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# Synthetic Antibiofilm Peptides

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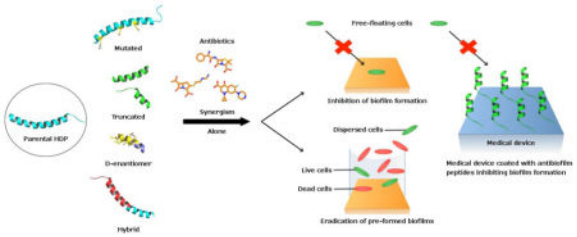
## Abstract

Bacteria predominantly exist as multicellular aggregates known as biofilms that are associated with at least two thirds of all infections and exhibit increased adaptive resistance to conventional antibiotic therapies. Therefore, biofilms are major contributors to the global health problem of antibiotic resistance, and novel approaches to counter them are urgently needed. Small molecules of the innate immune system called host defense peptides (HDPs) have emerged as promising templates for the design of potent, broad-spectrum antibiofilm agents. Here, we review recent developments in the new field of synthetic antibiofilm peptides, including mechanistic insights, synergistic interactions with available antibiotics, and their potential as novel antimicrobials against persistent infections caused by biofilms.

## Graphical Abstract

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## 1. Introduction

It has now been well established that bacteria are found not only as planktonic, free-swimming cells, but can also engage in a developmental cycle that allows them to form sessile, surface-associated multicellular communities called biofilms [1–4]. Biofilms are the predominant lifestyle of bacteria as they account for at least two thirds of all infections in humans and are found in many different natural environments. Indeed, biofilms are known to form in diverse environmental niches, including hydrothermal hot springs and deep-sea vents, freshwater rivers and rocks. Biofilms are formed when planktonic bacteria encounter certain environmental signals that are not yet completely understood. This process entails a complex adaptation that involves numerous regulatory gene networks, which translate the input signals into gene expression changes thus allowing the spatial and temporal organization of individual bacterial cells into biofilm aggregates [1–4].

Biofilm development begins with bacteria associating with a surface and forming microcolonies that, over time, turn into mature biofilm colonies. Bacteria within biofilms are encapsulated in a self-produced extracellular matrix made of various components that include polysaccharides, proteins, extracellular DNA, lipids and water [1–4]. One of the most significant characteristics of biofilms is their increased resistance to stress signals, including biocides and antibiotics used in industrial and clinical settings, as well as UV damage, metal toxicity, anaerobic conditions, acid exposure, salinity, pH gradients, desiccation, bacteriophages, amoebae, etc. [1–4]. Biofilms are also estimated to be 10 to 1000-times more resistant to conventional antibiotics than planktonic (free-swimming) bacteria. This has led to the recognition that biofilms are major contributors to chronic infections, which are highly resistant to antimicrobial therapies and are a major concern in hospitals worldwide. Moreover, currently available antibiotics have been shown to extensively damage the host microbiota, thus allowing reinfection by opportunistic pathogens that can form biofilms, and further intensifying the selective pressure towards antibiotic resistance [3].

In this daunting scenario, host defense (antimicrobial) peptides (HDPs) have emerged as a promising alternative to traditional antibiotics for the treatment of persistent infections caused by biofilms [5]. HDPs constitute the major component of the innate immune system of most living organisms, including mammals, insects, bacteria and fungi. In conferring protection to the organism from microbial attack, these molecules exhibit multiple mechanisms of action and, consequently, a low potential to select for resistance in bacteria [6]. In recent years, HDPs have been used as scaffolds that represent excellent starting points for the design of peptide libraries. Synthetic peptides derived from HDPs have been produced either by *de novo* synthesis or by modification of natural templates and have been optimized for improved biological functions and reduced size, which in turn reduces production costs [6,7]. Synthetic peptides have been obtained that present different functional sequences and adopt α-helical or β-hairpin conformations, show remarkable antimicrobial activity, low hemolysis and cytotoxicity, as well as optimized cell selectivity [6–9]. It is now possible to select for the biological function of choice in peptide templates (e.g., antibiofilm activity) through iterative design and structure-activity studies [6–8]. This review provides an overview of recent work describing the antibiofilm properties of

synthetic peptides and their parent peptides, highlighting their promise as next-generation antimicrobials.

## 2. Antibiotic resistance and bacterial biofilms

Antibiotic resistance is one of the greatest challenges of our time. It has recently been estimated that, if no new antibiotics are discovered by 2050, 10 million people will die worldwide as a result of antibiotic-resistant infections. Currently, drug-resistant infections lead to the death of at least 25,000 people per year in Europe, which costs the European Union 1.5 billion euros annually [10]. In addition, resistant bacteria infect >2 million people per year in the USA leading to 23,000 deaths, although if we include sepsis for which the main treatment is antibiotics the death rates rise to 210,000 and 5 million worldwide [11]. Despite our inability to treat these recalcitrant infections, only a few antibiotics have been approved for human use in recent years. However these new antibacterials, which include the vancomycin derivative oritavancin (licensed in 2014), are mostly active against Gram-positive bacteria but not Gram-negative organisms. Indeed, no new classes of antibiotics have been approved to treat Gram-negative organisms since the discovery of fluoroquinolones >50 years ago.

Most persistent infections in humans are caused by biofilms, which are prevalent in device-related infections, infections on body surfaces (skin and soft tissue, lung, bladder, endocarditis, etc.) and chronic infections [1–4]. Therefore, biofilms play a fundamental role in infectious diseases as they can form on any given body or implanted device surface and persist after treatment with a wide range of diverse antimicrobial agents [1]. Indeed bacteria in biofilms are between 10 and 1000-fold more resistant to treatment with most conventional antibiotics compared to their planktonic counterparts, which substantially hinders their treatment in the clinic [1–4]. Biofilm cells can also withstand host immune responses (both innate and adaptive), being particularly resistant to phagocytosis.

Unfortunately, none of the antibiotics currently available in the clinic have been purposely designed to inhibit biofilms [11,12], since their development was centered on exploiting their ability to target planktonic bacteria. Even today, antibiotic development pipelines rarely test the susceptibility of recalcitrant biofilm cells or utilize animal models in which bacteria form biofilm infections.

## 3. Peptides of the innate immune system

HDPs are evolutionarily conserved small molecules of the innate immune system that provide a first line of defence to virtually all organisms on Earth against microbial infections. HDPs were originally termed antimicrobial peptides (AMPs), before their functions as immunomodulatory and antibiofilm agents were recognized. For the purpose of this review, we will account for these additional biological functions and will refer to these peptides as HDPs. HDPs are typically composed of short chains (12–50) of positively charged and hydrophobic amino acid residues. In addition, they present a hydrophobicity that ranges from 40% to 60%. It is recommended to keep the percentage of hydrophobic amino acids within that range, as it has been described that excess hydrophobicity tends to drastically decrease the antimicrobial activity and enhance the hemolytic potential of several

HDPs, including synthetic variants such as lipopeptides [13]. Despite these commonalities in physical properties, it is worth mentioning that antimicrobial, antibiofilm and immunomodulatory peptides have distinct structure-activity relationships and in particular antibiofilm and immunomodulatory activities can occur without substantial antimicrobial activity against planktonic bacteria [14–18].

Such physical characteristics allow HDPs to interact with membranes and translocate into negatively charged bacterial and host cells, thus enabling their diverse biological properties. These include their ability to directly kill planktonic microorganisms through their well-studied antimicrobial activity, modulation of the immune system to control infections by means of their immunomodulatory properties, and their antibiofilm activity that enables them to inhibit and disperse biofilms (Figure 1). This article focuses on the most recently identified function of these peptides: their ability to target drug-resistant bacterial biofilms. One of the first examples of an HDP with antibiofilm properties was the human cathelicidin LL-37, which was shown to inhibit biofilm formation in *P. aeruginosa* [16] at one-sixteenth the MIC and to inhibit and disperse preformed biofilms. This initial study triggered the field of antibiofilm peptides and promoted many subsequent studies that focused on exploiting the biofilm inhibitory potential of HDPs.

## 4. Synthetic antibiofilm peptides

In recent years, much attention has been given to the discovery of novel bioactive compounds with desired antimicrobial properties. As described above, HDPs constitute promising blueprints to generate synthetic peptide candidates that can counteract microbial infections caused by both bacteria and fungi in either their planktonic or biofilm lifestyles. Despite the fact that peptides can be isolated from numerous sources, and despite the discovery of several lead peptides over the years, significant hurdles have restricted their subsequent success in clinical trials. These include largely unexplored toxicities, long amino acid sequences that increase production costs, degradation by host proteases, and limited understanding about the structure-function relationships of these peptides [6]. Researchers have attempted to solve some of these limitations by performing physicochemical modifications to peptides, such as deletion and/or substitution of amino acid residues, cyclization, design of retro-inverso peptides and the use of D-enantiomer amino acids [18], sequence truncations [19] and construction of hybrids [20], or by computational methods [5–7, 21, 22]. Examples of synthetic peptides with antibiofilm properties that have been described to date are outlined in Table 1.

Several studies have performed comparative studies in order to understand the functional divergence between naturally occurring peptides, synthetic peptides and conventional antibiotics. For example, de la Fuente-Núñez et al [14] performed a peptide library screen for small cationic peptides with antibiofilm activity. Intriguingly, this study revealed that there was absolutely no concordance between antimicrobial (vs. planktonic bacteria) and anti-biofilm activity and the most interesting peptide was active against *Burkholderia cenocepacia*, which is completely resistant to the antimicrobial activity of peptides. A 9-mer (-mer signifies number of amino acids) antibiofilm peptide named 1037 (KRFRIRVRV-$NH_2$) was identified that exhibited good activity vs. biofilms constructed from Gram

negatives *P. aeruginosa* and *B. cenocepacia* and Gram positive *Listeria monocytogenes* but displayed low activities against the same strains when grown in broth culture with minimal inhibitory concentration (MIC) values ranging from 25 to >608 μg.mL$^{-1}$. Indeed, 1037 inhibited biofilm formation by 50% at one-twentieth the MIC or less, 10 μg.mL$^{-1}$. This was confirmed under flow cell conditions wherein 20 μg.mL$^{-1}$ of peptide 1037 caused a strong decrease in biofilm thickness of *P. aeruginosa* PAO1 and PA14 biofilms [14]. Analysis of the transcriptomic profile of *P. aeruginosa* biofilms treated with 1037 revealed that peptide treatment led to down- and up-regulation of 138 and 260 genes, respectively [14]. Genes related to flagella and quorum sensing were down-regulated, similar to results obtained in a prior study using peptide LL-37 [16]. These genes are intrinsically involved in swimming and twitching motilities processes, which are related to different stages of biofilm development.

As mentioned, these studies showed that one could reduce the size of antibiofilm peptides from 37 amino acids in LL-37 [16] to as few as 9 amino acids [14]. Intriguingly, the anti-biofilm activity of LL-37 was subsequently also found to be more broad spectrum since LL-37 and derivatives effectively prevented biofilm formation and eradicated pre-formed biofilms of *Staphylococcus epidermidis* [23]. Another study identified peptides derived from the related (67% identity to LL-37), 34-mer murine cathelicidin-related antimicrobial peptide (CRAMP) that inhibited biofilm formation by the fungus *C. albicans* at concentrations that did not affect planktonic growth [24]. Lastly, it is worth mentioning that recent Phase I/II human clinical trials [25] revealed an ability of LL-37 to enhance healing of hard-to-heal venous leg ulcers, although this efficacy could in part be related to the anti-inflammatory activity of LL-37 [26].

Additional studies have also successfully modified the sequence of LL-37 to introduce certain advantages. For example, Nagant et al. designed a library of twelve 19- to 31-mer (-mer = number of amino acids) truncated fragments of LL-37, and eight of these peptides significantly inhibited *P. aeruginosa* biofilm formation [19]. Among these active peptides, an N-terminal fragment (LL-31) and a C-terminal fragment (LL7-37) were the most efficient at inhibiting biofilm formation, since they reduced biofilm biomass by approximately 70% at a concentration of 5 μM (~12.5 μg.mL$^{-1}$). Furthermore, at levels ranging from 15 to 313 μg.mL$^{-1}$, these two peptides were capable of combating pre-formed biofilms, killing biofilm cells as revealed by propidium iodide uptake assays. LL-37 tends to be somewhat toxic and, intriguingly cytotoxicity assays revealed that removing the first six N-terminal amino acid residues in LL7-37 made this peptide much less toxic than LL-37 [19].

Other LL-37 analogues were studied for their ability to eradicate multidrug-resistant *S. aureus* from *in vitro* models of thermally-wounded skin. Thus, Haisma and coworkers [27], generated fourteen 24-mer peptides based on the LL-37 analogue peptide P60.4Ac. Peptide P10 was shown to be more effective than either the wild-type peptide LL-37 or P60.4Ac, being able to kill >99% of five *S. aureus* strains at ~3.4 μg.mL$^{-1}$. P10 was also the best peptide at inhibiting biofilm development of a multidrug-resistant (MDR) clinical isolate of *S. aureus*, causing a 50% biofilm reduction ($EC_{50}$) at 6.19 μg.mL$^{-1}$, while the same reduction was achieved by wild-type LL-37 and P60.4Ac at 15.27 and 8.54 μg.mL$^{-1}$, respectively. At ~9.9 μg.mL$^{-1}$, P10 was shown to also cause a decrease of ~90% in the

number of viable bacterial cells within preformed biofilms, revealing its potential to both inhibit and eradicate biofilm cells. These findings, coupled with the lack of cytotoxic effects of these peptides, led the authors to test the topical application of these three peptides on human skin equivalents (HSEs) infected with MDR *S. aureus*. Again, P10 exhibited the most potent activity, reducing the number of viable cells by ~99% [27]. Other studies explored the impact of the chirality of LL-37 by creating a D-peptide analog D-LL-37. Interestingly [28] both α-helical LL-37 and D-LL-37 were able to equivalently inhibit cellular attachment and biofilm formation by *P. aeruginosa*. The authors also determined that these peptides down-regulated the expression of two genes (*rhlA* and *rhlB*) that are part of the quorum-sensing circuitry in *P. aeruginosa* and therefore might be interesting targets for antibiofilm agents since quorum sensing contributes to biofilm establishment [28]. As expected, in contrast to LL-37, the proteolytically-stable peptide D-LL-37 was not degraded in the presence of trypsin, which likely makes it more stable when administered *in vivo* (e.g. in wounds) since proteases abound at infected, inflammatory sites. Indeed, treatment with D-LL-37 led to increased protection (~60% survival) in *Galleria mellonella* against *P. aeruginosa* infections. The level of protection was comparable to groups treated with ciprofloxacin (~60% survival), and substantially superior to wax moths treated with L-LL-37 (~15% survival) [28].

Other templates were also employed. For example, Gopal et al. [29] performed antimicrobial and antibiofilm assays, comparing the efficacy of different antimicrobial agents against *P. aeruginosa* and *S. aureus* strains isolated from otitis media/cholelithiasis patients. They showed that a 19 amino acid pleurocidin analogue peptide, NRC-16 (GWKKWLRKGAKHLGQAAIK-$NH_2$), generated by amino acids substitutions, had MICs vs. planktonic bacteria that were generally between 2.17 and 17.4 $\mu g.mL^{-1}$ against several Gram-negative and Gram-positive bacteria, as well as fungi. These results were very similar to those obtained with the well-known bee-venom peptide melittin. The antibiofilm activities of both NRC-16 and melittin indicated minimal biofilm inhibitory concentration (MBIC) values ranging from 8 to 35 $\mu g.mL^{-1}$ against five clinical strains of *P. aeruginosa*. In contrast, all six antibiotics (ampicillin, chloramphenicol, levofloxacin, ciprofloxacin and piperacillin) tested in this assay presented MBIC values ranging from 278 to >1.110 $\mu g.mL^{-1}$, clearly demonstrating their inability to combat biofilms [29]. In addition, even though NRC-16 and melittin revealed similar antimicrobial and antibiofilm potential, they were completely divergent in their cytotoxicity vs. human red blood cells (hRBCs), HaCat and RAW-264.7 cells. NRC-16 was nontoxic at concentrations as high as 326.25 $\mu g.mL^{-1}$, while melittin showed to be extremely hemolytic even at concentrations as low as 21 $\mu g.mL^{-1}$ [29]. Hemolysis is often used as an indicator of peptide-induced toxicity, but is somewhat artificial in that the blood cells are suspended in saline rather than the natural matrix serum. Recognizing the obstacle of cytotoxicity in the use of melittin as an antibiofilm agent, Almaaytah and colleagues [30] developed a new hybrid peptide that combined the α-helical regions of melittin and another highly effective but cytotoxic antimicrobial peptide (i.e., BMAP-27) with the objective of improving or maintaining antibacterial activities, but enhancing the therapeutic index. Thus, a 21 amino acid hybrid, cationic peptide named BMAP27-melittin (KFKKLFKKLSPVIGAVLKVLT), was generated. This peptide had MICs of 2 to 17 $\mu g.mL^{-1}$ against planktonic bacteria when

tested against nine wild-type and four antibiotic-resistant strains that included *S. aureus*, *P. aeruginosa* and *Acinetobacter baumannii* [30]. Moreover, BMAP27-melittin had a minimal biofilm eradication concentration (MBEC) of 23.56 μg.mL$^{-1}$ against *S. aureus* and *P. aeruginosa* biofilms, while exhibiting minimal toxicity at antimicrobial concentrations when compared with its parent peptides [30].

Mataraci and Dosler [31] also constructed a hybrid peptide, named CAMA, by combining the N-terminal region (1–7) from cecropin-A and the N-terminal region (2–9) from melittin-A, both derived from insects. CAMA had 2-fold lower MIC values against methicillin-resistant *S. aureus* (MRSA) when compared with two other naturally occurring peptides (i.e., indolicidin and nisin). Antibiofilm assays further revealed that, at 8 μg.mL$^{-1}$, CAMA was highly effective at inhibiting MRSA attachment to wells and biofilm formation (~85–90%) [31]. A very recent study investigated the effect of a series of 15-mer peptides composed of six lysines and nine leucines on *P. aeruginosa* biofilms [32]. Treatment with sub-MIC concentrations of the peptides reduced biofilm growth. More specifically, significant but minor inhibition occurred at concentrations as low as 50 ng/mL, although generally higher concentrations ranging from 0.25 to >1 fold the MIC were required to achieve at least 50% biofilm inhibition. Another study [33] reported that the chemically synthesized cyclic lipopeptide battacin was membrane-lytic and exhibited antibiofilm activity against *P. aeruginosa*, *S. aureus*, and *P. syringe* pv. actinidiae. These findings thus demonstrate that it is possible to combine biologically promising parts of different well-known peptides, natural or synthetic, to generate improved peptide candidates that effectively contain biofilm infections. However, MBIC values were generally in the range of 10–40 μg.mL$^{-1}$, which is likely insufficient for clinical development, and unlike 1037 [14] there was no preferential activity vs. biofilms cf. planktonic cells.

Although the peptides presented above revealed clear advantages when compared with their parent molecules, many of these peptides are quite long or chemically complex and thus expensive to produce. For this reason, our own efforts and those of other groups are focusing on the design and synthesis of smaller peptides. Synthetic 12-mer peptide 1018, loosely derived from a cattle neutrophil HDP, bactenecin, potently inhibited and eradicated biofilms formed by a broad range of both Gram-negative pathogens including *P. aeruginosa*, *E. coli*, *A. baumannii*, *Klebsiella pneumoniae*, and *S. enterica* as well as the Gram positive MRSA, at concentrations well below its MIC [34]. The anti-biofilm activity of peptide 1018 was shown to be concentration-dependent, as treatment with very low peptide levels (0.8 μg.mL$^{-1}$) led to increased dispersal of bacteria from biofilms, while higher concentrations (10 μg.mL$^{-1}$) led to death of cells within biofilms [34]. The mechanism of action is described below. Peptide 1018 was subsequently shown to induce killing of bacteria present within oral multispecies biofilms [35], therefore identifying a promising role of this agent for plaque disinfection in dentistry. It is worth mentioning that, like LL-37, peptide 1018 (also termed IDR-1018) is a potent modulator of innate immunity and as such is able to suppress inflammation and enhance protective immunity in several animal infection models [36].

More recently, we designed 12-mer D-enantiomeric and retro-inverso peptides based on the physicochemical properties of active antibiofilm peptides [18]. These design features

included the use of only 9 of the 20 natural amino acids (V, R, L, I, A, W, F, K, Q), 4 charged residues (most commonly R), 7 or 8 hydrophobic residues, and no more than 1 glutamine (Q). The use of D-amino acids makes these peptides protease-resistant, thus overcoming one of the main limitations of L-form cationic peptides, which are susceptible to degradation by host proteases [37, 38]. These peptides also provided enhanced biological activities *in vitro* even though they work by the same mechanism as 1018 (promoting ppGpp degradation) [18]. Among several designed peptides, DJK-5 and DJK-6 were reported as the best broad-spectrum antibiofilm agents of the study, since their $MBIC_{50}$ values against *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae* and *S. enterica* ranged from 0.5 to 2 $\mu g.mL^{-1}$ (except for one outlier for each peptide), well below their MICs of 16 $\mu g.mL^{-1}$ [18]. Furthermore, at 2.5 $\mu g.mL^{-1}$ these peptides eradicated pre-existing biofilms [18]. These peptides showed enhanced activity in animal models cf. 1018, since both DJK-5 and DJK-6 conferred protection to the invertebrate organisms *Caenorhabditis elegans* and *Galleria mellonella* from otherwise lethal *P. aeruginosa* biofilm infections [18]. This study demonstrated that D-enantiometic peptides could be used to treat biofilms *in vivo*, a key finding that encourages future development of these peptides for applications in humans. Importantly, the peptides were shown to be non-toxic in the same invertebrate studies. In a parallel study performed by Ribeiro et al. [39], it was demonstrated that DJK-6 was a promising peptide in preventing the formation of biofilms, as well as eradicating preexisting biofilms (at 2 – 4 $\mu g.mL^{-1}$) formed by carbapenemase-producing *K. pneumoniae* clinical isolates (KpC isolates) [39].

### 4.1 Synergy with conventional antibiotics

As mentioned above, conventional antibiotics are quite ineffective at clearing biofilm-associated infections and biofilm cells are between 10 and 1,000 fold more resistant to conventional antibiotics [1–4]. Some peptides have been described that enhance antibiotic action to prevent biofilm formation and eradicate mature biofilms [18, 40]. This approach also serves to reduce the selective pressure for the development of resistance exerted by each individual agent as very low concentrations are used. For example, checkerboard titration and flow cell experiments demonstrated that peptide 1018 synergized with different classes of conventional antibiotics to prevent and eradicate existing biofilms [40]. Indeed, when the peptide was added in the presence of low levels of the antibiotics ceftazidime, ciprofloxacin, imipenem, or tobramycin, the concentration of antibiotic required to eradicate biofilms was reduced by up to 64-fold. This included biofilms formed by *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, *S. enterica* and MRSA [40] Similar results were obtained with the D-enantiomeric peptides DJK-5 and DJK-6, which exhibited synergistic interactions with the antibiotics ceftazidime, imipenem, ciprofloxacin or tobramycin [18]. Indeed, it was observed that 42.5% of the combinations revealed synergy or nearly synergy, with excellent synergy shown e.g. when 0.1 $\mu g.mL^{-1}$ of DJK-5 was combined with 0.04 $\mu g.mL^{-1}$ of ciprofloxacin to completely prevent and eradicate existing *P. aeruginosa* biofilms, representing a 10-fold decrease in concentration compared to the DJK-5 $MBIC_{50}$ and the ciprofloxacin MIC (ciprofloxacin was not able to completely eradicate biofilms even at 100-fold the MIC). Several other combinations of DJK-5 and DLK-6 with different antibiotics led to the eradication of *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae* and *S. enterica* [18]. Intriguingly, Ribeiro et al. demonstrated similar findings, reporting that

peptide DJK-6 enhanced the activity of the β-lactam antibiotics meropenem, imipenem and cefepime to prevent biofilm formation by carbapenemase KpC-producing clinical isolates [39]. The combination of this peptide at 0.125 μg.mL$^{-1}$ with only 0.06 μg.mL$^{-1}$ of meropenem was capable of eradicating mature biofilms formed by these KpC isolates. This represents at least a 16-fold decrease in the concentration of antibiotic required to eradicate such biofilms [39]. It would be interesting to determine whether peptides such as DJK-6 act as β-lactamase inhibitors, as this has been shown to be an activity of certain peptides [41].

Synergistic properties have also been described for hybrid peptides. Gopal and colleagues [20] recently reported that four chimeric peptides, namely CAMA, CAME, HPMA and HPME designed based on the residues 1–12 from melittin-A (ME), 1–12 from magainin-2 (MA), 1–8 from cecropin-A (CA) and 2–9 from the *Helicobacter pylori* ribosomal protein LI (HP), showed MIC values ranging from 5.12 to 28.78 μg.mL$^{-1}$ against nineteen strains of MDR *A. baumannii* isolated from patients with cholelithiasis. Five currently used antibiotics belonging to different structural classes (i.e., ampicillin, cefotaxime, ciprofloxacin, erythromycin and tobramycin) were able to exert little to no inhibitory activity against the resistant strains [20]. In contrast, addition of all four peptides to the antibiotics led to synergistic effects against planktonic cells of this bacterial pathogen. When tested for their abilities to prevent MDR *A. baumannii* biofilm formation, the peptides showed MBIC values from 11.06 to 115.1 μg.mL$^{-1}$. However, HPMA when combined with ciprofloxacin drastically decrease their MBIC values [20].

Mataraci and Dosler also investigated the effects of combining cationic peptides with antibiotics to treat MRSA biofilms [31, 42]. The authors used different classes of antibiotics (daptomycin, linezolid, teichoplanin, azithromycin, and ciprofloxacin) combined with the following cationic peptides: indolicidin, CAMA [cecropin (1–7)-melittin A (2–9) amide], and nisin. Synergy against MRSA biofilms was found in nearly all cases between the different combinations of peptide plus antibiotic. The same group obtained similar results with *P. aeruginosa* biofilms [43]. Further, another study unveiled the interactions of the macrolide antibiotic azithromycin with peptide LL-37 against MDR isolates of *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii*, which led to multi-log-fold synergies [44].

Together, these findings clearly show that synthetic peptides can be used to potentiate the activity of otherwise ineffective antibiotics to treat biofilms. This approach substantially reduces the likelihood of potential side effects and the selective pressure for the development of drug resistance, as it decreases the concentrations of both peptides and antibiotics.

### 4.2 Applications in biomaterials

Biofilm infections are also very prominent in wounds, tissues and the bloodstream, accounting for numerous cases of medical device-associated infections, which are a major concern in hospital environments [1, 45, 46]. Within this context, different studies have proposed coating strategies in order to incorporate antimicrobial and antibiofilm compounds onto biomedical device surfaces with the aim of inhibiting initial bacterial attachment, and therefore biofilm formation (Figure 2) [47–57]. Surface coating with peptides has been reported with promising biocompatibility (comparing the antimicrobial activity of the

peptide coated surface and its cytotoxicity under clinically relevant conditions) [47,50], as well as antibiofilm properties both *in vitro* and *in vivo*.

For example, Gao et al [50] utilized polymer brush methods of coating titanium and other surfaces to make peptide-coated surfaces with potent antimicrobial activity vs. *P. aeruginosa* and *S. aureus*. Conversely, peptides could also be presented non-covalently on calcium phosphate coated titanium surfaces to enable antimicrobial activity [49]. Forbes and coworkers [55] compared the antimicrobial/antibiofilm potential of the human apolipoprotein E peptide (apoEdp), its tryptophan-rich analogue (apoEdpL-W) and commonly used antimicrobials (chlorhexidine, polyhexamethylene biguanide (PHMB) and triclosan) when incorporated onto hydrogels [poly (2-hydroxyethyl methacrylate) (pHEMA) and polyethylene glycol (PEG)] and non-porous polymers [polyurethane (PU) and polydimethylsiloxane (PDMS)]. The association levels were measured and both apoEdp and apoEdpL-W were more stable when present in the hydrogels (97% of initial association; 50% and 80% of retention after three washes, respectively) when compared to the non-porous polymers (15% of initial association; ~3.5% of retention after three washes). It was observed that PEG coated with apoEdp could decrease the survival of *S. aureus* and *P. aeruginosa* planktonic cells to <10%, which corresponded with the activity of apoEdpL-W associated with pHEMA against these same strains. More efficient results were obtained for apoEdpL-W associated with PEG, where it could entirely eradicate *S. aureus* and *P. aeruginosa* planktonic cells, similarly to chlorhexidine and PHMB [55]. In addition, hydrogels coated with apoEdpL-W decreased the viability of *S. aureus* pre-existing biofilms more efficiently than hydrogels coated with apoEdp [55]. This can be explained due to the higher level of absorption and retention caused by the superior hydrophobicity of apoEdpL-W when compared to the parent peptide, which might have influenced a slower transition through the hydrogel matrixes thus improving the antimicrobial and antibiofilm potential of this peptide [55].

By exploiting the characteristics of tryptophan-rich peptides and the salt-tolerant properties of arginine-rich peptides, Kim and colleagues [57] engineered two tryptophan-arginine-rich peptides, named WR11 (WFWKWWRRRRR-NH2) and CWR11 (CWFWKWWRRRRR-NH2), based on wild type jelleine-I from *Apis mellifera*. The rationale behind the addition of a cysteine residue in the N-terminal region of WR11 to generate CWR11 was to enable the immobilization of this peptide on polydimethylsiloxane (PDMS) slides (involving PEG and the allyl glycidyl ether) by sulfhydryl coupling (PDMS-AGE-PEG-CWR11), as used previously for polymer brush attachment [50]. WR11 displayed antimicrobial activities against *E. coli*, *S. aureus* and *P. aeruginosa* under 5.63, 3.09 and 8.36 $\mu g.mL^{-1}$, respectively, while CWR11 presented only a slight two-fold increase in the MIC values, largely at higher salt-concentrations [57]. Structurally, CWR11 was also characterized as an environment-dependent peptide, presenting higher helical contents in the presence of anionic conditions, such as Gram-negative bacterial surfaces, to which the peptide was able to adhere and disrupt. After being immobilized, CRW11 was once again evaluated against *E. coli*, *S. aureus* and *P. aeruginosa* planktonic bacteria, revealing a high bactericidal potential after being in contact with these strains for three hours. In addition, CRW11-immobilized

also displayed promising antibiofilm activities against *E. coli*, without being cytotoxic towards mammalian cells [57].

Although the above-mentioned coating strategies have proved be useful, immobilized synthetic peptides may lose their functionality over time. To solve this, researchers have proposed additional coating strategies, in which HDPs are incorporated into degradable hydrogels, nanoparticles (associated or not with colloidal carriers), as well as other film architectures that allow controlled and temporal release of antimicrobial agents in the sites of infection. These systems hold several advantages when compared to the ones previously described: i) under these conditions, the peptides are more stable both *in vitro* and *in vivo*; ii) increased effectiveness of peptide treatment, as both the levels of peptide that reach the infection sites and the time of exposure to the peptide are increased [58]. These strategies are particularly relevant in the context of biofilm-related infections, since the sustained-release of HDPs could serve to either protect a surface (*e.g.*, tissues) from biofilm formation or act more effectively on pre-formed biofilms.

Some examples of the approaches described above include a study by Shukla et al. [59] that showed for the first time the incorporation and release of the peptide ponericin G1 from thin films constructed based on a layer-by-layer assembly (Figure 2B). Among the three assemblies proposed, the one consisting of poly 2 (β-amino ester), alginic acid (polyanion) and ponericin G1 presented the highest index of peptide incorporation, as well as peptide release (65% after ~24 h) when compared with assemblies using chondroitin and dextran sulfates (polyanions). These properties seemed to also have an influence on the antimicrobial assays performed against *S. aureus*, in which ponericin G1 released from alginic acid films for a period of 10 days presented MIC values ranging from 15 – 30 $mg.mL^{-1}$, similar to those obtained using single ponericin G1 (11 – 22 $mg.mL^{-1}$). The two other assemblies did not affect the antimicrobial activity against *S. aureus*. Moreover, agar plates coated with poly 2/alginic acid/ponericin G1 completely (100%) inhibited initial attachment of *S. aureus* planktonic cells, thus preventing biofilm formation [59].

More recently, Angelo et al. [58] evaluated the ability of engineered poly (lactide-co-glycolide) (PGLA) nanoparticles (NPs) containing the cationic peptide colistin in eradicating pre-formed *P. aeruginosa* biofilms (Figure 2C). The authors showed that chitosan (CS)-modified NPs were able to release 50% of the encapsulated colistin in 6 h. This percentage was 10-fold higher than that obtained with poly (vinyl alcohol) (PVA)-modified NPs. However, after NP burst, both NP systems were able to sustain colistin release during ~15 days. Since *P. aeruginosa* biofilms are of great concern in lung infections (e.g., in cystic fibrosis patients), the engineered NPs were embedded into microparticles that contained inert carriers such as lactose and mannitol, following a specific spray-drying protocol [58]. This strategy represents a promising alternative for inhaled-based treatment [60]. Moreover, a recent report showed that delivery of high doses of colistin (160 mg of colistin solution, twice a day) through nebulization could be a good strategy to deliver intact peptides to conductive airways [58,61]. Further, it has been posed NPs containing colistin embedded into microparticles could be a promising alternative to the more traditional inhalation-based treatment [58]. When tested against pre-formed biofilms of *P. aeruginosa*, 7.5 and 15 $mg.mL^{-1}$ of free colistin caused a reduction of 90% of biofilm biomass in the

first 24 h. On the other hand, these same concentration of colistin/PVA NP and colistin/CS NP could only reduce biofilm biomass by 50 and 25%, respectively [58]. In contrast, the antibiofilm properties of free colistin decreased after 48 h, and were completely lost at 72 h, thus allowing biofilm regrowth. On the other hand, the activity of encapsulated colistin was sustained for 72 h. Importantly, it was also shown that both colistin/PVA NP and colistin/CS NP could penetrate inside *P. aeruginosa* biofilms, allowing the release of colistin *in situ*, therefore increasing the effectiveness of the treatments [58].

Medical device-associated infections are of great concern for public health, being one of the causes of recurrent surgeries, prolonged administration of antibiotics, and eventually patient death [62]. Therefore, the study of new alternatives and strategies for peptide immobilization to biomaterial surfaces that are able to retain their pharmacological potential and activity, as well a the development of nano and micro systems able to sustain the release of HDPs in the sites of infection, could be interesting strategies to help overcome such obstacles.

### 4.3 New concepts on mode of action against biofilms

Synthetic cationic peptides have been shown to kill bacteria through many different mechanisms [6] For instance, they have been shown to alter cytoplasmic membrane permeability, inhibit cell division septum formation, and inhibit a series of cellular processes including the synthesis of cell wall, nucleic acid, protein and enzymatic activity [63]. The multifunctional nature of peptide action has been proposed to be one of the bases for their low propensity to select for resistance in bacteria [6]. However, it is worth noting that while some peptides have similar inhibitory concentrations for planktonic and biofilm cells, strong antibiofilm peptides do not necessarily work well against planktonic cells and vice versa [14, 16, 18, 32, 64]. Insights into the mechanism of action of antibiofilm peptides were recently revealed. Peptide 1018 was shown to bind *in vitro* to the second messenger stress-induced nucleotide ppGpp and *in vivo* to stimulate its degradation in stressed cells [34]. This nucleotide is part of the stringent stress response in bacteria [65] and we were able to show that *E. coli*, *P. aeruginosa*, *S. enterica* and *S. aureus* cells unable to make this nucleotide exhibited biofilm-deficient phenotypes when grown in flow cells within 3 days, while overexpression of ppGpp rendered *P. aeruginosa* and *E. coli* cells resistant to peptide 1018 [34]. Similarly, antibiofilm D-enantiomeric peptides also targeted the intracellular signal (p)ppGpp [18]. In addition to its role in biofilm formation, ppGpp is also key in regulating the formation of persister cells [66, 67] that are extremely tolerant to antibiotic action, making ppGpp an attractive target for new antimicrobials. Along these lines, work by Chen et al demonstrated that certain antimicrobial peptides that contain Trp and Arg residues caused detachment of pre-formed biofilms and were used to efficiently treat persister cells [68].

Additionally, van Hoek and colleagues discovered a new mechanism of action for peptide LL-37 in its ability to interact with the cytoplasmic acyl carrier protein AcpP from *Francisella novicida, E. coli* and *B. anthracis* [69]. The study further found that the sheep cathelicidin peptide SMAP-29 facilitated binding of LL-37 to AcpP. This study identified a

novel intracellular target for LL-37 that may contribute to the broad-spectrum activity of this peptide.

### 4.4 Rational design

The biological activities of peptides can be optimized through structure-activity relationship studies based on previously collected experimental data. This approach involves the rational or semi-random design of peptides through mutation of individual amino acid residues in order to enhance the chemical and biological properties of parent peptides (e.g. reference 14).

Another strategy consists on the large-scale synthesis of peptide mutants derived from template peptides using the SPOT synthesis peptide array approach [7]. Such an approach was described in the recent study of Haney et al. [22] in which they used two starting peptide sequences (IDR-1002 - VQRWLIVWRIRK-$NH_2$ and IDR-HH2 - VQLRIRVAVIRA-NH2) that had been previously characterized as immunomodulatory peptides. The authors generated peptide arrays on cellulose membranes and eluted 100 variants of each peptide to subsequently systematically assess and improve the immunomodulatory and antibiofilm activities of the parent synthetic peptides. Single amino acid substitution libraries of both IDR-1002 and IDR-HH2 were generated substituting the nine constituent amino acids of both peptides (R, K, Q, G, A, W, V, L, I) at every position along the length of each peptide. All derived peptides were then evaluated using a 96-well plate assay for their antibiofilm activity against a MRSA clinical isolate. The results obtained served to generate substitution matrices for both peptides IDR-1002 and IDR-HH2, some of which showed equivalent or enhanced biological activities and informed the design of next generation peptides with improved antibiofilm activity. The authors then assessed the biological activity profiles of the next generation peptides and identified one lead peptide that demonstrated improved therapeutic potential.

## 5. Conclusions

Synthetic cationic peptides represent one of the most promising alternatives to overcome the problem of antibiotic resistance. These molecules exhibit a wide range of biological functions that include antimicrobial activity against planktonic cells, ability to modulate the immune system and antibiofilm properties. The antibiofilm activity represents an excellent strategy to counter antibiotic resistance, since biofilms exhibit pronounced increased resistance to most conventional antimicrobials prescribed by clinicians. Rational design approaches have allowed the design and synthesis of new peptides with improved, broad-spectrum, biological functions. Indeed, the increasing interest in peptides with antibiofilm activity has prompted the creation of an open-access, manually-curated database called BaAMPs [70] that can be accessed at http://www.baamps.it. The next challenge will be to design Gram-negative or Gram-positive-specific peptides, or peptides that selectively kill the pathogen of interest while leaving microbiome bacteria unaffected. In addition, some of these antibiofilm peptides can potentiate the action of available antibiotics, thus lowering the amount of antibiotic required and therefore decreasing the likelihood of resistance development. Studies focusing on the mechanisms of action of such peptides have revealed, for instance, that they act on the stressed-like nature of biofilms producing nucleotide

ppGpp. The next step in the field will be to confirm the activity of the identified lead peptides in animal models of biofilm infections. Some success has been achieved to date (for example, references 18, 28 and 71) but much work remains to be done to definitely establish these molecules as real alternatives to currently available antibiotics.

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## Highlights

- Bacteria tend to live in multicellular communities called biofilms that exhibit increased adaptive resistance to antibiotics.
- Naturally occurring host defense peptides (HDPs) represent excellent templates for engineering novel synthetic peptides with optimized biological activities.
- Bio-inspired synthetic peptides have potential for human health applications as they synergize with conventional antibiotics, can be used in biomaterials and exhibit activity in animal models.

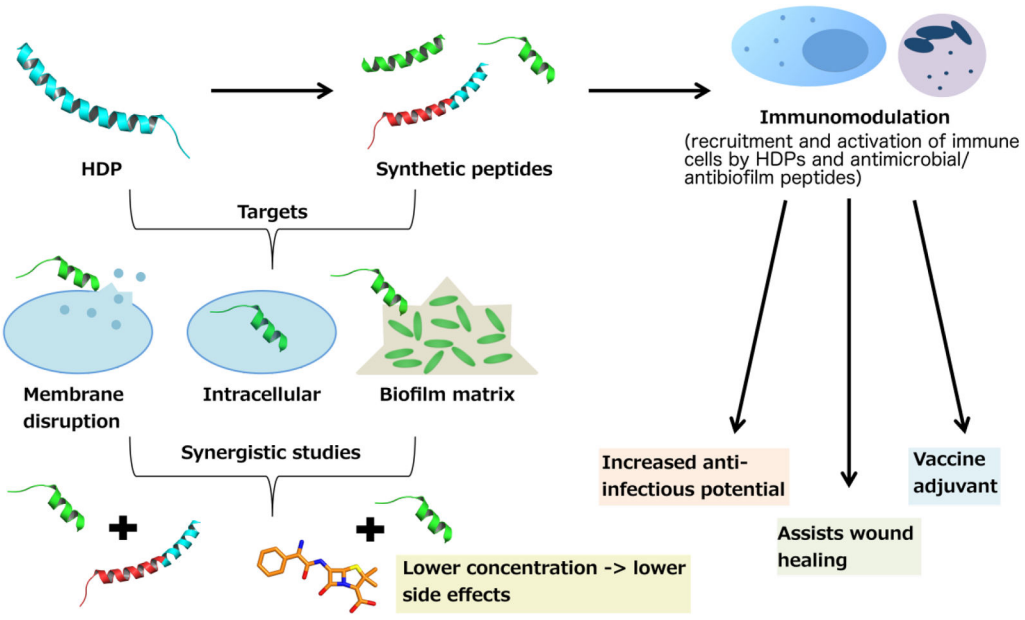


**Figure 1. Potential biotechnological uses of HDPs and their synthetic analogues**

HDPs and their derivatives can act both by direct killing of biofilms (alone or in combination with conventional antibiotics), being able to cause damage to the membrane of the targets cells, as well as by interfering with the homeostasis of the intracellular environment; and by immunomodulation, where the peptides posses the ability to recruit and activate cells from the immune system, facilitating bacterial clearance and increasing wound healing.

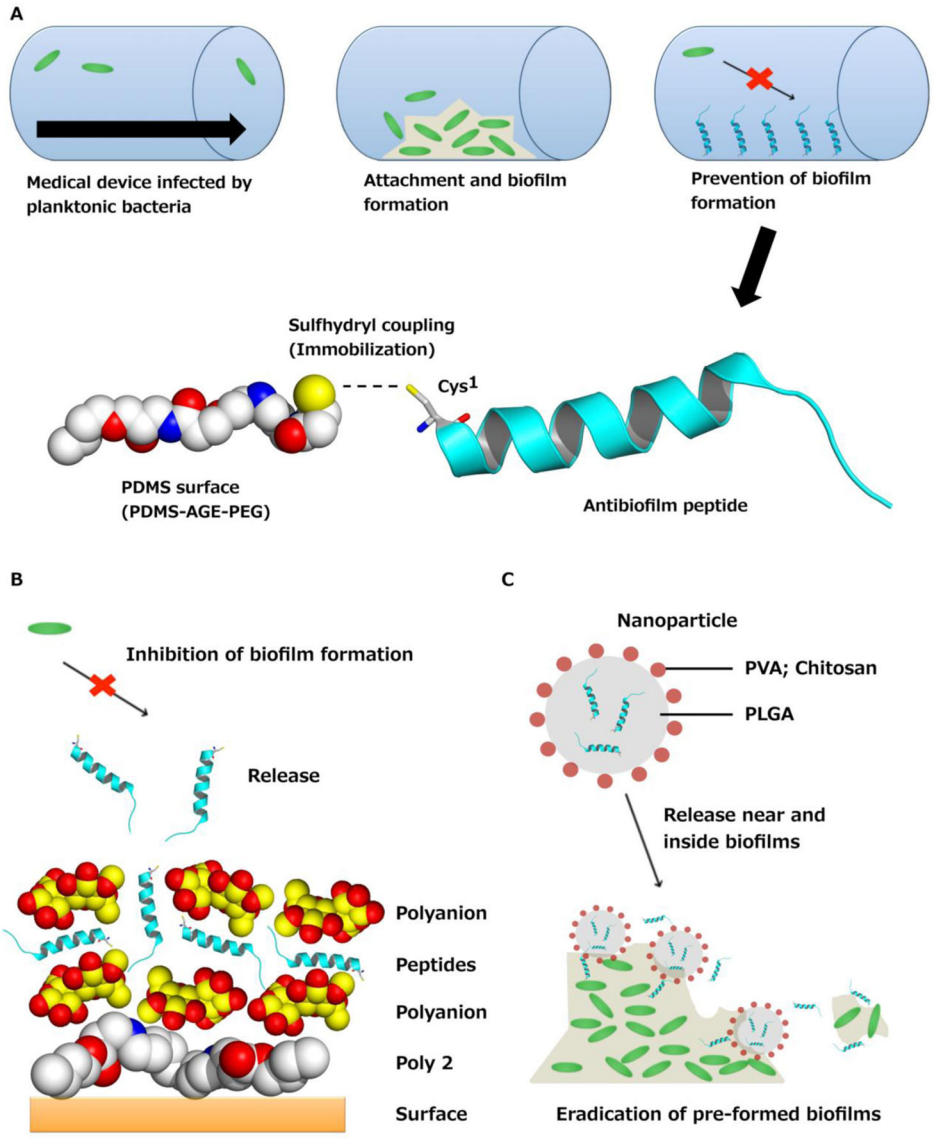


**Figure 2. Medical device coating strategy, layer-by-layer assembly and nanoparticle engineering to counteract biofilm formation**

**(A)** Representation of bacterial biofilm formation within a medical device before (top middle) and after coating with antibiofilm peptides (top right). The polymer illustrated below is a fusion of polydimethylsiloxane (PDMS), allyl glycidyl ether (AGE) and maleimide – polyethylene glycol (PEG) – amine whose maleimide group is used for a sulfhydryl coupling with the antibiofilm peptide containing a cysteine residue at the N-terminus. **(B)** Layer-by-layer construction consisting of the poly 2 (β-amino ester) and alginic acid (polyanion) in which HPDs are incorporated and subsequently released. **(C)** Engineered poly (lactide-co-glycolide) (PGLA) nanoparticles (NPs) coated with hydrophilic polymers (chitosan; PVA), which are able to optimize the efficiency of entrapment and modulate surface properties. HDPs are encapsulated within NPs, and are then released near and/or inside pre-formed biofilms.

**Table 1**

Synthetic peptides with antibiofilm activity

| Synthetic Peptide | Precursor | MBIC | Antibiofilm activity against | Type of Activity | Reference |
|---|---|---|---|---|---|
| 1037 | LL-37 | 5 – 10 μg.mL$^{-1}$ | *P. aeruginosa*, *L. monocytogenes* | Inhibition/Eradication | [14] |
| 1018 | Bactenecin | 0.8 – 10 μg.mL$^{-1}$ | *P. aeruginosa*, *E. coli*, *A. baumannii*, *Klebsiella pneumoniae*, *S. enterica* and MRSA | Inhibition/Eradication | [34] |
| AS10 | CRAMP | 0.47 μg.mL$^{-1}$ | *C. albicans* | Inhibition | [24] |
| Battacin | Lipopeptides | 5 – 51 μg.mL$^{-1}$ | *P. aeruginosa*, *P. syringe* pv. actinidiae, *S. aureus* | Inhibition/Eradication | [33] |
| BMAP27-melittin | Melittin | 23.56 μg.mL$^{-1}$ | *S. aureus*, *P. aeruginosa* | Inhibition/Eradication | [30] |
| CAMA | Cecropin-A and Melittin-A | 14.16 μg.mL$^{-1}$ | MRSA | Inhibition/Eradication | [31] |
| DJK-5 and DJK-6 | Synthetic analog of active antibiofilm peptides | 0.5 to 8 μg.mL$^{-1}$ | *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae* and *S. enterica* | Inhibition/Eradication | [18] |
| DJK-6 | Same as above | 2.0–4.0 μg.mL$^{-1}$ | *K. pneumoniae KpC* | Inhibition/Eradication | [39] |
| LL7-31 and LL7-37 | HDP LL-37 | 15 – 313 μg.mL$^{-1}$ | *P. aeruginosa* | Inhibition/Eradication | [19] |
| NRC-16 | Pleurocidin | 8 – 35 μg.mL$^{-1}$ | *P. aeruginosa* | Inhibition | [29] |
| P10 | P60.4Ac | 6.19 μg.mL$^{-1}$ | MRSA | Inhibition/Eradication | [27] |
| P318 | CRAMP | 0.45 μg.mL$^{-1}$ | *C. albicans* | Inhibition | [24] |