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Perspectives on polymeric nanostructures for the therapeutic application of antimicrobial peptides.

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ABSTRACT

Antimicrobial peptides (AMPs) are a class of promising anti-infective molecules but their therapeutic application is opposed by their poor bioavailability, susceptibility to protease degradation and potential toxicity. The advancement of nanoformulation technologies offers encouraging perspectives for the development of novel therapeutic strategies based on AMPs to treat antibiotic resistant microbial infections. Additionally, the use of polymers endowed *per-se* with antibacterial properties, stands out as an innovative approach for the development of a new generation of drug delivery systems in which an enhanced antimicrobial action could be obtained by the synergic combination of bioactive polymer matrices and drugs. Herein, the latest AMPs drug delivery research is discussed.

KEYWORDS

Antimicrobial peptides, drug delivery, polymers, nanostructures.

INTRODUCTION

Severe diseases related to microbial infections and contaminations are of great concern in various areas, such as medical devices, drugs, hospitals, dental office equipment, food industry, packaging and storing, etc. Generally, these infections are treated with antimicrobial agents and are susceptible to their action. Particularly problematic are microorganisms that rapidly and easily gain resistance to conventional antimicrobial drugs, making them insusceptible to conventional drugs and leading to difficult pathogen eradication [1]. At least one mechanisms of resistance, but often more than one, has been developed for each one of the 17 different classes of antibiotics produced to date. Moreover, the ability of bacteria to develop simultaneous resistance to two or more antibiotic classes has made the treatment of such infections extremely difficult, very costly and associated, in many cases, with high morbidity and mortality [2]. The alarming rise in antibiotic resistance, has led to the selection of pan drug-resistant microorganisms (PDR) that are resistant to all available antibiotics. The inability to treat infections caused by such pan-resistant bacteria, is keeping infectious diseases among the major public health issues and poses substantial challenges to the human welfare [3]. Bacterial resistance causes only in the European Union about 25.000 deaths per year, with an overall societal cost of €1.5 billion per year [3, 4].

Development of multiple drug resistance enforces administration of high-dose of conventional antibiotics leading to adverse side effects and intolerable toxicity. Alternative strategies to treat microbial diseases such as the use of potent and/or specific antimicrobial systems would help to mitigate, treat and/or eradicate these infections, with an improvement in the state of well-being. Among them, novel molecules and nanoscale materials have emerged as novel antimicrobial agents. In this context, the development of polymeric nanoformulations as carrier for a new class of anti-infective drugs, such as AMPs, will be presented and discussed. After a brief description of the major and more conventional classes of antimicrobial polymer-based materials, this review will focus on polymeric materials, with emphasis on those intrinsically endowed with antimicrobial activity, as fundamental constituents of AMPs loaded nanocarriers, towards the development of a novel therapeutic approach for the treatment of antibiotic resistant infections.

Antimicrobial polymer-based materials

It is generally assumed that an antimicrobial polymer is a polymer exerting a bactericidal activity. This generates a wide and heterogeneous category of polymers with different chemical composition and active towards various microorganisms. Some antimicrobial polymers are highly attractive candidates for the development of new antimicrobial nanostructured delivery systems, as they would likely be able to confer to the nanosystems itself a biological activity, opening up possibilities for antibacterial synergism between the nanocarrier and the loaded cargo. In fact, antimicrobial biomaterials could exert their own antimicrobial activity when utilized for the formulation of bactericidal drug delivery systems, as well as resist to microbial colonization when employed for the development of biomedical devices [5, 6]. As an instance, anti-infective biomaterials have progressively become a primary strategy to prevent medical device-associated infections, after the latest achieved improvements in terms of aseptic techniques, sterility control and antimicrobial prophylaxis [7].

The use of antimicrobial polymers holds promises to enhance the efficacy of some existing antimicrobial agents, minimize their residual toxicity, increase their efficiency and selectivity and

prolong their lifetime [8]. The requirements that antimicrobial biomaterials need to cover are very broad, primarily depending on the material application. The ideal antimicrobial polymer should be easily and inexpensively synthesized, stable in long-term usage and at the temperature of its application, not decomposing to and/or emitting toxic sub-products, not toxic or irritating and biocidal to a broad spectrum of pathogenic microorganisms in brief times of contacts [9].

Many factors are known to affect the bactericidal activity of antimicrobial polymers, comprising, among the others, molecular weight, hydrophilic-hydrophobic ratio, carried charge and, if the polymer brings antimicrobial moieties, the key role of the spacer length.

The first and most widely investigated antimicrobial polymers are charged polymers inhibiting the adhesion of bacteria (negatively charged polymers) or their growth (positively charged polymers). Among those, important observations were performed on acidic or quaternarized acrylic and methacrylic polymers [10] and quaternary ammonium salts (QAS) pending from various polymer types (polysiloxanes [11]; polyethyleneimine [12], poly(oxazoline)s [13] and polystyrene [14]), with the bactericidal activity strictly affected by the adopted spacers (amphiphilic balance, length of the alkyl chain, balance of cationic moieties and hydrophobic groups). Variations in the amphiphilic balance lead to different affinities to the bacterial membranes favor the diffusion through the membranes and influence the bactericidal activity/cytotoxicity ratio. Alternatively to antimicrobial polymers with pendant quaternary ammonium/phosphonium salt (QAS/QPS), cationic polymers containing QAS or QPS in the backbone have been deeply investigated. Such polymers, known as linear or comb-like ionene polymers, are capable of forming complexes with heparin and DNA, accompanied by adhesion, aggregation and lysis of bacterial cells. Additionally, ionenes with rigid spacers exhibited stronger interaction with phospholipid bilayers; longer hydrophobic segments, with lower charge densities, exhibited more effectively biocidal ability versus yeast protoplast than those with higher charge densities [15].

Further than QAS containing polymers, several antimicrobial structures have been investigated through the years. Significant roles play guanidine containing polymers; polymers mimicking natural peptides, such as arylamide and phenylene ethynylene backbone polymers and polynorbornene derivatives; halogen polymers, such as fluorine- and chlorine-containing polymers and polymeric N-halamines; polymers containing phospho and sulfo derivatives; organometallic polymers; and phenol/benzoic acid derivative polymers [6] (Table 1).

Polymer-based materials, exerting a microbicidal action can be obtained by chemical modification of polymer and addition of low molecular weight antimicrobial compounds, addition of metal nanoparticles (polymer nanocomposites), insertion of oxides or inclusion of antimicrobial modified inorganic systems [16].

Antimicrobial agents containing reactive functional groups (hydroxyl, carboxyl or amino groups) can be covalently linked to a variety of polymerizable derivatives. Most of the synthesized drug-bearing monomers are acrylic types of pharmaceutically active molecules that can be copolymerized obtaining different drug contents and hydrophobic/hydrophilic branches. Otherwise, antimicrobial agents have been immobilized on synthetic preformed polymers or naturally occurring polymers [9].

The combination of polymeric-based materials with antimicrobial inorganic systems has also been widely studied. Silver nanoparticles are probably the metal particles most used as antimicrobial agent in polymeric nanocomposites. Silver and its compounds are well known for their broad antimicrobial spectrum against bacteria, fungi and viruses [17]. Several antimicrobial polymer nanocomposites have been prepared by mixing preformed particles with polymers. Nanocomposites

of polyamide [18] and polypropylene [19] containing silver powder were produced by melt processing; multilayer films with antimicrobial properties were prepared from polyethylene (PE)/silver nanocomposites by the dispersion of silver powders in the polymeric solution. The Ag^+ release and the subsequent antimicrobial activity were found to be dependent on the silver nanoparticles content and on the deposition method [20]. Polymeric nanotubes and nanofibers [21] with silver nanoparticles have been prepared by chemical oxidation polymerization of rhodanine. The synthesized materials showed excellent antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*, caused by the combined activity of silver and rhodanine. Also copper particles are known for their antimicrobial activity [22] and were employed for the preparation of polypropylene (PP) nanocomposites by melt mixed method. Composites with only 1% (v/v) of Cu were able to kill 99.9% of bacteria after 4 h of contact [23]. Several studies were also focused on the incorporation of copper nanoparticles onto the surface of polymers. Copper ion implantation by plasma immersion was employed to create antibacterial surface on PEs, exhibiting excellent long-term antibacterial effects against *E. coli* and *S. aureus* [24].

The insertion of oxides in polymers allows for photocatalytic disinfection or photo-killing, an emerging powerful technologies for bacterial killing [25]. Titanium dioxide TiO_2-anatase is the most broadly used photocatalytic, generating energy-rich electron-hole pairs able to degrade cell components of microorganisms. Among several possibilities, titanium can be supported on polymers. Zinc oxide (ZnO) is also a photocatalyst, with a bactericidal mechanism of action similar to TiO_2-anatase. ZnO nanoparticles have been incorporated into thermoplastic polymers polyamide 6 and low density PE [26]: in both cases the nanocomposites showed great antimicrobial activity with low content of ZnO, 1% (w/w), which enhanced as the ZnO increased in the nanocomposite.

Montmorillonite is an antimicrobial modified inorganic system explored in combination with polymeric materials. Organo-montmorillonite with antimicrobial properties was prepared from Na^+-montmorillonite and chlorhexidine acetate [27]. This was blended with polydimethylsiloxane (PDMS) to produce nanocomposites films. The antibacterial activity was investigated by mean of the inhibitory zone tests, revealing a strong inhibition of the growth of *E. coli* and *S. aureus*. Commercially available organoclay montmorillonites modified with cationic surfactants were also introduced into polymers such as nylon-6, rendering biocidal polymeric nanocomposites [28].

Antimicrobial peptides

Antimicrobial peptides (AMPs) are a large and diverse group of molecules, utilized as nature's antibiotics and produced constitutively or in response to infections in virtually every organism [29]. Generally they are small (with a varying number of amino acids from 5 to over a hundred) mainly cationic and amphipathic with a considerable diversity in sequence and structure. Based on their molecular masses, secondary and tertiary structures and amino acid composition, AMPs can be classified into various categories comprising peptides with α-helix structures (e.g. human cathelicidin), peptides with β-sheet structures stabilized by disulfide bridges (e.g. human defensins), peptides with extended structures (e.g. the bovine AMP indolicidin); and peptides with loop structures, like cyclic defensins found in *Rhesus macaques*. At present more than 2500 AMPs from both living prokaryotic and eukaryotic organisms have been reported. Updated databases [30] of AMPs structures and activities are available on line at: *http://aps.unmc.edu/AP/main.php* [105] and *http://www.biomedicine.org.ge/dbaasp* [106] The mechanisms of the antimicrobial action of AMPs are complex and still not fully understood. It is widely accepted that most cationic AMPs establish

electrostatic interactions with the negatively charged phospholipid head groups of bacterial membranes followed by insertion in and disruption of the lipid bilayer. Several models of AMP-membranes interaction have been proposed (Figure1). In the "barrel-stave" model, AMPs assemble to form pores across the membrane so that their hydrophobic moieties face the lipid bilayer, while their hydrophobic parts face the pore's lumen. In the "carpet model" peptides cover the outer side of the membrane like a carpet, and then reorient themselves and place in the hydrophobic core of the membrane forming micelle-like units and acting like a detergent [31]. Moreover, it is suggested that some AMPs do not cause cell membrane disruption, but rather act by crossing the membrane and accumulating in the cytoplasm of bacterial cells where they interfere with the activity of intracellular target.

In the era of antibiotic resistance, AMPs are widely considered among the most promising lead compounds for the development of new anti-infective drugs. Indeed, the potential advantages of AMPs as antimicrobial drugs are numerous (Figure 2). They usually exhibit a fast and broad-spectrum of activity against a wide range of microorganisms, including Gram-negative and Gram-positive bacteria, protozoa, yeast, fungi and viruses [32] (Table 2). Notably, many AMPs are also active against multi-drug-resistant strains [33-35] and synergize when tested in combination with conventional antibiotics [36]. Furthermore, their interactions with bacterial components usually do not involve specific protein binding sites and thus, they are believed to induce resistance at much lower rate than conventional antibiotics. Beside their direct antimicrobial properties, AMPs often display immunomodulatory activities like chemotaxis, modulation of cytokine and chemokine expression, leukocyte activation and others, suggesting that, *in vivo*, they may also indirectly participate to the eradication of an infection [37]. Many AMPs can promote healing processes by stimulating cellular proliferation or angiogenesis, thus potentially contributing to tissue repair during the course of an infection [38]. Finally, AMPs can be chemically manipulated (by amino acid substitution, introduction of D or non-natural amino acids, expression as fusion proteins, combination of different functional domains etc.) to develop derivatives of natural peptides or *de novo* designed molecules with improved pharmacological profiles [ref 39]. Critical features for peptide design are optimal size, charge, hydrophobicity, the positioning of shape-modifying amino acids (proline, glycine) and the presence of amino acids with affinity for membrane interfaces (tryptophan) [40].

Despite these numerous desirable characteristics, many limitations still hamper AMP-pharmaceutical development (Figure 2). For instance, the unspecific action of AMPs on the bacterial membranes could potentially be harmful to mammalian cell membranes as well, determining a low cytocompatibility, at therapeutic concentrations. Inactivation by biological fluids [41], sensitivity to host and/or bacterial proteases [42] (with consequent decrease of their antimicrobial potency in physiological environments) and high manufacturing costs are additional limits in the use of AMPs as future drugs. Thus, the current challenge in AMP therapeutic development is to produce them at a reasonable cost as well as to overcome the obstacles that still hamper their clinical employment, especially as systemic drugs. In this regard, the entrapment of AMPs in suitable micro-nanostructures, possibly endowed with their own antimicrobial activity, may represent a promising approach with the potential of minimizing the peptides toxicity towards mammalian cells [43], protecting them by proteolysis and un-wanted interactions with biological fluids, and ensuring a controlled and long-lasting release of the entrapped molecules (Figure 2).

Clinical applications of AMPs: topical and local delivery

In spite of the potential possibilities offered by the discovery of thousands of natural peptides and the design of millions of synthetic peptide, relatively few AMPs, selected on the basis of promising outcomes of *in vitro* and animal studies, have actually proceeded into clinical trials. Both positive and negative experiences have been observed in clinical trials.

Pexiganan (MSI-78), a synthetic 22-amino acid analogue of Magainin II, was the first antimicrobial peptide to undergo commercial development. It had demonstrated excellent *in vitro* broad-spectrum activity against 3109 bacterial clinical isolates, with no selection of resistant mutants after repeated passages at sub-inhibitory concentrations. Two Phase III clinical trials involving 835 diabetic patients with infected foot ulcers showed an improvement in 90% of patients using either Pexiganan or conventional oral Ofloxacin. Eradication of pathogens was obtained in 82% of the Ofloxacin recipients compared to 66% of Pexiganan recipients, at the end of the therapy. In 1999, FDA approval was denied not because of a lack of activity but rather for an inability to demonstrate an advantage over existing therapeutics (i.e. non-equivalence) [44].

Another negative result involves Nisalpin™, the trade name for bacterial Nisin, subjected to Phase I clinical trials on *Helicobacter pylori* infections with encouraging results, but then abandoned.

Despite these negative experiences, there is ample cause for optimism around AMPs, at least as topical/local agents. Cationic peptides Polymyxin B and Gramicidin S, considered too toxic for systemic use, have been used in combination for many years in various topical formulations, including wound creams and eye/ear drops. Several attempts have been made in order to reduce polymyxins and gramicidins toxicity, unsuccessfully to date [45]. Clinical efficacy has been observed with MX-226/Omeganan in the prevention of catheter-associated infections, but issues with clinical trial design and endpoints have precluded licensure to date. Other peptides that advanced into Phase III clinical-efficacy trials are the pig Protegrin derivative IB-367, Iseganan, indicated for the treatment of oral mucositis, and the human bactericidal permeability protein derivative $rBPI_{23}$, Neuprex, indicated for treating sepsis [46]. In addition to the above peptides, many other molecules are proceeding through discovery, development and clinical trials [47] (Table 3).

The potential systemic applications of AMPs has been limited by several major issues such as the poor pharmacokinetics related to their susceptibility to proteases and other clearance mechanisms, unknown systemic toxicity and cost of goods. At present, clinical trials aimed at exploiting the direct antimicrobial activity of AMPs have been restricted to topical applications for the treatment of surface infections.

In addition, the high cost of manufacturing peptides has limited both the testing and development of large numbers of variants and the potential clinical targets to which these molecules can be applied. However, increasingly practical recombinant DNA expression strategies, the use of peptide array and advanced computational strategies are starting to impact on the cost of goods [48]. Regarding AMPs lability to proteases, leading to potentially unfavourable pharmacokinetics, several solutions have been proposed, including: the use of unusual or D- (rather than natural L-) amino acids (which renders peptides protease-resistant), the use of non-peptidic backbones (peptidomimetics), formulations to improve stability (e.g. liposomes, nanoparticles) or the chemical modification of peptides to create protease-resistant (and/or less toxic) pro-drug molecules [49]. Although AMPs seem to have a lesser ability to disrupt eukaryotic membranes, lacking negatively charged lipids on

the surface, systemic toxicity is an issue yet to be properly addressed. Research on antimicrobial peptides urgently needs careful investigations aimed at evaluating subtle toxicities associated with systemic peptide such as apoptosis induction and mast-cell degranulation.

Nanostructures for the delivery of antimicrobial peptides

The formulation of peptides and proteins into nanocarriers has been extensively studied over the past decades and among those, few AMPs have been included into drug delivery nanostructures [50]. The most commonly applied nanoformulation techniques are easily classified into two main categories, whether they involve the *in-situ* polymerization of the macromolecules (emulsion and dispersion polymerization, interfacial polymerization/polyaddition/polycondensation) or more commonly, the direct use of preformed polymers. Due to their lability, the retention of activity, structural identity and stability of the peptides after encapsulation are basic concerns in the development of peptide-loaded nanostructures. Reasonably, the less aggressive technics are selected, e.g. emulsion-solvent evaporation, phase separation, salting out, dialysis methods, ionotropic gelation and self-assembling [51, 52].

AMPs have been formulated into several nanostructures up to date, including lipid nanovesicles, nanofibers, coated metallic nanoparticles, self-assembled structures, hydrogels and polymeric nanoparticles (Figure 3).

Lipid nanovesicles and liposomes, can be employed for the delivery of water-soluble, lipid-soluble or amphiphilic molecules due to the presence of both lipidic and aqueous phases in their structure [53]. Commonly, they are obtained by thin film hydration techniques frequently associated to additional sonication or membrane extrusion. Considering that the main mechanism of action of AMPs toward bacteria is the destabilization of the phospholipid membrane, the use of lipid vesicles for the delivery of AMPs introduces several questions regarding the stability of the carrier itself and the obtained antimicrobial activity of the formulation. Indeed, lipid composition of the vesicles affects the interaction with the biological membranes, favouring the repulsion, adhesion, fusion or internalization of the carrier. Lipid composition can also affect its susceptibility to perturbation caused by the loaded AMP, favouring then its release from the carrier itself. These aspects were investigated by varying the composition of phosphatidylcholine (PC) based vesicles loaded with Nisin, for the inhibition of *Listeria monocytogenes* growth for milk conservation applications, and reviewed in Malheiros *et.al*. [54]. Food application of Nisin was further investigated by preparing liposomes made with marine lecithin (ML) or soy lecithin (SL). SL improved Nisin loading, physical stability monitored at 4 °C revealing pore-formation by the AMP and fusion phenomenon after 20 weeks. Antimicrobial assay revealed that blend of unencapsulated/free and encapsulated nisin (1:1) exhibited a better control of *L. monocytogenes* as compared to free or 100% encapsulated nisin alone [55]. Recently, the LL-37 (human AMP) has been encapsulated in nanostructured lipid carriers (NLCs), produced by the melt-emulsification method. LL-37 has a broad spectrum of antimicrobial activity, but it is also able to modulate wound healing by participating in angiogenesis, epithelial cell migration and proliferation, and immune response. The described carrier showed lack of *in vitro* cytotoxicity and the encapsulated LL-37 maintained its bioactivity, as it was evidenced by assessing the antimicrobial activity against *E. coli* and the *in vivo* wound

healing repairing, in a full thickness wound db/db mice model [56]. Similarly, pegylated-liposomes loaded with LL-37 were evaluated against herpes simplex virus 1 (HSV-1) and compared to analogous liposomes loaded with indolicidin. The LL-37 liposomes were rapidly taken up by human keratinocyte cell line (HaCaT), remained intact within the cells, and the release of the active peptide within the cytoplasm was followed by the migration of the vesicles' lipids to the plasma membrane. Furthermore, in 3D epidermis model (immortalized primary keratinocytes) liposomal LL-37 treatment was able to protect the epidermis by inhibiting HSV-1 infection, and without cytotoxic induction [57].

Additional lipidic formulations have been investigated for lung delivery applications. Anionic dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) (3:1 molar ratio) liposomes encapsulated high levels of the cationic α-helical AMP CM3 were delivered by nebulization to the lungs of rats chronically infected with *Pseudomonas aeruginosa*. A reduction in the AMP toxicity and an enhanced protection of the peptide against proteolytic degradation were observed [58].

Nanofibers, polymeric fibres arranged into woven or no-woven meshes has a large specific surface area, which increase by reducing nanofibers diameter. Their functionality is generally affected also by the interfibres spaces and structure geometry, affecting eventual permeability, swellability, mass transport and degradability as well as mechanical properties. Such advantages, together with high drug loading and flexibility in materials selection, suggested the use of nanofibrous constructs as drug delivery devices, dressings, coatings, and tissue regeneration applications [59]. Concerning the loading of AMPs, electrospinning has been the mostly applied. Both natural and synthetic polymers can be employed for their development, such as chitin and chitosan, silk fibroin, poly(L-lactic acid), poly(vinyl alcohol) and polyurethane. Also in this field, the development of antimicrobial meshes exploiting the activity of AMPs represents an alternative to the use of antibiotic eluting devices [60].

AMPs loaded electrospun meshes have been investigated for various applications, including food preservation, prevention of microbial adhesion on surfaces and wound dressing. The use of Nisin for food applications has been investigated not only by liposomal formulations, but also as loaded into phosphorylated soybean protein isolate/poly(l-lactic acid)/zirconium dioxide (Nisin-PSPI/PLLA/ZrO2) nanofibrous membranes. The device displayed controlled release and good antimicrobial activity against *Staphylococcus aureus* [61]. Also the AMP Pleurocidin has been evaluated as food preservative. Its loading into poly(vinyl alcohol) electrospun nanofibers provided a sustained release after a temperature dependent burst release. Interesting, the inhibition activity toward *E. coli* was assessed in a real food system [62]. Electrospun polyethylene oxide nanofibers were developed for the encapsulation of Plantaricin 423, for food and medical applications. The peptide maintained its antimicrobial activity after electrospinning and the loaded nanofibers successfully inhibited the growth of *Enterococcus faecium* and *Lactobacillus sakei* [63].

Another strategy envisages the immobilization of the AMP on preformed electrospun meshes. Magainin II (Mag II) was covalently immobilized on poly(lactide-co-glycolide) (PLGA) and PLGA/gelatin electrospun fibrous membranes. In this case bacterial adhesion tests revealed that the attachment and survival of microorganisms were inhibited [64]. This approach gives the possibility to confer multiple functions to a membrane device, as investigated with a bilayer membrane for tissue engineering application [65].

Antimicrobial wound-dressings based on the incorporation of AMPs into polyelectrolyte multilayer films were obtained by alternate deposition of polycation (chitosan) and polyanion (alginic acid

sodium salt) over cotton gauzes. The AMPs (β-Defensin-1, Dermaseptin, Cys-LC-LL-37, Magainin 1) used in this work provided a good antimicrobial effect without cytotoxicity to human dermal fibroblasts at the tested concentrations [66].

Metallic nanoparticles (MNPs) could serve as potential nanocarriers for AMPs, as well. Metallic nanoparticles, based on silver as an instance, have been known for their intrinsic antimicrobial properties and used in the medical field for antimicrobial applications for years [67]. The exact mechanisms of MNPs toxicity against bacteria are not completely understood. However, NPs action may result in bacterial cell wall or membrane damage or be responsible for detrimental changes in cellular organelles. Generally, the good antibacterial properties of nanostructured materials arise from their superior surface area, providing greater contact with bacterial cells [68]. In most cases, antibacterial nanostructures are able to attach to the bacterial surface by electrostatic interactions and disrupt its integrity. The mechanisms of NPs toxicity depend on NPs composition, surface modifications and intrinsic properties and on the target bacterial species [69]. Multifunctional nanoparticles can be created by mean of entrapment, coupling or absorption of specific molecules like AMPs onto MNPs, thus combining the antimicrobial activity of both the nanoparticles and the selected AMP. As an instance, magnetic nickel nanoparticles uniformly coated with a nanolayer biofilm of polyacrylic acid were used to immobilize the antimicrobial peptide LL-37. Nanoparticles coupled to a critic concentration of LL-37 peptide were able to effectively inhibit *E. coli* propagation [70].

Currently, high attention is devoted to *self-assembly structures* that can be formed by natural or synthetic peptides in determined conditions. Self-assembly peptides exhibit several attractive features for drug delivery applications. Short peptides, ranging from single di-peptides to small linear/cyclic peptides, can self-assemble in nanotubular structures [71]. Nanotubes can be internalized by cells through endocytosis, upon spontaneous conversion into vesicles. Self-assembled cyclic peptides usually have numerous alternating D and L amino acids and stack through extensive intermolecular hydrogen bonding to form extended cylindrical structures. The ability to adjust the outer surface properties enables nanotube arrangement in a variety of different environments, such as in bulk solution, in the solid state and as transmembrane pores in the bacterial membrane, possibly acting as efficient ion channels. The cationic peptide KSL, active against a wide range of microorganisms, was selected as a model to catalyze self-immobilization on bionanocomposites (silica or titania nanoparticles). A self-encapsulating method was developed and sustained diffusion of active peptide was achieved in order to deliver a controlled dose of KSL over an extended period of time. The developed AMP-loaded nanoparticles retained biocidal activity against *Staphylococcus epidermidis* and *Staphylococcus aureus*, protected the peptide from proteolytic degradation and facilitated a continuous release of the AMP over time. The effect towards *S. aureus* suggested that the developed KSL bioinorganic nanoparticles exert a stronger biocidal effect compared to the free peptide [72].

Antimicrobial core-shell structured nanoparticles were obtained by self-assembly of the synthetic amphiphilic peptide CG_3R_6TAT. The formation of nanoparticles was found to strongly enhance the peptide antimicrobial activity compared to the unassembled peptide counterpart. The developed nanoparticles exhibited a broad spectrum of antimicrobial activities, which efficiently inhibited the growth of various types of drug-sensitive and drug-resistant Gram-positive bacteria with low minimum inhibitory concentration (MIC) values, yet inducing relatively low haemolysis. Moreover, they displayed a high therapeutic index against *S. aureus* infections in a mouse model and were able to cross the blood-brain barrier (BBB) in a *S. aureus*-induced meningitis rabbit model, suppressing

bacterial growth in the brain. These nanoparticles may provide an efficient antimicrobial tool for the treatment of brain infections and other infectious diseases [73]. Moreover, some AMPs are able to naturally form self-assembled nanostructures, such as Linocin M18, which spontaneously forms 20-30 nm particles, Iturin A, which has a great propensity to self-associate in 150 nm-vesicles, and Lactacin F, giving 25-50 nm micelles [50]. Self-assembly propensity of AMPs could be exploited to develop more efficient pharmaceutical forms of these peptides, improving their stability and pharmacokinetics.

Hydrogels have been investigated for AMPs delivery, as well. Antimicrobial hydrogels could have a massive impact in wound healing: when infections prevent tissue regeneration at the site of injury, biocompatible hydrogels carrying AMP could accelerate the healing by allowing cells attachment and infiltration. The synthetic peptide PXL150, exhibiting a broad-spectrum antimicrobial activity, was incorporated into a hydroxypropyl cellulose gel for topical treatment of infected wounds at surgical sites [74]. PXL150, a novel short synthetic AMP active against Gram-positive and Gram-negative strains, including methicillin-resistant *S. aureus* (MRSA), was slowly released from the hydrogel *in vivo* on the wound site.

Regarding *polymeric NPs*, PLGA nanoparticles have successfully proved to be efficient carriers for large biomolecules such as vaccines and proteins, for the treatment of various diseases [75]. In fact, PLGA is fully biodegradable, biocompatible, has versatile degradation kinetics and is approved by the European Medical Agency and Food and Drug Administration as an excipient for parenteral products. Chereddy et al. [76] developed PLGA NPs by mean of the emulsion-solvent evaporation technique for the encapsulation of the antimicrobial peptide LL-37 for wound healing applications. LL-37 exerts different functions like broad antimicrobial activity, modulation of pro-inflammatory response, promotion of wound healing and angiogenesis [76]. The PLGA-based sustained delivery of LL-37 significantly improved the wound healing activity, compared to PLGA or LL-37 alone. The healing effect of PLGA-LL-37 NPs included higher re-epithelialization, granulation tissue formation, immunomodulation and improved angiogenesis. Wound healing promotion by PLGA-based drug delivery systems was found to be dependent on the sustained release of bioactive LL-37 as well as on the intrinsic activity of lactate released from PLGA. Recently, D'Angelo et al. [77] reported a method to engineer PLGA nanoparticles loaded with a model cationic peptide, namely colistin, for lung delivery in cystic fibrosis. The surface of PLGA nanoparticles was engineered with polyvinyl alcohol or chitosan to promote colistin diffusion through artificial mucus. Moreover the embedding of nanoparticles in lactose microparticles allowed obtaining a dry powder with promising properties for inhalation.

Poly(lactic acid) (PLA) was investigated as well: nisin was encapsulated in poly(L-lactic acid) nanoparticles prepared by mean of the precipitation method, maintaining its sustained antimicrobial activity for up to 45 days [78]. Niece et al. [79] investigated the employment of poly(alkylacrylic acid) polymers grafted with poly(alkylene oxides) for the development of cationic AMPs delivery systems. Poly(alkylacrylic acid)s have pH-dependent conformational properties that promote membrane penetration and endosomal escape, while the poly(alkylene oxides) chains provide steric stabilization and reticuloendothelial system (RES) protection. Protective polyelectrolyte complexes were formed with a model cationic AMP, KSL-W. The binding and release characteristics of the peptide from the nanocomplexes could be tuned by varying graft density, polymer backbone and charge ratio. Depending on the graft density and charge ratio, these peptide/copolymer nanostructures were able to provide substantial peptide protection from degradation in human

plasma for up to 24 hours, retaining from 25 to 100% of the peptide biological activity against planktonic *S. aureus*.

Natural polymers-based systems have been investigated for AMPs formulation. Gelatin is a denatured protein derived from collagen, greatly and safely used in pharmaceuticals, cosmetics, and food products [80]. A slow release system based on biodegradable freeze-dried cationic gelatin microspheres loaded with the small and angiogenic α-helical cationic peptide AG-30, was developed as potential treatment for ischemic diseases [43]. AG-30 displays its antimicrobial activity by inducing the lysis of bacterial cells without affecting eukaryotic cells and at the same time is able to induce angiogenesis, thus allowing the concomitant killing of bacteria and enhancement of endothelial cell growth. The slow-release formulation in gelatin microspheres was effective in protecting the peptide form proteases degradation *in vivo*, allowing its prolonged delivery in a mouse ischemic hind limb model, for angiogenic and antimicrobial treatment.

A water-soluble glycogen-like α-D-glucan derived from plants, namely Phytoglycogen (PGG), was selected for the development of nanoparticles for the controlled release of nisin [81]. PGG polysaccharide nanoparticles subjected to β-amylolysis and subsequent succinate- or octenyl succinate-substitution, combined or not with β-dextrin (PGB), were employed to develop the novel nisin loaded nanocarriers. PGB-based nanoparticles showed enhanced ability to retain AMP activity with respect to the PGG-based ones, regardless of the substitution with succinate or octenyl succinate. The increase of nisin loading achieved with the surface thinning of nanoparticle by β-amylolysis lead to a prolonged activity of the formulation against *L. monocytogenes*.

Improved features have been achieved by using antimicrobial polymers for AMP encapsulation. Chitosan has been known for years for its antibacterial properties, which along with its good biocompatibility and biodegradability, make it an excellent candidate for AMPs delivery [82, 83]. Chitosan nanoparticles were recently developed for the controlled release of the AMP temporin B (TB) [84]. The AMP was released from the NPs *in vitro* in a controlled and linear manner, and the encapsulation of temporin B in chitosan NPs proved to significantly reduce the peptide's cytotoxicity towards mammalian cells. Additionally, the TB-loaded nanocarrier evidenced a sustained antibacterial action against various strains of *S. epidermidis*, including four clinical isolates for at least 4 days, with up to 4-log reduction in the number of viable bacteria compared to plain chitosan NPs and plain TB. The developed TB-loaded nanocarriers combined the antimicrobial properties of chitosan with those of the loaded AMP: a sustained antibacterial activity was ensured by an initial "burst" effect of the intrinsic antimicrobial polymer, combined with the gradual release of TB that further reduced the viable bacteria. Chitosan NPs would act as carrier for the encapsulated TB, delivering it directly to the bacterial surface while preventing its inactivation by interaction with medium components or dead bacteria; the achievement of a high local concentration of the peptide would be addressed by the release of TB at the bacterial surface, rapidly causing cell death. If this was the case, peptide concentrations below those needed to ensure a bactericidal effect of the free peptide, would be sufficient to cause cell death.

These results suggest that the development of targeted nanoparticles loaded with AMPs could maximize their bactericidal effect, releasing the peptide directly to the site of action (bacterial membrane), allowing for minimum peptide concentration and maximum microbicidal activity. NPs targeting could be achieved by STAMPs (Specifically Targeted Antimicrobial Peptides) conjugation on the NPs surface [85]. A typical STAMP molecule consists of two functionally independent moieties conjoined in a linear peptide sequence: a non-specific antimicrobial peptide serves as the killing moiety while a species specific binding peptide comprises the target moiety.

The target moiety provides the specific binding to a selected pathogen and facilitates the targeted delivery of the attached AMP. Nowadays, STAMPs are available for *Streptococcus mutans* and against *Pseudomonas* spp [86, 87].

Difficulties in generating or obtaining adequate amount of peptides together with the unavailability of established animal models strongly limited pre-clinical studies with encapsulated AMPs. To date, only very limited number of formulations have undergone *in vivo* pre-clinical or clinical trials. Examples comprise the AG-30 loaded gelatin microspheres for angiogenic and antimicrobial purposes [43], the PXL 150/hydroxypropyl cellulose gel for the treatment of wound infections [74] and cartridges of immobilized Polymyxin B for septic shock therapy, which showed good results in pre-clinical [88] or clinical trials [89].

Future perspective

The encapsulation of AMPs in nanocarriers represents an effective tool for controlling their exposure to different environmental stresses typically encountered in biological systems, thus improving the peptides stability, efficacy and biodistribution. Nowadays, AMPs have been formulated in nanostructures by various methods based on different synthetic and natural materials. Investigations on nanoparticles-encapsulated AMPs generally showed advantages in comparison to free peptides action, in terms of efficacy, stability and systemic toxicity. These promising results should encourage intensive efforts converging on extended preclinical/clinical investigations for the development and characterization of AMPs-loaded antibacterial nanostructures.

Moreover, antimicrobial polymers should be primarily considered for the development of AMPs drug delivery systems multifunctional platforms, presently focusing on new antimicrobial lead-compounds, should additionally deal with nanotherapeutics and nanotools as adjuvant approaches to prevent and combat microbial infections. A combined and enhanced antimicrobial action should be obtained, being not only the loaded drug, but also the material and the delivery system, bioactive.

Studies focusing on the mechanism of action of AMPs-loaded antibacterial nanotools should also be highly encouraged. AMPs interactions with bacterial membrane have already been investigated by mean of fluorescent labelling and electron microscopy analysis, X ray scattering, solid state nuclear magnetic resonance (NMR) and differential scanning calorimetry [90, 91]. The mechanism of bacterial membrane interaction with loaded antibacterial nanotools is a highly interesting topic of study, potentially useful for the development of improved and highly efficient antibacterial nanotools. Moreover, fluorescent labelling of AMPs or antimicrobial nanotools could give precious information about intracellular trafficking and target of AMPs, which could lead to practical improvements in clinical practice of infectious diseases.

In addition to basic research, more efforts should be focused on pre-clinical and clinical studies. The application and administration route of AMPs-loaded nanostructures should be carefully evaluated during the ideation and development of these therapeutic nanotools and particular attention should be devoted to clinical trials involving the developed systems, as that was the critical and limiting phase up to date.

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Executive Summary

Executive summary
With the dramatic rise in bacterial resistance, novel antimicrobials are urgently needed • Alternative strategies to conventional antibiotics to treat microbial infections are currently widely investigated. • Among those novel strategies, the employment of non conventional nanotools (e.g. AMPs-delivery systems) seems very promising.
Antimicrobial polymers • Antimicrobial polymers are highly attractive candidates for the development of antimicrobial nanotools, capable of conferring to the nanosystems itself a biocidal activity. • Antimicrobial polymers usually display an improved and prolonged antimicrobial activity compared to conventional antibacterial agents, also preventing antibiotic resistance. • Besides naturally occurring antimicrobial polymers, microbicidal materials can be obtained by chemical modification and addition of low molecular weight antimicrobial compounds, addition of metal nanoparticles, insertion of oxides and inclusion of antimicrobial modified inorganic systems.
Antimicrobial peptides (AMPs) • AMPs exhibit a broad-spectrum activity against a wide range of microorganisms, including drug resistant-isolates • Most cationic AMPs interact through electrostatic forces with the negatively charged phospholipid headgroups on the bacterial membrane and cause cell disruption. • Clinical applications limited up to date to topical/local therapies, mainly due to their poor pharmacokinetics, protease degradation and potential for systemic toxicity.
Nanotools fot the delivery of AMPs • Encapsulation of AMPs in suitable nanocarriers may improve the potential of these therapeutic proteins by protecting the peptides, improving the molecules stability, reducing their systemic toxicity and enhancing their therapeutic activity by sustained and targeted delivery. • Antimicrobial peptides have been encapsulated/formulated into several nanostructures up to date, including lipid nanovesicles, nanofibers, metallic nanoparticles, self-assembled structures, hydrogels and polymeric nanoparticles. • Future research should be aimed at the development of **multifunctional antibacterial nanoplatforms**, based on the combination of the bactericidal activities of antimicrobial polymers-based nanosystems and loaded antimicrobial peptides.

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Papers of special note have been highlighted as:
* = of interest
** = of considerable interest

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Figure Legends:

Figure 1. *Mechanisms of bacterial membrane disruption by AMPs.*

Figure 2. *Opportunities and limits offered by antimicrobial peptides (AMPs) as future antibiotics, and possibilities offered by nanomaterials as delivery systems to improve AMPs' clinical potential. The development of AMPs as new drugs may largely benefit from the use of nanotechnologies, improving their delivery to the infectious site, stability in biological fluids, cyto-compatibility, and penetration trough mucus and epithelial barriers. In addition, nanoparticles may be endowed with an inherent antimicrobial activity that may sum/synergize with that of the loaded AMPs.*

Figure 3. *Schematic representation of nanostructures developed for the delivery of AMPs.*

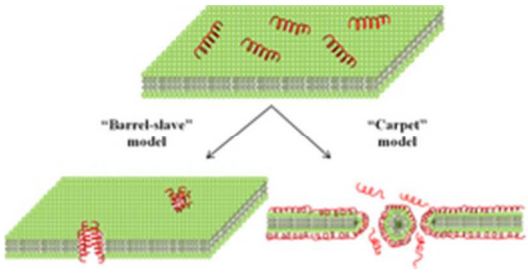


Figure 1: Mechanisms of bacterial membrane disruption by AMPs.
22x11mm (300 x 300 DPI)

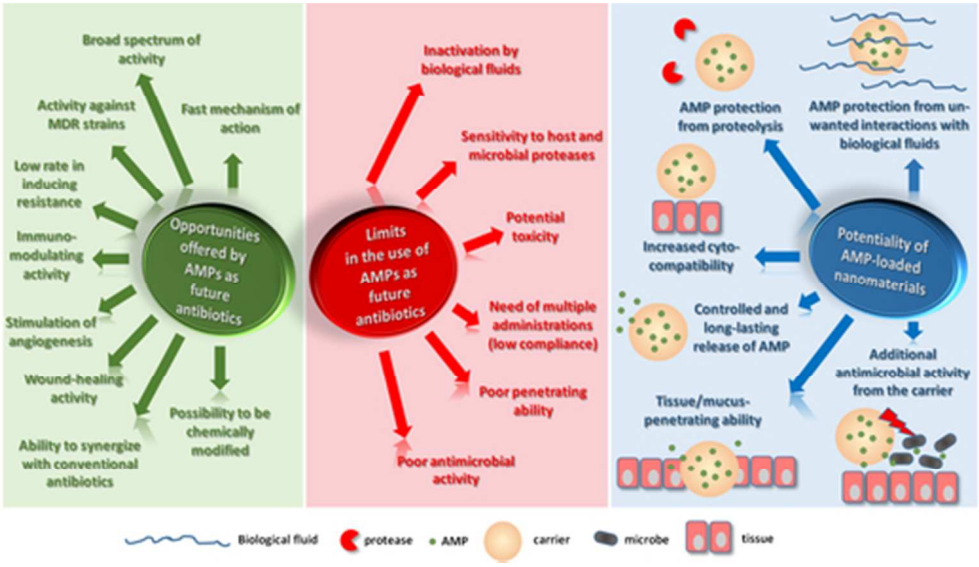


Figure 2: Opportunities and limits offered by antimicrobial peptides (AMPs) as future antibiotics, and possibilities offered by nanomaterials as delivery systems to improve AMPs' clinical potential. The development of AMPs as new drugs may largely benefit from the use of nanotechnologies, improving their delivery to the infectious site, stability in biological fluids, cyto-compatibility, and penetration trough mucus and epithelial barriers. In addition, nanoparticles may be endowed with an inherent antimicrobial activity that may sum/synergize with that of the loaded AMPs
44x25mm (300 x 300 DPI)

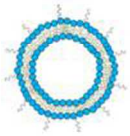
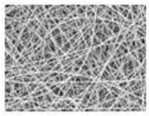
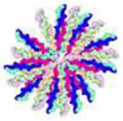
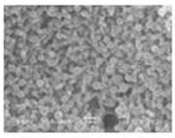
Nanostructures		AMPs	Applications
	Lipid nanovesicles	Nisin	Nisin-loaded PC nanovesicles for the inhibition of *L. monocytogenes* growth for milk conservation applications [54].
		CM3	CM3-loaded DPMC/DMPG liposomes for *P. aeruginosa* infections treatment in lung delivery applications [58].
	Nanofibers	Plantaricin 423	Plantaricin 423-loaded PEO nanofibers for growth inhibition of *E. faecium* and *L. sakei* for food and medical applications [63].
		Magainin II	PLGA and PLGA/gelatin electrospun fibrous membranes with covalently immobilized Magainin II [64].
	Metallic nanoparticles	LL-37	LL-37-coupled nickel NPs for *E. coli* propagation inhibition [68].
	Self-assembly structures	KSL	KSL-self-immobilised silica NPS for the inhibition of *S. aureus* and *S. epidermidis* growth [70].
		CG_3R_6TAT	Self-assembled CG_3R_6TAT NPs for the treatment of *S. aureus* infections in *S. aureus*-induced meningitis rabbit models [71].
	Hydrogels	PXL150	Hydroxypropyl cellulose hydrogels incorporating PXL150 for topical treatment of MRSA infected wounds at surgical sites [72].
	Polymeric nanoparticles	LL-37	LL-37-loaded PLGA NPs for *E. coli* growth inhibition and wound healing applications [74].
		KSL-W	Poly(alkylacrylic acid)/poly(alkylene oxides) polyelectrolyte complexes loaded with KSL-W for the treatment of *S. aureus* infections [76].
		AG-30	AG-30-loaded gelatin microspheres for angiogenic and antimicrobial treatment [77].
		Nisin	Nisin-loaded PLA NPs for the inhibition of *L. delbrueckeii* growth as food preservative [75].
			Nisin-loaded PGG NPs for the prolonged inhibition of *L. monocytogenes* growth [78].
		Temporin B	TB-loaded CS NPs for the prolonged inhibition of *S. epidermidis* growth [81].

Figure 3: Schematic representation of nanostructures developed for the delivery of AMPs
154x181mm (300 x 300 DPI)

Tables

Table 1. *Main classes of polymers displaying antimicrobial activity.*

Class of antimicrobial polymer	Examples	Properties
Guanidine containing polymers	Oligo/poly-guanidines, polybiguanidines [92, 93].	High water solubility, excellent biocidal efficiency, wide antimicrobial spectrum and non-toxicity
Organometallic polymers	Organotin derivatives, silver polymeric complexes, metal (Mn, Co, Cu, Zn) resin complexes [6] either in the backbone chain or in the pendant groups.	Broad spectrum biocides against both bacteria and yeasts
Polymers with quaternary nitrogen atoms	Polymers containing aromatic or heterocyclic structures [94], acrylic and methacrylic polymers [95], cationic conjugated polyelectrolytes [96], polysiloxanes [97], hyperbranched and dendritic polymers [98], oxazolines [99]	Broad spectrum activity, effective against drug-resistant bacteria, rapid biocidal action
Polymeric Synthetic Mimics of Antimicrobial Peptides (polymeric SMAMPs)	Arylamide and phenylene ethynylene backbone polymers, polynorbornene derivatives [100]	Excellent antimicrobial activity, tunable selectivity and toxicity towards mammalian cells
Halogen polymers	Poly(urethanes) containing ciprofloxacin/norfloxacin, perfluoroalkyl acrylate, chlorophenyl methacrilates, poly(N-halamines) [101, 102]	High chemical, thermal, aging and weather resistance, antimicrobial activity associated with their surface activity and their high hydrophobicity
Phospho-/sulpho-derivatives polymers	Poly(styrene sulfonic acid), poly(sulfobetaine methacrylate) [103]	Viral replication inhibition (HIV-1, HSV-1), long-term active against bacterial biofilms
Phenol/benzoic acid derivatives polymers	Benzaldehyde derivatives, ferulic acid copolymers [104]	Broad spectrum inhibitory activities, bactericide, fungicide and algaecide, antioxidant

Table 2. *Different functions of AMPs and examples of peptides with those activities [33-35, 106].*

Activities of AMPs	Example AMPs
Broad spectrum antibacterial	Protegrin, IB-367, MSI-78, Indolicidin, CEMA, Gramicidin S, Magainin II, Polyphemusin, Defensin MV
Anti Gram-negative bacterial	Polymixin B, Colistin, Hinnavin II, Bactenecin 5
Anti Gram-positive bacterial	Nisin, Isoform 5, Andropin, Rugosin A, Temporin C, Protegrin I
Synergy with conventional antibiotics	CEMA, Magainin II, MSI-78, IB-367, Human β-Defensin 3, Temporin L
Antifungal	OdVP1-2-3, Protegrin, CEMA, Indolicidin, Gramicidin S, Polyphemusin, Bactrocerin-1, Drosomycin
Synergy with conventional antifungal	Indolicidin, Hepcidin 20
Anti endotoxin	CEMA, Indolicidin
Antiviral (HIV, HSV, Dengue-2)	Indolicidin, Polyphemusin, Protegrin, RScp
Anticancer	CEMA, Indolicidin, Lasioglossins
Synergy with conventional anticancer agents	Indolicidin
Wound healing	Magainins, PR39
Antiparasite	Magainin II, Indolicidin, Phylloseptins, BMAP-18
Antimalarial	Meucin-24, Gambicin, Phylloseptin-H1

Table 3. *Peptides in commercial development [104, 107].*

Company	AMP or AMP-based drug	Stage of development	Application
Ceragenix (US)	CSA-13 (AMP-mimic peptide)	Preclinical	Anti-infective
Helix Biomedix(US	HB-50 (synthetic Cecropin-analog)	Preclinical	Anti-infective
Helix Biomedix (US)	HB-107 (Cecropin 19-aa fragment)	Preclinical	Anti-infective
Novacta Biosystems Ltd. (UK)	Mersacidin	Preclinical	Gram-positive infections
Novozymes A/S (DK)	Plectasin (fungal defensin)	Preclinical	Systemic anti-Gram-positive, especially pneumococcal and streptococcal infections
Inimex (CDN)	IMX942 (synthetic bactenecin-analog)	Phase II	Nosocomial infections, febrile neutrophenia
Lytix Biopharma	LTX-109	Phase I-IIa	MRSA nasal decolonization and skin infection
AM-Pharma (NL)	hLF-1-11 (derived from human lactoferrin	Phase II complete	Allogeneic bone marrow stem cell transplantation-associated infections
OctoPlus (NL)	OP-145 (synthetic LL-37 analog)	Phase II complete	Chronic bacterial middle ear infections
Polymedix (US)	PMX-30063 (peptidomimetics)	Phase II complete	Anti-infectives, antimicrobial polymers and coating materials
Xoma (US)	XOMA 629 (derived from permeability-increasing protein)	Phase IIa	Impetigo
Zengen (USA)	CZEN-002	Phase IIb	Vulvovaginal candidiasis
Pacgen Biopharmaceuticals (CDN)	PAC-113 (synthetic histatin-analog)	Phase II complete	Oral candidiasis
Migenix (CDN)	Omiganan pentahydrochloride/CP-226/MX-226/CLS001 (Bactolysin analog)	Phase IIIb/Phase II	Prevention of catheter-related infections; dermatology-related infections
RX Generic drugs (US)	Polymixin B-Colistin-Colomycin (prodrug)	Available on the market	Gram-positive skin infections
Cubist Pharmaceuticals (US)	Daptomycin (lipopeptide)	Available on the market	Gram-positive skin infections