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Design and Assessment of Anti-Biofilm Peptides: Steps Toward Clinical Application

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Abstract
Highly antibiotic resistant, microbial communities, referred to as biofilms, cause various life-threatening infections in humans. At least two-thirds of all clinical infections are biofilm associated, and antibiotic therapy regularly fails to cure patients. Anti-biofilm peptides represent a promising approach to treat these infections by targeting biofilm-specific characteristics such as highly conserved regulatory mechanisms. They are being considered for clinical application and we discuss here key factors in discovery, design, and application, particularly the implementation of host-mimicking conditions, that are required to enable the successful advancement of potent anti-biofilm peptides from the bench to the clinic.

Biofilms: Environmentally Shaped and Phenotypically Diverse

In their natural habitats, microorganisms predominantly grow in biofilms, which are highly structured communities embedded in a self-produced matrix [1–5]. The formation of biofilms is considered to be an adaptive stress response ensuring survival in rapidly changing environments [3–6]. Triggered by external attack, challenging physical conditions or nutrient limitation, planktonic cells undergo major gene expression changes regulated by several interconnected networks [4, 6]. Even though these regulatory networks are poorly understood, there is evidence suggesting nucleotide signaling pathways, stress adaptations, and two component regulators play crucial roles [1, 2, 4, 7].

The biofilm growth mode follows a developmental sequence of multiple stages [1, 2, 4]: Initially motile cells attach reversibly to a surface before developing into microcolonies, which become effectively irreversibly bound due to the production of an extracellular matrix containing polysaccharides, proteins, and extracellular DNA (eDNA). Over time these cell aggregates can grow to develop mature biofilms with highly variable architecture from surface coatings to 3D-structured colonies. Mature biofilms can at least partially disperse, releasing planktonic cells to colonize suitable new environments and reinitiate the biofilm-lifecycle. Intriguingly, research has shown that the architecture of mature biofilms depends not only on the microorganism but also on the environment in which it is formed [8]. The human host offers highly diverse environments in which biofilms can thrive.

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Clinically Relevant Biofilms

Biofilms can form on various biological surfaces or medical devices in the human body and are associated with at least two-thirds of all clinical infections [1, 2, 4, 5]. Medical device-related infections involve, for example, implants, catheters, and prosthetics [3, 4]. Biological surfaces in the human body prone to biofilm infections include among others teeth, bones, skin, upper respiratory tract, ears, lungs, and urinary tract [4]. Furthermore, biofilm-like aggregates residing unattached in mucus layers, in abscess infections, or even intracellularly within host cells have been reported in several studies [4, 5]. The complexity increases even further when taking into account that clinically relevant biofilm infections are usually polymicrobial [1, 3, 5, 9]. Since biofilm growth is accompanied with a substantial increase in adaptive resistance (up to 1,000-fold) to essentially all conventional antibiotics, the treatment of these infections is severely challenging for physicians [2–4, 6]. As a result of the complete lack of selective anti-biofilm drugs in the clinic, treatment is rather aggressive and involves either the removal of an implanted medical device, surgical debridement, or administration of stringent antibiotic combination therapies [2–4]. Innovative treatment strategies specifically targeting biofilms are urgently needed, and peptides, both natural and synthetic, represent a promising class of anti-biofilm compounds [1, 4, 10].

Origin and Characteristics of Anti-Biofilm Peptides

Anti-biofilm peptides are a subset of host defense peptides (HDPs), also referred to as antimicrobial peptides (AMPs) [3, 11]. HDPs are evolutionarily conserved in all kingdoms of life and act as a first line of defense against invading pathogens [11]. Accordingly, HDPs can exhibit a wide variety of different activities, sometimes even within the same small peptide [11, 12]. Most of these activities provide direct (anti-biofilm, antimicrobial) or indirect (immunomodulatory/anti-inflammatory) protection against pathogens such as bacteria, fungi, viruses, and parasites [11, 12]. Despite their diversity in origin and function, many natural HDPs share common features [3, 11, 12]. Typically the sequence is composed of 12–50 amino acid residues. Their structure is associated with a high proportion of hydrophobic residues (~50%) and a positive net charge of +2 to +9 resulting from the presence of multiple cationic residues (Arg/Lys) [3, 11], allowing them to fold into amphipathic structures, often upon membrane interaction. Nevertheless, HDPs vary remarkably in their specific amino acid composition and primary structure. Identification of new HDP sequences from natural sources represents a major research focus in the search for anti-biofilm compounds [11]. The AMP Database (ADP) currently harbors more than 2,900 natural peptides, but only 33 are classified as anti-biofilm peptides (June-2018) [13].

The first identified anti-biofilm peptide was human cathelicidin LL-37, which is able to inhibit and disperse preformed *Pseudomonas aeruginosa* biofilms at one-sixteenth the minimal inhibitory concentration (MIC) [14]. The characterization of LL-37 as an anti-biofilm peptide encouraged the discovery and design of numerous peptides with anti-biofilm activity [11]. Whereas some of those peptides resemble conventional antibiotics and exhibit anti-biofilm activity at concentrations exceeding the MIC, other peptides, like LL-37, are active against biofilms at concentrations less than or equal to the MIC. This observation implies that anti-biofilm peptides target biofilm-specific characteristics rather than ubiquitous bacterial structures and suggests a mechanism of action distinct from antimicrobial activity against planktonic bacteria. We define an anti-biofilm peptide as one having selective activity against bacteria when growing in biofilms as opposed to the same free living (planktonic) bacteria.

Here, we highlight the potential of anti-biofilm peptides emphasizing the mechanism of action, peptide activity assessment in host-like conditions, design, synergy with other drugs, and delivery strategies for future clinical applications.

Mechanisms of Anti-Biofilm Peptides: Not Only Membrane Disruption

Most mechanistic studies have focused on membrane-disrupting properties that were originally proposed to explain antimicrobial activity against planktonic bacteria [11, 12]. The cationic residues within the AMP sequences initiate their passage through the bacterial cell envelope by electrostatic interactions with the anionic bacterial surface. When encountering the cytoplasmic membrane, the peptides bind to the anionic headgroups of phospholipids and then insert into the membrane and perturb its integrity, interfere with membrane-related functions (e.g., cell wall growth), or translocate across the membrane. To explain the disruption of cytoplasmic membrane integrity, the barrel-stave, torroidal-pore, aggre-

gate, and carpet models have been proposed and have been compared in detail [11, 12]. However, to date, there is little evidence that general membrane disruption is the mechanism of anti-biofilm action.

Biofilm-specific targets are slowly becoming increasingly recognized and investigated [15–19]. Original studies with LL-37 [14] indicated that this peptide acted by decreasing the attachment of bacterial cells, stimulating twitching motility, and influencing 2 major quorum sensing systems (Las and Rhl). However, these mechanisms were quite unsatisfying, since each of these processes (attachment, motility, quorum sensing) is quite distinct in other bacterial species. Study of the broad spectrum synthetic bactenecin derivatives 1018 and DJK-5 provided insights into the mode of action of anti-biofilm peptides [15]. The basis for this activity was found to be their ability to directly target the highly conserved stringent stress response through binding to and triggering the degradation of the signaling molecule ppGpp [16, 17]. The stringent stress response is activated in nutrient-limiting conditions and is considered to play an important role in bacterial biofilm formation and maintenance [1, 19]. In Gram-negative bacteria, intracellular ppGpp concentrations are controlled by 2 key enzymes: RelA, which is exclusively involved in synthesis, and SpoT, which is able to synthesize or degrade the signaling molecule [1, 19]. Recently, in vivo studies using a murine cutaneous abscess model of *P. aeruginosa* supported the peptide's effect on the stringent stress response pathway and demonstrated that treatment with DJK-5 and 1018 suppressed *spoT* promotor activity, consistent with the possibility that treatment with both peptides interferes with intracellular ppGpp homeostasis [19].

Other studies have shown that peptide P1 disrupts the architecture of *Streptococcus mutans* biofilm and can significantly decrease viable biofilm bacteria in an in vitro saliva-coated hydroxyapatite disc tooth model compared to a control peptide [15]. Although P1 can directly bind to bacterial cells, it shows no bactericidal activity. Therefore, the authors hypothesized that this peptide might impair secretion of and/or interaction between the extracellular polymers in the matrix [15]. Another study investigated eDNA, a common component of the biofilm matrix, as a potential target for two homologous fish HDPs piscidin-1 and piscidin-3, which carry C-terminal copper binding motifs [18]. Extraction of eDNA from *P. aeruginosa* biofilms treated with either of the peptides showed copper-dependent degradation of eDNA. Interestingly, based on previous studies using microbial and model membranes, both peptides seem to follow membrane-disrupting mechanisms, suggesting that peptides can act through multiple mechanisms [18].

Such mechanistic studies of anti-biofilm peptides emphasize their potential for clinical application. For example, targeting conserved regulatory pathways provides these peptides with extremely broad-spectrum anti-biofilm activity against multidrug resistant and clinically relevant Gram-positive and Gram-negative pathogens [11]. Unlike conventional antibiotics, these compounds have been shown to interfere with secondary messenger molecules such as ppGpp [15] and can have multiple mechanisms of action, which may decrease the possibility of resistance development in future therapies. More recently there is evidence that such peptides can also target fungal biofilms [20], but the basis for this activity is currently unknown.

In vivo Activity of Anti-Biofilm Peptides

In order to translate anti-biofilm peptides successfully from the bench to the clinic, efficacy in animal models of human diseases represents an important benchmark and allows a far more realistic prediction of a compound's activity shaped by pharmacokinetics and metabolism in the complex host environment [21]. In vivo activity of anti-biofilm peptides is most commonly tested in murine skin infection and wound-healing models [19, 22–25].

Synthetic bactenecin derivatives 1018 and DJK-5, which have potent in vitro anti-biofilm activity, do not clear murine cutaneous abscess infections of MDR Gram-positive and Gram-negative pathogens, but they visually reduce abscess size and peptide DJK-5 modestly affects bacterial burden [19, 23, 25]. Furthermore, the synthetic anti-biofilm peptide 3002 exhibits improved in vitro activity, whereas in vivo efficacy in the same infection model against methicillin resistant *Staphylococcus aureus* (MRSA) is not altered compared to its parental peptide 1018 [25]. Accordingly, the D-enantiomeric peptide D-BMAP18, that is highly active against *P. aeruginosa* and *Stenotrophomonas maltophilia* in vitro, was unable to treat pulmonary infections in mice and in bronchoalveolar lavage fluid [26]. Inconsistencies between in vitro and in vivo efficacy might be explained by physiological conditions within the host influencing stability and toxicity of anti-biofilm peptides and thus indirectly interfering with their activity. For instance, 1018 has been shown to precipitate in vitro in the presence of mucin and ions as well as when injected subcutaneously into mice [27, 28]. In another study, topical application of the rationally de-

signed peptide DRGN-1 significantly reduced wound size in a mixed biofilm-infected murine wound model [22]. In vitro and in vivo wound-closure activity supported the hypothesis that the peptide not only directly targets bacteria but also affects the host by modulating keratinocyte cell migration and proliferation [22].

Potent in vitro activity of anti-biofilm peptides does not necessarily translate into complete eradication of infections in vivo [19, 22–26]. The complex host environment is most likely able to affect in vivo efficacy by interfering with the activity, stability, and toxicity of anti-biofilm peptides. Additionally, potential immunomodulatory activities likely play an important role in vivo that cannot be evaluated when assessing anti-biofilm activity in vitro. Critically clinical development requires evidence of in vivo efficacy in animal models; therefore, research has to address these potential issues.

Design of Anti-Biofilm Peptides

Peptide design can be used to alter the sequences of peptides to provide excellent activity spectra while addressing potential issues such as stability and toxicity. Recently used strategies to optimize anti-biofilm peptides include alteration of the amino acid composition, creation of hybrid peptides, and the design of structurally and functionally related compounds referred to as peptidomimetics [22, 24, 25, 27, 29–33].

Alteration of the amino acid composition is by far the most studied approach and can be applied to modulate activity, stability, solubility/aggregation behavior, and cytotoxicity of anti-biofilm peptides [22, 24, 25, 27, 29, 33]. Using peptides with known sequences and functions as templates allows for the development of modified peptides with improved properties by testing various amino acid substitutions using systematic or random design principles [12]. For instance, Chung et al. [22] designed peptide DRGN-1, that significantly reduced wound size in a mixed biofilm-infected murine wound model, by rearranging only the first 2 N-terminal amino acids serine and proline. Both parental and altered peptides showed similar secondary structures and only minor differences in their interactions with the bacterial membrane. Therefore, the authors hypothesized that removing serine from the N-terminus interferes with a signal for proteolytic degradation, and thus spares the peptide derivative from degradation by the mitochondrial ClpXP system. In another study, substitutions of multiple amino acid residues in the sequence of LL-37-inspired peptide OP-145 resulted in the production of synthetic peptides with improved stability to plasma proteases [24]. Recently, the synthesis of a series of 1018 derivatives with amino acid substitutions, mainly in the hydrophobic stretch, allowed the investigation of sequence features associated with aggregation [11, 27]. The identification of peptide sequences with decreased aggregation tendency but similar immunomodulatory activity suggested that precipitation can be avoided by the rational design of peptides.

Due to the continuous increase in information on sequences and structures of effective anti-biofilm peptides, computational modeling strategies, such as quantitative structure-activity relationship (QSAR) modeling, have become increasingly attractive. Based on experimental data, QSAR models identify the relationship(s) between chemical structure and biological activity by defining molecular descriptors for each individual peptide sequence. These models can then be used to predict the anti-biofilm activity of peptide sequences in silico [11, 12, 25, 29]. Haney et al. [25] developed a QSAR model based on the experimentally evaluated in vitro anti-biofilm activity of 96 single amino acid variants of 1018 against MRSA. Subsequent synthesis and analysis of chosen peptides with predicted anti-biofilm activity showed 85% accuracy and resulted in the in silico identification and subsequent synthesis of anti-biofilm peptide 3002. This peptide exhibited potent in vitro anti-biofilm activity against MRSA at an eightfold decreased concentration compared to 1018 [25]. Rajput et al. [33] went a step further and used any literature that described chemical compounds inhibiting biofilms, including anti-biofilm peptides, to generate a QSAR model that demonstrated 80% accuracy in identifying the anti-biofilm activity of unknown chemicals. Furthermore, Sharma et al. [29] created 6 different models for the prediction of effective anti-biofilm peptides based on machine learning tools and implemented them in a predictive tool launched on the freely accessible webserver design Peptides Against Bacterial Biofilms.

An alternative approach to improve anti-biofilm peptides is through the design of hybrid peptides. Generally, hybrid peptides combine the functional domains of multiple peptides in order to increase activity and reduce cytotoxicity [31, 34]. Yu et al. [31] engineered the hybrid peptide TIH3F by fusing a truncated cathelicidin sequence with a trypsin inhibitor loop. While retaining very low in vitro cytotoxicity against human erythrocytes and human umbilical vein endothelial cells, the stability of the anti-biofilm peptide in the presence of 20% serum and physiological salt concentrations was significantly improved.

Peptidomimetics refer to compounds that mimic the structural features and/or biological activity of natural HDPs [35]. Accordingly, there are many possible modifications ranging from the incorporation of unnatural amino acids (of which more than 700 are known [36]) and peptides with altered backbones, to entirely different chemical structures. These modifications and structural differences are often able to resolve stability and cytotoxicity issues [30, 32, 35]. Raman et al. [30], for example, designed a β-peptide with anti-fungal and anti-biofilm activity against *Candida albicans,* which remained structurally stable and active in synthetic urine media. A peptidomimetic of 5 residues containing only arginine and biphenylalanine also showed anti-biofilm activity against multiple Gram-negative and Gram-positive bacteria, and exhibited very low toxicity to human erythrocytes and Vero monkey kidney epithelial cells, purportedly due to its short size and low hydrophobicity [32]. Conversely one study showed that enantiomeric peptides with D amino acids tended to have potent activity while being protease resistant [16, 17].

Peptide design and engineering is a powerful tool to produce anti-biofilm peptide sequences with increased activity and stability but decreased cytotoxicity under physiologically relevant conditions. Furthermore, engineering anti-biofilm peptides suitable for the treatment of specific polymicrobial biofilm communities in defined environments has been proposed as a strategy in order to overcome challenges in the human host [37]. In this regard, peptides have been shown to work against oral polymicrobial communities [38].

Synergistic Combination Therapy to Fight Biofilm Infections

Well-designed, anti-biofilm peptides on their own can have significant efficacy in vitro and in vivo [16–19, 23]. Many biofilms are accessible to topical treatment in the form of creams, aerosols, or direct application to sites that have been subjected to surgical debridement or drainage. Indeed this matches the known successful applications of peptides in clinical trials where evidence of efficacy via local treatment has been obtained [12, 39]. However, peptides tend to be expensive drugs and although they are thought to engender very low resistance, there is always the possibility this will arise during therapy. To reduce peptide concentrations and their drawbacks, the synergistic effects of peptides in combination with other antimicrobial agents are being tested to reduce the amount of each agent required, while increasing efficacy and decreasing costs [10]. In vitro anti-biofilm peptides show strong synergy with a wide range of antibiotics reducing antibiotic concentrations by up to 64-fold while simultaneously reducing peptide concentrations [40]. Similarly combination therapy has been applied to treat biofilms with natural and synthetic peptides in vitro and in vivo and includes the use of peptides in combination with biologically active materials such as enzymes, anti-quorum sensing molecules, antibiotics, and other peptides [41–43]. Dosler et al. [42] showed that the hybrid peptide cecropin A(1–7)-melittin A(2–9) amide (CAMA) synergized with tobramycin, ciprofloxacin, and colistin to treat *P. aeruginosa* biofilms, decreasing the amount of peptide by tenfold and the amount of antibiotic by up to 8-fold. Although these experiments were performed in synthetic lab media, other research has shown that peptide LL-37 led to a 6 log-fold reduction in bacteria when combined with the antibiotic azithromycin against *P. aeruginosa*, *Acinetobacter baumannii,* and *Klebsiella pneumoniae* when grown in tissue culture media with 20% human serum [43]. Furthermore, our lab has recently shown synergy between peptides DJK-5, 1018, 1002, and HHC-10 and antibiotics ciprofloxacin, gentamicin, meropenem, and erythromycin, effectively decreasing abscess size and bacterial load in a mouse model of high density ESKAPE (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) pathogen infections [44]. This is of particular interest, since DJK-5 targets the stringent response regulating biofilm formation and maintenance, which has been shown to play a role in abscess formation and susceptibility to antibiotics/persistence [10, 23, 44].

Treating high-density bacterial infections or biofilms with a combination of anti-biofilm peptides that can target specific regulatory mechanisms and conventional antibiotics is a promising approach to improve clinical therapies, reduce cost of goods, and minimize resistance development.

Challenges Facing Clinical Application of Anti-Biofilm Peptides

Their broad spectrum of activity and synergy with conventional antibiotics make anti-biofilm peptides especially attractive for clinical applications [16–18]. However, future research must address some important barriers limiting the use of these peptides therapeutically. For instance, antimicrobial agents are often tested in lab-

Table 1. Host-mimicking conditions for anti-biofilm treatment testing

Host environment	Host-mimicking condition	Bacteria	Treatment	Reference
Tooth	Saliva coated HA discs	*S. mutans*	P1	[15]
Blood/tissue	PBS with human plasma	*P. aeruginosa*, *S. aureus*	SAAP-148	[24]
Skin	Ex vivo epithelial skin cell model	MRSA, *A. baumannii*	SAAP-148	[24]
Lung	Brochoalveolar lavage fluid	*P. aeruginosa* *S. maltophilia*	D-BMAP18	[26]
Urinary tract	Synthetic urine media	*C. albicans*	β-peptide-1	[32]
Blood/tissue	Tissue culture media with human serum	*P. aeruginosa*, *A. baumannii*, *K. pneumoniae*	Azithromycin Colistin	[43]
Lung	Cystic fibrosis airway mucus	*P. aeruginosa*	Colistin Liposomes	[48]
Lung	Lung epithelial cell model with and without fetal bovine serum	*P. aeruginosa*	Colistin DJK-5	[50]
Blood	Whole human blood, plasma and serum	*E. coli*	R-1, R-11	[51]
Lung	Airway mucus	*P. aeruginosa*	Amikacin Liposomes	[70]
Lung	Artificial sputum medium	*P. aeruginosa*	Lin-SB056-1 EDTA	[72]
Lung	Human airway epithelial cells	*P. aeruginosa*	WLBU2	[73]

oratory conditions such as Mueller Hinton broth (MHB), and other nutrient rich laboratory media, which is not physiologically relevant [10, 43], while many AMPs have been tested for efficacy in phosphate buffer, which is also non-physiological. Many parameters such as carbon and nitrogen sources, iron availability, and divalent cation concentrations differ significantly in lab media or buffers from those in an infected host [10, 45, 46]. HDPs exposed to components of plasma, serum, saliva, mucus, or urine for example, often exhibit very different activity levels from those in lab media for various reasons [24, 27, 46–49]. Furthermore, the environment in which a bacterium grows can substantially affect the biofilm architecture and alter anti-biofilm peptide binding and/or penetration [45].

To overcome the lack of transferability from in vitro to in vivo activity, research on anti-microbial agents needs to address the influence of host conditions on biofilm structures and peptide activity [2, 45, 50]. The introduction of physiologically relevant host materials into antimicrobial bioassays can partially address this issue, but we have to be wary of the influence of host molecules on traditional detection methods such as optical density and fluorescence. Host-mimicking in vitro conditions that have been used to test the effects of anti-biofilm treatments against biofilm forming pathogens are summarized in Table 1. The alternative activity of AMPs in whole blood, plasma, and serum compared to conventional media conditions has been demonstrated with AMPs RP-1 and RP-11, designed based on antimicrobial platelet proteins. In biomatrices, these peptides were found to have increased antimicrobial activity against *Escherichia coli*, reducing bacterial loads by up to 3 log-fold at concentrations that were not effective in MHB [51]. *P. aeruginosa* biofilms, when grown in a 3D epithelial cell model mimicking the human lung, exhibited decreased susceptibility to the peptide antibiotic colistin and D-enantiomeric peptide DJK-5 in the presence of fetal bovine serum compared to serum-free conditions [50]; intriguingly, the combination of DJK-5 with tobramycin substantially reduced the formation of *P. aeruginosa* biofilms in this 3D model. Recently, de Breij et al. [24] designed an LL-37-derived peptide, SAAP-148,

with bactericidal and anti-biofilm activity at 12.8 μM against *P. aeruginosa* and *Staphylococcus aureus* in 50% human plasma. Even though the peptide's activity was negatively influenced by human plasma, the effective concentrations in physiological conditions were still lower than those of clinical phase peptides such as Omiganan and Pexiganan that required >102.4 and 25.6 μM respectively. Furthermore, the peptide also showed efficacy against *S. aureus* and *A. baumannii* in human skin ex vivo and murine in vivo models [24]. Ultimately clinical development will require the evidence of in vivo efficacy in animal models as mentioned above.

Despite some promising discoveries of peptides with anti-biofilm activity under physiologically relevant conditions, there are still a number of barriers limiting clinical use. These limitations include stability and efficacy in the host environment, possible peptide-induced toxicity toward host cells, aggregation-induced toxicity, and high production costs.

The stability of anti-biofilm peptides under physiological conditions depends on their sensitivity to degradation by proteolytic enzymes and inhibition by salts, proteins, and ions in the host environment [49, 52, 53]. Bacteria can protect themselves from AMPs such as human LL-37 and beta-defensins, by producing peptide-degrading enzymes such as aureolysin in *S. aureus* and elastase in *P. aeruginosa* [53]. Additionally, digestive enzymes produced by mammals, such as trypsin and chymotrypsin, can cause the cleavage of amino acids that are important for the structure and function of the anti-biofilm peptides [53]. One solution to such toxicities is to design peptides with altered backbones (peptidomimetics) or D-enantiomeric residues as discussed above. Alternatively judicious formulations can also influence the in vivo stability of peptides. Moreover, a large issue influencing the efficacy and toxicity of peptides in vivo is their tendency to precipitate or aggregate in these conditions. Cationic amphipathic peptides such as β-amyloid are known to have a strong tendency to aggregate into fibrils leading to the inflammatory brain disorder Alzheimer's [27]. We consider this to be likely a class property of the anti-biofilm, antimicrobial, and immunomodulatory peptides mentioned here, since they have the potential to form visible aggregates under conditions containing high concentrations of particular salts or serum proteins, and this can reduce in vitro immunomodulatory or anti-biofilm activity [53]. Haney et al. [27] studied the aggregation of immunomodulatory/anti-biofilm peptide 1018 in various anion and cation solutions. Peptide 1018 aggregated (and/or formed hydrogels) in a concentration-dependent manner in buffers containing certain anions, particularly phosphate, benzoate, citrate, and nitrate, according to the Hofmeister series of anions, whereas altering the cation had little effect except at very high concentrations. It also co-precipitated serum proteins in tissue culture medium [27] and demonstrated precipitation in vivo using a mouse skin injection model [28]. This latter observation might explain the toxicity of certain peptides due to their precipitation in the blood when applied intravenously. It was further demonstrated that peptide aggregation interfered with its immunomodulatory activities against peripheral blood mononuclear cells [27, 49]. In studies with other peptides, concentration-dependent aggregation can trigger enhanced immunogenicity which might pose a major problem for clinical use of peptide treatments by inducing chemokine activity and causing allergic responses or cytotoxic effects [54]. Again appropriate formulation can influence aggregation as well as cytotoxicity [28]. Early peptide work assessed cytotoxicity in terms of its ability to cause red blood cell lysis. More recently, cytotoxicity has been determined by measuring the release of cytosolic lactate dehydrogenase from lysed isolated primary human/mammalian cells or cell lines. However, the results of these assays likely do not reflect the overall cytotoxicity of peptides within the host and, in our experience, aggregation is a more serious concern.

Finally, synthesis methods involving expensive intermediates and many steps, and high therapeutic doses, raise the costs for large scale production of anti-biofilm peptides substantially, when compared to conventional antibiotics, and thus limit clinical applications [24]. In order to develop more cost-effective synthesis strategies for the production of anti-biofilm and AMPs, research focuses on recombinant expression systems in bacteria, yeast, and plants [55].

Strategies to address the limitations of anti-biofilm peptides including design and synergistic combination therapies were highlighted above and are summarized in Figure 1. One of the most profound methods of counteracting these issues is therapeutic delivery methods involving more sophisticated formulation methodologies.

Formulation and Therapeutic Delivery of Anti-Biofilm Peptides

As discussed above, peptide design strategies and novel peptide discovery have made some inroads into overcoming potential limitations of therapeutic treatments

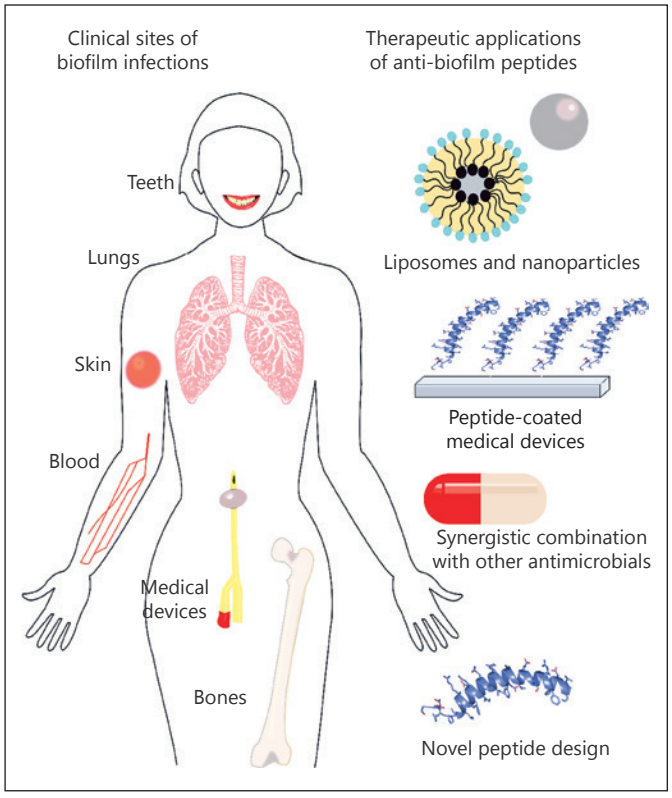

Fig. 1. Strategies for therapeutic applications of anti-biofilm peptides to treat clinically relevant biofilm infections.

against biofilm infections [3, 12]. A common method for increasing bioavailability, thus reducing therapeutic amounts, overcoming aggregation and other physical limitations of drugs such as excessive hydrophobicity, and decreasing any toxic effects involves advanced formulation. Thus, beyond making peptide modifications, increased pharmacological research endeavors are needed to find innovative ways of delivering these agents, to enhance solubility, avoid toxicity, have more targeted delivery to the site of infection, and ultimately to increase effectiveness in the host environment. Due to unknown systemic toxicities and aggregation issues as well as the cost of goods, most AMP treatments to date have focused on topical applications, which allow the use of smaller amounts of peptide applied to confined sites [10]. Anti-biofilm peptides will also likely be applied topically, since biofilms generally occur locally rather than systemically. By formulating anti-biofilm peptides with polymers or gels, tethering anti-biofilm peptides to biomedical devices, or delivering anti-biofilm peptides within nanocarriers such as nanoparticles or liposomes, peptide efficacy might be improved.

One potential method of improving topical application of anti-biofilm peptides to infections is through the use of gel formulations. By administering peptides to infected wounds in viscous gel-like polymer delivery systems, the peptides will become localized at the site of infection and exhibit increased resistance to exogenous proteases [56]. For example, topical delivery of the LL-37 derivative P60.4Ac in a water-based hypromellose gel decreased the peptide's cytotoxic effects and allowed for the killing of greater than 85% of MRSA biofilm in a human epidermal model [57]. The peptide-gel formulation retained antimicrobial activity and had increased ability to eradicate biofilm on epidermal cells, while reducing cytotoxicity, as compared to peptide dissolved in phosphate buffered saline [57]. This same gel formulation was successfully used by de Breij et al. [24] to administer anti-biofilm peptide SAAP-148 to MRSA and *A. baumannii* biofilms in human skin and murine models without any systemic toxicity or irritation.

Biofilm infections often occur on biomedical devices such as catheters and prosthetics [3, 4]. These infections can be difficult to remove and often require complete replacement of the biomedical device, which is not always successful in preventing an infection from re-occurring [58]. Biofilms on biomedical devices can be limited by coating the device surface with peptides [59]. Yu et al. [60] tethered an AMP to polyurethane in a mouse catheter model infected with *P. aeruginosa*. The coating led to a 4 log-fold reduction in bacterial load compared to uncoated material and also reduced bacterial load in urine, without having toxic effects on eukaryotic cells. By coating biomedical devices with AMPs, the treatment can prevent initial biofilm formation and is localized to the site of infection, which potentially reduces systemic selective pressures for resistance.

Immobilizing anti-biofilm peptides on nanoparticles has also been explored as a novel approach to peptide delivery [61]. Metal nanoparticles such as nickel, silver, and gold have been used as antimicrobial treatments in vitro and have potent activity against biofilms [62, 63]. Silver nanoparticles, for example, have been used to eradicate up to 99% of *A. baumannii* biofilms in vitro [63]. Nanoparticles also have potential to be synergistically linked to antimicrobial agents to decrease treatment load and toxicity while increasing effectiveness of the treatment [62, 64]. Chen and colleagues, for example, designed nickel magnetic nanoparticles linked to LL-37 and used them to treat established *E. coli* biofilms in vitro [64]. Similarly, the cationic lipopeptide antibiotic colistin was attached to nanoparticles made of poly(lactide-

co-glycolide) and used to treat pre-established *P. aeruginosa* biofilms, resulting in 90% biofilm reduction within 24 h. Though the anti-biofilm effects have only been tested in vitro so far, the nanoparticle-peptide combination is being engineered for lung delivery through an inhalation method and showed promising penetration through a mucus layer mimicking the conditions of the lungs of cystic fibrosis patients [48].

Lipid-based vesicles composed of lipid bilayers encapsulating an aqueous center are one of the most widely utilized nanotechnology applications for delivering treatments to biofilm infections [4, 65]. These liposomes are nontoxic to mammalian cells, and have shown efficacy for drug delivery in mouse models [65, 66]. Liposomes can have many different phospholipid compositions, sizes, and surface properties, which determine their ability to encapsulate the drug payload, their stability in the host environment, and their interactions with biological molecules [66]. Because their lipid bilayer structure mimics the cell membrane, and may allow fusion with bacterial membranes, depending on their characteristics, the encapsulated compounds have increased bioavailability and biocompatibility and can release entrapped drugs into the cell membranes or interior of microorganisms [67]. To target cells within a biofilm, the liposomes must have appropriate surface characteristics including charge and lipid composition. Accordingly, using liposomes with a cationic charge has been suggested to prolong contact between the anti-biofilm agent and the biofilm, allowing for more effective treatment [66]. Such liposomes would not however, be readily usable in the context of cationic peptides, as it is necessary to use negatively charged lipids to obtain good loading of cationic peptides; however, addition of polycationic peptides to anionic liposomes would confer increased positive charge. Unilamellar and multilamellar vesicle liposomes by themselves have been shown to have anti-biofilm activity against *S. aureus* and *P. aeruginosa* biofilm formation [68], although more promising anti-biofilm applications are associated with the encapsulation of anti-biofilm agents [66]. Indeed, liposomes encapsulating antibiotics have shown efficacy in inhibiting the formation of *S. aureus* biofilms [69, 70]. The cationic aminoglycoside antibiotic amikacin was loaded into neutral liposomes composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and cholesterol and designed for inhalational use to treat *P. aeruginosa* biofilms. Fluorescently labeled liposome formulations were measured for their ability to diffuse through patient lung mucus samples and *P. aeruginosa* biofilms, and antibiotic release was monitored over time. It was found that more than 60% of the liposomes administered were able to diffuse through mucus layers and penetrate biofilms, exhibiting sustained release of antibiotic. In a rat model of *P. aeruginosa* lung infection using inoculated agar beads, liposomal formulations were able to reduce bacterial loads by 2 log-fold with an estimated dose of 6 mg/kg, whereas the same concentration of free amikacin had no effect [70]. Additionally, this treatment was proposed to be biofilm specific due to the ability of liposomes to bind to charged mucins at the sites of bacterial biofilm infection and increase drug release in response to rhamnolipid-mediated liposome burst [70].

Overall, gel-based formulations, tethering techniques, nanoparticles, and liposomes have the potential to act as a safe delivery system for anti-biofilm peptides to the site of infection and can also increase peptide potency and stability.

Future Perspectives for Discovery, Design, and Application of Anti-Biofilm Peptides

The recalcitrant, adaptive nature of microbial biofilms is a serious problem in tissue and medical device-related infections. Natural and synthetic anti-biofilm peptides are promising agents for the treatment of biofilm infections, with low-resistance profiles and broad spectrum activity against pre-formed biofilms [4, 11]. These peptides can be designed to improve their anti-biofilm specificity and stability inside the host, while reducing cytotoxicity and aggregation [22, 25, 29, 32, 35]. Furthermore, peptide design has the potential to engineer peptide sequences and structures that can specifically target the regulatory mechanisms behind biofilm formation, such as ubiquitous secondary messengers that trigger adaptive responses to diverse disease-related niches [16, 17, 23]. By exploiting these mechanisms, anti-biofilm peptides can become more targeted to specific infections and more efficacious against the adaptive biofilm growth state. The design of peptides with multiple, distinct anti-biofilm mechanisms combined in one molecule has enormous potential, possibly allowing the creation of a peptide sequence with activity and stability in different disease-related environments and thus the treatment of a variety of biofilm-related diseases. Although computational design strategies for anti-biofilm structure and function can be fine-tuned to a certain extent, the effectiveness of peptide treatments against human infections has to take into account the environment in which the peptides will be used clinically. This includes the environmental conditions

shaping the biofilm architecture and biofilm susceptibility, as well as the relevant serum, salt, and matrix components present that may inhibit the activity of an anti-biofilm peptide in the host environment. Initial screening of anti-biofilm activity as well as mechanistic studies should be done in conditions mimicking the host environment to avoid wasting time and money associated with animal studies and clinical trials with peptides that might not even be active in these conditions. Similarly, we must develop standard and high throughput methods for screening anti-biofilm activity both in vitro and in vivo. In this regard, a new abscess animal model that has been adapted to use with all ESKAPE pathogens [53, 71], and a recently described human skin model [24], offer relatively simple primary screening methods for treatment of biofilm infections.

Furthermore, although research focused on anti-biofilm peptide design has the potential to modify and select for defined activity and performance, many of the issues facing peptide efficacy cannot be addressed by peptide design alone. Anti-biofilm peptides will probably not represent a stand-alone solution, but in our opinion have greater promise as adjunctive therapies with other antimicrobial agents [41, 42]. Combining anti-biofilm peptides with antibiotics, other peptides, or completely different antimicrobial compounds, may enable targeting of multiple aspects of microbial biofilm development and can improve the treatment efficacy, reduce the effective concentration of each antimicrobial agent required for treatment, and ultimately reduce the cost of effective therapeutic treatment. Finally, the development of delivery methods for anti-biofilm peptides, such as gel formulations, surface tethered biomedical devices, and nanocarriers, can increase peptide localization and decrease cytotoxic effects by improving delivery and biocompatibility [56].

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Disclosure Statement

The authors declare that there are no conflicts of interest associated with the manuscript.

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