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Antimicrobial peptides in infected wounds

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Abstract

Wound healing is a fundamental process to re-establish tissue integrity. Microbial infections, however, may hinder this process and compromise our health. The increasing resistance of microorganisms colonizing infections to conventional antibiotics has raised many concerns. Hence, new treatment options have been researched and new biomolecules uncovered. As known, multicellular organisms are endowed with an arsenal of host-defense molecules, the Antimicrobial Peptides (AMPs) that fight microbial invaders and modulate the host's immune response. In recent years, research has been focused on the development of such molecules with lower toxicity and improved activity compared to their endogenous counterparts for potential applications in wound healing. The present work offers a review over AMPs involved in wound healing and used against infected wounds, their potentialities and limitations, and highlights their mode of action. The challenges with the use of AMPs and the current strategies to prevent those challenges are also enumerated.

Introduction

Skin is the largest organ in the human body. It works as a physical barrier, protecting our integrity from environmental threats. Inevitably wounds and traumas occur, compromising the skin defenses against pathogens and putting at risk our health [1]. In normal conditions, the skin heals by following a well-organized set of stages, hemostasis, inflammation, proliferation and remodeling, in a process that may last few hours or days. However, under certain diminished health conditions, such as diabetes, infection, etc., the healing process may be delayed in a stage, usually at inflammation, and lead to the appearance of chronic wounds. Chronic wounds are a result of gradual tissue degradation in which biochemical agents, like proteolytic enzymes, are involved becoming very difficult to treat. Chronic wounds are characterized by flawed tissue, debris impair healing, bacterial colonization (biofilms), prolonged inflammation, and moisture imbalance [2,3]. Hence, accelerate healing is vital to the human body as a mean to prevent wound chronicity and treat chronic wounds.

In many chronic wound patients, the immune system is compromised. As such antimicrobial agents must be added to their treatment to fight infections. Traditional wound healing drugs include antiseptics, ointments, antibiotics, growth factors, cytokines, plant derivates and even metal nanoparticles. However, these have been proven difficult to translate into successful therapies for chronic wounds [4]. Considering the limitations of the previous strategies, such as high cost, low availability, reduced stability, specific and low antimicrobial activity, and release/delivery issues, Antimicrobial Peptides (AMPs) have been established as potential biomolecules for the healing of infected wounds [5,6].

AMPs are cationic, low molecular weight molecules and an integral part of the innate immune system, being present in many multicellular organisms, including insects, bacteria, vertebrates, plants and humans. AMPs display a broad spectrum of antimicrobial activity, including microorganisms from resistant

strains, are bactericidal, their activity is not inhibited by biological fluids, exudates or biofilms, and act quickly at multiple sites within microbial cells reducing chance of resistance. Since most microorganisms frequently found colonizing the wounded site are potentially pathogenic, infection control is critical [7-10].

The present work offers a review over the most common AMPs involved in wound healing and used against infected wounds, their potentialities and limitations. It also uncovers the most problematic aspects with skin infections and highlights the AMPs mode of action towards the pathogens colonizing the wounded site. Finally, a reflection over the recent advances in wound healing and the expectations for the future is provided.

Antimicrobial peptides in wound healing

At each stage of the healing process, the wounded site is invaded by biomolecules responsible for inducing the consecutive phases. Between those, many antimicrobial agents that are part of our innate immune system, including AMPs, are attracted and activated to protect our system against foreign invaders. Upon tearing of the skin, proteases are activated and release Heparin Binding-Epidermal Growth Factors (HB-EGF) and amphiregulin that possess antimicrobial activity and are responsible for instigating the expression of epidermal AMPs later in the healing process [11]. During hemostasis, the complement and coagulation cascades are activated which results in the cleavage of many proteins like fibrinogen or thrombin. Fragments from those proteins give rise to many AMPs including the C3a, known for its antimicrobial activity [12].

In the inflammatory stage, the wounded site is invaded by neutrophils followed by monocytes and lymphocytes. Neutrophils are the most important "producers" of AMPs during inflammation; they contain α-defensins (human neutrophil peptides, HNPs) in azurophil granules, cathelicidins in specific granules (or large granules in rudiments), and calgranulins in the cytosol [2,13]. The defensins antibacterial and antiviral activity is mainly exerted in the neutrophil phagolysosome, but they are also responsible for boosting bacterial phagocytosis by macrophages and possess chemotactic activity towards monocytes, T cells and immature dendritic cells [14,15]. After release from the granules, cathelicidins are processed by the proteinase 3 into the antimicrobial peptide hCAP-18 or more commonly known the LL37, which is endowed with great antimicrobial activity [16]. Cathelicidins are also responsible for recruiting monocytes to the wounded site, for inducing the expression of the Vascular Endothelial Growth Factor (VEGF), and for causing the transactivation of the Epidermal Growth Factor (EGFR) and thus promoting keratinocytes migration [17-19]. The most common calgranulins found in the cytosol is the potent antifungal agent S100A8/S100A9 [20]. This AMP enhances phagocytosis and induces neutrophil chemotaxis and adhesion. It also mediates pro-inflammation by binding to the toll-like receptor 4 (TLR-4) or the Receptor for Advanced Glycation End products (RAGE) and induces the expression of the cytokine interleukin 10 (IL-10) [21-24].

During proliferation, most AMPs are obtained from the epidermal keratinocytes, like hBD-2, hBD-3, RNase7 and psoriasin. At this stage the LL37 and S100A8/S100A9 together with the previous reach their peak of expression. As many defensins have the same ancestral gene, neutrophils (inflammation) and keratinocytes (proliferation) share many of the same AMPs and antimicrobial proteins. However, their expression is dependent on the healing phase [25,26]. For instance, the expression of hBD-3 is induced by the EGFR activation in epidermal keratinocytes upon injure, while the expression of S100A8/S100A9 can be both induced by the activation of growth factors during injury or by pro-inflammatory cytokines, thus linking growth and tissue regeneration with AMPs expression [26,27]. Epidermal AMPs involved in wound healing display a broad spectrum of antibacterial activity, with nBD-3 and RNase 7 being extremely effective against Staphylococcus aureus, psoriasin against Escherichia coli and calgranulins against Candida albicans [20,28,29]. Aside from protecting the wounded site from foreign invaders, the expression of these AMPs both during proliferation and inflammation points to the manifestation of other non-antimicrobial functions conducted by these AMPs. It has been reported that hBD-2 activates dendritic cells through TLR-4, being a chemoattractant towards immature dendritic cells and memory T cells, and has also been shown to stimulate proliferation, migration, and cytokine production of epidermal keratinocytes [30]. hBD-3 and psoriasin have also shown chemoattractant properties, with the first also being associated with the activation of mast cells with increase of vascular permeability and the second expression of keratinocyte differentiation markers, promoting proliferation of endothelial cells [25,31]. Tissue remodeling is the last stage of the healing process. Even though currently there are no evidences of AMPs being produced during this phase, the increase expression of the highly antimicrobial collagen type VI, characteristic of this phase, protects the connective tissue of the skin [32].

Wound infections: From single colonies to biofilms

Skin infections are some of the most common bacterial infections in humans. In hospitalized patients, bacterial skin infections are the 28th most common diagnosis, with some of those infections already revealing resistance to antibiotics [33]. However, not all wounds containing bacteria are considered infected; in fact, a wound that contains non-replicating bacteria is said to be contaminated, and those wounds containing replicating bacteria, but without causing cellular damage to the host, are said to be colonized. A wound is only considered infected when replicating bacteria invade the tissue and cause damage. Indeed, a major advance in the prevention and management of wound infection has been this understanding, that the mere presence of organisms in a wound is not an indication of infection and may not be more important than the level of bacterial growth. Bacterial infections can range from superficial, in which antibiotic administration may not be required, to complicated, in which biofilm formation is observed and systemic sepsis is a major problem with a lethal outcome [34,35]. In cases of skin rupture (most common in hospitalized patients recovering from surgery, burns or trauma), the chance of infection increases drastically. Wounds of surgical or traumatic origin, in which the host immune response is compromised, and the tissue is devitalized (i.e ischemic, hypoxic or necrotic), bacteria colonization is inevitable since these wounds offer optimal conditions for microbial growth. In these situations, infection has been defined as the product of entrance, growth, metabolic activity and resultant pathophysiologic effects of microorganisms in the wound bed. Since the human body is constantly surrounded by potentially pathogenic microorganisms, even in the absence of clinical infection, a delicate balance must exist between the host resistance and the actions of the bacteria to maintain our integrity. In fact, wound contaminants may originate from the environment, the surrounding skin (Staphylococcus epidermidis, micrococci, skin diphtheroids, and propionibacteria are common in the normal skin microflora), and even from endog-

enous sources involving mucous membranes [36-38]. In minor, healing wounds only a relatively small number of bacteria will take residence, while in devitalized tissues or chronic wounds colonization and establishment of a wide variety of endogenous microorganisms will occur, slowing the healing process. Bacteria loads in excess of 10^5 microorganisms/g of tissue are considered to inhibit healing; this number depends, however, on the immune system of the host and the type of bacteria species. The primary microorganisms causing delayed healing and infection in both acute and chronic wounds are Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis and β-hemolytic Streptococcus bacteria [39]. Infected wounds may severely compromise the health of an individual. As such, efficient healing is essential in restoring the epidermal barrier natural characteristics and, with that, its bacterial resistance skills.

Over the years, to aid with the healing process different biomolecules, drugs and ointments together with appropriate dressings have been proposed and researched. Silver, in ionic or nanocrystalline forms, has been used as an antimicrobial agent in the treatment of burns. Lately, the incorporation of silver in dressings has widen its use to other wound types that are either colonized or infected [40]. Povidone or cadexomer iodine, chlorhexidine, polyhexamethyl biguanide and honey, all antiseptic agents, have also been used to impregnate dressings for acute and chronic wound care [41]. However, the rising of antibiotic-resistant infection agents has increased the need for new alternatives and more efficient therapies. Because of the AMPs ability to act at multiple sites within microbial cells in a very short time (smaller than the microorganism replicating cycle), bacteria are less likely to develop resistance; also AMPs display a broad spectrum, including resistant strains, are bactericidal and not just bacteriostatic, and their activity is not inhibited by body fluids, wound exudates or biofilms [2,7,10,42,43]. More importantly, AMPs are present in each phase of healing process contributing actively to the wound healing. Recently, studies have been conducted with the purpose of immobilizing AMPs onto the surface of wound dressings, to generate bioactive dressings, with promising results [10].

Antimicrobial peptides: Action mode against skin pathogens

Over 2500 AMPs with different origins and applications have been identified. Even though they present a broad antimicrobial action, AMPs can be classified by their primary target microorganisms: (i) Antibacterial, which target bacterial cell membranes; (ii) Antiviral, which penetrate the viral envelope neutralizing their action; (iii) Antifungal, which act by targeting the cell wall or the intracellular organelles; and (iv) Antiparasitic, which kill by direct interaction with the cell wall. Most AMPs are small, cationic peptides composed of over 50% hydrophobic residues that enable them to fold into an amphiphilic conformation to better interact with the microorganisms' cell membrane. AMPs mostly kill by disrupting the microorganism cell membrane integrity, which task can be accomplished in a matter of seconds after initial contact. For most cases the AMPs action starts with electrostatic attraction between the anionic cell wall of the microorganisms colonizing a wound and the cationic AMPs, which conformation then adapts to a cell membrane-water interface [2,44,45]. Once the interaction is established several models describing the AMPs transmembrane mechanisms of action against pathogens have been proposed. Table 1 and Figure 1 provide specific details describing the most common models used by AMPs.

Table 1: Description of AMPs transmembrane mechanisms of action against infectious pathogens [2,46].

Model	Action Mode
Toroidal pore	Transmembrane pore model in which the peptides insert perpendicularly in the lipid bilayer, inducing a local curvature. After binding to the phospholipid head groups, the AMPs align and insert into the membrane and cluster into unstructured bundles that interact with water molecules generating channels within the membrane. As the pores are transient upon disintegration, some peptides may enter the cytoplasm and potentially target intracellular components.
Barrel stave	Transmembrane pore model in which the AMPs are initially oriented parallel to the membrane, forming staves, and eventually insert perpendicularly to the plane of the lipid bilayer, forming a barrel. This mechanism promotes lateral peptide-peptide interactions, with the hydrophobic regions interacting with the membrane lipids and the hydrophilic forming the lumen of the channels. The barrel-stave model is most common with AMPs with a minimum length of 22 residues, if with an α-helical structure, or 8 residues, if with a β-sheet structure.
Carpet-like	Transmembrane non-pore model in which the AMPs coat the microbial membrane up to saturation, forming a "carpet", which leads to unfavorable interactions and consequent loss of membrane integrity. As the membrane integrity is lost a detergent-like effect is accomplished in which the membrane gives rise to wormholes, causing the abrupt lysis of the microbial cell, and micelles are formed. The carpet model does not require specific peptide-peptide interactions nor the peptide to insert into the hydrophobic core to form transmembrane channels or specific peptide structures.

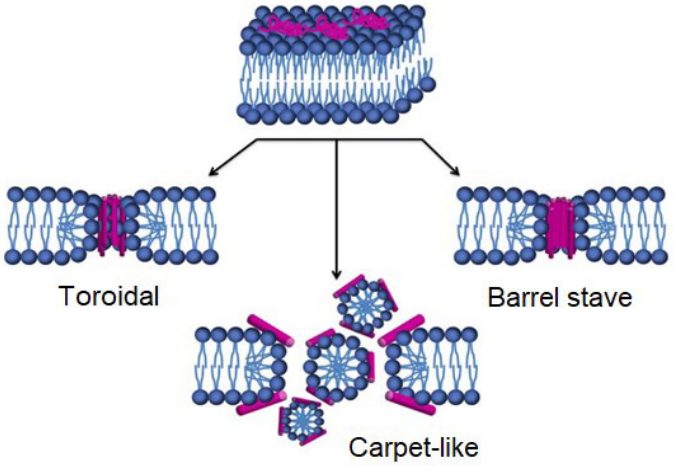

Figure 1: Schematic representation of the most common AMPs transmembrane cell action models: Toroidal, barrel stave and carpet-like [47].

Aside from the models described above, in which AMPs kill bacteria via membrane permeabilization, there are others that act via non-membrane targets. Even though these are not as common some AMPs action mode consists in targeting the bacteria cell wall or intracellular organelles. In the first case, AMPs may inhibit the cell wall synthesis by interacting with specific

precursor molecules, for instance the lipid II. Here, the AMPs bind to the negatively charged pyrophosphate sugar moiety of lipid II leading to membrane disruption [48]. In the second case, AMPs interact with the cytoplasmic membrane first and then accumulate intracellularly, blocking critical cell processes, like protein/nucleic acid synthesis and disruption of enzymatic/protein activity [49]. Additionally, AMPs may also recruit and activate immune cells that instigate microorganisms elimination and that intervene in the different healing phases [50]. As seen earlier, AMPs can induce a variety of immune responses during wound healing, including attraction, activation and differentiation of white blood cells, stimulation of angiogenesis, reduction of pro-inflammatory cytokines expression, etc.

Challenges in wound healing: Antimicrobial peptides bioavailability

In chronic wounds the excessive release of pro-inflammatory cytokines delays wound healing, retaining the process in the inflammatory phase. Hence, the AMPs action becomes most important, since their activity to neutralize bacteria is not direct but rather through the inhibition of pathogenicity factors or by controlling the host immune response. Even though AMPs can successfully retain their antimicrobial activity for millions of years and their immunomodulatory properties stay unaffected in contact with bacteria, they possess important limitations that hamper their clinical use, such as high production costs, potential toxicity and unknown pharmacokinetics. Indeed, one of the greatest challenges with the use of AMPs in wound healing is their availability in the market. So far very few AMPs have been thoroughly characterized and accepted in clinical trials, and from those even fewer have been approved by the US Food and Drug Administration (FDA). Most AMPs in clinical trials are analogues of natural AMPs and the majority is limited to topical applications, due to systemic toxicity, susceptibility of the peptides to protease or enzymatic degradation and rapid kidney clearance [45]. To circumvent these issues and improve the AMPs efficacy, different strategies have been proposed including the chemical modification of AMPs by including non-natural or D-amino acids in their structure, shortening the peptides lengths, or induce amidation at the N-terminus to avoid peptide degradation [51], the use of delivery vehicles, like liposome encapsulation [52], or the functionalization at the surface of wound dressings for a topical delivery [10]. Several AMPs have been synthesized and produced with promising topical effects, both in vitro and in vivo, on infected wounds [53]. Between the many that have been researched in skin infections, the ones listed in Table 2 have shown the most promising results, with some like pexiganan, already undergoing clinical trials.

Table 2: Primary sequences and activities of selected natural- and synthetic-origin AMPs, most commonly employed in wound healing.

AMPs	Sequence	Activity	Ref.
LL37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Promotes neovascularization, Migration and proliferation of epithelial cells and is antimicrobial.	[54]
Pexiganan	GIGKFLKKAKKFGKAFVKILKK	Fights infection and stimulates migration of cells during tissue remodeling (already in phase III of clinical trials).	[55]
Tiger 17	WCKPKPKPRCH	Promotes keratinocytes migration and proliferation, fibroblasts proliferation and re-epithelialization.	[56]
Esculentin-1-a(1-21)NH_2	GIFSKLAGKKIKNLLISGLKG	Promotes keratinocytes migration and skin re-epithelialization and is antimicrobial.	[57]
AH90	ATAWDFGPHGLLPIRPIRIRPLCG	Stimulates the expression of transforming growth factors and angiogenesis and prevents excessive inflammation.	[58]
CW49	APFRMGICTTN	Promotes macrophage recruitment during inflammation and upregulates pro-angiogenic proteins during tissue formation.	[59]

Antimicrobial efficacy of bioactive, fibrous dressings: In vitro evaluation

Synthetic and natural-original polymers have been processed in the form of fibrous mats or porous dressings and used as base substrates for the incorporation of bioactive molecules, like AMPs, for applications in skin regeneration and wound healing. In 2002, Kenawy et al. reported for the first time the incorporation of antibiotics within nanofiber meshes produced via electrospinning [60]. Metal oxide nanoparticles, like silver, zinc oxide and titanium dioxide have been widely researched for their antimicrobial potentialities and large surface area. AgNPs, for instance, have been loaded onto Polycaprolactone (PCL), Poly Vinyl Alcohol (PVA), chitosan or gelatin for topical and systemic administration with successful results against both Gram-positive and Gram-negative bacteria [61-63]. More recently, AMPs, which amino acids-based composition can be easily immobilized onto polymeric surfaces and structurally modified, have also been incorporate within polymeric matrices for wound healing applications. For instance, the inverse-Crabrolin AMP has been incorporated into PCL nanofibers with successful results against Escherichia coli and Bacillus subtilis [64]. The AMP motif Cys-KR12 originated from the human cathelicidin peptide LL37 has been immobilized onto electrospun silk fibroin nanofiber membranes, via click chemistry, and demonstrated great antimicrobial action against four pathogenic bacterial strains (Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, and Pseudomonas aeruginosa), and to promote the keratinocytes and fibroblasts proliferation and differentiation [53]. Lysozyme and nisin AMPs were functionalized onto poly(acrylic acid) and PVA electrospun mats, revealing the capacity to completely eliminate Staphylococcus aureus bacteria colonies, and free the affected area within 14 days of incubation [65]. These are only some of the examples of the incorporation of AMPs into wound dressings and their potentialities to fight microorganisms in infected wounds. Most of the previous studies implemented antimicrobial efficacy tests used in textile engineering, since most bioactive dressings are formed of nanofibrous, porous polymeric matrices, resembling fabrics. Generally, these antimicrobial tests are classified in qualitative, being the most common the agar diffusion method in which it

is also included the "halo" and "parallel streak" methods, and quantitative, in which both dynamic tests ("shake flask") and intimate contact tests are included. Many bacterial strains may be employed; however, the Gram-positive bacteria Staphylococcus aureus and the Gram-negative bacteria Escherichia coli or Klebsiella pneumoniae are the standard.

Agar Diffusion Method ("Halo" and "Parallel Streak")

The agar diffusion method is a qualitative or semi-qualitative test in which an antimicrobial agent in solution form or immobilized onto a fibrous surface diffuses into the surrounding agar forming a zone of bacterial inhibition. The standards AATCC 147-2004, JIS L 1902-2002 and ISO 20645:2004 are used to attain such results. In case of an antimicrobial solution, after spreading the bacteria along the agar plates, the solution can be poured onto punched-out wells of 6 mm diameter or injected into 6 mm diameter cellulose discs. In case of a fibrous dressing, the "halo" method can be employed by mixing the bacteria with the agar, leaving it to solidify, and then pressing gently the mats' against the agar. The "parallel streak" method requires for the formation of 3 to 5 lines above the agar using the bacteria inoculum and then covering partially the lines with the mats. Either way, the plates are then incubated at 37ºC for 18-24 h.

The diffusion of the antimicrobial agent along the agar and the formation of a zone of inhibition are indicatives of the agent antimicrobial efficiency, while the size of the zone attests to its potency (Figure 2). However, it should be noticed that in many cases, when the antimicrobial agent is strongly attached to the dressing and there is no leaching a zone of inhibition does not appear. Instead, the samples should be removed, and the zone underneath analyzed. If a transparent, clear zone is observed, then the dressing is endowed with antimicrobial action by contact.

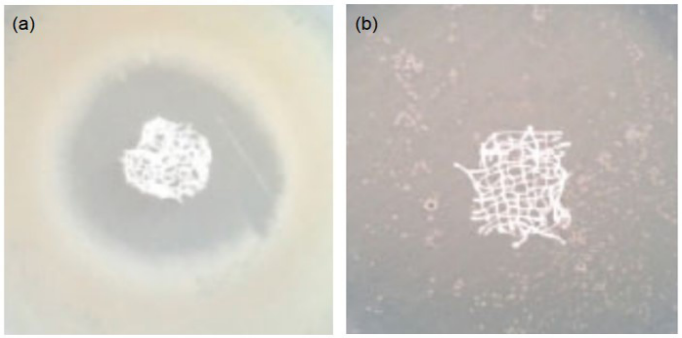

Figure 2: Formation of zone of inhibition against *S. aureus*.

(a) Chitosan (natural-origin polymer with antimicrobial properties) coated cotton gauze

(b) Bare cotton gauze. Data collected from the application of the standard ISO 20645: 2004 [66].

Dynamic Method ("Shake Flask")

The dynamic contact method, also known as "shake flask" method, follows the guidelines of the ASTM E2149-01, in which the efficacy of an immobilized antimicrobial agent will be evaluated under dynamic environment. Briefly, the functionalized dressing is immersed in a bacteria suspension and submitted to regular shaking. After specific time periods aliquots of bacteria suspension are collected and cultured to determine bacteria concentration. This method is both used to tests non-leaching and leaching antimicrobial agents due to its ease of process and simplicity of results treatment. It has been used for instance in nanofibrous dressings loaded with tetracycline [67], amoxicillin [68], AgNPs [69] and even in chitosan polymeric blends [70], or to confirm the efficiency of antimicrobial nanofibrous filtration membranes of poly(vinyl alcohol) and poly(catechol) blends [63].

Intimate Contact Method

This test is designed for antibacterial examination of textiles under intimate contact with bacteria suspensions. It follows the guidelines of the standards AATCC 100-2004 or the JIS L 1902-2002 (quantitative option). Here, a small volume of bacteria inoculum is fully absorbed by a dressing sample and incubated in a humidified environment at 37ºC for 24 h. For hydrophobic surfaces intimate contact between bacteria and surface may be accomplished using a sterile glass slide of equal size as the sample gently pressed along the testing surface. After this period, a buffer is introduced, and the samples are submitted to strong shaking so the bacteria can be eluted and counted. This methodology is best suited for non-leaching antimicrobial agents, since those leaching may quickly surround the cells and kill all bacteria in a very short period of incubation. For wound dressings, the dynamic methods are the most appropriated since, when implanted, they are likely to be surrounded very quickly by biological fluids, wound exudates or blood, which will effectively interact with the antimicrobial agent.

Conclusions

The evolution of therapies for skin infections has been constant. However, in the last decades, the advances have been more important, being now possible to fight infections while accelerating healing using bioactive dressings loaded with AMPs, that still possess the abilities of traditional bandages (wound management and protection against repeated trauma). The inclusion of AMPs within wound dressings responds to an urgent need for more effective therapies to treat infected acute and chronic wounds, colonized by antibiotic resistant pathogens. This is still a fairly recent strategy and, as such, very little researched. It is still necessary to understand the real impact of long-term therapies using functionalized AMPs in our innate immune system, and the ability to control and manage the release of such antimicrobial agents upon contact with open wounds. The functionalized AMPs stability in physiological environment, their side effects, life-spam, and tunable performance, should be carefully investigated prior to entering the general market. In fact, despite the great efforts made by many researches, only a small number of AMPs are now available in the market or are in clinical trials. Nevertheless, it is expected that in few decades these limitations and concerns to be put to rest as new discovers are being made every day on the structure, properties and benefits of AMPs for skin infections.

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