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Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria

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Abstract
Biofilm-associated infections represent one of the major threats of the modern medicine. Biofilm-forming bacteria are encased in a complex mixture of extracellular polymeric substances (EPS) and acquire properties that render them highly tolerant to conventional antibiotics and host immune response. Therefore, there is a pressing demand of new drugs active against microbial biofilms. In this regard, antimicrobial peptides (AMPs) represent an option taken increasingly in consideration. After dissecting the peculiar biofilm features that may greatly affect the development of new antibiofilm drugs, the present article provides a general overview of the rationale behind the use of AMPs against biofilms of medically relevant bacteria and on the possible mechanisms of AMP-antibiofilm activity. An analysis of the interactions of AMPs with biofilm components, especially those constituting the EPS, and the obstacles and/or opportunities that may arise from such interactions in the development of new AMP-based antibiofilm strategies is also presented and discussed.

Keywords: biofilm; antimicrobial peptides; extracellular DNA; antibiofilm drugs; biofilm matrix; biofilm exopolysaccharides; biofilm proteins

Contents:

1. Introduction: medical importance of biofilm-associated infections

Over the last decades, microbiologists and infectious disease specialists have experienced a radical change in the way to face infections [1]. The classical, acutely evolving and antibiotic treatable infectious diseases that have caused millions of death till the mid of the last century (e.g. tetanus, diphtheria, cholera) have progressively left the scene to infections characterized by chronic development that often alternates with phases of acute exacerbations, refractory to antimicrobial treatments, and displaying undefined pathogenic mechanisms [1] (Figure 1). While the classical, acutely evolving infections are thought to involve independent, free-floating (planktonic) microbial cells, extensive *in vitro* and *in vivo* studies have provided the notion that many types of chronic infections are sustained by sessile microbial aggregates known as biofilms. An exhaustive definition of biofilms, that takes into consideration the peculiar attributes of biofilm organisms, has been proposed by Donlan and Costerton in 2002: "a biofilm is as microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" [2]. Hallmarks of biofilm-associated infections are i) a dramatically reduced susceptibility to commonly used antibiotics, despite cells isolated from the biofilm may result susceptible to the same drugs, and ii) a high capacity to resist the clearance by host innate and adaptive immune responses [3-5]. Both of these factors play a major role in treatment failure and persistence of the infections caused by sessile microorganisms.

Overall, biofilm-associate infections may be subdivided into two main categories [6,7]. The first one involves biofilm formation on host tissues (e.g. epithelia, mucosal surfaces, teeth). Examples of this type of infections are pulmonary infections in cystic fibrosis (CF) patients, foot ulcer infections in diabetic patients, chronic otitis media or rhinosinusitis, chronic prostatitis, recurrent urinary tract infections, and dental caries or periodontitis. The second type of infections may arise from microbial colonization of abiotic surfaces such as those of indwelling medical devices (e.g. central venous or urinary catheters, joint or dental prostheses, heart valves, endotracheal tubes, intrauterine devices, dental implants and many others) [6,7]. A crucial feature of biomaterial-associated infections is that microbial cells may detach from biofilms and disseminate to the surrounding tissues or to the bloodstream, further exacerbating the clinical outcome of the infection [1].

With the increased use of medical devices in health care procedures, biofilm-associated infections have emerged as a major problem in different clinical disciplines. It is estimated that up to 80% of microbial infections in the human body involve biofilm formation, greatly contributing to morbidity and mortality, especially in hospital settings [8,9]. Indeed, the management of biofilm-associated infections is problematic as they are difficult to prevent, diagnose, and treat.

Undoubtedly, one of the most medically relevant biofilm property is the severely reduced susceptibility to antimicrobials, which is considered a multifactorial process [4]. Beside the classical resistance mechanisms, due to the acquisition of mobile genetic elements, several other biofilm-specific resistance mechanisms have been proposed. These include: reduced diffusion or sequestration of antimicrobials through the extracellular matrix, low growth rate of biofilm cells, presence of dormant cells virtually tolerant to all drugs ("persisters") [3,4,10]. The treatment of biofilm-associated infections is so burdensome that often the only option is to remove the colonized medical device or to undergo surgical debridement of the biofilm-infected tissue [8,10].

Given the intrinsic resistance of biofilms to antimicrobial therapy, particularly pressing is the discovery of new compounds able to target not only planktonic cells, but also specific features of

the sessile lifestyle. In this regard, various innovative antibiofilm approaches have been proposed over the last few years aimed at limiting microbial adhesion to biotic and abiotic surfaces, targeting microbial signals that modulate the switch to the biofilm mode of growth, or dislodging cells from established biofilms [4,11].

Recent work has highlighted that among future antibiofilm strategies, the possible use of antimicrobial peptides (AMPs), also referred to as host defense peptides, may represent a promising approach [12-15]. The present review critically analyzes the possible use of AMPs to prevent biofilm formation or to treat established biofilms. After pointing out the peculiar properties of biofilms that may greatly impact on the development of new antibiofilm therapeutic strategies, the article provides an overview of the multiple mechanisms of the AMPs' antibiofilm action. Finally, the article focuses on the interactions of AMPs with biofilm components (e.g. those that constitute the extracellular matrix) of medically relevant microorganisms and the possible obstacles and/or opportunities that may arise from such interactions in the development of new AMP-based antibiofilm strategies.

2. Antibiofilm drug-development: properties of an "ideal" antibiofilm agent

Biofilms are microbial communities that display unique characteristics compared with their planktonic counterparts. These characteristics must be accurately considered when evaluating the potential of biofilm prevention or control strategies (Figure2). The properties that could allow an "ideal" antibiofilm agent to target optimally the biofilm lifestyle are listed below.

1) Display rapid killing ability. Biofilms are highly dynamic entities that develop according a well-defined step-by-step process which roughly involves an initial adhesion phase followed by a maturation phase and a dispersal phase [2] (Figure 2). The adhesion phase involves a primary attachment of free-floating cells to a conditioned surface by weak and reversible long-range interactions (e.g. electrostatic and hydrophobic interactions, steric hindrance, van der Waals forces, hydrodynamic forces and others). Conditioning occurs when a foreign body is exposed to body fluids and its surface is modified by the adsorption of host molecules (e.g. albumin, lipids, extracellular matrix molecules, complement, fibronectin, inorganic salts). Following the primary phase, loosely bound microorganisms stably attach to the substratum by short-range and more specific molecular interactions between bacterial surface structures (e.g. pili, fimbriae, fibrillae, capsule etc.) and host molecules (e.g. fibronectin) that function as receptors [16]. The adhesion step is highly influenced by a number of factors that include the associated flow conditions, local environment, bacterial properties as well as the surface properties of the biomaterial/host tissue [17]. Maturation of biofilms is associated with the production of EPS that constitutes a large proportion of the biofilm biomass and plays a major role in the establishment of the biofilm phenotype. A final step or dispersal phase involves the detachment of clusters of cells or single cells and colonization of surrounding sites. Due to the described temporal biofilm heterogeneity, an ideal antimicrobial agent against biofilms should be able to act in a fast way, to face a rapidly changing entity, and to target cells before they stably enter in the biofilm community and switch to the biofilm phenotype.

2) Act in different microenvironments and target slow growing or even non-growing cells. Mature biofilms are also spatially highly heterogeneous as gradients of oxygen, nutrients, pH, and waste material are established due to the reduced diffusion of gasses and molecules through the extracellular matrix (Figure 2). The establishment of microenvironments that differ for physicochemical characteristics may impair the activity of several antibiotics. For instance,

aminoglycosides fail to act in anaerobiosis and at low-pH [18], conditions found in the deep biofilm layers.

The establishment of gradients implies that cells in the biofilm's periphery are directly in contact with oxygen and nutrients while those sited in the deepest biofilm layers may experience anoxia, lack of nutrients and acidic conditions. This generates a spatial and metabolic heterogeneity of the bacterial population that may include rapidly as well as slowly growing cells. Notably, environmental conditions within a biofilm may induce the occurrence of dormant, non-dividing cells at high rate [4,10], the so-called "persisters", that constitute a small fraction of essentially invulnerable cells believed to play a key role in biofilm recalcitrance to antibiotics [4].

Thus, an "ideal" antibiofilm agent should be able to act in disparate environmental niches and to target sub-populations of cells with different growth rate, including persisters. Molecules acting on multiple targets or able to synergize with antimicrobials displaying different mechanisms of action, could be suitable to accomplish this goal. For instance, combination of an antibiotic acting on metabolically active cells (i.e ciprofloxacin) with one able to target non-dividing cells of *Pseudomonas aeruginosa* biofilms (i.e. colistin), was described to ensure higher biofilm killing rates *in vitro* as compared to the antibiotics used alone [19]. The same combination also demonstrated some clinical efficacy in the early eradication therapy of intermittent airway colonization by *P. aeruginosa* in CF patients [20].

3) Penetrate the extracellular matrix and/or interfere with its production. Typically, biofilm communities are encased in a self-produced matrix of EPS that have been defined "the house of biofilm cells" [21]. EPS represents a major biofilm component accounting for up to 90% of the total biofilm dry biomass [22]. It contributes to maintain biofilm architecture providing a highly hydrated environment and favoring cell-to-cell and cell-to-surface adhesion. The major EPS components are polysaccharides, proteins, lipids and extracellular DNA (eDNA) distributed in a non-homogeneous pattern [22]. Occasionally also host molecules may enter in the composition of extracellular matrix as is the case of salivary glycoproteins in the oral biofilm.

The matrix plays a central role in the biofilm resistance mechanism to antibiotics [3,4]. It constitutes essentially a diffusion barrier that delays or prevents the interaction of antimicrobial agents with microbial cells. Depending on the charge of EPS and the drug, the latter may be sequestered or repulsed with consequent decrease of the bioactive concentration. Of note, sub-inhibitory concentrations of some antibiotics may even induce matrix synthesis, as in the case of beta-lactam induction of alginate synthesis in *P. aeruginosa* biofilms [23] or vancomycin induction of slime synthesis in coagulase-negative staphylococci [24].

Thus, ideally, an antibiofilm agent should be able to penetrate the matrix and/or to inhibit/interfere with its accumulation. The heterogeneity of matrix composition across different species/strains and the environmentally modulated expression of matrix synthesis [25], may render this requirement particularly difficult to be satisfied.

4) Interfere with bacterial cell communication machinery. Cells in biofilm communicate and coordinate their behavior through the secretion of signal molecules known as auto-inducers [26]. When the concentration of such molecules reaches a critical level (Quorum, Q) it is sensed by the population members (Sensing, S) that coordinate their behavior in a cell-density dependent manner [26]. Different types of acyl homoserine lactones function as signal molecules in Gram-negative bacteria, while in Gram-positive ones QS-mediated gene expression is mainly triggered by small peptides [5,26]. Interestingly, different biofilm-related properties are under the control of QS signals. Depending on the microbial species, signal molecules may promote biofilm formation [27] or, rather, favor biofilm dispersal [28]. Currently, the possibility to interfere with QS signals is an extensively investigated research area for biofilm control [29]. As QS often regulates also the expression of virulence traits, the employment of antagonists or quenchers of QS signals could

allow accomplishing the double goal of inhibiting biofilm formation and down-regulating pathogen's features involved in the pathogenic process. Nevertheless, as QS quenchers not necessarily ensure that infectious bacteria are eradicated, their therapeutic use as a single treatment seems less feasible than their utilization in combination with sterilizing agents (e.g. antibiotics). In this regard, the study by Christensen *et al*. demonstrated a synergistic antibacterial efficacy of early combination treatment with tobramycin and QS inhibitors against *P. aeruginosa* in an intraperitoneal foreign-body infection mouse model [30].

5) Modulate host response to biofilm. Biofilms are not only recalcitrant to antibiotics, but also evade host immune-responses [31,32]. *In vitro* studies demonstrated that antibodies or phagocytic cells at most enter the interstitial voids (water channels) that intercalate the microcolonies in a mature biofilm, but barely penetrate the deep biofilm layers [1,33]. Phagocytic cells seem not only to be unable to physically engulf the biofilm structures but also to be impaired in their activities [34,35].

In vivo biofilms are much less investigated than biofilms obtained *in vitro*; they are usually smaller in physical dimensions, lack mushroom-like structures, are embedded in host material, and are continuously exposed to host defense reactions [36]. Confocal microscopy images of *P. aeruginosa* biofilms produced in the lung of CF patients have shown that microcolonies are mostly very compact, only rarely perforated by holes resembling water channels, and with phagocytic cells mainly surrounding the biofilms [37]. The study of the *in vivo* immune response to biofilms is still a poorly investigated research area with the potential to disclose interesting aspects of the biofilm-host interaction that does not necessarily resemble those known to act against planktonic bacteria [32].

The clinical outcome of biofilm-associated infections is often exacerbated by the intense host pro-inflammatory response, to the persistent microbial stimulus, that may greatly contribute to the tissue damage. Interestingly, by employing a mouse model of biofilm-mediated prosthetic implant infection, Prabhakara *et al.* reported that suppression of Th1/Th17 pro-inflammatory immune response prevents the development of a *Staphylococcus aureus* chronic biofilm infection [38]. In apparent contrast, Hanke and coworkers found that *S. aureus* biofilms skew macrophage differentiation toward an anti-inflammatory phenotype, the alternatively activated type 2 macrophages that support T regulatory cell responses [39]. In the same study, treatment of established biofilm infections with local administration of macrophages with a pro-inflammatory phenotype, the classically activated type 1 macrophages that support Th1 cell responses, significantly reduced catheter associated biofilm burden. The authors suggest that targeting macrophages' pro-inflammatory activity can overcome the local immune inhibitory environment created by the biofilm and represent a novel immuno-therapeutic antibiofilm strategy [39].

The nature of the biomaterial is also important for the host response to the combined presence of bacteria and biomaterial [40]. For instance, murine or human macrophages exposed to various biomedical polymers including polydimethylsiloxane (PDMS), expanded polytetra fluoroethylene (ePTFE), and alginate react by secreting pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α , and IL-12 [41].

It might be possible that, similarly to other persistent infections [42,43], a balance between pro-inflammatory and anti-inflammatory immune cell responses is needed for an optimal control of biofilm infections. Thus, ability to recruit immune cells and/or modulate the host immune response would be an added value of an ideal antibiofilm drug.

6) Synergize with other conventional and unconventional antimicrobial compounds. It is quite unlikely that a single drug may exhibit all the above reported features at once. In this regard, combinatorial therapeutic strategies may represent a valid approach to target the numerous features of the biofilm mode of life. Several innovative combinatorial approaches are being

investigated in *in vitro* and *in vivo* models and will be hopefully translated to the clinical use in the years to come [11]. Examples include the use of antibiotics in combination with compounds able to i) digest or destabilize the biofilm matrix [44,45]; ii) inhibit QS signals [30]; iii) interfere with pathways leading to persistency [46,47]; iv) promote ROS production to increase bacterial membrane permeability [48]; v) rise the pH to enhance activity of aminoglycosides [49]; vi) render antibiotics more accessible into biofilms [50,51].

3. Rational of using antimicrobial peptides as therapeutic strategies against microbial biofilms

In the last decade, growing interest has been devoted to the possible use of AMPs as antibiofilm agents [12-15]. A PubMed survey on papers published since the year 2005 yielded 856 results using "antimicrobial peptides AND biofilms" as keywords, while the number of the corresponding papers was only 107 in the previous decade. Such interest is probably justified by the fact that, on a rational basis, AMPs may have the potential to exert activity against biofilms as they display many, if not all, the properties of an "ideal" antibiofilm drug outlined in the previous paragraph (Figure 2).

For instance, they overall exert a fast killing ability. Our previous studies focused on the antibacterial properties of the human beta-defensin-3 (hBD3) against multidrug-resistant nosocomial bacterial strains, demonstrated that the peptide is bactericidal against a wide variety of Gram-positive and Gram-negative bacteria in 1 to 20 minutes, depending on the species [52]. Similar short killing times were obtained by us also testing a number of frog-skin derived peptides against bacterial strains isolated from hospitalized patients [53]. This feature is a direct consequence of AMPs' main mechanism of action, namely, permeabilization of bacterial membranes [54]. Membrane integrity is essential for the survival of bacteria irrespective of the metabolic stage of the cell. Thus, AMPs may have the potential to kill not only metabolically active cells but also slow growing or even persister cells. In a recent study, cationic membrane-penetrating peptides containing various numbers of arginine (Arg) and tryptophan (Trp) repeats were demonstrated to be effective in killing planktonic persister cells of *Escherichia coli* HM22, a hyper-persister producer [55]. Some of the Trp/Arg containing AMPs were also able to disperse and kill preformed biofilms harboring high percentages of persister cells. Bacteria within biofilms resemble stationary phase bacteria and are generally less sensitive to antibiotics than log-phase bacteria. To assess whether this was the case also for AMPs, in another recent study the effects of LL-37-derived peptides were evaluated on biofilm-cells obtained by mechanical disruption of mature *S. aureus* biofilms [56]. Interestingly, two LL-37 analogues (P60.4Ac and P10) significantly reduced the number of biofilm-derived cells at a concentration as low as 1.6 μM after 4 h incubation, suggesting the potential of such peptides to kill also bacteria released from the biofilm during the dispersal phase.

Many AMPs are effective against multi-drug-resistant bacteria probably due to their prevalent mode of action on bacterial membranes that is different from that of the large majority of conventional drugs [52,53]. This characteristic is particularly relevant since, in the actual post-antibiotics era, a growing number of conventional drugs have lost their effectiveness due to the rapid spread of resistant microorganisms.

Although most AMPs act, principally, by the electrostatic attraction to negatively charged bacterial surfaces followed by membrane disruption, their antibacterial activity may also involve interference with metabolic processes or with different types of intracellular targets that may result in inhibition of cell wall, nucleic acid or protein biosynthesis [54,57]. The complex, often-multimodal, antimicrobial action of AMPs renders more difficult for microbes to develop durable resistance mechanisms, offering another notable advantage of AMPs over conventional

antibiotics. In addition, this property may allow AMPs to target different biofilm sub-populations and, in mixed biofilms, even different microbial species. In some cases, AMP activity may be also modulated by environmental conditions. For example, we demonstrated that, similarly to other histidin-rich peptides, the antimicrobial properties of the human liver-derived peptides hepcidin 25 and hepcidin 20 (hep-20) against clinically relevant bacterial and fungal strains are highly enhanced and quickened at acidic pH [58-61]. Besides this, we found that pH influences the mode of hepcidin 25 and hep-20 action on *E. coli* cells and model membranes, with a predominant membrane permeabilizing effect at acidic pH and a plausible effect on intracellular target(s) at neutral pH [62]. Acidic microenvironments may very well exist in the deep layers of a mature biofilm, due to the production of metabolites by bacteria, while at the biofilm periphery or in the close proximity of the water channels pH might be neutral. Acidic conditions may also originate in pathological conditions associated with biofilm formation such as those establishing in the dense mucus of the airway surface of CF patients. Ability of AMPs to target biofilm-specific features has also been demonstrated. For instance, AMPs may act as QS inhibitors, down-regulators of extracellular matrix biosynthesis or interferers of regulatory pathways that lead to the persister phenotype (see next paragraph).

AMPs can also act as immunomodulators, recruiting polymorphonuclear cells, lymphocytes or dendritic cells at the site of infection, enhancing the activities of host immune cells and modulating the release of pro or anti-inflammatory cytokines [63]. Importantly, many AMPs interact with high affinity with the lipopolysaccharide (LPS) or endotoxin on the cell wall of Gram-negative bacteria or in suspension and thus have the potential to neutralize the toxic effect of this molecule which is one the major mediators of septic shock [64]. In addition, AMPs can promote healing processes by stimulating cellular proliferation or angiogenesis, thus potentially contributing to tissue repair during the course of biofilm infections [15].

Additional AMP-properties that may reveal useful in the development of AMPs as antibiofilm agents include the possibility: i) to immobilize them on biomaterial surface to inhibit microbial adhesion [65,66]; ii) to encapsulate them into natural or synthetic polymeric carriers as delivery systems [67]. In the case of biomaterial-associated infections, the possibility of releasing the active molecule directly at the site of implant in a controlled manner may allow not only to prevent biofilm formation, but also offer the unique advantage to eradicate bacteria in peri-implant tissue [40,68]; iii) to chemically manipulate them (amino acid substitution, introduction of D or non-natural amino acids, expression as fusion proteins, combination of different functional domains, others) in order to improve their effectiveness against planktonic and/or biofilm cells [69,70].

Although all the above mentioned characteristics are found among AMPs, they are not necessarily exhibited by a single AMP molecule. Therefore, strategies aimed at combining AMPs with compounds acting with different mechanisms of action and targeting distinct biofilm features might represent a valid therapeutic approach to improve the AMP antibiofilm potential. For instance, it is well established that AMPs may synergize with conventional and unconventional compounds, thus reducing the active antibiotic concentrations and possible side effects [47,71,72]. We recently focused on the study of the antibiofilm properties of the frog-skin derived peptide temporin B (1Tb) in combination with conventional antibiotics or unconventional antimicrobial compounds such as cysteine or EDTA. A striking ability of the peptide to kill both forming and mature *S. epidermidis* biofilms was observed, especially when it was used in combination with cysteine or EDTA. Interestingly, temporin B in combination with EDTA was able to eradicate mature *S. epidermidis* biofilms formed *in vitro* on silicon catheters suggesting the possible use of temporin B-EDTA combinatorial strategies in the lock therapy of central access devices colonized by *S. epidermidis* biofilms (Maisetta *et al*. manuscript in preparation).

4. Difficulties in testing AMP-anti biofilm properties

Due to the rational beyond the use of AMPs as novel antibiofilm drugs, an increasing number of AMPs with different chemical-physical properties and origins have been tested over the last years against forming or mature biofilms of a variety of pathogenic or environmental microbial species [73]. In this regard, it should be noted that testing AMP antibiofilm properties has inherent difficulties that may render comparison among different AMPs cumbersome. A given AMP may or may not exert activity depending on the experimental conditions adopted that, in turn, may greatly influence the ability of clinical isolates to form biofilms. For instance, we recently tested *in vitro* the ability to form biofilm of different bacterial species/strains isolated from biopsies of patients suffering from chronic rhinosinusitis. We found that the type of medium used might have a dramatic impact on biofilm biomass evaluated as crystal violet (CV) staining after 24h culture. Overall, rich media containing high glucose or sucrose concentrations, human plasma, and/or salts promote biofilm formation, but not for all the species (Di Luca *et al*. unpublished observations). On the other hand, in the presence of complex mixture of molecules/ions the antibacterial activity of many AMPs is inhibited [74]. Strain-to-strain variability in the susceptibility to AMPs is also evidenced in many studies [75,76], adding complexity to the evaluation of the antibiofilm properties of a given AMP. A vast heterogeneity in the methods used to evaluate AMP-antibiofilm properties across different studies is another important issue emerging from the analysis of the literature. While some methods use as read out the biofilm biomass without taking into account the vitality of biofilm-embedded cells, others measure the biofilm metabolic activity, the number of biofilm-associated viable cells, or the amount of specific biofilm components [73]. It should be stressed here that the above mentioned methods are not always equivalent. For instance, staining of biofilms with CV is a commonly employed assay for evaluating biofilm biomass upon AMP treatment. CV stains bacterial cells and components of the extracellular matrix (e.g. polysaccharides and proteins), but does not distinguish between living and dead cells. Measurement of metabolic activity by XTT, MTT or alamar blue assays is widely used as a parameter of cell viability in biofilm studies, the latter being an obviously important parameter when evaluating the activity of antimicrobial agents against biofilms. Nevertheless, in the case of biofilms, reduction of metabolic activity does not necessarily imply that cells are dead as biofilm-associated bacteria may enter in a reversible dormant status and down-regulate active cell processes as a survival-response to stressful conditions. In this regard, assessment of vitality by counting colony forming units (CFU) on agar plates might be more appropriate, although dislodgement of biofilm-embedded cells might also be problematic in the case of particularly sticky biofilms. Microscopic techniques, including confocal laser scanning microscopy and scanning electron microscopy, are widely used in biofilm research as they offer the unique possibility to obtain valuable information on the biofilm structure, its three-dimensional organization and, in the case of mixed biofilms, on the relative abundance of the different microbial species. In addition, the information obtained from microscopic images may aid in the elucidation of the mechanisms of antimicrobial action of active agents and, if used in conjunction with labeling techniques that identify the various biofilm components (e.g. microbial cells, distinct EPS components), in the identification of the possible molecular targets of the active molecules.

Recently, attempts have been made to establish the minimum information that needs to be reported to guarantee the interpretability and independent verification of experimental results

involving biofilms [77]. Valuable initiatives aimed at establishing international networks with the aim, among others, to identify optimal and standardized *in vitro* microbiological tests suitable for predicting AMP-activity *in vivo* against medical device infections (e.g. www.ipromedai.net) are ongoing. Nevertheless, a consensus on standardized operating protocols for assessing AMP anti-biofilm activity is still missing, generating possible interpretation mistakes.

Further difficulties may arise when testing antibiofilm activity of AMPs *in* vivo, as several variables may greatly influence the peptide's performances in a host. These include the presence of body fluid components (e.g. proteases, plasma proteins, nucleic acids), expression of additional virulence factors by the infectious microorganisms, host immune responses, short half-life of the peptides, type of the surface on which the biofilm is formed (mucosal/epithelial surface) and, in the case of biomaterial associated infections, the physicochemical properties of the biomaterials [41]. In this regard, de Breij *et al*. demonstrated that the substrate used for biofilm formation (plastic surface *versus* bronchial epithelial cells) can impact bacterial gene expression in *Acinetobacter baumannii* [78]. These observations underscore the importance of using biological matrices for studies of medical biofilm growth and for predicting the effectiveness of new therapeutic agents in *in vivo* settings [79].

In the attempt to facilitate the comparison among different AMPs, some of us have recently collaborated at the development of the first database entirely focused on AMPs tested on microbial biofilms [73]. The database is freely accessible online at the site www.baamps.it and for each peptide provides, in a pre-organized framework, relevant information that cannot be separated from the evaluation of its antibiofilm properties. The information provided includes: i) sequences, physiochemical properties and origin of the tested AMPs; ii) stage of biofilm considered; iii) active concentrations and corresponding biofilm reduction; iv) experimental conditions used to evaluate the activity; v) methods used to evaluate biofilm reduction; vi) microbial species/strains towards which activity was evaluated; vii) experimental models (*in vitro*, *in vivo*). Furthermore, all the experimental data regarding a given AMP are directly linked to the doi of the corresponding original article, allowing users to reach the on-line article page, if further information are required. Scientists working in the field of AMPs and biofilms may upload their own results following a simple registration procedure, thus contributing to keep updated the database and to avoid interpretation mistakes.

Currently the database includes 209 peptide sequences from 7 different sources tested *in vitro* and/or in *in vivo* against 112 different microbial species/strains of clinical or environmental relevance.

5. Unravelling the mechanisms of the antibiofilm activity of AMPs

Although the study of the AMP antibiofilm mechanisms of action is still a relatively poorly investigated area of research, it appears that AMPs have the potential to act on multiple targets and stages of biofilm formation [12]. For instance, some peptides can interfere with the early events of biofilm formation by preventing adhesion of bacterial cells to the substrate or to other cells, or by killing cells before they stably become part of the biofilm architecture [80-82]. Others may act on established biofilms by killing mature biofilm cells, or by causing their detachment [55,81,83]. Interference with QS or other regulatory signals, dysregulation of genes involved in motility, modulation of the immune system, interference with matrix synthesis/accumulation are other examples of AMP-antibiofilm mechanisms of action [75,82-85].

The concentration at which a peptide exerts its antibiofilm activity is an important parameter that may be suggestive of its mechanism of action. AMP-ability to inhibit biofilm formation or reduce/eradicate mature biofilms at concentrations equal or higher than its minimal

inhibiting concentration (MIC) against the corresponding planktonic cells, may suggest that the peptide acts by a classical "microbicidal" effect. On the other hand, a growing number of peptides show activity against biofilms at concentrations much lower than their inhibitory concentrations. In this case, their antibiofilm effect is likely to rely, exclusively or in part, to "non classical" mechanisms of actions targeting the biofilm mode of growth.

In this second group of peptides, falls the human cathelicidin LL-37 whose antibiofilm properties have been extensively investigated [56,85-87]. Interestingly, Overhage and coworkers assessed the mechanisms of *P. aeruginosa* biofilm inhibition by LL-37 by microarray technology and demonstrated that the peptide affects biofilm formation by decreasing the attachment of bacterial cells, stimulating twitching motility, and influencing two major QS systems (Las and Rhl), leading to the down-regulation of genes essential for biofilm development [85]. Dysregulations of genes involved in biofilm formation and/or motility, only partially overlapping with those caused by LL-37, were also observed treating *P. aeruginosa* biofilms with the small cationic peptide 1037 [84], suggesting that different peptides may target common set of genes controlling biofilm properties.

HBD3 is another AMP possibly acting against biofilms by a "non classical" mechanism of action. Real-time polymerase chain reaction experiments demonstrated that the peptide interferes with the expression of *ica*A and *ica*D genes [82], which are part of the *ica* operon responsible of the synthesis of PIA (polysaccharide-intercellular-adhesin), the major extracellular polysaccharide produced by a large fraction of staphylococcal strains (see paragraph 6.3). In the same study hBD3 was also demonstrated to up-regulate the expression of *ica*R (a transcriptional repressor of the *ica* operon expression) resulting in a marked attenuation of biofilm production.

Interestingly, while evaluating the antibiofilm properties of hep-20, a peptide with structural similarity with hBD3, our group evidenced that in the presence of hep-20 *S. epidermidis* strains develop biofilms with an altered architecture and reduced amount of extracellular matrix [75]. This effect was observed at peptide concentrations unable to kill bacterial cells in biofilm like conditions (i.e. stationary phase cells in 50% Tryptone Soy broth, 0.25% glucose) and was directed against *S. epidermidis* strains with an extracellular matrix made mainly of PIA, as well as strains producing protein-dependent biofilms (PIA-negative strains, see paragraph 6.4). The observation that the inhibitory effect paralleled a strong reduction of biofilm metabolic activity suggested that it might be due to a peptide-driven down-regulation of active cell processes such as protein or polysaccharide biosynthesis [75]. Alternatively, due to its cationic nature, hep-20 could intercalate between the negatively charged bacterial cells interfering with the interactions of EPS components either reciprocally or with the cell-wall, thus reducing the amount of EPS that accumulates. A striking ability of hepcidin to bind DNA has been recently reported [88]. As eDNA is involved in biofilm development (see paragraph 6.1), it is tempting to speculate that binding of hep-20 to DNA may play a role in the destabilization of biofilm structure and accumulation of matrix components.

Another interesting "non-classical" mechanism of antibiofilm action has been demonstrated for the synthetic cationic peptide IDR-1018 [83]. At concentrations that did not affect planktonic growth, the peptide completely prevented biofilm formation and led to the eradication of mature biofilms of clinically relevant bacterial species, including *P. aeruginosa*, *E. coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, methicillin resistant *S. aureus*, *Salmonella Typhimurium* and *Burkholderia cenocepacia*. The mechanism of action involved the inhibition of a widely conserved stress response, the so-called stringent response, mediating (p)ppGpp synthesis in response to environmental signals such as nutritional limitations [83]. A summary of the possible mechanisms of action of AMPs against biofilms is depicted in Figure 3, while selected examples of AMPs exerting antibiofilm properties *in vitro* or *in vivo* are reported in Table 1 and Table 2 [89-95], respectively.

With the growing medical relevance of biofilm-associated infections and the still complete absence of licensed antibiofilm therapies, overall AMPs represent promising lead compounds for future antibiofilm strategies. Although some recent studies aimed at identifying specific structural features needed for the explication of an optimal antibiofilm activity [69,70,81,84,87,96], intensive structure-activity studies are still required for AMPs exploitation as antibiofilm drugs. Structural requirements optimal for antimicrobial activity against planktonic cells, not necessarily may coincide with those required for an optimal antibiofilm effect [84], suggesting that the selection of peptides with antibiofilm potential should not exclusively be based on the screening of their antimicrobial properties against floating microbes.

6. Interactions of antimicrobial peptides with biofilm components and their role in biofilm resistance to AMPs

Despite the numerous features underlined above render AMPs promising antibiofilm agents, AMPs' clinical use is still hampered by several drawbacks that lower their translational potential. These may include potential toxicity at the therapeutically efficacious doses, poor stability in biological fluids, high production costs, potential development of resistance mechanisms, and/or unwanted interference with host-immune responses [5,63,97]. In addition to these general obstacles, other biofilm-specific features may generate further impediments to the exploiting of AMPs as anti-biofilm therapeutics. In this regard, interaction of AMPs with polymers of the biofilm extracellular matrix is considered to play a major role [98].

Therefore, this paragraph provides an overview of the possible interactions of AMPs with biofilm components of medically relevant microorganisms and on the possible role of such interactions in biofilm-resistance to AMPs, with special emphasis to EPS components (Figure 4).

6.1 Interaction of AMPs with extracellular DNA, a common component of a variety of bacterial and fungal biofilms

Extracellular DNA (eDNA) is a major component of the biofilm matrix of many pathogenic bacterial and fungal species [22]. It mainly derives from cells undergoing lysis, but active mechanisms of secretion have been suggested as well [99,100]. It has been proposed that eDNA may act as an intercellular connector that stabilizes and maintains the biofilm architecture [22]. In addition, it may constitute a flexible pool of genes that bacteria in the biofilm exchange by mechanisms of horizontal gene transfer with possible acquisition of virulence traits and/or resistance determinants [99]. In *P. aeruginosa* biofilms the release of eDNA is under the control of QS signals [101] and it may facilitate the twitching motility-mediated biofilm expansion by maintaining coherent cell alignments [102]. A role of eDNA as nutrient source [103,104] or cation chelator [105] has also been proposed.

Of note, ability of sub-optimal antibiotic concentrations to promote eDNA release by biofilm cells has been reported. Kaplan *et al*. demonstrated that sub-inhibitory concentrations of beta-lactam antibiotics induce eDNA release and, in turn, cell aggregation and biofilm formation in some strains of *S. aureus* [106]. Similarly, biofilms of *S. epidermidis* pre-exposed to sub-inhibitory concentrations of vancomycin were found to contain higher concentrations of eDNA as compared to untreated biofilms and to impede penetration of the same antibiotic [107]. These observations may have clinical relevance as, due to the barrier effect played by the EPS, sub-optimal drug concentrations may very well be established within a biofilm.

Due to the crucial importance of eDNA in promoting cell aggregation and biofilm stabilization, several DNA-targeting antibiofilm strategies have been proposed [108]. For instance, recombinant human DNase I was shown to prevent the formation and cause the detachment of

staphylococcal biofilms at clinically achievable concentrations and to increase the survival of *S. aureus*-infected *Caenorhabditis elegans* nematodes treated with tobramycin compared with control nematodes treated with tobramycin alone [109]. Similarly, DNAse I was reported to prevent biofilm formation of *P. aeruginosa* and to dissolve mature biofilms (12, 36, and 60 hours old) of the same species [100]. In a very recent study, ciprofloxacin-loaded poly(lactic-co-glycolic acid) nanoparticles were functionalized with DNase I and their antibiofilm activity was assessed against *P. aeruginosa* biofilms [110]. Interestingly, DNase I-activated nanoparticles not only prevented biofilm formation from planktonic bacteria, but they also successfully reduced established biofilm mass, size, and living cell density. Aerosolized recombinant human DNase I has been also evaluated in clinical trials for the treatment of CF patients [111].

It is well established that by virtue of their cationic nature, several AMPs have DNA-binding properties. Therefore, sequestration of AMPs by matrix-associated eDNA has been proposed as a biofilm-specific mechanism of resistance to AMPs (Figure 4A). In this regard, Jones *et al.* analyzed the effect of eDNA on the antimicrobial activity of hBD3 against biofilms of nontypeable *Haemophilus influenzae* (NTHI), a Gram-negative bacterium often involved in chronic infections of the airway and otitis media in children [112]. They found that pre-incubation *in vitro* of physiological concentrations of recombinant hBD3 with genomic DNA of NTHI abrogated the ability of the peptide to prevent biofilm formation of NTHI, while this ability was restored when biofilms were established in the presence of both DNase I and hBD3. DNase I, but not RNase A, also increased hBD3 killing of biofilm-associated NTHI, suggesting that removal of eDNA from the biofilm restores the ability of hBD3 to alter NTHI biofilm formation through rescue of its antimicrobial activity. The same study provided evidence that when NTHI-induced biofilms were established in the middle ear of an adult chinchilla (*Chinchilla langinera*), eDNA co-localized with cBD1, the orthologous of hBD3, with the peptide-eDNA complexes mainly localized at the periphery of the biofilm. As hBD3 is expressed in the mammalian middle ear, these observations shed light not only on a possible mechanism of biofilm resistance to AMPs, but also suggest that neutralization of important effectors of innate immunity by biofilm components may contribute to the pathogenesis and persistency of biofilm infections. On the other hand, as eDNA seems crucial to maintain the structural integrity of biofilms formed by multiple bacterial species, the DNA-binding properties exhibited by many AMPs could be exploited as a therapeutic option to sequester this integral structural component from biofilm EPS.

It should be pointed out here that beside the possible interaction of AMPs with biofilm eDNA, also their interaction with host DNA may be involved in neutralization of their antimicrobial activity. For instance, high levels of host DNA are present in the viscous airway surface fluid of CF patients [113]. Despite high levels of antibacterial mediators (e.g. defensins, cathelicidins, lactoferrin, lysozyme) can be present in the airway fluid of these patients, their lung defenses are defective. Peptide binding and neutralization by eDNA has been suggested to contribute, among other factors, to this process [114]. However, the interaction of AMPs with extracellular host DNA may also enhance antimicrobial defense. Recently it has been discovered that upon encountering bacteria, neutrophils release mesh-like structures called neutrophil extracellular traps (NETs). These web-like traps contain a backbone consisting of DNA/histones associated with AMPs (e.g. defensins, lactoferrin, calprotectin and others) that is capable of capturing and eliminating microbes [115].

Indirect mechanisms of eDNA interference with biofilm susceptibility to AMPs have been also described (Figure 4B) [116]. For instance, Mulcahy *et al*. reported that in *P. aeruginosa*, DNA ability to bind and sequester cations, including magnesium, in the surrounding environment induces the PhoPQ and PmrAB two-component systems [105]. Such systems regulate the cationic antimicrobial peptide (CAP) resistance operon *PA3552–PA3559* whose induction leads to the

expression of genes involved in LPS modifications (addition of aminoarabinose to lipid A), which, in turn, reduce the outer membrane permeability to CAPs. Induction of the CAP resistant operon *PA3552–PA3559* by eDNA was dose-dependent, was abolished by addition of excess Mg^{++} and, importantly, occurred not only in planktonic cultures, but also in biofilms of *P. aeruginosa*. Finally, biofilms supplemented with eDNA were 8-fold more resistant to CAP (polymyxin B, colistin) and 64-fold more resistant to aminoglycoside (gentamycin, tobramycin) than biofilms without exogenous DNA. As suggested by the authors, altogether these results reveal a novel mechanism of biofilm-associated resistance to antimicrobials in which the presence of DNA in the extracellular matrix of biofilms creates a localized cation-limited environment that is detected by *P. aeruginosa* leading to the induction of LPS modification genes and resistance to antimicrobials.

Similar eDNA-mediated induction of the PhoPQ/PmrAB systems and consequent resistance to AMPs was demonstrated also in *Salmonella enterica* serovar Typhimurium [117]. This suggests that such a resistance mechanism may be widely used by bacteria to evade host innate immune responses or antimicrobial therapy in DNA-rich environments like biofilms or the lung of CF patients,.

Again, the DNA-binding properties of AMPs with particularly high affinity to DNA could be exploited to saturate the cation-binding ability of eDNA and to possibly prevent the eDNA-induced resistance of biofilms as well as planktonic bacteria to antimicrobials.

6.2 Interaction of AMPs with alginate and other polysaccharides, major components of biofilms of *P. aeruginosa* and other pulmonary pathogens

Exopolysaccharides are a major fraction of the biofilm matrix of a wide variety of microbial species [22]. Most of them are long molecules, linear or branched, with molecular masses ranging from 0.5×10^6 to 2×10^6 daltons. They can be homopolysaccharides or, more often, heteropolysaccharides that consist of a mixture of neutral and charged sugar residues [22]. Most of the known exopolysaccharides are polyanionic (e.g alginate of *P. aeruginosa*), but positively charged exopolysaccharides also exist (e.g. staphylococcal PIA).

Polysaccharides play essential roles in the biofilm lifestyle. Together with other EPS components, they allow the initial colonization of biotic and abiotic surfaces by planktonic cells, and the long-term attachment of mature biofilms to surfaces, maintain a highly hydrated microenvironment around biofilm organisms, form the skeleton and mediate the mechanical stability of biofilms, allow the accumulation of nutrients and the stabilization of extracellular enzymes. Not least, they confer resistance to non-specific and specific host defenses during infection and tolerance to various antimicrobial agents [22].

P. aeruginosa is an opportunistic pathogen responsible for a wide variety of both acute and chronic infections. Beyond its intrinsic resistance to many conventional drugs, its ability to form biofilms is considered one of the main pathogenicity trait of the bacterium [118]. *P. aeruginosa* pulmonary infections are particularly relevant in CF patients in which the bacterium can persist for decades by switching to the biofilm mode of growth. About 80% of CF patients suffer from chronic *P. aeruginosa* infections that represent the major cause of morbidity and mortality in these patients [118]. It has been shown that in the lung of CF patients *P. aeruginosa* undergoes complex adaptation process driven by genetic variation and acquires phenotypic diversity including the ability to synthetize high amounts of the extracellular polysaccharide alginate (mucoid strains) [118]. Alginate is a high-molecular-mass, linear polymer composed of D-mannuronic acid and L-guluronic acid [119]. Polysaccharides other than alginate are produced by non-mucoid *P. aeruginosa* strains, the first to colonize the lung of CF patients [119]. These polysaccharides include Pel, mainly composed of glucose, and Psl, a repeating pentasaccharide consisting of D-mannose, L-rhamnose, and D-glucose [119]. Mucoid and non-mucoid *P. aeruginosa* strains differ

by the qualitative composition of their polysaccharides in the biofilm matrix, predominantly alginate or Psl/Pel, respectively [119].

Besides *P. aeruginosa*, other pathogens produce considerable amount of extracellular polysaccharides. Among them, there are members of the *Burkholderia cepacia* complex, also involved in pulmonary infection in CF patients, or *K. pneumoniae*, an important cause of nosocomial infections in infants or immunocompromised patients [120,121].

Interaction of AMPs with extracellular polysaccharides of biofilm EPS is still a relatively poorly investigated area of research. A major contribution in this area has been provided by Chan and coworkers who demonstrated that alginate induces a α-helical conformation in a series of synthetic cationic AMPs, provided that the average core sequence hydrophobicity exceeds a "hydrophobicity threshold" [122,123]. Through a combination of experiments measuring release of the fluorescent dye calcein from phospholipid vesicles, peptide interactions with vesicles in the presence and absence of alginate, and affinity of peptides for alginate as a function of net peptide core hydrophobicity, the authors show that alginate both binds and promotes the self-association of the cationic peptides. As α-helical conformation is typically induced in membrane-active peptides upon their interaction with membrane bilayers, the authors suggest that alginate might function as an "auxiliary membrane" for the bacteria encased in a biofilm. By forming complexes with peptides competitively with the bacterial membranes, alginate might entrap AMPs before they can reach their bacterial target and constitute for encased bacteria a protective environment against the innate host defense (Figure4C).

Further insights on peptide-polysaccharide interactions were provided by Herasimenka *et al*. [124] who studied the interaction of two cathelicidins, LL-37 and SMAP-29, with three bacterial polysaccharides, respectively produced by *P. aeruginosa* (alginate), *B. cepacia* (cepacian) and *K. pneumoniae* (capsular polysaccharide K40). Circular dichroism experiments showed that all these polysaccharides induced α-helical conformation in the two peptides although at different extent. Fluorescence measurements also indicated the formation of peptide-polysaccharide complexes. Interestingly, the authors proposed a model in which, at low polysaccharide/AMP ratio, one AMP molecule is induced to assume a helical conformation via interaction of its polar cationic surface with the anionic polysaccharide. This would in fact result in a hydrophobic surface exposed to the aqueous medium. The interaction of polar solvent molecules with the non-polar surface of the α-helix leads to an energetically unfavorable configuration that would promote complexation with a second peptide molecule, which is induced in turn to assume a helical conformation [124,125] (Figure5).

Of note, Benincasa *et al.* investigated the *in vitro* effect of different polysaccharides from lung pathogens on the antimicrobial activity of a panel of structurally diverse AMPs from mammals [126]. These included two peptides, LL-37 and hBD3, released in the human alveoli, as well as peptides from other mammals, i.e. SMAP-29, PG-1 and Bac7(1-35). All the polysaccharides investigated (alginate, cepacian and K40) were able to inhibit at variable extent the antibacterial activity of the peptides against an *E. coli* reference strain. The less inhibiting polysaccharide was cepacian that even at high concentrations (500 μg/ml) caused an increment of the MIC value of not more than 2-4fold as compared to that obtained in its absence. Alginate exerted a potent inhibitory effect on SMAP-29, LL-37, Bac7(1-35) and hBD3 causing a MIC increase of 8-32 fold, in the concentration range 100-500 μg/ml. The inhibitory effect of K40 was also potent against PG-1, with a 8-32-fold MIC increment , while its effect was modest, although still significant, on the other peptides [126]. Inhibition of peptides' killing ability by polysaccharides was overall fast and paralleled the polysaccharides' ability to inhibit *E. coli* inner membrane permeabilization. The differences among the various polysaccharides in the extent of the inhibitory effect on the AMPs tested could not be explained simply based on ionic interactions between the negatively charged

polysaccharides and the cationic AMPs. This suggests that, albeit charge-mediated interactions are important, other structural features of both interactors may play a role in the formation of the complexes [126,127].

Altogether, these results point out that inhibition of AMP antibacterial activity by extracellular polysaccharides may protect the pathogens from host defenses during the course of an infection. The same mechanism could also represent an obstacle to the development of AMPs as antibiofilm agents. Intensive structure-function studies will constitute the basis for the design of optimized peptides with low tendency to interact with extracellular polysaccharides and/or high ability to penetrate them. In this regard, it is worth noting that peptide modifications aimed at optimizing antimicrobial activity against planktonic cells not necessarily match those required for optimizing the antibiofilm properties. For instance, while it has been reported that increasing the average core hydrophobicity of a cationic peptide can improve its antimicrobial activity [128], this same approach may promote peptide-peptide interaction in the weakly hydrophobic alginate, with consequent peptide aggregation and inactivation [126]. Thus, a balance between antimicrobial activity and alginate permeability may represent a better strategy in future design of peptides active against *P. aeruginosa* biofilms. Of note, Yin *et al*. recently demonstrated that although D-isomers of cationic AMPs are slightly more active than their corresponding L-isomers (because bacteria lack proteases to hydrolyze the unnatural D-isomers), D-isomers are relatively more affine to alginate than their L-isomer counterparts [129]. These observations are relevant for the design of peptides with antibiofilm activity and further support the view that optimization of the antibiofilm potential of AMPs may require specific measures.

6.3 Interaction of AMPs with PIA of staphylococcal biofilms

S. aureus represents a major cause of health-carerelated and community-associated infections. The worldwide emergence of multidrug-resistant strains such as methicillin-resistant *S. aureus* (MRSA) has highly contributed to the spread of this bacterium [130]. In addition, *S. aureus* often forms matrix-encased biofilms on tissues and medical devices, which confers an additional level of drug resistance and further complicates the treatment [130]. *S. epidermidis*, another member of the *Staphylococcus* genus, has long been considered an innocuous colonizer of the human skin, but it is now clear that it is also frequently involved in nosocomial infections mostly occurring in patients with implanted medical devices such as intravascular catheters, prosthetic heart valves or orthopedic implants [131]. Ability of *S. epidermidis* to colonize and form biofilms on a variety of biotic and abiotic surfaces is considered the major virulence factor of the bacterium [132].

After attachment of staphylococcal cells by bacterial surface-attached proteins to tissue or indwelling medical devices, which have been coated with host plasma proteins, staphylococcal biofilms develop and mature by the establishment of multiple interactions among biofilm cells [133]. This phase of intercellular aggregation in staphylococci is mainly mediated by PIA, a polymer of β-1,6-linked N-acetyl-glucosamine with partially N-deacetylated amine groups also called PNAG (poly-N-acetylglucosamine). Deacetylation of around 10-20% of the N-acetylglucosamine residues produces free amino groups that become positively charged at neutral to acid pH, resulting in a positive net charge of the otherwise neutral PIA molecule. The quite unusual net positive charge of PIA promote intercellular interactions by binding to the negatively charged surface of bacterial cells.

The biosynthesis of PIA is regulated by genetic elements located in the *ica* (intercellular adhesin) operon which comprises four genes *ica*A, *ica*D, *ica*B and *ica*C. A gene, *ica*R, located upstream of *ica*ADBC, encodes a transcriptional repressor of the *ica*ADBC-operon [133]. Of note, *ica* gene expression and PIA production may also be subjected to environmental-driven regulation.

For instance, it has been reported that anaerobic conditions induce PIA expression in both *S. aureus* and *S. epidermidis* [134]. Mechanisms of phase variation regulating the on/off switching of the genes involved in PIA production have also been proposed [135,136]. Isogenic mutants of wild-type biofilm-forming strains in which the *ica* locus has been mutated or deleted fail to form biofilms *in vitro*, demonstrating that the *ica* genes, and therefore PIA production, are required for biofilm formation [137].

Experimental evidences support the view that staphylococcal PIA plays a crucial role in bacterial protection against major components of the human innate immune system, including host defense peptides [138]. An *ica*-negative *S. epidermidis* mutant strain, lacking PIA production, was demonstrated to be significantly more susceptible than wild-type strain to hBD3 and LL-37 suggesting that the positive net charge of PIA may cause electrostatic repulsion of positively charged peptides (Figure 4D). The importance of PIA positive charge in resistance to cationic peptides is supported by the observation that inactivation of the *ica*B gene, whose product is responsible for PIA deacetylation, reduces the positive charge of the polymer and increases the susceptibility of the *ica*B mutant strain to cationic peptides, at levels comparable to those obtained for the *ica*-negative mutant, devoid of PIA [139]. Remarkably, compared to wild-type *S. epidermidis*, the *ica*B mutant strain, with nondeacetylated PIA, was highly impaired in its ability to form biofilms and to establish device-related infection in a murine model [139]. However, electrostatic repulsion seems to explain only in part the mechanism by which PIA protects biofilms from AMPs, as it was reported to protect biofilm cells also from the anionic dermicidin, a peptide secreted by human epithelia [138].

Despite the evidence that PIA, and possibly other staphylococcal polysaccharides, may play an inhibitory role on the antimicrobial activity of AMPs, ability of AMPs to inhibit PIA biosynthesis/accumulation has also been demonstrated. As reported in paragraph 5, hBD3 was shown to down-regulate the expression of *ica*A and *ica*D genes and up-regulate that of *ica*R [82], while hep-20 was demonstrated to cause the formation of *S. epidermidis* biofilms with an altered architecture and a reduced amount of PIA at sub-inhibitory concentrations [75]. Interestingly, *S. epidermidis* biofilms obtained in the presence of hep-20, were found to be more susceptible to vancomycin than control biofilms; the antibiotic, used at sub-lethal concentrations, caused a statistically significant reduction of biofilm-associated viable cells, as compared to biofilms not pre-treated with hep-20. This observation suggests that interference with extracellular matrix production/accumulation by hep-20, may improve diffusion of conventional antibiotics (e.g., vancomycin) through the biofilm layers.

Although the biosynthetic machinery for PIA production has been mainly investigated in staphylococci, homologous systems are present in other pathogenic, biofilm-forming microorganisms, such as *E. coli*, *Yersinia pestis*, *Aggregatibacter actinomycetemcomitans*, and *Bordetella pertussis*, [98,140]. This suggests that the use of AMPs able to interfere with PIA biosynthesis/accumulation, alone or in combination with conventional antibiotics, could be a strategy to target biofilms of different medically important microorganisms.

6.4 Interaction of AMPs with biofilm or host proteins

The biofilm matrix contains also a considerable amount of extracellular proteins [22]. Among these there are enzymes involved in the degradation of EPS (e.g. DNases, proteases, lipases, polysaccharide- or oligosaccharide-degrading enzymes), with a role in nutrient acquisition or in the detachment and dispersal of biofilm cells, as well as non-enzymatic matrix proteins that exert structural functions, playing adhesive roles in the cell-to-surface or cell-to cell interactions. Proteins of the extracellular bacterial appendages such as flagella, type IV pili or fimbriae may also be part of the biofilm matrix and act as additional structural elements by interacting with other

EPS components. For example, type IV pili of *P. aeruginosa* bind DNA and possibly work as inter-connecting structures [141].

In staphylococci, although PIA is essential for biofilm formation of *ica* positive strains, significant matrix composition variation has been reported across clinical isolates and it is now clear that also *ica*-negative strains may exhibit marked biofilm-forming abilities [142,143]. Biofilm accumulation in these isolates is protein-dependent as their biofilms are sensitive to protease treatment, but resistant to polysaccharide-degrading enzymes [142,143]. A wide variety of proteins has been identified in protein-based biofilm matrices in staphylococci [144]. Examples are the *S. aureus* surface protein C and G (SasC and SasG), the clumping factor B (ClfB), the biofilm-associated protein (Bap), or the fibronectin/fibrinogen-binding proteins (FnBPA and FnBPB). In *S. epidermidis*, a protein named accumulation-associated protein (Aap) contributes to both the primary attachment phase and the establishment of intercellular connections by forming fibrils on the cell surface. Staphylococcal peptides able to disrupt interaction of biofilm matrix molecules with the bacterial surface have also been described. Among them, there is a family of short peptides called phenol-soluble modulins (PSMs), characterized by an amphipathic α-helical structure and surfactant-like properties, the production of which is strictly regulated by the *agr* (accessory gene regulator) locus [145]. Among others, PSMs includes the PSMβ1 and PSMβ2 that at low concentrations facilitate the formation of channels in the biofilm structure, thus promoting biofilm formation, while at high concentrations cause the detachment of biofilm cells independently of the nature (exopolysaccharidic or proteinaceous) of the biofilm [146]. Interestingly, PSMβ1 and PSMβ2 were found to promote dissemination of biofilm cells from colonized catheters in a mouse model of device-related infection, while the use of antibodies against PSMβ inhibited bacterial spread from the device [146]. These observations may have important implications for the development of antistaphylococcal therapeutic strategies. For instance, structural non-toxic analogs of the surfactant-like *S. epidermidis* β subclass of PSMs, could be employed to promote dispersal of biofilm cells and favor their targeting by other bactericidal agents used in combination. On the other hand, specific PSM-inhibitors could aid in interfering with biofilm detachment phase and prevent dissemination of biofilm-associated infections.

Interestingly, by using proteomic approaches, recently Gil and coworkers characterized the exoproteome of exopolysaccharide-based and protein-based biofilm matrices produced by two clinical isolates of *S. aureus* [147]. They found that, independently of the nature of the biofilm matrix, a common set of secreted proteins is contained in both types of exoproteomes. Notably, immunization with a biofilm matrix exoproteome extract effectively reduced biofilm formation and the number of cells in the surrounding tissues in an *in vivo* model of mesh-associated biofilm infection, suggesting the potential of using extracellular proteins for antibiofilm vaccine development [147].

The possible interactions between biofilm exoproteins and AMPs and the eventual role of these interactions in biofilm protection from host defense peptides is still poorly investigated. One could expect that, similarly to what has been shown following interaction of AMPs with serum proteins [74], specific and/or unspecific AMPs-exoprotein interactions may occur with possible inhibitory effects on AMP-activity (Figure4E). In this regard, a role of the *Vibrio cholera*e biofilm-associated extracellular matrix protein Bap1 in cross-resistance to AMPs has been recently demonstrated by Duperthuy *et al*. [148]. Based on an array of experimental data the authors propose a model according to which growing a *V. cholera* strain with sub-lethal concentrations of the cationic polypeptide polymyxin B induces the release of outer membrane vesicles (OMVs) able to bind Bap1 at high levels through the OMV-associated major outer membrane protein OmpT [148]. Bap1 then serves as an adapter protein between LL-37 and the OmpT on the surface of the

OMVs. Following LL-37 binding by Bap1, the concentration of free LL-37 is reduced to sub-lethal concentrations, leading to the apparent resistance and survival of *V. cholerae*.

Degradation of AMPs by biofilm matrix exoproteases might also be possible. For instance, our group has previously shown that proteases secreted by *Porphyromonas gingivalis*, an oral pathogen found in the sub-gingival biofilm of patients suffering from periodontitis, may degrade hBD3 and inhibit the antibacterial activity of the peptide which is found at lower levels in the crevicular fluid of patients than in healthy controls [149,150].

It should be mentioned here that often also host proteins might enter in the constitution of the biofilm matrix. For instance, salivary proteins and glycoproteins are included in the extracellular matrix of oral biofilms and are used as endogenous nutrients by plaque bacteria [151]. Similarly, the airway fluid of CF patients contain significant amount of filamentous (F)-actin that is released together with DNA from neutrophils and other cells that undergo lysis as the result of inflammation [114]. Such host components may also interact with AMPs and cause loss of their antibacterial function [114,152].

An in-depth characterization of the biofilm matrix proteome and of the structure/function relationships of matrix proteins/peptides will provide further insights into biofilm formation and facilitate the development of AMP-based anti-biofilm therapeutics aimed at inhibiting cell-to-cell interactions involved in biofilm accumulation.

7. Conclusions and future directions

Nowadays biofilm infections represent a serious threat for human health. Guidelines to drive the clinical and laboratory diagnosis of biofilm-associated infections have recently been elaborated [6,153], and represent a valuable milestone in the fight against biofilm infections. Nevertheless, to date the diagnosis and treatment of biofilm infections in clinical settings is far from being satisfactory.

In particular, identification of new therapeutic strategies to combat biofilm-associated infections represents one of the main challenges of the modern medicine. Integrated and multidisciplinary approaches will be necessary in the years to come to translate the huge amount of data obtained from extensive biofilm research into the clinic and to solve the numerous obstacles that still hamper the successful management of biofilm-associated infections [7].

In this regard, AMPs may represent a promising therapeutic approach although their interaction with EPS components may neutralize their antimicrobial action representing a possible obstacle for the development of these molecules as antibiofilm drugs. Nevertheless, the observation that many peptides may exert their antibiofilm activity with mechanisms that go beyond a direct microbicidal effect suggests that their use, alone or in combination with other conventional or unconventional drugs, may represent an effective strategy to target biofilm cells. AMP-EPS interactions could even be exploited for the design of AMP-based antibiofilm strategies aimed at sequestering essential EPS components, thus interfering with the establishment and maintenance of biofilm architecture. Alternatively, specifically designed antibiofilm peptides could be employed to interfere with signaling pathways involved in the synthesis of EPS components.

Extensive structure-function studies are desirable to identify the minimal structural features required for an optimal antibiofilm effect, since those known to enhance antimicrobial activity against planktonic cells not necessarily can be applied to biofilms. In other's [69] and our (Batoni *et al*., submitted) experience, subtle changes in the amino acidic sequence of a peptide may greatly affect its antibiofilm activity. In this context, computational approaches [154,155] implemented with large biofilm-oriented AMP-datasets [13,73], may help to predict novel peptide sequences specifically active against biofilms, while physicochemical inspired molecular modeling

methods may provide insights on the AMPs antibiofilm mechanisms of action and/or interaction with EPS components.

The study of possible combination strategies is another research field that is worth investigating as the heterogeneity of microbial biofilms might require targeting cells in different metabolic state or environmental niches. Promising combinatorial strategies include the use of AMPs with: i) other AMPs; ii) conventional drugs used for anti-infective therapy; iii) compounds that can dissolve the biofilm matrix (e.g. DNAase, matrix-disrupting enzymes); iv) inhibitors of QS or other signal pathways; v) anti-inflammatory or mucolytic agents (e.g. ibuprofen, salicylic acid, N-acetyl-cisteine) [156].

Finally, the advent of nanotechnology in the area of infectious diseases may offer further opportunities to optimize antibiofilm AMP-activity. In particular, encapsulation of peptides or proteins in nanocarriers is emerging as a promising technology to overcome the poor stability of the active molecules in physiological medium, avoid their unwanted interactions with biofilm matrix components and deliver them directly to their microbial targets [157]. In this regard, our group has recently developed a delivery system based on the use of chitosan nanoparticles loaded with the antimicrobial peptide temporin B [67]. We found that beyond the intrinsic antimicrobial activity of either chitosan nanoparticles or temporin B alone, the loaded nanocarrier exhibited a highly enhanced and long-lasting microbicidal activity against a number of clinical isolates of *S. epidermidis*, while reducing the toxic potential of the encapsulated peptide against mammalian cells. Further characterization of the developed delivery system against microbial biofilms is underway.

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Figure legends

Figure 1: Comparison of the main features of infections caused by planktonic cells (on the left) and by sessile aggregates of microorganisms known as biofilms (on the right). The two types of infections greatly differ for important aspects regarding prevention modalities, diagnosis, therapy and clinical outcome. Along with the increased use of medical devices in health care procedures, biofilm-associated infections have emerged as an alarming reality of modern medicine. The different colors of bacterial cells represent the heterogeneity of the metabolic status of microorganisms within a biofilm or, in mixed-species biofilms, the diversity of the cells constituting the community. The shadowed area in the biofilm represents the extracellular polymeric substance.

Figure 2: Properties of the biofilm life-style (red text) and "ideal" features of an optimal antibiofilm drug (black text). Many of these features are exhibited by naturally occurring host defense peptides or their optimized derivatives. The three main steps of the biofilm life-cycle (attachment to a surface, maturation and dispersal) are depicted.

Figure 3: Main possible mechanisms of the antibiofilm activity of AMPs based on classical bactericidal effects or on the interference with essential attributes of the biofilm life-style. Coexistence of more than one mechanism is also possible. See text for details.

Figure 4: Examples of interactions of AMPs with extracellular polymeric substances of the biofilm matrix such as extracellular DNA (A, B), polysaccharides (C, D), and proteins (E). **A)** High levels of host or bacteria-derived extracellular DNA can bind and neutralize AMPs (blue helix); **B)** AMPs can displace LPS-stabilizing cations (Mg) and disrupt membrane integrity. Extracellular DNA sequesters cations from the membrane and generates a cation-limited environment. This activates the PhoPQ/PmrAB systems that activate the cationic antimicrobial peptide (CAP) resistance operon, leading to the production of aminoarabinose (orange) and polycation spermidine (red) that mask the negative charges and protect the outer membrane from AMP damage [116]; **C)** Negatively charged alginate might entrap AMPs before they can reach their bacterial target and constitutes a protective environment against the innate host defense; **D)** polysaccharide-intercellular-adhesin (PIA)/poly-N-acetylglucosamine (PNAG) due to their positively charged ($NH3^{+}$) free amino groups may cause electrostatic repulsion of positively charged AMPs; **E)** *V. cholerae* outer membrane vesicles (OMVs) bind Bap1 at high levels through the major outer membrane protein OmpT. On the surface of OMVs Bap1 binds LL-37, reducing the concentration of free LL-37 to sub-lethal

concentrations leading to the survival of the bacteria [148]. Degradation of AMPs by biofilm matrix exoproteases might represent another mechanism by which biofilm bacteria resist to AMPs.

Figure 5: Model of the interaction of one EPS chain with two AMP-molecules based on circular dichroism and fluorescence spectroscopy data, as proposed by Foschiatti *et al*. [125]. Reproduced with permission from John Wiley & Sons Inc., http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2958.2009.06707.x/full#f6

Table 1

Key examples of antimicrobial peptides acting against biofilms in *in vitro* models.

Peptide (source)	Amino acid sequence [a]	Charge [b] (pH 7)	Pho (CCS)	Microbial species/strain	Proposed antibiofilm mechanism of action	Ref.
D,L-K_6L_9 (de novo)	LKLLKKLLKKLLKLL-NH_2	5.975	1.86	*P. aeruginosa* PAO1	Decrease of bacterial attachment by surface adhesion	[81]
All L-K_6L_9 (de novo)	LKLLKKLLKKLLKLL-NH_2	5.975	1.86	*P. aeruginosa* PAO1	Decrease of bacterial attachment by binding to bacteria	[81]
Seg6D (de novo)	LLLLLKKKKKLLLL-NH_2	5.975	1.86		Degradation of established biofilms by direct bacterial killing	[81]
Seg6L (de novo)	LLLLLKKKKKLLLL-NH_2	5.975	1.86	*P. aeruginosa* PAO1	Degradation of established biofilm by bacterial detachment	[81]
LL-37 (human)	LLGDFFRKSKEKIGKEFKRIV QRIKDFLRNLVPRTES	5.98	-1.84	*P. aeruginosa* PAO1, PA14, *Burkholderia cenocepacia* 4813; *Listeria monocytogenes* 568	Decreased attachment of bacterial cells, stimulation of twitching motility, influence on two major QS systems (Las and Rhl), leading to the down-regulation of genes essential for biofilm development	[85]
1037 (modified)	KRFRIRVRV-NH_2	4.976	-2.55	*P. aeruginosa* PAO1,	Inhibition of swimming and swarming motilities and stimulation of twitching motility	[84]
IDR-1018 (modified)	VRLIVAVRIWRR-NH_2	3.976	0.66	*P. aeruginosa PAO1*, PA14; *E. coli 0157*; *A. baumannii SENTRY C8*, *K. pneumoniae ATCC13883*, *S. aureus MRSA SAP0017*, *S. enterica* sv Typhimurium 14028S, *B. cenocepacia IIIa 4813*	Binding and promotion of degradation of the signal for biofilm formation and maintenance (p)ppGpp	[15, 83]
hBD-3 (human)	GIINTLQKYYCRVRGGRCAV LSCLPKEEQIGKCSTRGRKCC RRKK	10.792	-3.11	*S. epidermidis* ATCC35984; *S. aureus* ATCC43300; methicillin-resistant *S. epidermidis* MRSE287	Decreased expression of *icaA* and *icaD* gene expression and up-regulation of *icaR* expression (transcriptional repressor of the *ica* operon)	[82]
hep-20 (human)	ICIFCCGCCHRSKCGMCCKT	2.971	-1.64	*S. epidermidis* ATCC3594, *S. epidermidis* clinical isolates	Interference with matrix PIA and protein production/accumulation	[75]

[a] Underlined amino acids are D-enantiomers.

[b] Net charge at neutral pH and hydrophobicity (pho) were calculated using the Baamps database [73] (not considering the terminal modification).

CCS: combined consensus scale.

Table 2
Examples of antimicrobial peptides tested against biofilms in different *in vivo* models.

Peptide (source)	Sequence	Charge[a] (pH 7)	Pho (CCS)	Microbial species/strain	Experimental model	Ref.
Citropin 1.1 (frog)	GLFDVIKKVASVIGGL-NH2	0.97	1.15	*S. aureus* strain Smith diffuse	Reduction of biofilm bacterial load and bacteremia in a rat model of central-venous-catheter infection	[89]
BP2 (modified)	GKWKLFKKAFKKFLKILAC	6.944	0.06	*S. epidermidis* (ATCC3594)	Treatment and prevention of biomaterial-associated infection in the mouse model	[90]
Novispirin G10 (modified)	KNLRRIIRKGIHIIKKYG	7.21	-1.64	mucoid *P. aeruginosa* clinical isolate NH57388A	Intratracheal administration in a rat model of lung infection	[91]
				P. aeruginosa clinical isolate 2	Treatment of infected burns in a mouse model	[92]
OP-145 (modified)	Ac-IGKEFKRIVERIKRFLRELVRPLR-NH_2	5.979	-1.408	*P. aeruginosa* PAO1	Treatment of biofilm-related sinusitis in a rabbit model	[93]
DASamP1 (de novo)	FFGKVLKLIRKIF	3.97	2.2	*S. aureus* USA 300 LAC*::Lux*	Suppression of early biofilm formation in a mouse model of catheter-associated infection	[94]
Tet-20 (de novo)	KRWRIRVRVIRKC	6.94	-2.83	*S. aureus*	Biofilm resistance of Tet-20 conjugated titanium implants in a rat infection model	[95]

[a]Net charge at neutral pH and hydrophobicity (pho) were calculated using the Baamps database [73] (not considering the terminal modification).
CCS: combined consensus scale

Types of infections prevalent until the mid-twentieth century	Types of infections prevalent nowadays
• Acute development	• Chronic progression, often recurrent
• Controlled by antibiotics and vaccines	• Highly recalcitrant to antimicrobial therapy; no vaccine available
• Defined pathogenic mechanisms; severe symptoms	• Undefined pathogenic mechanisms; generally mild symptoms, but highly debilitating
• Involvement of specific highly pathogenic etiological agents, cultivable from infected tissues	• Possible involvement of microorganisms making part of the normal flora; culture from infected tissues often negative
	• large aggregates of bacteria surrounded by an extracellular matrix visible on tissues or medical devices at the site of infection.

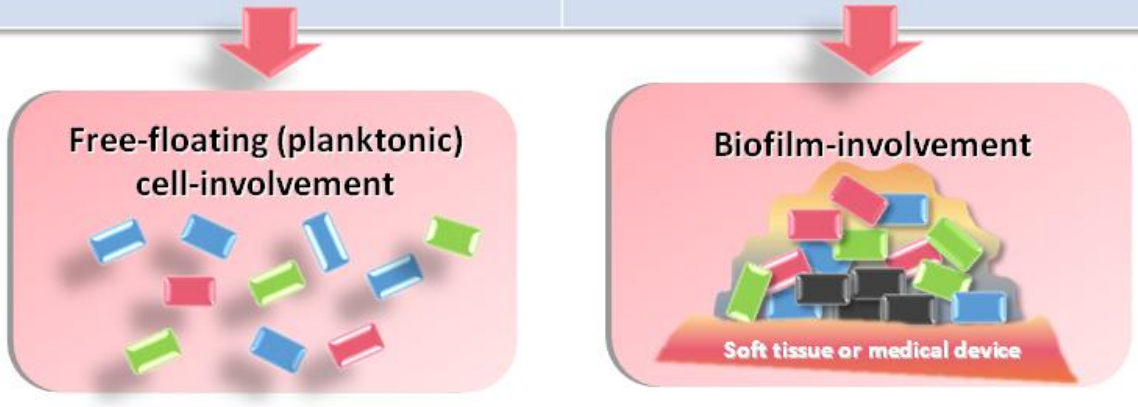


Fig. 1

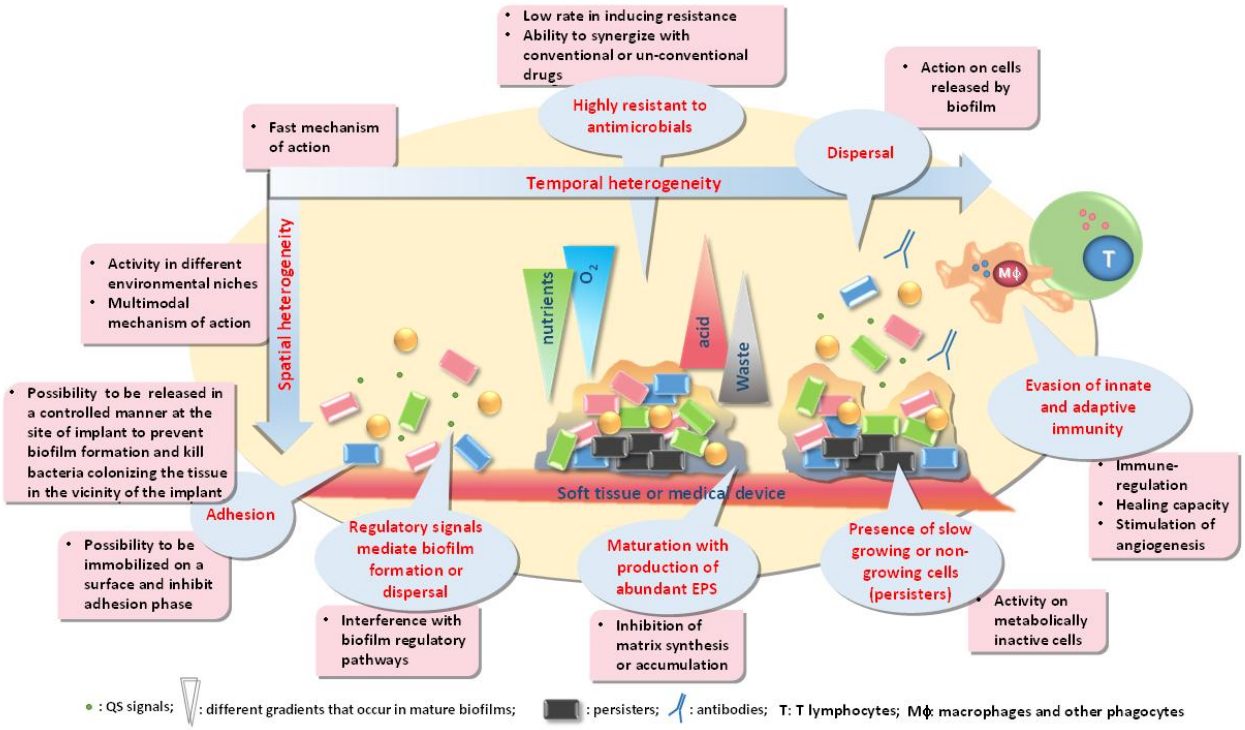


Fig. 2

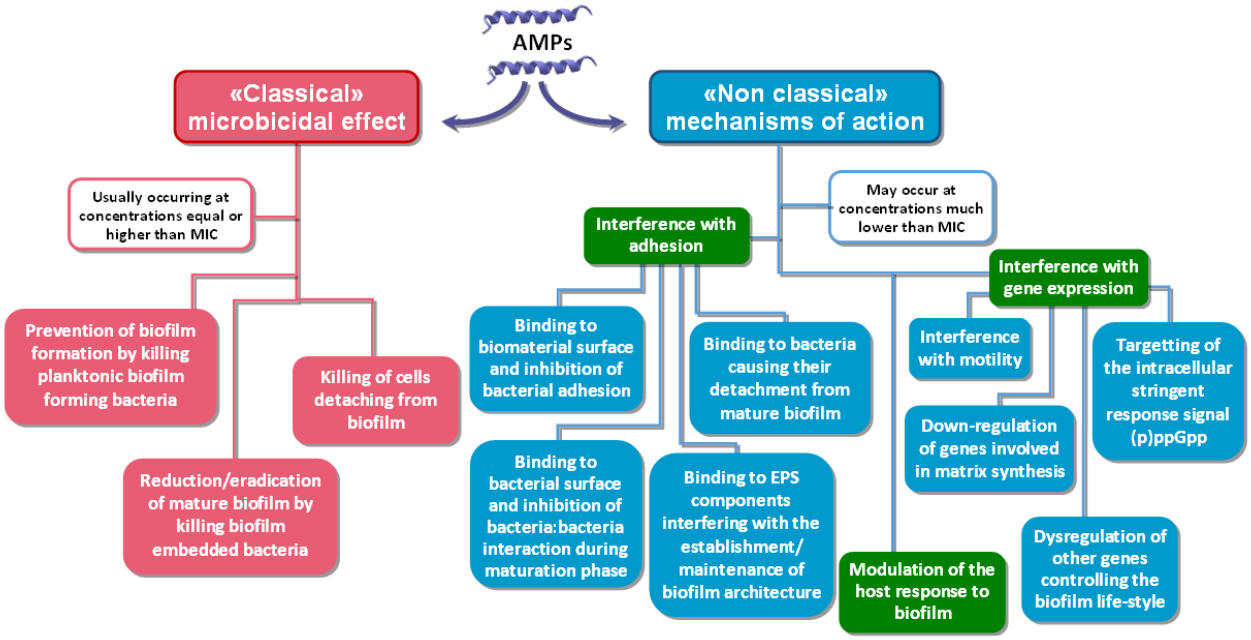


Fig. 3

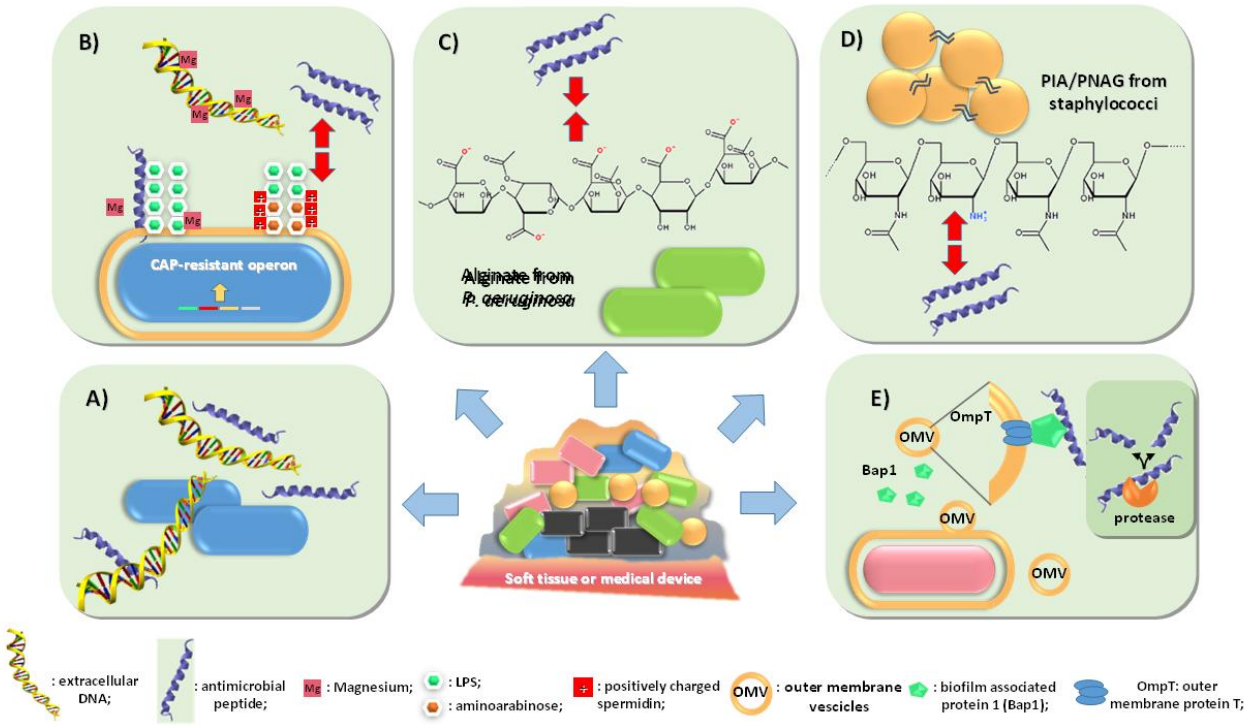


Fig. 4

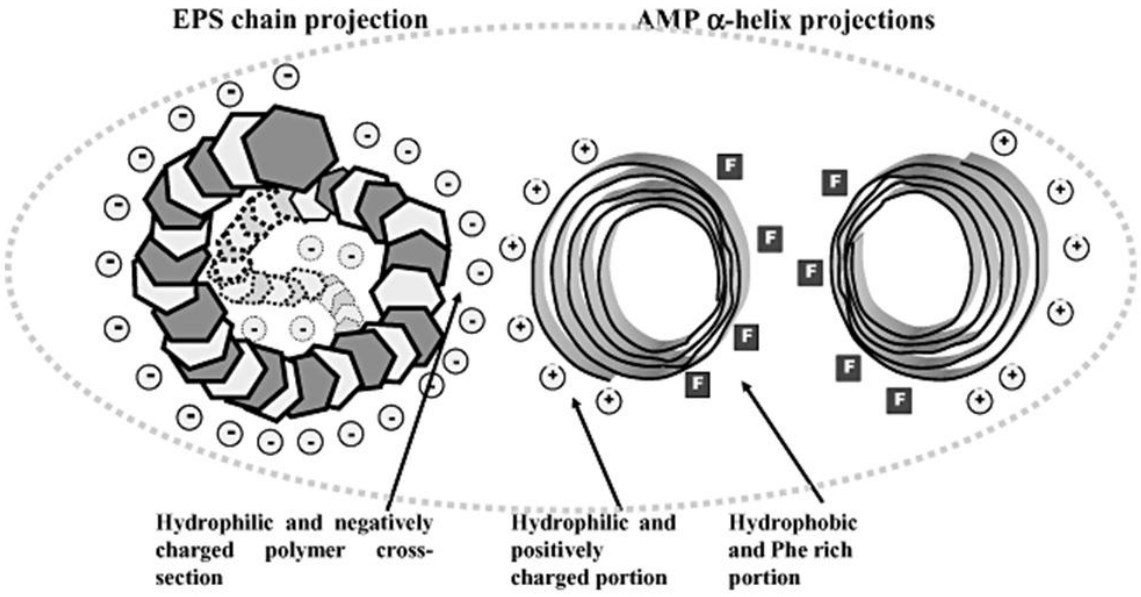
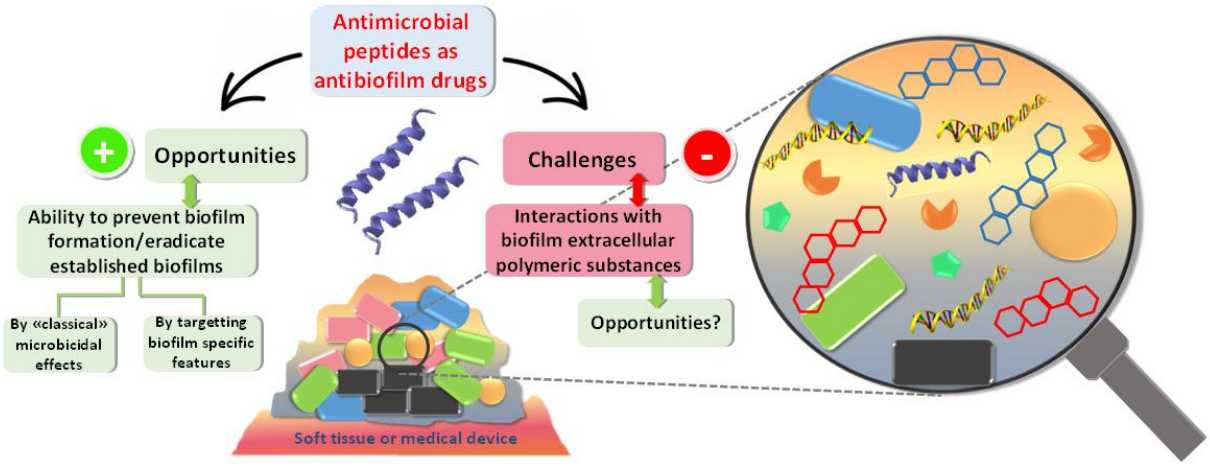


Fig. 5



Graphical abstract

Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria

G. Batoni, G. Maisetta, S. Esin

HIGHLIGHTS

Biofilm-associated infections are one of the major threats of the modern medicine

AMPs may represent a promising therapeutic approach for biofilm-associated infections

AMP-activity may rely on bactericidal effect or interference with biofilm life-style

Interaction with polymers of biofilm matrix may hamper AMPs' antibiofilm activity