobial peptides,

(III) future research should also aim to broaden the scope of application of phages by promoting modified phages as delivery vehicles of other antibiofilm agents, and

(IV) efficacy and safety protocols should be prepared to develop an established phage-based method to eliminate biofilms.

Acknowledgment

Disclosure

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