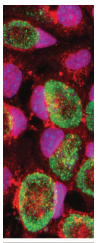

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MINIREVIEW

Treatment of microbial biofilms in the post-antibiotic era: prophylactic and therapeutic use of antimicrobial peptides and their design by bioinformatics tools

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This is a comprehensive review of antimicrobial peptides against biofilm-growing bacteria. The review gives an in-depth view of the mechanisms of antimicrobial peptides and is essential for all researchers working with these.

Keywords
microbial biofilm; antibiotic resistance; antimicrobial peptides; surface coating; biofilm simulations; AMP modeling and design.

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Abstract

The treatment for biofilm infections is particularly challenging because bacteria in these conditions become refractory to antibiotic drugs. The reduced effectiveness of current therapies spurs research for the identification of novel molecules endowed with antimicrobial activities and new mechanisms of antibiofilm action. Antimicrobial peptides (AMPs) have been receiving increasing attention as potential therapeutic agents, because they represent a novel class of antibiotics with a wide spectrum of activity and a low rate in inducing bacterial resistance. Over the past decades, a large number of naturally occurring AMPs have been identified or predicted from various organisms as effector molecules of the innate immune system playing a crucial role in the first line of defense. Recent studies have shown the ability of some AMPs to act against microbial biofilms, in particular during early phases of biofilm development. Here, we provide a review of the antimicrobial peptides tested on biofilms, highlighting their advantages and disadvantages for prophylactic and therapeutic applications. In addition, we describe the strategies and methods for *de novo* design of potentially active AMPs and discuss how informatics and computational tools may be exploited to improve antibiofilm effectiveness.

Introduction

In the majority of chronic infections, microorganisms are rarely found as planktonic organisms. Rather, they gather in biofilm communities as a consequence of complex developmental processes emerging in response to environmental changes. Thus, biofilms can be considered as a physiological state of a broad spectrum of microorganisms, including pathogens, typically attached to biotic (e.g. tissues) or abiotic sites (Costerton *et al.*, 1999). A biofilm is constituted of single or multiple organism species, such as fungi, bacteria, and viruses, existing at a phase or density interface, and encased in a self-secreted extracellular matrix. This matrix mainly consists of hydrated polysaccharides, proteins, glycopeptides, extracellular DNA, and lipids (Donlan & Costerton, 2002). Biofilms tend to be polymicrobial. Besides competition for nutrients and space, the cohabitation may also promote cooperative interactions such as horizontal gene transfer, metabolic cooperation, and other synergies, resulting in improved survivability of the microorganisms and resistance to antimicrobial agents (Yang *et al.*, 2011; Wolcott *et al.*, 2013). For example, it has been found that dental biofilms contain more than 500 different bacteria taxa (Whittaker *et al.*, 1996) and that the co-existence of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* plays a key role in the progression of chronic periodontitis, considered a clear polymicrobial biofilm etiology disease (Zhu *et al.*, 2013b). The co-existence between bacteria and fungi in polymicrobial biofilm was also proved. The polymorphic opportunistic fungus *Candida albicans* and the bacterial pathogen *Staphylococcus aureus* can be co-isolated from several diseases and from the surfaces of various biomaterials, including dentures, voice prostheses, implants, endotracheal tubes, and feeding tubes (Shirtliff *et al.*, 2009).

According to several estimates, most infections in the developed world are characterized by the involvement of

biofilms. In the clinical environment, microbial biofilms are a perpetual source of nosocomial infections, accounting for over 65% of hospital-acquired infections, via colonization of medical devices and implants such as catheters, prosthetic heart valves, and joint replacement (Costerton *et al.*, 1999; Wenzel, 2007; Bryers, 2008). After formation, a biofilm cannot be easily eliminated by standard clinical procedures, and the infection often can only be eradicated by the removal of the infected implant, thus increasing the trauma to the patient and the treatment cost (Römling & Balsalobre, 2012). Biofilm-related microorganisms are also responsible for some chronic diseases in humans such as endocarditis, osteomyelitis, otitis media, urinary tract infections, and lung colonization in patients with cystic fibrosis (Hall-Stoodley *et al.*, 2012; Römling & Balsalobre, 2012). Although infection incidence has been reduced by aseptic surgical techniques and prophylactic systemic antibiotic therapy, it has become clear that microorganism colonization by biofilms still has an enormous impact on medicine and represents a serious hazard for human health.

Due to the physiological properties of biofilm phenotype, bacteria and fungi in the communities become highly resistant to many traditional therapies, exhibiting much higher antibiotic/antifungal resistance levels (up to 1000-fold) compared with those normally observed during planktonic growth (Costerton *et al.*, 1999; Mah & O'Toole, 2001). Several mechanisms have been proposed to explain the phenomenon of drug resistance within biofilms, including (1) delayed/suppressed penetration of the antimicrobial into the extracellular matrix, due to the presence of biofilm-typical exopolysaccharide hindering antibiotic diffusion into the biofilm by electrostatic repulsion and/or sequestration; (2) presence of metabolically inactive nondividing 'persister' cells able to survive the antimicrobial attack, preventing complete elimination of the colony; (3) increased ability to exchange mobile genetic elements encoding resistance, thanks to cell vicinity (Høiby *et al.*, 2010; Mah, 2012).

Great research efforts have been directed at developing effective antimicrobial agents able to overcome drug resistance in biofilms. Antimicrobial peptides (AMPs) have emerged as an attractive target area from which to source new antibiofilm technology solutions. Indeed, AMPs are active against a wide range of infectious microorganisms and against metabolically inactive cells, having as main mechanism of action the permeabilization of the cellular membrane. For the same reason, they induce bacterial resistance at lower rates with respect to common antibiotics, because emergence of resistance to bilayer-disruptive AMPs would entail changing membrane composition and organization, a 'costly' process in evolutionary terms (Zasloff, 2002). Over the past decades, a large number of naturally occurring AMPs have been identified and evaluated as antimicrobial molecules, especially against planktonic cells. An updated list can be found at the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). In recent years, AMPs have also been tested as new therapeutic agents in biofilm-related infections. Although no biofilm-active AMP has so far reached clinical and commercial use, substantial developments can be anticipated from future design and optimization aimed at improving AMP antibiofilm activity, minimizing cytotoxicity, reducing proteolytic degradation, or activity inhibition and promoting synergy with conventional antibiotics (Fjell *et al.*, 2012; Maccari *et al.*, 2013).

This review presents a survey of antimicrobial peptides employed in fighting microbial biofilms, in connection with both prophylactic and therapeutic strategies, especially in healthcare settings. The review also covers computational tools that are expected to play an important role in the design and optimization of AMPs.

AMP classification and mechanisms of action

AMPs are small evolutionally conserved molecules found in virtually every life form, from multicellular organisms to bacterial cells. In higher organisms, AMPs play a major role in innate immunity as a part of the first defense line directly against invading pathogens or by modulating the immune response (Hancock & Scott, 2000). For example, anuran tissues are the source of more than 1000 different AMPs (Novković *et al.*, 2012), some of them active, alone or in synergy with antibiotics, even against emerging opportunist pathogens as *Stenotrophomonas maltophilia* (Maisetta *et al.*, 2009).

AMPs can be classified according to their physiochemical properties (net charge, hydrophobicity, amphipathicity) and their derived characteristics, such as secondary structure (see Fig. 1a) and solubility (Park & Hahm, 2005). Among the most abundant and widespread AMPs in nature, cationic alpha-helical AMPs are able to perturb the cytoplasmic membrane and determine cell death by osmotic shock. Some of the most studied AMPs in this group are cecropin, magainin, the human cathelicidin LL-37, and their derivates (Hancock & Rozek, 2002). Anionic AMPs have also been described (Harris *et al.*, 2009). Most of them adopt an amphiphilic alpha-helical conformation. However, the mechanisms underlying their antimicrobial activity are unclear. For example, the mode of action of dermcidin, one of the best-studied human anionic AMPs, is still controversial, although experimental studies demonstrated its assembly into an oligomeric state to form a pore across the lipid bilayer (Paulmann *et al.*, 2012).

A second structural class includes peptides such as those belonging to mammalian defensin and protegrin families with several intramolecular disulfide bonds stabilizing an amphipathic beta-sheets conformation. Peptides in this class have been found to possess greater selectivity toward microbial cell membranes compared with their alpha-helical counterparts of equal charge and hydrophobicity (Jin *et al.*, 2005).

Not all AMPs belong to the above-mentioned classes; some AMPs lack a well-defined secondary structure. Usually such peptides contain rather uncommon amino acids, such as proline and/or glycine or tryptophan or histidine. Indolicidins, initially isolated from bovine neutrophils, are particularly important AMPs in this class and display high tryptophan and proline content. Indolicidins' mechanism of action is twofold, including both initial activity on the

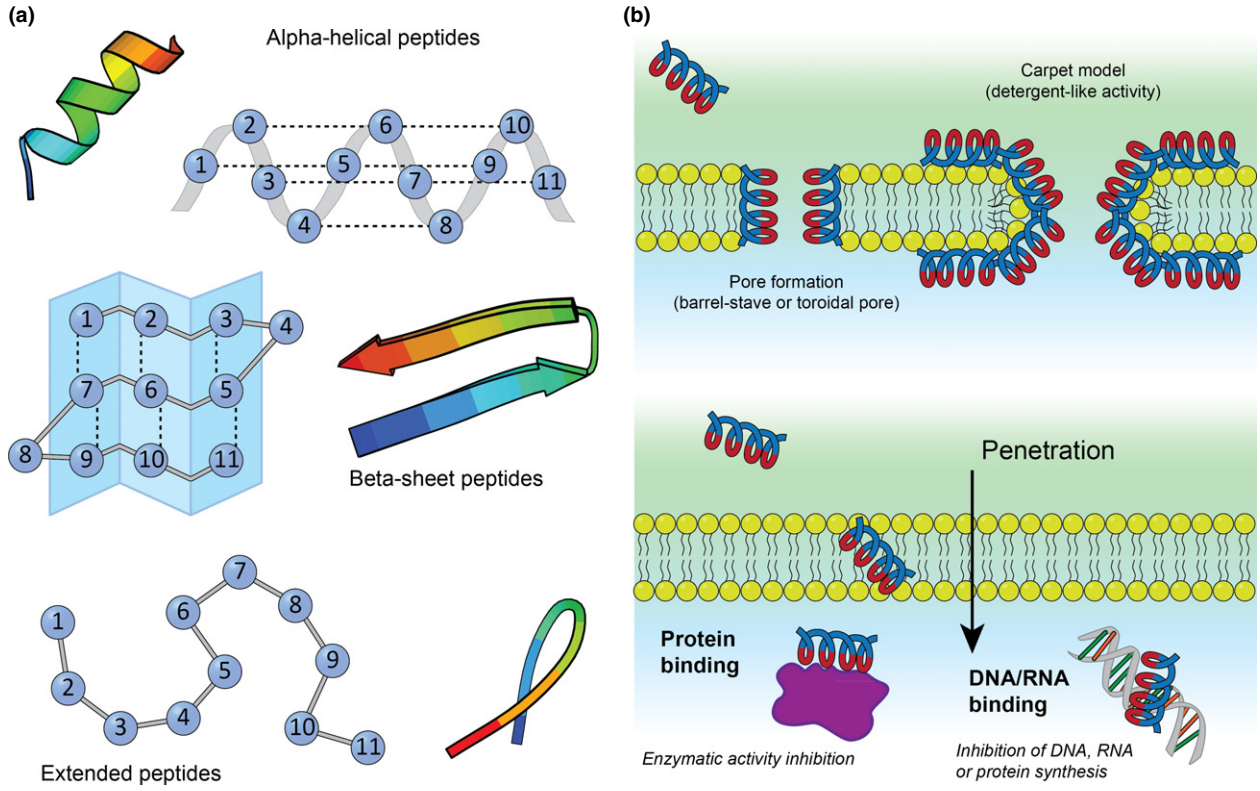

Fig. 1 Peptide classification strategies. (a) AMPs can be distinguished into three structural classes: alpha-helical AMPs, beta-sheet AMPs, and extended AMPs, a particular class usually with uncommon amino acid composition, such as proline and/or glycine or tryptophan or histidine amino acids. (b) On the basis of the mechanism of action, AMPs can be roughly classified into two families: membrane-targeting peptides (top panel) and peptides with intracellular targets (bottom panel).

cytoplasmic membrane and intracellular activity in preventing DNA replication (Friedrich *et al.*, 2001).

In fact, another way to classify AMPs is on the basis of their molecular targets: membrane-targeting peptides and intracellular-targeting peptides (Fig. 1b). Membrane perturbation activity is usually determined by at least three mechanisms (Bahar & Ren, 2013). The best-characterized models, the 'barrel-stave' and the 'toroidal-pore' models, rely on the peptide ability to form ordered transmembrane channels/pores, while in the so-called carpet model, the peptides disrupt the bilayer in a detergent-like manner, eventually leading to the formation of micelles (Shai & Oren, 2001). However, it has been found that some AMPs can cross the lipid bilayer without provoking any damage to the cell membrane and target intracellular components by binding to DNA, blocking enzyme activity, or inhibiting the synthesis of protein, cell wall, and nucleic acids. For example, buforin II, a partial alpha-helix amphipathic peptide, was shown to inhibit the cellular functions by binding to nucleic acids after penetrating the cell membranes, resulting in rapid cell death (Park *et al.*, 1998).

AMPs active against microbial biofilm

In the last 10 years, interest in biofilm treatment by AMPs has been increasing dramatically. The number of articles on AMPs and biofilm has reached more than 60 in 2012 and more than 70 in 2013, whereas only a few (< 5) articles were published in 2002–2003 (source: ISI Web of Science).

As mentioned in the Introduction, AMPs mechanisms of antimicrobial activity result in a low rate of induced resistance, crucial against biofilms, where the risk to select resistant strains is certainly higher, and in efficacy against a wide range of microorganisms, particularly suitable to treat biofilms with polymicrobial character (Batoni *et al.*, 2011). Other mechanisms of action include inhibition of protein synthesis, binding with DNA, and detoxification of lipopolysaccharide (LPS). In addition, AMPs can interact with polysaccharide components of the matrix and disaggregate biofilms (Mah & O'Toole, 2001; Park *et al.*, 2011; Mah, 2012). AMPs, in particular cationic alpha-helix peptides, are able to synergize with antibiotics and to be active even against multidrug-resistant microorganisms. Current anti-biofilm control can be either prophylactic or therapeutic (Fig. 2). The prophylactic strategy aims at preventing biofilm development by killing planktonic cells potentially able to form biofilm, either hindering their adhesion to a surface or inhibiting the growth of attached cells in the early biofilm stage. The therapeutic approach targets the more difficult tasks of reduction or eradication of mature biofilms (Jorge *et al.*, 2012).

A number of natural, semi-synthetic, and synthetic AMPs have been proven active against microbial biofilms. Recent

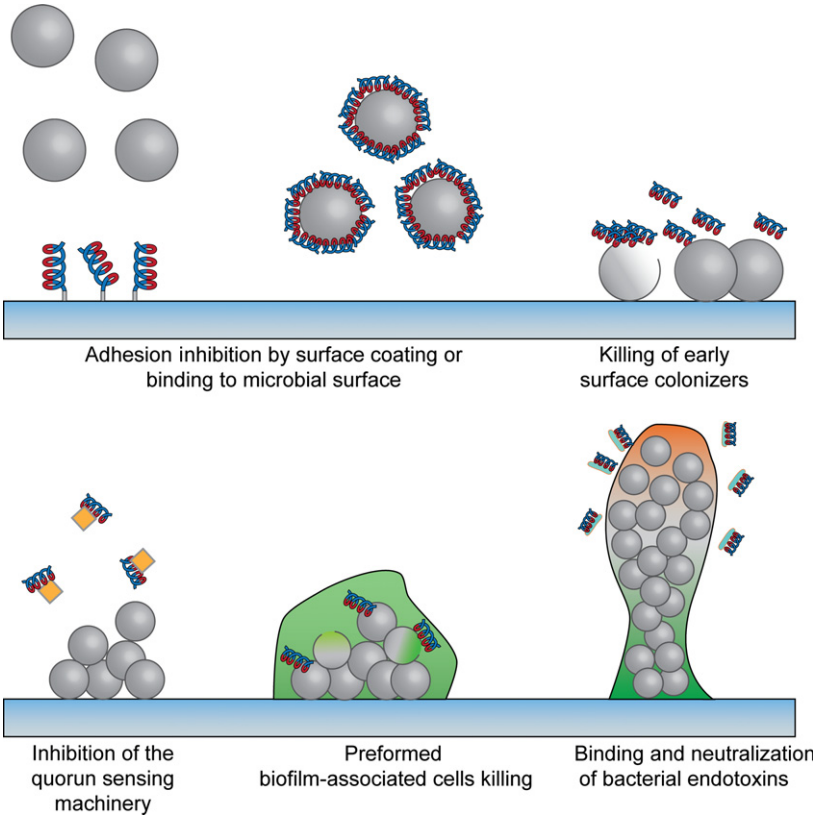

Fig. 2 Mechanism of action of AMP-mediated antibiofilm strategies. Top panel, biofilm formation inhibition strategies. AMPs can prevent the initial biofilm adhesion by coating of medical device surface or by interacting with microbial surface. Biofilm maturation can be prevented by killing the early surface colonizers. Bottom panel, biofilm eradication strategies. AMPs can bind to the quorum-sensing molecules to inhibit bacteria communication. AMPs can kill preformed biofilm by penetrating the biofilm matrix and killing biofilm-associated cells. AMPs may bind and neutralize bacterial endotoxin released by biofilm-associated cells.

studies of AMPs tested against *in vitro* and *in vivo* biofilm models are summarized in Tables 1 and 2, respectively, whereas the most characterized AMPs exerting an antibiofilm activity are reported below.

Lactoferrin

Lactoferrin is an abundant multifunctional iron-binding protein of the innate immune system found in several mammal biological fluids (especially in milk), which is known to exert a broad-spectrum antimicrobial activity against bacteria, fungi, protozoa, and viruses. An antibiofilm activity of lactoferrin and its derivatives was also described (Singh *et al.*, 2002; Ammons *et al.*, 2009).

In vitro studies demonstrated that lactoferrin at 20 μg mL^{-1} (sub-MIC value) is able to inhibit *P. aeruginosa* biofilm formation by preventing surface adhesion and stimulating bacterial motility (Singh *et al.*, 2002). In addition, the oral administration of lactoferrin to mice challenged with *Escherichia coli* determines a reduction in *E. coli* bacterial cells from the lower intestine, suggesting that lactoferrin inhibits the bacterial adhesion to epithelial cells and intestinal mucosa *in vivo* (Giugliano *et al.*, 1995).

The antibiofilm mechanism of lactoferrin and its derivatives still remains to be elucidated, although some researchers have linked it to its iron-chelating nature. Iron ions participate in a large number of biological processes in microorganisms and are essential for biofilm formation, where they regulate surface motility, stabilize the polysaccharide matrix, and are considered critical for transition from planktonic to sessile existence (Weinberg, 2004). Thus, iron sequestration by lactoferrin was proposed as a potential means to impair biofilm development in *Klebsiella pneumoniae*, *P. aeruginosa*, and *E. coli* (Chhibber *et al.*, 2013). However, lactoferrin is also known to penetrate biofilm matrix and directly interact with the cellular membrane compromising cell integrity (Ammons *et al.*, 2009). More details about the antibiofilm properties of lactoferrin are available in the recent review by Ammons and Copié (Ammons & Copié, 2013).

Cathelicidins and derivatives

One of the most well-known families of antimicrobial peptides is cathelicidins. LL-37, the only human member of cathelicidins, is derived proteolytically from the C-terminal end of the human CAP18 protein. Together with other cathelicidin derivatives, this peptide displays killing activity against a broad range of Gram-negative and Gram-positive bacteria *in vitro* (Turner *et al.*, 1998; Dean *et al.*, 2011; Kanthawong *et al.*, 2012) and exerts a robust antibiofilm effect at subinhibitory concentrations (Jacobsen & Jenssen, 2012).

LL-37 strong inhibition of bacterial biofilm formation was evaluated for the first time *in vitro* on *P. aeruginosa* and was proven already effective at very low sub-MIC concentration (0.5 μg mL^{-1}). Microarray assays demonstrated that LL-37 affected biofilm formation by stimulating twitching motility,

Table 1 Example of AMPs active against microbial biofilm

Name	Organism	Antibiofilm activity			Minimal active concentration (μM*)	Reference
		A	G	P		
MUC7 20-mer	*S. mutans*			X	25	Wei *et al.* (2006)
C16G2	*S. mutans*			X	25	Eckert *et al.* (2006b)
M8-33	*S. mutans*			X	25	Eckert *et al.* (2006b)
M8G2	*S. mutans*			X	25	Eckert *et al.* (2006b)
AAP2	*S. mutans*			X	40	Li *et al.* (2010)
Lys-a1	*S. mutans*		X		7.55	Da Silva *et al.* (2013)
Indolicidin	*S. aureus*		X		0.08	Mataraci & Dosler (2012)
				X	335.78	
		X			0.84	
Nisin	*S. aureus*		X		0.05	Mataraci & Dosler (2012)
				X	183.09	
		X			0.46	
Cecropin A (1-7)-Melittin	*S. aureus*	X			0.45	Mataraci & Dosler (2012)
			X		0.04	
				X	361.55	
$F_{2,5,12}W$	*S. epidermidis*		X		10	Molhoek *et al.* (2011)
				X	10	
Hepcidin 20	*S. epidermidis*	X			50	Brancatisano *et al.* (2014)
G10KHc	*P. aeruginosa*			X	23.45	Eckert *et al.* (2006a)
1026	*P. aeruginosa*		X		1.27	De la Fuente-Núñez *et al.* (2012)
1037	*P. aeruginosa*		X		4.07-123.70	De la Fuente-Núñez *et al.* (2012)
LL-25	*P. aeruginosa*		X		10	Nagant *et al.* (2012)
LL-31	*P. aeruginosa*		X		5	Nagant *et al.* (2012)
LL7-37	*P. aeruginosa*		X		5	
NRC-16	*P. aeruginosa*		X		16	Gopal *et al.* (2013)
KR-12	*A. baumannii*	X			81.48	Feng *et al.* (2013)
			X		81.48	
KR-20	*A. baumannii*	X			25.94	Feng *et al.* (2013)
			X		25.94	Feng *et al.* (2013)
KS-30	*A. baumannii*	X			17.57	Feng *et al.* (2013)
			X		17.57	Feng *et al.* (2013)
KSL-W	*C. albicans*		X		19.12	Theberge *et al.* (2013)

A, inhibition of adhesion; G, inhibition of growth; P, eradication of preformed biofilm.
*Minimal concentration determining 50% reduction of biofilm compared with untreated control.

Table 2 Example of AMPs active against microbial biofilm *in vivo* model

Name	Organism	Model	Peptide concentration	Reference
DASamP1	*S. aureus*	Mouse catheter-associated biofilm	Repeated injections of 200 μg of peptide into and different sites surrounding the catheter	Menousek *et al.* (2012)
LL-37 derivative peptide	*P. aeruginosa*	Rabbit sinusitis biofilm	2.5 mg mL^{-1}	Chennupati *et al.* (2009)
IB-367	*S. aureus*/*E. faecalis*	Rat CVC-associated biofilm formation	10 μg mL^{-1}	Ghiselli *et al.* (2007)
Tachyplesin III	*P. aeruginosa*	Rat urethral stent biofilm formation	10 μg mL^{-1}	Minardi *et al.* (2007)
Citropin 1.1	*S. aureus*	Rat CVC-associated biofilm formation and mature biofilm	10 μg mL^{-1}	Cirioni *et al.* (2006a)
BMAP-28	*S. aureus*	Rat CVC-associated biofilm formation	10 μg mL^{-1}	Cirioni *et al.* (2006b)
DD_{13}-RIP	*S. aureus*	Rat graft-associated biofilm formation	10–50 μg mL^{-1}	Balaban *et al.* (2004)

thereby reducing the attachment of bacterial cells, and by influencing two major quorum-sensing systems (Las and Rhl), leading to the down-regulation of genes essential for biofilm development (Overhage *et al.*, 2008). To evaluate the effect of LL-37 on the established 2-day-old *P. aeruginosa* biofilms, 4 μg mL^{-1} peptide was incubated for 48 h prior to staining and microscopy analysis. Compared with the 50-μm-thick untreated biofilm, confocal microscopy

images of this 2-day LL-37-treated biofilm revealed a reduced thickness of 20 μm and the lack of a characteristic architecture of the mature biofilms (Overhage *et al.*, 2008).

The inhibitory effects of LL-37 on biofilm formation and initial attachment of *Staphylococcus epidermidis* have been reported (Hell *et al.*, 2010). The biofilm mass was reduced to 42% in the presence of 1/32MIC (1 μg mL^{-1}) of LL-37 (Hell *et al.*, 2010). Recently, LL-37 and its truncated variant, LL-31, were also found to be strongly active against *B. pseudomallei* biofilm (Kanthawong *et al.*, 2012).

Pompilio *et al.* (2011) evaluated antibiofilm effects of three different cathelicidin-derived peptides (SMAP-29, BMAP-28, and BMAP-27) against *S. aureus, P. aeruginosa*, and *S. maltophilia* strains isolated from cystic fibrosis patients and compared them to those of tobramycin. They found that the cathelicidin-derived peptides, showing a faster kinetics and a rapid bactericidal activity against all the species tested in planktonic condition of growth, exhibit also an antibiofilm formation activity at sub-MIC concentration (ranging 2–8 μg mL^{-1}; Pompilio *et al.*, 2011).

Starting from NA-CATH, a snake cathelicidin, Dean *et al.* (2011) designed a new peptide, NA-CATH:ATRA1-ATRA1, which contains two mutations increasing hydrophobicity and enhancing the helical character, and presumably favoring peptide–membrane interaction. NA-CATH:ATRA1-ATRA1 exhibits antibiofilm activity against *S. aureus* at lower concentrations than both the parent peptide and LL-37. Both NA-CATH and NA-CATH:ATRA1-ATRA1 do not inhibit attachment of *S. aureus* cells, pointing to a different mechanism of action with respect to LL-37. The authors suggest that NA-CATH peptides may act internally on the bacteria, affecting the expression of genes essential for the development of biofilm (Dean *et al.*, 2011). The use of LL-37 to treat polymicrobial biofilm forming in wounds was recently reviewed (Duplantier & van Hoek, 2013).

Human β-defensin 3

Defensins are cationic peptides containing six cysteine residues that form three intramolecular disulfide bridges, resulting in a triple-stranded beta-sheet structure. In humans, two classes of defensins can be found: α-defensins and β-defensins. Among human defensins, β-defensin 3 (hBD-3) exhibits a strong broad-spectrum antibacterial activity and is resistant to trypsin and trypsin-like proteases action (Maisetta *et al.*, 2006, 2008). In recent studies, the antibiofilm properties of hBD-3 were tested (Huang *et al.*, 2012; Zhu *et al.*, 2013a) against methicillin-resistant *S. epidermidis* and *S. aureus* by evaluating bacterial adhesion, biofilm formation, and maturation on titanium, an orthopedic implant material. hBD-3 inhibited adhesion at 1MIC concentrations (4–8 μg mL^{-1}). However, higher concentrations (2–6 MIC) were needed to avoid biofilm maturation (Zhu *et al.*, 2013a). HBD-3 antibacterial efficacy was also tested on 3-week-old polymicrobial mature biofilm (composed of four oral bacterial species *Actinomyces naeslundii*, *Lactobacillus salivarius*, *S. mutans*, and *Enterococcus faecalis*) using confocal microscopy and dead/live fluorescent staining. Lee *et al.* (2013) observed that 24-h incubation of the biofilm with hBD-3 (50 μg mL^{-1}) resulted in a higher percentage of dead cells compared with both untreated samples and samples treated with common disinfectant solutions.

Histatin derivatives

Histatins are a family of low molecular weight, histidine-rich, cationic proteins produced and secreted by human parotid and submandibular-sublingual glands. Synthetic histatin analogs, exhibiting broad-spectrum antibacterial activity *in vitro,* were also active against complex mixtures of bacteria, such as those present in saliva and plaque. In particular, 100-mg mL^{-1} dhvar4 peptide, a synthetic histatin analog, determined a significant reduction in the number of CFUs in a model for oral biofilm especially of Gram-negative bacteria (Helmerhorst *et al.*, 1999).

Histatins are also active against fungal biofilms. In particular, activity of histatin-5 was tested against biofilms of *C. albicans*, a microorganism capable of colonizing polymeric surfaces of dentures and other prostheses introduced into the oral cavity. Treatment with histatin-5 significantly reduced the development of *C. albicans* biofilm grown on denture acrylic for 72 h, indicating that the peptide acts during late stages of biofilm development (Pusateri *et al.*, 2009). Recently, Tati *et al.* (2013) constructed a conjugate peptide using spermidine (Spd) linked to the active fragment of histatin-5 (Hst 54-15). They found that Hst 54-15-Spd was significantly more effective than histatin-5 alone in killing *C. albicans* and *Candida glabrata* in both planktonic cells and biofilm, retaining high activity in both serum and saliva. *Candida albicans* biofilm treated with Hst 54-15-Spd was reduced by 43% in mass, compared with only 24% reduction with the parental peptide. In *C. glabrata*, where histatin-5 showed no effect, Hst 54-15-Spd resulted in 41% mass reduction (Tati *et al.*, 2013). In addition, the effect of Hst 54-15-Spd for topical use was tested *in vivo*, in an immunocompromised mice challenged with oropharyngeal candidiasis. A 3–5 log-fold reduction in *C. albicans* colonies in tongue tissues suggests that Hst 54-15-Spd conjugates are good candidates as topical therapeutic agents for oral candidiasis (Tati *et al.*, 2013).

AMP prophylactic strategies

Designing and formulating drug delivery systems for AMPs has been a persistent challenge because of enzymatic degradation and their unfavorable physicochemical properties, such as molecular aggregation, serum sequestration, and cytotoxicity (Friedrich *et al.*, 1999; Park & Hahm, 2005; Yu *et al.*, 2011). The main target is to improve the local bioavailability from $< 1\%$ to at least 30–50%. Various strategies currently under investigation include chemical modification, formulation vehicles, and co-treatment with enzyme inhibitors or absorption enhancers. Entrapment of antibiotics in nanosized carriers has proven effective for treating infectious diseases, including antibiotic resistant ones (Huh & Kwon, 2011). Incorporation of AMP into polymeric nanocarriers has been recently reviewed (Sobczak

et al., 2013). These delivery strategies may be promising approaches also for AMP-based treatment for biofilms, although no specific example is currently available. Until now, the most pursued and successful strategy combining AMPs and biomaterials has been surface coating, a strategy that enables the direct administration to the implant site and takes advantage of the modular amino acidic composition of AMPs.

Biofilms are capable of colonizing almost every kind of material (metals, ceramics, and polymers). Such a pervasive proliferation may compromise medical devices, such as catheters, heart valves, stents, shunts, and fracture fixation devices (Hetrick & Schoenfisch, 2006; Matl *et al.*, 2008). A strategy to contrast biofilm formation on prosthetic materials involves surface coating with antimicrobial molecules. This approach has the advantage of delivering drugs directly to the implant site, resulting in locally high drug doses without exceeding the systemic toxicity levels (Zhang *et al.*, 2010).

AMPs are particularly indicated to work on solid surfaces, as such an arrangement resembles their natural application. As a consequence of covalent immobilization, AMPs can increase their long-term stability while decreasing toxicity and may preserve their optimal configuration. An effective strategy of immobilization requires careful consideration of different variables, such as the type of solid support, the immobilization methods, the surface density, the peptide sequence and secondary structure, and the introduction of spacers between peptide and surface. Different solid supports have been used for the production of AMP-coated surfaces, including metals, glass, and polymers (Haynie *et al.*, 1995; Bagheri *et al.*, 2009; Kazemzadeh-Narbat *et al.*, 2013).

For example, Tet-20 (a variant of scrambled versions of bovine cathelicidin Bac2A) tethered on an implant surface exhibited broad antimicrobial activities both *in vivo* (rats) and *in vitro* and appeared to be nontoxic to eukaryotic cells (Gao *et al.*, 2011). In another study, Yoshinari *et al.* (2010) evaluated the biofilm formation of *P. gingivalis* on a titanium sensor coated with histatin-5 and lactoferricin (a lactoferrin derivative peptide) and concluded that the coating strongly reduced *P. gingivalis* biofilm formation.

Chemical strategies to covalently bind peptides to surfaces may vary for each combination of substrate, linker, AMP, and orientation. One important factor is whether AMP activity is preserved upon immobilization. A pioneering study on immobilized AMPs analyzed the activity of some naturally occurring and designed AMPs tethered on a polyamide resin (Haynie *et al.*, 1995). Antimicrobial tests against several Gram-positive and Gram-negative bacteria showed that immobilized peptides retained their lethal activity, despite a highly reduced potential penetration depth, due to the short spacer used. Nonetheless, the coating strategy must be chosen carefully to preserve the original AMP structure, otherwise its activity may be compromised. For example, in Haynie *et al.* (1995), only AMPs retaining the original alpha-helical structure exhibited antibacterial activity. Beta-sheet peptides conjugated to a PEG-PS resin demonstrated an increased antimicrobial activity, presumably because of a more stable secondary structure, induced by the immobilization (Cho *et al.*, 2007).

Most of the AMP-coating studies in the literature present a spacer between the surface and the peptide (Bagheri *et al.*, 2009; Lim *et al.*, 2013). In particular, PEG spacer presents several advantages, such as its nonfouling characteristic and the ability to easily modulate the spacer length. Moreover, a flexible spacer may facilitate peptide contact with bacteria, hence increasing its antimicrobial activity. However, different peptides may require different strategies, as the suitability of a spacer can be directly associated with the mode of action of the specific AMP. For example, Hilpert *et al.* (2009) reported that cationic peptides directly immobilized to the surface with no spacer displayed bactericidal activity, probably due to the perturbation mechanism of the tested AMPs. These results suggest that a proper spacer needs be chosen to assure a sufficient mobility for pore-forming peptides, while it is not mandatory for membrane-perturbing AMPs.

Peptide orientation can be correlated with differential antimicrobial activities. Usually, the chain position and orientation is chosen following previously acquired experimental knowledge, or on the basis of experimental needs, such as the availability of functional groups suitable for a particular coupling chemistry. Hilpert *et al.* (2009) performed a systematic study of 122 short AMPs directly synthesized on a cellulose support modifying both the coupling strategy and peptide orientation. Interestingly, increased antimicrobial activity was observed as the cationic residues were placed close to the linker site, in the C-terminus. Furthermore, the positioning of hydrophobic residues proximal to the N-terminus was critical to the antimicrobial activity, presumably because the hydrophobic region is more prone to interact with the bacteria membrane.

Covalent AMPs immobilization to surfaces may offer important advantages, including long-term stability and low toxicity. However, a general strategy for AMP immobilization is not feasible, because the mechanism of action behind each AMP can influence the optimal conditions for efficient biofilm inhibition and/or attachment. Studies targeting single sequences will permit selection and development of effective specific AMP-coating strategies, allowing a wider use in health applications.

Biofilm resistance to AMPs

With respect to preventing surface adhesion, eradication of mature biofilm is a much more challenging target, because of enhanced drug resistance in the biofilm (Høiby *et al.*, 2010).

Although the development of resistance to AMPs is slower than to antibiotics, it is well established that bacteria adopt a variety of efficient strategies to resist even antimicrobial peptides in both planktonic and sessile lifestyle (Kraus & Peschel, 2006; Otto, 2006). Biofilm resistance to AMPs is multifactorial and can vary with the kind of microorganism present in the biofilm. Some mechanisms are in common with planktonic lifestyle and include efflux pumps, secreted proteases, or alterations of bacterial

surface aimed at increasing the net positive cell charge to minimize attraction of the typically cationic AMPs (Kraus & Peschel, 2006). Until now, very little is known about the resistance to AMP of bacteria with low metabolic activity and/or 'persister' cells in the biofilms. The biofilm resistance appears to be predominantly mediated by exopolysaccharides (EPSs) and other extracellular biofilm polymeric molecules that decrease the activity of AMPs, likely by preventing them from reaching the cytoplasmic membrane, their predominant target (Otto, 2006). *Staphylococcus epidermidis* and *S. aureus* produce an extracellular matrix mainly characterized by the presence of positively charged polysaccharide intercellular adhesin (PIA). Voung *et al.* (2004) found that PIA protects from cationic peptides such as hBD-3 and LL-37, but also from the anionic peptide dermcidin. This suggests that the mechanism by which PIA protects cells from AMPs activity is probably due to a combination of two effects against peptides with opposite charges: electrostatic repulsion or sequestration (Vuong *et al.*, 2004).

In *P. aeruginosa*, the best-studied polysaccharide component constituting the extracellular matrix is alginate, a negatively charged exopolymer. Chan *et al.* (2004) showed that alginate could provide a protective mechanism for the encased bacteria by binding cationic AMPs, inducing helix conformation, and promoting their self-association. In fact, hydrophilic alginate polymers contain a significant hydrophobic compartment, which can trap cationic AMPs behaving as an 'auxiliary membrane' (Chan *et al.*, 2004). Another example of resistance to AMPs mediated by EPSs is the presence of poly-γ-glutammic acid (PGA) capsule-like protective layer that characterizes some coagulase-negative staphylococci. In *S. epidermidis*, the *cup* gene locus, involved in the production of PGA, is up-regulated in the biofilm. Although PGA does not seem to be involved in biofilm formation, it might contribute to the biofilm-specific AMPs resistance in *S. epidermidis* (Kocianova *et al.*, 2005).

The presence of anionic extracellular DNA (eDNA) could represent another mechanism of resistance, especially to cationic molecules. Jones *et al.* (2013) have shown that at physiological concentration, hBD-3 binds eDNA, determining a drastic reduction of antimicrobial activity against nontypeable *Haemophilus influenzae* (NTHI) biofilm. The ability of hBD-3 to alter biofilm was rescued coincubating the peptide with DnaseI enzyme, tested at a concentration able to degrade these nucleic acids but low enough as to exclude any significant impact on resultant NTHI biofilm architecture. This result suggests that cationic peptides can be trapped by eDNA (Jones *et al.*, 2013).

Finally, the mechanism of biofilm resistance toward synergistic effects of antimicrobial peptides was also described. In a very recent report, Duperthuy *et al.* (2013) elucidated the mechanism of cross-resistance between polimixin B (PmB) and LL-37 in *Vibrio cholerae* biofilm. Bacteria incubated with sublethal concentration of PmB produce outer membrane vesicles (OMVs) containing high levels of Bap1, a biofilm-associated extracellular matrix protein. Bap1 protein electrostatically binds LL-37 to OMVs, thereby increasing the minimum inhibitory concentration of LL-37 against *V. cholerae* (Duperthuy *et al.*, 2013).

To overcome these limitations, innovative delivery strategies are being developed, based on entrapment of AMPs in nanostructured materials, which may be able to increase local AMP concentration, enhance peptide stability, and/or suppress peptide sequestration/repulsion by the biofilm matrix (Urban *et al.*, 2012).

Computational approaches to AMP design

Bioinformatics design and optimization of AMPs

In addition to targeted delivery, computational approaches may be valuable in optimizing AMPs to contrast the mechanisms of biofilm resistance. Traditional design and optimization studies of peptides are known to be expensive and time-consuming. A rational *in silico* approach to AMP design can drastically reduce production costs and the time required for the evaluation of activity and toxicity. In common statistical-based design strategies, a set of features describing compounds' activity is extrapolated from a dataset representing active and inactive molecules. Then, a mathematical model is applied to classify peptides based on their activity. Among the multitude of computer-assisted bioinformatics design strategies, two different categories can be distinguished.

The lexical model attempts to represent AMPs based on natural peptide sequence. AMP sequences in one-letter amino acid code are considered as 'text', and formal grammar rules are applied to identify text patterns in naturally occurring peptides. Some grammar rules are usually extrapolated from a dataset of known antimicrobial peptides (Loose *et al.*, 2006), such as amino acid frequency or particular motives occurrence (Fjell *et al.*, 2012). In these studies, amino acid systematic substitutions are generally operated to identify 'hot spots' in the primary sequence, important for the biological activity. An example of a successful template-based study is CM, a chimerical alpha-helical antimicrobial peptide obtained from the fusion of cecropin A and mellitin. These two natural AMPs firstly isolated from insects (Andreu *et al.*, 1992) were systematically combined to obtain a peptide with increased antimicrobial activity. However, these studies fail to account for the secondary structure, an important factor in antimicrobial activity (Fjell *et al.*, 2012; Salomone *et al.*, 2012). To introduce secondary-structure information, different strategies have been adopted. In particular, sequence alignment or position-specific scoring matrix (PSSM; Thomas *et al.*, 2010) allows to take into account particularly successful evolutionary conserved motives, sequence order, and amino acidic composition in bioactive peptides. For example, Wang *et al.*, (2011) reported that chemophysical information is combined with pseudo-amino acid composition to predict antimicrobial activity. The main drawback of these methods is the necessity to start from a template of existing sequences, limiting their use to natural amino acids.

Numerical analysis can be applied to a set of peptides with antimicrobial activity to derive a mathematical model

describing the relationship between chemophysical characteristics and biological activity. Quantitative structure activity relationship (QSAR) models have become an integral part of screening programs of small compounds thanks to their robustness and flexibility in analyzing compound's activity at different physiological conditions (Flower *et al.*, 2010; Lapins & Wikberg, 2010). In these models, chemophysical properties derived from AMPs primary sequence, such as polarity/hydrophobicity or net charge, are related to the biological activity in order to infer some prediction rules. Various classes of QSAR descriptors have been developed, generally named 3D QSAR or inductive QSAR (Pissurlenkar *et al.*, 2007), to account for intra- and intermolecular interactions. Moreover, the analysis of the correlation between QSAR variables along the primary sequence has been shown to account for secondary structure and amino acidic order information (Wold *et al.*, 1993). Advanced methods for data mining can be applied in connection to these QSAR variables to quantitatively or qualitatively discriminate between AMP and non-AMP sequences. For a detailed review on the subject, see Fjell *et al.* (2012).

Because of the huge number of possible combinations, a systematic exploration of the sequence space is unfeasible. Stochastic methods, such as genetic algorithms (GA), allow exploring a reduced, although still representative, number of possible candidates. GAs are evolutional algorithms that mimic Nature's adaptive approach to the environment, in which the process of evolution is performed through successive generations. Each potential AMP candidate is treated like an entity belonging to a population, and a fitness function is used as a measure of its biological activity. This value can reflect its predicted activity from a regression or a classification model. In the second case, it will express the confidence assigned to the prediction result. Various works apply genetic algorithms to AMP selection and optimization. In Fjell *et al.* (2011), a regression model was built with a training library of 1400 experimentally tested peptides to discriminate highly active AMPs. The training library was intentionally biased to mimic AMPs amino acidic composition. A neural network was trained on the basis of the tested antimicrobial activity against *P.aeruginosa* and used to screen a virtual library of 100 000 peptides. A set of 14 candidates was tested for their antimicrobial activity, demonstrating high accuracy in the algorithm prediction of antimicrobial activity.

In some cases, the simultaneous optimization of two or more conflicting features – the antimicrobial activity and a particular amino acidic composition, in order to be more resistant to degradation – can be required. A particular class of GA called multiobjective evolutional algorithms (MOEA) can be used to optimize different objectives separately, maintaining a set of good trade-off solutions (Coello *et al.*, 2007). The advantage of this technique is that final peptides can be screened on the basis of their biological activity as well as other chemophysical characteristics, without favoring one objective in particular. Recently, MOEA have been applied to design and optimize alpha-helical AMPs (Maccari *et al.*, 2013). Two *ab initio* alpha-helical AMPs and one optimized version of CM18 were designed, synthesized, and tested, together with an *ab initio* AMP with two norleucine residues.

The activity of AMPs is determined by a subtle combination of factors such as sequence, hydrophobicity, and position of cationic residue. Even though a large number of studies have attempted to establish more precise AMPs descriptors, a general statement is almost impossible to obtain due to the complexity of the target and to the mechanism of action lacking a well-defined characterization.

AMP design assisted by molecular modeling

The design of novel AMPs would greatly benefit from detailed understanding of the molecular mechanisms supporting their activity. A class of methods that has helped in unraveling such mechanisms is the simulations of their dynamics.

In molecular dynamics simulations (Leach, 2001), the motion of each molecule is predicted by the numerical solution of Newton's dynamic equation, as resulting from the interactions within the atoms of the molecule (intramolecular forces) and between different molecules (intermolecular forces). The detail with which each molecule is described can vary from the highest resolution possible in all-atom methods, in which each atom is taken into account, to different degree of coarse grain, in which the atoms are suitably grouped into interaction centers, sometimes also grouping different small molecules together (for example 3–4 water molecules together). The result of these simulations is a 'movie' recording the detailed dynamics of each molecule and how it interacts with the other components.

Several MD simulations studies have been aimed at investigating peptide–lipid bilayer interactions. In these studies, a patch of lipid bilayer (few hundreds of lipids), one or several copies of the studied peptide, and surrounding solvent molecules (water) are simulated for fractions of microseconds (Fig. 3a reports an example of these kinds of studies). Such systems are of sizes suitable for MD simulations (*c.* 100k atoms) and timescales on which some interesting mechanisms begin to unfold. From a survey of the literature, lipid-membrane computational studies emerge as very useful tools to complement and interpret experimental observations; detailed reviews of these studies are available (Matyus *et al.*, 2007; Gurtovenko *et al.*, 2010; Chen & Gao, 2012). For example, magainines, a well-characterized family of AMPs, also in terms of molecular-modeling studies, was shown by MD simulations to form rather disordered pores in POPC bilayers, with only one peptide completely spanning the membrane, the others being diffusely distributed on the rim of the pore with an orientation parallel to the bilayer (Kandasamy & Larson, 2004). This has led to a reassessment of the commonly accepted 'toroidal-pore' model, which in the original formulation assumed pores formed by symmetrically arranged peptides interacting with the lipid head groups. Disordered toroidal pores were also observed in MD simulations of melittin (Sengupta *et al.*, 2008) and cateslytin (Jean-François *et al.*, 2008). A certain degree of disorder was also observed in AMPs known to form barrel-stave channels in the

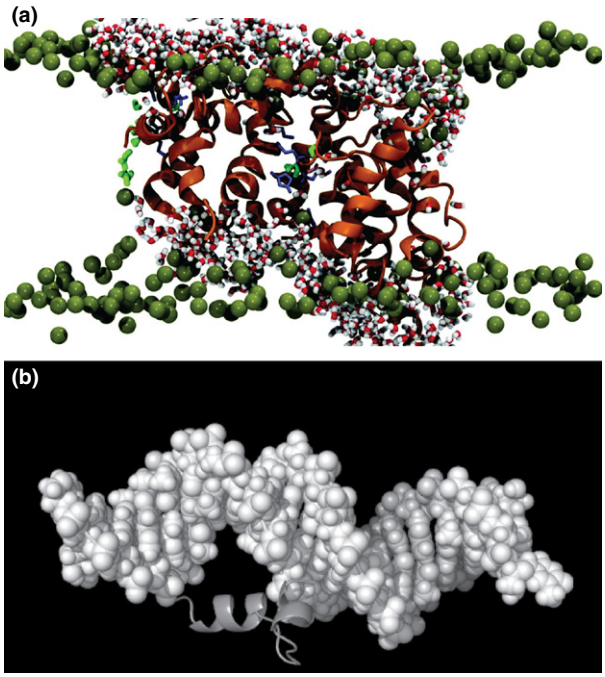

Fig. 3 (a) Snapshot from a molecular dynamics simulation study of pore formation by a cluster of 16 Maculatin 1.1 peptides (orange) in a lipid bilayer (DPPC phosphorus atoms shown as green spheres; Parton *et al.*, 2012). Nearby lysine and histidine side chains are shown in pale blue; nearby glutamic acid side chains are shown in pink. All other atoms are omitted for clarity. (b) computational model of the interaction of Buforin II (gray) with DNA (white; Uyterhoeven *et al.*, 2008). Reprinted (adapted) with permission from Parton *et al.* (2012), copyright (2012) American Chemical Society, and from Uyterhoeven *et al.* (2008) copyright (2008) Elsevier.

membrane, such as alamethicin (Peter Tieleman *et al.*, 2002; Dittmer *et al.*, 2009).

An explicit example of how MD simulations can assist AMP design is provided by a study by Tsai *et al.* (2009) on indolicidin, aimed at finding mutations that conserved antimicrobial activity but decreased hemolytic activity. The simulations of indolicidin absorption and insertion into models of erythrocyte and bacterial membrane suggested that perturbation of the former is assisted by the insertion of the hydrophobic Trp residue. By contrast, adsorption is already sufficient to destabilize the bacterial-membrane bilayer and is mediated by the cationic Lys residues. Mutations replacing the Trp residues by Phe and increasing the number of Lys lead to reduced hemolytic activity and enhanced antimicrobial activity.

All these studies were aimed at understanding the way in which AMPs perturb the cellular membrane. Specific studies of this kind on biofilm–peptide interactions have not yet been attempted, probably because the complexity of the biofilm greatly exceeds that of the lipid bilayer. Indeed, molecular dynamics studies would require a precise characterization of the morphology and of the molecular components of the biofilm, and the knowledge of the regions allowing AMP penetration (channels).

Possible routes to address biofilm–peptide interactions may involve singling out specific biofilm molecular components and studying the binding of AMP sequences to these molecules, either to destabilize the extracellular matrix by interfering with biofilm-specific molecular arrangements or to avoid extracellular sequestration of AMPs. For example, AMPs are known to interact with polysaccharide components of the matrix (Mah & O'Toole, 2001; Park *et al.*, 2011; Mah, 2012), although the details of these interactions are still obscure. In addition, AMP binding to eDNA may be exploited to interfere with eDNA structural roles and lead to biofilm perturbation, or it may be suppressed to avoid peptide sequestration. To the best of our knowledge, no computational study of AMP–polysaccharide or AMP–eDNA interactions in the context of biofilm has until now been performed. Elmore *et al.* addressed by computational modeling the related issue of Buforine II binding to nuclear DNA (Fig. 3b), which is believed to be at the basis of Buforine bacterial killing (Uyterhoeven *et al.*, 2008). Other developments may be fostered by experimental findings regarding the action of AMPs against specific biofilm components, or organized regions. Finally, one could think to bridge these molecular-modeling techniques with computational studies on biofilm morphology at the mesoscale. These studies are aimed at simulating biofilm growth taking into account variables such as cell mass and volume, nutrients consumption, fluid flow, and events such as biomass decay and biofilm detachment (Kapellos *et al.*, 2010). Such hierarchical multiscale approaches would in principle allow the researchers to investigate AMP diffusion in the biofilm, suggesting ways to circumvent peptide trapping and hence inactivation.

Conclusions

Microbial biofilms are responsible for several human infectious diseases, in particular chronic inflammatory and indwelling medical device-related infections (Costerton *et al.*, 1999).

Most antimicrobial agents at therapeutically achievable concentrations are not effective against these diseases, because of the unique characteristics of microorganisms living in biofilm lifestyle, such as slow-growing cells, EPS matrix, and antimicrobial tolerance biofilm-associated infections (Høiby *et al.*, 2010).

In recent years, several lines of research have addressed new strategies for the development of molecules active either alone or in synergy with conventional antibiotic/antifungal agents to effectively inhibit biofilm adhesion and growth and to promote biofilm dispersion and eradication.

The use of AMPs to contrast biofilm formation represents an attractive prophylactic and therapeutic approach, because of the nonspecific mechanisms of action, the low rate in inducing microbial resistance, and the ability to target even nongrowing or persister cells (Batoni *et al.*, 2011; Jorge *et al.*, 2012).

Increasing scientific literature on this topic has shown promising activity of AMPs against medical biofilms. Most of the tested peptides were suitable less in eradicating mature

biofilms than in preventing microbial adhesion, suggesting that their uses for topical applications and surface coating could be valid strategies to prevent microbial attachment on tissues and medical devices. To fully develop the potential of AMPs for biofilm therapies, future research will need to address the critical issue of biofilm-related resistance to AMPs. In particular, AMP repulsion or sequestration by the biofilm matrix and proteolytic degradation by microorganism proteases are known to limit AMP efficacy particularly against biofilm eradication.

The vast combinatorial space offered by peptides, further expanded by the use of non-natural amino acids, gives hope that yet more effective AMPs may be selected and designed specifically against biofilms. In this context, computational approaches may provide valuable guidelines for AMP rational design. Bioinformatics tools may help in analyzing large databases and predict novel sequences. More physical chemistry inspired molecular modeling methods can clarify AMP mechanisms of action on the bacterial membrane or even on the biofilm matrix, in connection with biofilm modeling tools aimed at unravelling the biofilm complex morphology and evolution.

References

Ammons MC & Copié V (2013) Mini-review: Lactoferrin: a bioinspired, anti-biofilm therapeutic. *Biofouling* 29: 443–455.

Ammons MCB, Ward LS, Fisher ST, Wolcott RD & James GA (2009) *In vitro* susceptibility of established biofilms composed of a clinical wound isolate of *Pseudomonas aeruginosa* treated with lactoferrin and xylitol. *Int J Antimicrob Agents* 33: 230–236.

Andreu D, Ubach J, Boman A, Wåhlin B, Wade D, Merrifield RB & Boman HG (1992) Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. *FEBS Lett* 296: 190–194.

Bagheri M, Beyermann M & Dathe M (2009) Immobilization reduces the activity of surface-bound cationic antimicrobial peptides with no influence upon the activity spectrum. *Antimicrob Agents Chemother* 53: 1132–1141.

Bahar A & Ren D (2013) Antimicrobial peptides. *Pharmaceuticals* 6: 1543–1575.

Balaban N, Gov Y, Giacometti A, *et al.* (2004) A chimeric peptide composed of a dermaseptin derivative and an RNA III-inhibiting peptide prevents graft-associated infections by antibiotic-resistant staphylococci. *Antimicrob Agents Chemother* 48: 2544–2550.

Batoni G, Maisetta G, Brancatisano FL, Esin S & Campa M (2011) Use of antimicrobial peptides against microbial biofilms: advantages and limits. *Curr Med Chem* 18: 256–279.

Brancatisano FL, Maisetta G, Di Luca M, Esin S, Bottai D, Bizzarri R, Campa M & Batoni G (2014) Inhibitory effect of the human liver-derived antimicrobial peptide hepcidin 20 on biofilms of polysaccharide intercellular adhesin (PIA)-positive and PIA-negative strains of *Staphylococcus epidermidis*. *Biofouling* doi:10.1080/08927014.2014.888062

Bryers JD (2008) Medical biofilms. *Biotechnol Bioeng* 100: 1–18.

Chan C, Burrows LL & Deber CM (2004) Helix induction in antimicrobial peptides by alginate in biofilms. *J Biol Chem* 279: 38749–38754.

Chen L & Gao L (2012) How the antimicrobial peptides kill bacteria: computational physics insights. *Commun Comput Phys* http://www.global-sci.com/issue/abstract/readabs.php?vol=11&page=709&issue=3&ppage=725&year=2012 (Accessed August 7, 2013).

Chennupati SK, Chiu AG, Tamashiro E, Banks CA, Cohen MB, Bleier BS, Kofonow JM, Tam E & Cohen NA (2009) Effects of an LL-37-derived antimicrobial peptide in an animal model of biofilm *Pseudomonas sinusitis*. *Am J Rhinol Allergy* 23: 46–51.

Chhibber S, Nag D & Bansal S (2013) Inhibiting biofilm formation by *Klebsiella pneumoniae* B5055 using an iron antagonizing molecule and a bacteriophage. *BMC Microbiol* 13: 174.

Cho W-M, Joshi BP, Cho H & Lee K-H (2007) Design and synthesis of novel antibacterial peptide-resin conjugates. *Bioorg Med Chem Lett* 17: 5772–5776.

Cirioni O, Giacometti A, Ghiselli R, Kamysz W *et al.* (2006a) Citropin 1.1-treated central venous catheters improve the efficacy of hydrophobic antibiotics in the treatment of experimental staphylococcal catheter-related infection. *Peptides* 27: 1210–1216.

Cirioni O, Giacometti A, Ghiselli R, Bergnach C *et al.* (2006b) Pre-treatment of central venous catheters with the cathelicidin BMAP-28 enhances the efficacy of antistaphylococcal agents in the treatment of experimental catheter-related infection. *Peptides* 27: 2104–2110.

Coello CA, Lamont G & Van Veldhuizen D (2007) Evolutionary Algorithms for Solving Multi-Objective Problems SE – Genetic and Evolutionary Computation. Springer, New York, NY citeulike-article-id:2242111.

Costerton JW, Stewart PS & Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284: 1318–1322.

Da Silva BR, de Freitas VAA, Carneiro VA, Arruda FVS, Lorenzón EN, de Aguiar ASW, Cilli EM, Cavada BS & Teixeira EH (2013) Antimicrobial activity of the synthetic peptide Lys-a1 against oral streptococci. *Peptides* 42: 78–83.

De la Fuente-Núñez C, Korolik V, Bains M, Nguyen U, Breidenstein EBM, Horsman S, Lewenza S, Burrows L & Hancock REW (2012) Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide. *Antimicrob Agents Chemother* 56: 2696–2704.

Dean SN, Bishop BM & van Hoek ML (2011) Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against *Staphylococcus aureus*. *BMC Microbiol* 11: 114.

Dittmer J, Thørgersen L, Underhaug J *et al.* (2009) Incorporation of antimicrobial peptides into membranes: a combined liquid-state NMR and molecular dynamics study of alamethicin in DMPC/DHPC bicelles. *J Phys Chem B* 113: 6928–6937.

Donlan RM & Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15: 167–193.

Duperthuy M, Sjöström AE, Sabharwal D, Damghani F, Uhlin BE & Wai SN (2013) Role of the Vibrio cholerae matrix protein Bap1 in cross-resistance to antimicrobial peptides. *PLoS Pathog* 9: e1003620.

Duplantier AJ & van Hoek ML (2013) The human cathelicidin antimicrobial peptide LL-37 as a potential treatment for polymicrobial infected wounds. *Front Immunol* 4: 143.

Eckert R, Brady KM, Greenberg EP, Qi F, Yarbrough DK, He J, McHardy I, Anderson MH & Shi W (2006a) Enhancement of antimicrobial activity against *Pseudomonas aeruginosa* by coadministration of G10KHc and tobramycin. *Antimicrob Agents Chemother* 50: 3833–3838.

Eckert R, He J, Yarbrough DK, Qi F, Anderson MH & Shi W (2006b) Targeted killing of *Streptococcus mutans* by a pheromone-guided "smart" antimicrobial peptide. *Antimicrob Agents Chemother* 50: 3651–3657.

Feng X, Sambanthamoorthy K, Palys T & Paranavitana C (2013) The human antimicrobial peptide LL-37 and its fragments possess both antimicrobial and antibiofilm activities against

multidrug-resistant *Acinetobacter baumannii*. *Peptides* 49: 131–137.

Fjell CD, Jenssen H, Cheung WA, Hancock REW & Cherkasov A (2011) Optimization of antibacterial peptides by genetic algorithms and cheminformatics. *Chem Biol Drug Des* 77: 48–56.

Fjell CD, Hiss JA, Hancock REW & Schneider G (2012) Designing antimicrobial peptides: form follows function. *Nat Rev Drug Discov* 11: 37–51.

Flower DR, Macdonald IK, Ramakrishnan K, Davies MN & Doytchinova IA (2010) Computer aided selection of candidate vaccine antigens. *Immunome Res* 6(suppl 2): S1.

Friedrich C, Scott MG, Karunaratne N, Yan H & Hancock RE (1999) Salt-resistant alpha-helical cationic antimicrobial peptides. *Antimicrob Agents Chemother* 43: 1542–1548.

Friedrich CL, Rozek A, Patrzykat A & Hancock RE (2001) Structure and mechanism of action of an indolicidin peptide derivative with improved activity against gram-positive bacteria. *J Biol Chem* 276: 24015–24022.

Gao G, Lange D, Hipert K *et al.* (2011) The biocompatibility and biofilm resistance of implant coatings based on hydrophilic polymer brushes conjugated with antimicrobial peptides. *Biomaterials* 32: 3899–3909.

Ghiselli R, Giacometti A, Cirioni O *et al.* (2007) Pretreatment with the protegrin IB-367 affects gram-positive biofilm and enhances the therapeutic efficacy of linezolid in animal models of central venous catheter infection. *J Parenter Enteral Nutr* 31: 463–468.

Giugliano LG, Ribeiro ST, Vainstein MH & Ulhoa CJ (1995) Free secretory component and lactoferrin of human milk inhibit the adhesion of enterotoxigenic *Escherichia coli*. *J Med Microbiol* 42: 3–9.

Gopal R, Lee JH, Kim YG, Kim M-S, Seo CH & Park Y (2013) Anti-microbial, anti-biofilm activities and cell selectivity of the NRC-16 peptide derived from witch flounder, *Glyptocephalus cynoglossus*. *Mar Drugs* 11: 1836–1852.

Gurtovenko AA, Anwar J & Vattulainen I (2010) Defect-mediated trafficking across cell membranes: insights from *in silico* modeling. *Chem Rev* 110: 6077–6103.

Hall-Stoodley L, Stoodley P, Kathju S, Høiby N, Moser C, Costerton JW, Moter A & Bjarnsholt T (2012) Towards diagnostic guidelines for biofilm-associated infections. *FEMS Immunol Med Microbiol* 65: 127–145.

Hancock REW & Rozek A (2002) Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol Lett* 206: 143–149.

Hancock RE & Scott MG (2000) The role of antimicrobial peptides in animal defenses. *P Natl Acad Sci USA* 97: 8856–8861.

Harris F, Dennison SR & Phoenix DA (2009) Anionic antimicrobial peptides from eukaryotic organisms. *Curr Protein Pept Sci* 10: 585–606.

Haynie SL, Crum GA & Doele BA (1995) Antimicrobial activities of amphiphilic peptides covalently bonded to a water-insoluble resin. *Antimicrob Agents Chemother* 39: 301–307.

Hell E, Giske CG, Nelson A, Römling U & Marchini G (2010) Human cathelicidin peptide LL37 inhibits both attachment capability and biofilm formation of *Staphylococcus epidermidis*. *Lett Appl Microbiol* 50: 211–215.

Helmerhorst EJ, Hodgson R, van't Hof W, Veerman EC, Allison C & Nieuw Amerongen AV (1999) The effects of histatin-derived basic antimicrobial peptides on oral biofilms. *J Dent Res* 78: 1245–1250.

Hetrick EM & Schoenfisch MH (2006) Reducing implant-related infections: active release strategies. *Chem Soc Rev* 35: 780–789.

Hilpert K *et al.* (2009) Screening and characterization of surface-tethered cationic peptides for antimicrobial activity. *Chem Biol* 16: 58–69.

Høiby N, Bjarnsholt T, Givskov M, Molin S & Ciofu O (2010) Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 35: 322–332.

Huang Q, Yu HJ, Liu GD *et al.* (2012) Comparison of the effects of human β-defensin 3, vancomycin, and clindamycin on *Staphylococcus aureus* biofilm formation. *Orthopedics* 35: e53–e60.

Huh AJ & Kwon YJ (2011) "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. *J Control Release* 156: 128–145.

Jacobsen AS & Jenssen H (2012) Human cathelicidin LL-37 prevents bacterial biofilm formation. *Future Med Chem* 4: 1587–1599.

Jean-François F, Elezgaray J, Berson P, Vacher P & Dufourc EJ (2008) Pore formation induced by an antimicrobial peptide: electrostatic effects. *Biophys J* 95: 5748–5756.

Jin Y, Hammer J, Pate M, Zhang Y, Zhu F, Zmuda E & Blazyk J (2005) Antimicrobial activities and structures of two linear cationic peptide families with various amphipathic beta-sheet and alpha-helical potentials. *Antimicrob Agents Chemother* 49: 4957–4964.

Jones EA, McGillivary G & Bakaletz LO (2013) Extracellular DNA within a nontypeable Haemophilus influenzae-induced biofilm binds human beta defensin-3 and reduces its antimicrobial activity. *J Innate Immun* 5: 24–38.

Jorge P, Lourenço A & Pereira MO (2012) New trends in peptide-based anti-biofilm strategies: a review of recent achievements and bioinformatic approaches. *Biofouling* 28: 1033–1061.

Kandasamy SK & Larson RG (2004) Binding and insertion of alpha-helical anti-microbial peptides in POPC bilayers studied by molecular dynamics simulations. *Chem Phys Lipids* 132: 113–132.

Kanthawong S, Bolscher JGM, Veerman ECI, van Marle J, de Soet HJJ, Nazmi K, Wongratanacheewin S & Taweechaisupapong S (2012) Antimicrobial and antibiofilm activity of LL-37 and its truncated variants against *Burkholderia pseudomallei*. *Int J Antimicrob Agents* 39: 39–44.

Kapellos GE, Alexiou TS & Payatakes AC (2010) Theoretical modeling of fluid flow in cellular biological media: an overview. *Math Biosci* 225: 83–93.

Kazemzadeh-Narbat M, Lai BFL, Ding C, Kizhakkedathu JN, Hancock REW & Wang R (2013) Multilayered coating on titanium for controlled release of antimicrobial peptides for the prevention of implant-associated infections. *Biomaterials* 34: 5969–5977.

Kocianova S, Vuong C, Yao Y, Voyich JM, Fischer ER, DeLeo FR & Otto M (2005) Key role of poly-gamma-DL-glutamic acid in immune evasion and virulence of *Staphylococcus epidermidis*. *J Clin Invest* 115: 688–694.

Kraus D & Peschel A (2006) Molecular mechanisms of bacterial resistance to antimicrobial peptides. *Curr Top Microbiol Immunol* 306: 231–250.

Lapins M & Wikberg JE (2010) Kinome-wide interaction modelling using alignment-based and alignment-independent approaches for kinase description and linear and non-linear data analysis techniques. *BMC Bioinformatics* 11: 339.

Leach A (2001) Molecular Modelling: Principles and Applications, 2nd edn. Pearson Education Limited, Harlow, UK, pp165–247, 353–406.

Lee J-K *et al.* (2013) Antibacterial efficacy of a human β-defensin-3 peptide on multispecies biofilms. *J Endod* 39: 1625–1629.

Li L, He J, Eckert R, Yarbrough D, Lux R, Anderson M & Shi W (2010) Design and characterization of an acid-activated antimicrobial peptide. *Chem Biol Drug Des* 75: 127–132.

Lim K, Chua RRY, Saravanan R, Basu A, Mishra B, Tambyah PA, Ho B & Leong SSJ (2013) Immobilization studies of an engineered arginine–tryptophan-rich peptide on a silicone surface

with antimicrobial and antibiofilm activity. *ACS Appl Mater Interfaces* 5: 6412–6422.

Loose C, Jensen K, Rigoutsos I & Stephanopoulos G (2006) A linguistic model for the rational design of antimicrobial peptides. *Nature* 443: 867–869.

Maccari G, Di Luca M, Nifosí R, Cardarelli F, Signore G, Boccardi C & Bifone A (2013) Antimicrobial peptides design by evolutionary multiobjective optimization. *PLoS Comput Biol* 9: e1003212.

Mah T-F (2012) Biofilm-specific antibiotic resistance. *Future Microbiol* 7: 1061–1072.

Mah TF & O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 9: 34–39.

Maisetta G, Batoni G, Esin S, Florio W, Bottai D, Favilli F & Campa M (2006) *In vitro* bactericidal activity of human beta-defensin 3 against multidrug-resistant nosocomial strains. *Antimicrob Agents Chemother* 50: 806–809.

Maisetta G, Di Luca M, Esin S, Florio W, Brancatisano FL, Bottai D, Campa M & Batoni G (2008) Evaluation of the inhibitory effects of human serum components on bactericidal activity of human beta defensin 3. *Peptides* 29: 1–6.

Maisetta G, Mangoni ML, Esin S *et al.* (2009) *In vitro* bactericidal activity of the N-terminal fragment of the frog peptide esculentin-1b (Esc 1-18) in combination with conventional antibiotics against *Stenotrophomonas maltophilia*. *Peptides* 30: 1622–1626.

Mataraci E & Dosler S (2012) *In vitro* activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin-resistant *Staphylococcus aureus* biofilms. *Antimicrob Agents Chemother* 56: 6366–6371.

Matl FD, Obermeier A, Repmann S, Friess W, Stemberger A & Kuehn K-D (2008) New anti-infective coatings of medical implants. *Antimicrob Agents Chemother* 52: 1957–1963.

Matyus E, Kandt C & Tieleman D (2007) Computer simulation of antimicrobial peptides. *Curr Med Chem* 14: 2789–2798.

Menousek J, Mishra B, Hanke ML, Heim CE, Kielian T & Wang G (2012) Database screening and *in vivo* efficacy of antimicrobial peptides against methicillin-resistant *Staphylococcus aureus* USA300. *Int J Antimicrob Agents* 39: 402–406.

Minardi D, Ghiselli R, Cirioni O *et al.* (2007) The antimicrobial peptide Tachyplesin III coated alone and in combination with intraperitoneal piperacillin-tazobactam prevents ureteral stent *Pseudomonas* infection in a rat subcutaneous pouch model. *Peptides* 28: 2293–2298.

Molhoek EM, van Dijk A, Veldhuizen EJA, Haagsman HP & Bikker FJ (2011) A cathelicidin-2-derived peptide effectively impairs *Staphylococcus epidermidis* biofilms. *Int J Antimicrob Agents* 37: 476–479.

Nagant C, Pitts B, Nazmi K, Vandenbranden M, Bolscher JG, Stewart PS & Dehaye J-P (2012) Identification of peptides derived from the human antimicrobial peptide LL-37 active against biofilms formed by *Pseudomonas aeruginosa* using a library of truncated fragments. *Antimicrob Agents Chemother* 56: 5698–5708.

Novković M, Simunić J, Bojović V, Tossi A & Juretić D (2012) DADP: the database of anuran defense peptides. *Bioinformatics* 28: 1406–1407.

Otto M (2006) Bacterial evasion of antimicrobial peptides by biofilm formation. *Curr Top Microbiol Immunol* 306: 251–258.

Overhage J, Campisano A, Bains M, Torfs ECW, Rehm BHA & Hancock REW (2008) Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect Immun* 76: 4176–4182.

Park Y & Hahm K-S (2005) Antimicrobial peptides (AMPs): peptide structure and mode of action. *J Biochem Mol Biol* 38: 507–516.

Park CB, Kim HS & Kim SC (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem Biophys Res Commun* 244: 253–257.

Park S-C, Park Y & Hahm K-S (2011) The role of antimicrobial peptides in preventing multidrug-resistant bacterial infections and biofilm formation. *Int J Mol Sci* 12: 5971–5992.

Parton DL, Akhmatskaya EV & Sansom MSP (2012) Multiscale simulations of the antimicrobial peptide maculatin 1.1: water permeation through disordered aggregates. *J Phys Chem B* 116: 8485–8493.

Paulmann M *et al.* (2012) Structure-activity analysis of the dermcidin-derived peptide DCD-1L, an anionic antimicrobial peptide present in human sweat. *J Biol Chem* 287: 8434–8443.

Peter Tieleman D, Hess B & Sansom MSP (2002) Analysis and evaluation of channel models: simulations of alamethicin. *Biophys J* 83: 2393–2407.

Pissurlenkar RRS, Malde AK, Khedkar SA & Coutinho EC (2007) Encoding type and position in peptide QSAR: application to peptides binding to class I MHC molecule HLA-A*0201. *QSAR Comb Sci* 26: 189–203.

Pompilio A, Scocchi M, Pomponio S, Guida F, Di Primio A, Fiscarelli E, Gennaro R & Di Bonaventura G (2011) Antibacterial and anti-biofilm effects of cathelicidin peptides against pathogens isolated from cystic fibrosis patients. *Peptides* 32: 1807–1814.

Pusateri CR, Monaco EA & Edgerton M (2009) Sensitivity of *Candida albicans* biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. *Arch Oral Biol* 54: 588–594.

Römling U & Balsalobre C (2012) Biofilm infections, their resilience to therapy and innovative treatment strategies. *J Intern Med* 272: 541–561.

Salomone F, Cardarelli F, Di Luca M, Boccardi C, Nifosì R, Bardi G, Di Bari L, Serresi M & Beltram F (2012) A novel chimeric cell-penetrating peptide with membrane-disruptive properties for efficient endosomal escape. *J Control Release* 163: 293–303.

Sengupta D, Leontiadou H, Mark AE & Marrink S-J (2008) Toroidal pores formed by antimicrobial peptides show significant disorder. *Biochim Biophys Acta* 1778: 2308–2317.

Shai Y & Oren Z (2001) From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. *Peptides* 22: 1629–1641.

Shirtliff ME, Peters BM & Jabra-Rizk MA (2009) Cross-kingdom interactions: *Candida albicans* and bacteria. *FEMS Microbiol Lett* 299: 1–8.

Singh PK, Parsek MR, Greenberg EP & Welsh MJ (2002) A component of innate immunity prevents bacterial biofilm development. *Nature* 417: 552–555.

Sobczak M, Dębek C, Olędzka E & Kozłowski R (2013) Polymeric systems of antimicrobial peptides–strategies and potential applications. *Molecules* 18: 14122–14137.

Tati S, Li R, Puri S, Kumar R, Davidow P & Edgerton M (2013) Histatin 5-spermidine conjugates have enhanced fungicidal activity and efficacy as a topical therapeutic for oral candidiasis. *Antimicrob Agents Chemother* http://www.ncbi.nlm.nih.gov/pubmed/24247141 (Accessed December 11, 2013).

Theberge S, Semlali A, Alamri A, Leung KP & Rouabhia M (2013) *C. albicans* growth, transition, biofilm formation, and gene expression modulation by antimicrobial decapeptide KSL-W. *BMC Microbiol* 13: 246.

Thomas S, Karnik S, Barai RS, Jayaraman VK & Idicula-Thomas S (2010) CAMP: a useful resource for research on antimicrobial peptides. *Nucleic Acids Res* 38: D774–D780.

Tsai C-W, Hsu N-Y, Wang C-H, Lu C-Y, Chang Y, Tsai H-HG & Ruaan R-C (2009) Coupling molecular dynamics simulations with experiments for the rational design of indolicidin-analogous antimicrobial peptides. *J Mol Biol* 392: 837–854.

Turner J, Cho Y, Dinh NN, Waring AJ & Lehrer RI (1998) Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob Agents Chemother* 42: 2206–2214.

Urban P, Jose Valle-Delgado J, Moles E, Marques J, Diez C & Fernandez-Busquets X (2012) Nanotools for the delivery of antimicrobial peptides. *Curr Drug Targets* 13: 1158–1172.

Uyterhoeven ET, Butler CH, Ko D & Elmore DE (2008) Investigating the nucleic acid interactions and antimicrobial mechanism of buforin II. *FEBS Lett* 582: 1715–1718.

Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR & Otto M (2004) Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell Microbiol* 6: 269–275.

Wang P, Hu L, Liu G *et al.* (2011) Prediction of antimicrobial peptides based on sequence alignment and feature selection methods. *PLoS One* 6: e18476.

Wei G-X, Campagna AN & Bobek LA (2006) Effect of MUC7 peptides on the growth of bacteria and on *Streptococcus mutans* biofilm. *J Antimicrob Chemother* 57: 1100–1109.

Weinberg ED (2004) Suppression of bacterial biofilm formation by iron limitation. *Med Hypotheses* 63: 863–865.

Wenzel RP (2007) Health care-associated infections: major issues in the early years of the 21st century. *Clin Infect Dis* 45(suppl 1): S85–S88.

Whittaker CJ, Klier CM & Kolenbrander PE (1996) Mechanisms of adhesion by oral bacteria. *Annu Rev Microbiol* 50: 513–552.

Wolcott R, Costerton JW, Raoult D & Cutler SJ (2013) The polymicrobial nature of biofilm infection. *Clin Microbiol Infect* 19: 107–112.

Wold S, Jonsson J, Sjörström M, Sandberg M & Rännar S (1993) DNA and peptide sequences and chemical processes multivariately modelled by principal component analysis and partial least-squares projections to latent structures. *Anal Chim Acta* 277: 239–253.

Yang L, Liu Y, Wu H, Hóiby N, Molin S & Song Z (2011) Current understanding of multi-species biofilms. *Int J Oral Sci* 3: 74–81.

Yoshinari M, Kato T, Matsuzaka K, Hayakawa T & Shiba K (2010) Prevention of biofilm formation on titanium surfaces modified with conjugated molecules comprised of antimicrobial and titanium-binding peptides. *Biofouling* 26: 103–110.

Yu H-Y, Tu C-H, Yip B-S, Chen H-L, Cheng H-T, Huang K-C, Lo H-J & Cheng J-W (2011) Easy strategy to increase salt resistance of antimicrobial peptides. *Antimicrob Agents Chemother* 55: 4918–4921.

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415: 389–395.

Zhang L, Pornpattananangku D, Hu C-MJ & Huang C-M (2010) Development of nanoparticles for antimicrobial drug delivery. *Curr Med Chem* 17: 585–594.

Zhu C, Tan H, Cheng T, Shen H, Shao J, Guo Y, Shi S & Zhang X (2013a) Human β-defensin 3 inhibits antibiotic-resistant *Staphylococcus* biofilm formation. *J Surg Res* 183: 204–213.

Zhu Y, Dashper SG, Chen Y-Y, Crawford S, Slakeski N & Reynolds EC (2013b) Porphyromonas gingivalis and *Treponema denticola* synergistic polymicrobial biofilm development. *PLoS One* 8: e71727.