The battle against antimicrobial resistant bacterial infections: next stage development of antimicrobial peptides

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General introduction

1. Major threats for effective treatment of bacterial infections

In 1928, Alexander Fleming revolutionized the (medical) world with the discovery of the antibiotic penicillin [1]. Initially, Fleming's discovery recieved little attention. In the 1940s however, penicillin could be produced in large quantities due to efforts of Chain and Florey. As a result penicillin entered the military theater at the end of World War II [2]. By the 1950s, a wide variety of antibiotic classes with different modes of action was discovered [3, 4]. These antibiotics became one of the greatest medical advances of the 20th century for the treatment and prevention of bacterial infections, saving millions of lives yearly. Also today, advanced medical care heavily depends on the use of these antibiotics, for example during transplantations and surgery. However, bacteria and other pathogens have evolved in response to the (ab)use of these antibiotics, resulting in the selection of antimicrobial resistant (AMR) strains. Already in 1945, Fleming noted the danger of resistance development during his Nobel lecture: "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body" [5]. Bacteria can acquire resistance to antibiotics through mutation of genes encoding for enzymes, efflux pumps and/or processes altering their cell wall in effect selecting for optimal settings to remove the antibiotic from their system or through genetic exchange mechanisms with other bacteria [6]. AMR is one of the greatest public health threats we face as global community, as it might lead to a scenario where simple infections, such as skin wound infections or bladder infections, can no longer be treated with modern antibiotics. Currently, in the United States and Europe alone about 50,000 deaths annually are caused as a result of AMR and for 2050 it is estimated that this number will increase to 10 million people globally [7]. AMR strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* among others are increasingly encountered and constitute a major threat to the public health systems [8]. Moreover, biofilm and/or persister formation by these pathogens further hampers the efficacy of antibiotics. Approximately 80% of the chronic and recurrent bacterial infections are biofilm-associated [9]. Biofilms protect bacteria from actions of environmental stressors like antibiotics and effectors of the immune system, and are also a breeding unit for resistance development [9, 10]. **Figure 1** provides an overview of the different stages involved in biofilm formation: the biofilm life-cycle. Biofilm formation is initiated after irreversible attachment of planktonic bacterial cells to a surface, which could be human skin or medical devices like catheters or implant materials [11]. After attachment, biofilms develop as (poly) microbial communities embedded in self-produced extracellular matrix composed of polysaccharides, protein and DNA, that protects the bacteria from hostile factors [12]. Within deeper layers of the biofilm, bacterial cells might change to a metabolically inactive state, so called persister cells [13]. These persister cells are more resistant

to antibiotics that have bacterial targets involved in metabolism, while also actively removing antibiotics from their system via efflux pumps [14]. Additionally, bacteria can hide inside host cells to prevent removal by the immune system and/or antibiotics [15]. The increasing failure of antibiotics and the lack of new antibiotic development with a mode of action different from current antibiotics, highlights the need for novel therapeutic agents.

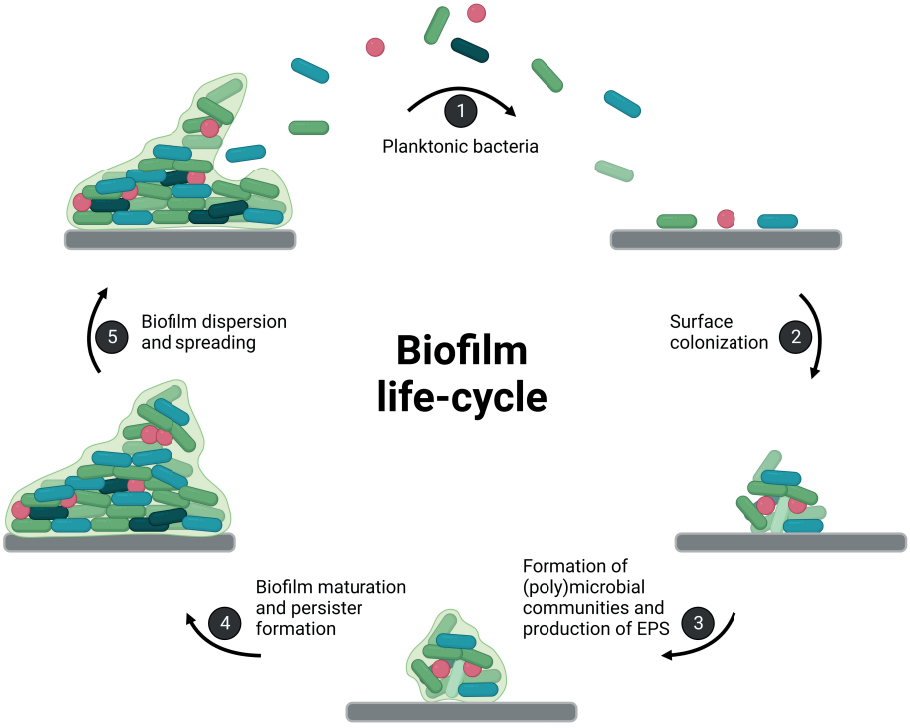

Figure 1. The biofilm life-cycle describes the steps involved in bacterial biofilm formation. Free floating planktonic bacteria (1) bind irreversibly to a surface, e.g. human skin or medical devices (2). These bacteria start to form (poly)microbial communities and produce extracellular polymeric substances (EPS) that forms the biofilm matrix around the bacteria (3). As the biofilm develops and maturates, some bacteria residing deeper inside the biofilm might change to a metabolically inactive state, so called persister cells (4). Over time, bacteria can disperse out of the biofilm, revert to a planktonic state and start to colonize new surfaces (5). represent *Staphylococcus* bacteria, polymicrobial bacteria and persister cells. Figure is based on Sahli et al. [16].

2. Antimicrobial (host defense) peptides

Antimicrobial peptides (AMPs) are considered promising alternatives to antibiotics. AMPs are a class of small peptides that span 10-60 amino acids and are part of the innate immune response in a great variety of organisms, including humans [17]. These peptides exhibit a wide range of biological activities, which include antibacterial, antibiofilm, antiviral, antifungal, anticancer, wound healing and immunomodulatory activities [18]. Until today >3000 AMPs have been identified;

an online database reporting potent AMPs originating from natural sources can be consulted at https://aps.unmc.edu/. Since AMPs are a diverse group of peptides, they can be classified based on i) source, ii) activity, iii) structure and iv) amino acid-rich species [19]. The best studied structural classes of human AMPs are cathelicidins and defensins [20]. Two most common characteristics of these AMPs include a predominant cationic charge at physiological pH and ≥30% hydrophobic residues [21, 22]. The cationic charge of these peptides drives the initial electrostatic interaction with the polyanionic outer surface of bacterial cells, i.e. lipopolysaccharide or wall-associated teichoic acids for Gram-negative or Gram-positive bacteria, respectively [22]. AMPs are typically unstructured in aqueous solution, but in presence of a

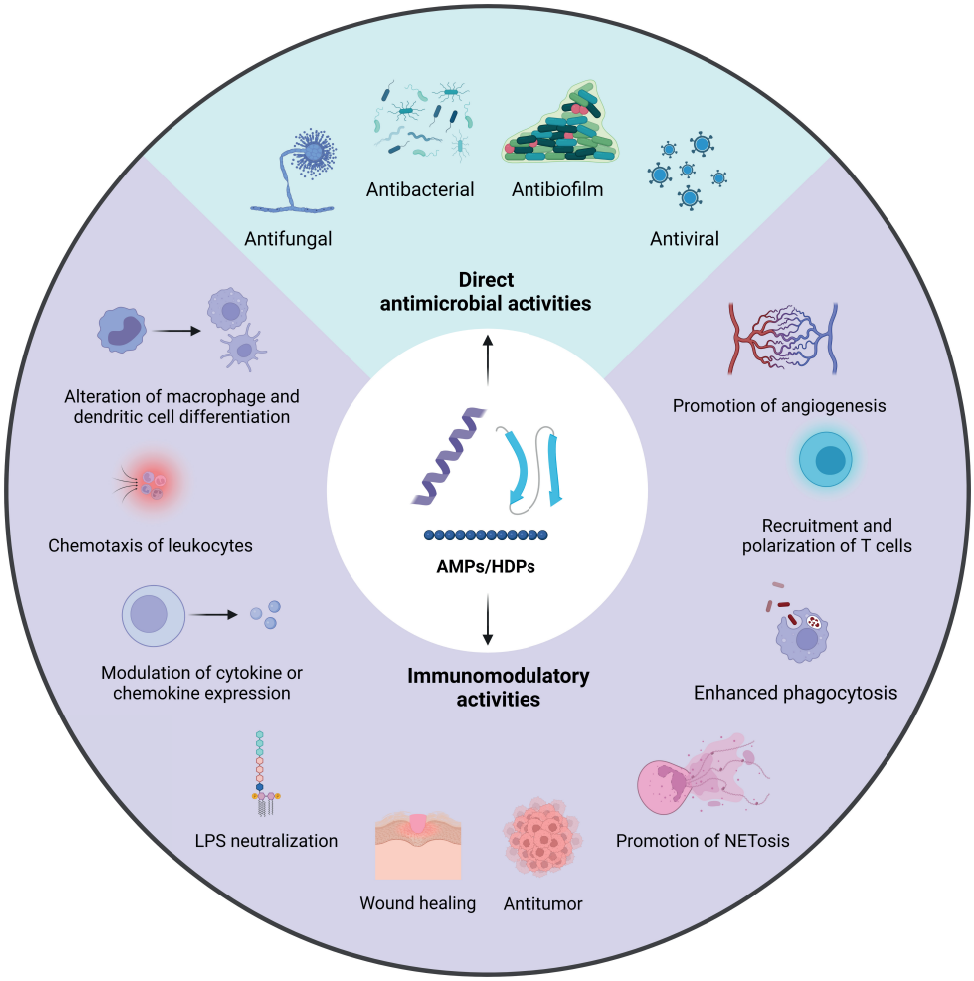

Figure 2. Overview of the antimicrobial and immunomodulatory activities of AMPs/HDPs. Direct antimicrobial activities include antifungal, antibacterial and antiviral activities. The variety of immunomodulatory activities range from effects on cells of the innate and adaptive immunity, but also comprise effects on tumors and wound environment.

biological membrane, these peptides fold into an 'active' amphipathic secondary structure, where hydrophobic residues are oriented opposite from cationic or polar residues [21, 23]. Although the mechanism of action of AMPs is diverse, generally direct interactions with and disruption of the bacterial membrane play a key role. Multiple destabilization models have been proposed to describe these interactions [21]. In addition, AMPs can induce bacterial cell death by acting on intercellular targets after cellular internalization, which can affect DNA/RNA synthesis, protein synthesis or protein folding [24]. Importantly, the risk of resistance development by bacteria in response to AMPs is considered low due to their unspecific and sometimes multifaceted mode of action [17, 25]. It was long thought that direct antimicrobial activities were the primary function of natural AMPs, however, recently this consensus has changed, and immunomodulatory properties are now considered the primary role of natural AMPs [26, 27], hence their alternative name host defense peptides (HDPs). The immunomodulatory properties of HDPs are diverse and include i) chemotaxis of leukocytes, ii) alteration of macrophage and dendritic cell differentiation, iii) modulation of cytokine or chemokine expression [26-29]. Moreover, several HDPs have shown to stimulate angiogenesis and promote wound healing [30, 31]. An overview of the direct antimicrobial and immunomodulatory activities of AMPs/HDPs is described in **Figure 2**.

3. Development of synthetic antimicrobial and antibiofilm peptide (SAAP)-148

Development of novel antimicrobial peptides has been guided primarily by rational design principles, and is increasingly combined with computer-aided methods like artificial intelligence for better structure-activity relationship predictions [32]. The only family member of human cathelicidins is LL-37, which has moderate broad-spectrum antimicrobial activities [33, 34], and is well-known for its immunomodulatory activities [29, 35-37]. LL-37 has served as starting point for development of AMPs with improved bactericidal and antibiofilm activities. First, a library of LL-37 derivates, where the core antimicrobial region was maintained, was synthesized and hit compound OP-145 (also named P60.4Ac) showed to have improved antimicrobial activities compared to LL-37 [38]. OP-145 was also effective in prevention of *S. aureus* implant-associated infection in rabbits when incorporated in a biodegradable implant coating [39] or *S. aureus* infections on human epidermal models when incorporated in a hypromellose gel [40]. Moreover, OP-145 successfully cured chronic otitis media in 47% of the cases in a phase II clinical trial [41]. Despite these successes, antimicrobial activity of OP-145 was shown to be reduced in presence of biological fluids, like human plasma, wound fluid or urine [38]. Therefore, in an attempt to further improve antimicrobial and antibiofilm activities under these physiological conditions, a second library of LL-37 derivates was synthesized, which resulted in lead peptide SAAP-148. SAAP-148 is a potent broad-spectrum agent, able to

eradicate multidrug resistant bacteria of the ESKAPE panel (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* species) and capable to completely eradicate *S. aureus* and *A. baumannii* biofilm infections from murine skin [42]. Moreover, SAAP-148 can eradicate *S. aureus* persister cells in antibiotic-exposed maturated biofilms simulating prosthetic joint infection [43]. SAAP-148 is a membrane disruptive AMP; it's mode of action is ascribed to insertion into the bacterial membrane followed by membrane thinning, permeabilization and leakage, resulting in bacterial cell death within minutes [42]. The improved antimicrobial activity of SAAP-148 is a result of superior ability to disrupt the bacterial membrane compared to OP-145 [44]. Importantly, SAAP-148 showed almost no resistance development [42], indicating the therapeutic potential of this AMP for treatment of antimicrobial-resistant bacterial infections.

4. Limitations of antimicrobial peptides and SAAP-148

Until date, only seven AMPs (including (cyclic) lipo- and/or glycopeptides) have been admitted to the clinic: Gramicidin, Daptomycin, Colistin, Vancomycin, Oritavancin, Dalbavancin and Telavancin [45]. Pharmacokinetic and pharmacodynamic properties related to the peptidic nature of AMPs have hampered their success rate in clinical trials [46]. Challenges for further development of AMPs include i) relatively high hemolytic and cytotoxic activities at antimicrobial concentrations resulting in low selectivity towards bacterial cells over mammalian cells [47-50], ii) limited proteolytic stability due to peptide degradation by proteolytic enzymes produced by the host or bacteria [49-51], iii) limited bioavailability due to binding to plasma and/or serum proteins [51, 52], iv) short systemic half-life [45, 49], v) limited penetration into tissue [51, 53] or bacterial biofilms [54] and vi) expensive production costs especially for long peptide sequences [49-51]. Also SAAP-148 suffers from limitations related to its peptidic nature, such as a small therapeutic window and short half-life due to (plasma) protein binding [55, 56]. Although SAAP-148 has proven successful in treatment of superficial skin wound infections in vivo [42], its antibacterial activities were limited in deeper skin wounds, such as surgical wound infections in rats [55]. Factors contributing to the reduced activity of SAAP-148 in this in vivo surgical wound infection model were related to i) components within the wound micro-environment (e.g. proteins, proteases, etc.), ii) inadequate or relatively slow release from the hydrogel or wound dressing and iii) re-colonization due to relatively high bacterial load in the wounds [55].

5. Strategies to improve antimicrobial peptides

Several strategies to minimize the peptide-related limitations of SAAPs and AMPs in general can be considered. Such strategies include AMP chemical lead-optimization, combination therapy and innovative peptide delivery systems (**Figure 3**).

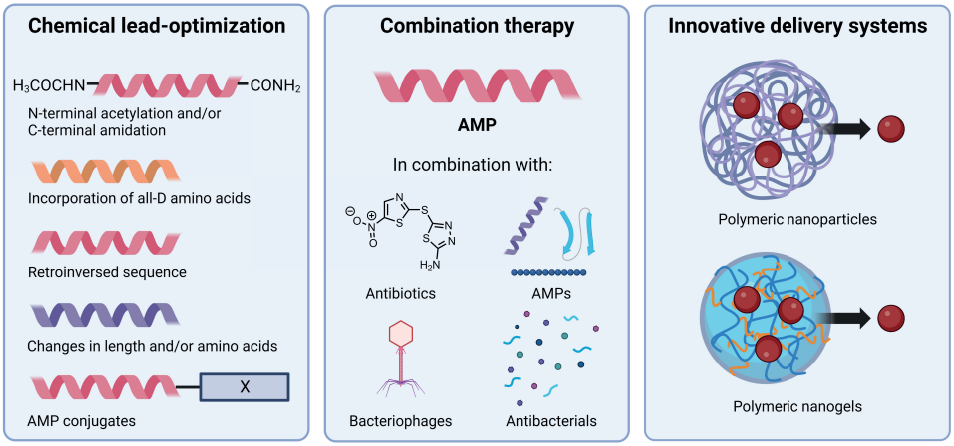

Figure 3. Three strategies are described that can be used to further improve AMPs like SAAP-148. First, chemical lead-optimization of peptides includes strategies, such as N- or C-terminal capping, incorporation of D-amino acids or changes in length and/or amino acid sequence. Second, AMPs can be combined with agents like antibiotics, AMPs, bacteriophages or other antibacterials that may have synergistic activities. Third, innovative delivery systems are used to encapsulate AMPs and can be applied at the site of infection, where they release their content over time.

5.1. Chemical lead-optimization of antimicrobial peptides

Post-translational modifications of natural AMPs are very common and synthetically changing the AMP backbone is one of many strategies that can be investigated in order to improve pharmacokinetic and pharmacodynamic properties of synthetic AMPs. Post-translational modifications occurring in natural AMPs include N-terminal acetylation, C-terminal amidation, incorporation of D-amino acids, disulfide bridge formation and cyclization among others [57]. The most simple synthetic modification is end-capping of AMPs, where N-terminal acetylation greatly improves proteolytic stability of the AMP (sometimes at the cost of antimicrobial activity), while C-terminal amidation generally improves antimicrobial activities of the AMP [58-60]. Moreover, substitution of L-amino acids by D-amino acids, thereby changing chirality of the AMP, is a useful strategy to improve proteolytic stability of AMPs [61-64]. The same holds for retro-inversed AMPs, that have a reversed sequence and thus reversed chirality but where the same amino acid orientation in 3D space is remained [63]. Furthermore, changes in length and/or amino acid sequence of AMPs might also allow for improved properties [64, 65]. Alternatively, AMPs can be conjugated to other molecules to further improve their pharmacokinetic and pharmacodynamic properties. The most extensively studied conjugation method is PEGylation, the process of coupling a polyethylene glycol chain to AMPs. Generally, PEGylation of AMPs results in decreased hemolytic and cytotoxic activities, improved proteolytic stability, reduced binding to serum proteins and improved solubility [66-70]. Nevertheless, reduced antimicrobial activity for PEGylated AMPs is observed,

especially upon conjugation to sufficiently long PEG chains [70, 71]. Conjugation of AMPs to shorter PEG chains allows for similar advantages, while minimizing the loss of antimicrobial activity [72, 73]. In addition, conjugation of AMPs to cell penetrating peptides (CPPs) or penetration enhancers has proven successful for increasing internalization into cells or improved tissue penetration [74, 75]. CPPs are a special group of peptides capable of crossing cellular membranes via endocytosis and/or direct translocation [74, 76, 77]. CPPs often originate from naturally occurring amphiphatic (antimicrobial) peptides, and some examples include penetratin [78], HIV-1 Tat [79] and W/R [80]. Model amphiphilic peptide (MAP) is a special CPP that has additional capabilities as penetration enhancer by modulation of tight junctions [81]. Notably, all beforementioned modifications might allow for improved pharmacokinetic and pharmacodynamic properties of synthetic AMPs. Nevertheless, it is important to carefully review their antimicrobial activities, as these modifications might undesirably decrease antimicrobial activities of the parent peptide.

5.2. Combinations of antimicrobial peptides with other therapeutic agents

Alternatively, antimicrobial activities of AMPs can be enhanced by combining these peptides with other therapeutic agents. Drug combinations can show synergism, additive effect, no interaction or antagonism [82]. Synergy between two drugs occurs when the combined effect of the two drugs is greater than the expected additive effect of these drugs. Synergistic AMP-drug combinations are less likely to induce resistant bacteria [83, 84], and thus have the advantage of further reduction of AMR development. Moreover, these combinations reduce the individual drug dosage and in effect reduce cytotoxic side effects [85]. Reasonably, favorable combinations of AMPs with first-line antibiotics would allow for more effective and shorter therapies compared to current antibiotic regimens. Membrane-disruptive AMPs have been successfully combined with current antibiotics, thereby facilitating antibiotic penetration into the bacterial cytosol allowing the antibiotic to reach its target [86-88]. Likewise, SAAP-148 has shown to synergize with classical antibiotics, such as teicoplanin [89] and demeclocycline [90]. Alternatively, AMPs have also been shown to synergize in combination with a second AMP [85, 91], and addition of a third AMP to the combination further improved these synergistic activities [85]. Moreover, a recent study on bacteriophages showed that these bacterial viruses are able to synergize with AMPs [92]. Finally, other antibacterial agents could also synergize with AMPs, like phage endolysins [93], histones [94] or silver nanoparticles [95]. In the context of bacterial biofilms, AMPs could be combined with agents that inhibit biofilm formation (e.g. inhibitors of quorum sensing [96, 97]) or degrade and/or disaggregate the biofilm matrix (e.g. matrix degrading enzymes [98] and chelating agents [99-102]). Conjugates of AMPs and above mentioned antimicrobials could

also be considered, as conjugates may have several advantages of combined application of two agents [103].

5.3. Nano-scaled delivery systems for antimicrobial peptides

Another strategy to improve the therapeutic potential of AMPs is encapsulation of these peptides in drug delivery systems (DDS). Since the introduction of the first controlled-release drug-formulation in the 1950s [104], the field of drug delivery technology has advanced significantly, with nowadays a wide range of DDSs available for the encapsulation of AMPs. These DDSs can be categorized in inorganic materials (e.g. metal-based nanoparticles, mesoporous silica nanoparticles, etc.) and organic materials (e.g. lipid-based nanoparticles, polymeric nanoparticles, polymeric nanogels, etc.) [105]. Nano-scaled DDSs will be the main focus of this thesis, which are usually defined by a particle size ranging from 1 to 100 nm [106], although the same terminology is also used for larger particles up to 500 nm. However, since efficacy and usefulness of DDSs do not only depend on particle size [107], in this thesis nano-scaled DDSs are defined by a particles size ranging from 1 to 500 nm. Several limitations of AMPs can be circumvented by nano-scaled DDSs through i) protection of AMPs from proteolytic enzymes and prevention of binding to plasma and/or serum proteins thus improving the stability and bioavailability of AMPs [108-110], ii) sustained release of AMPs, which reduces the cytotoxicity associated with AMPs [111, 112], iii) assisted transport of AMPs across cellular membranes thus improving intracellular uptake [112, 113], and iv) improved biofilm penetration and intracellular retention [16, 112-114]. Physicochemical properties of these nano-scaled DDSs (e.g. material composition, particle size, surface charge, etc.) affect their pharmacokinetic profile and cellular interaction [115]. Thus, for a certain application one delivery system might be preferred over another. For cutaneous application on skin wounds, nanogels have received great interest as versatile DDS due to their unique properties resulting from the combined features of nanoparticles and hydrogels. These soft nano-scaled particles are formed when water-soluble polymers (natural or synthetic) are cross-linked in three-dimensional space and nanogels have the ability to absorb high amounts of water or biological fluids into the formed network while maintaining their structure [116, 117]. The great amount of hydrophilic groups in the polymeric backbone of nanogels allows for highly efficient AMP encapsulation and provides high biocompatibility [118]. Nanogels are considered as ideal DDSs for skin wound infections as i) their high water content allows for prevention of wound dehydration and creates a moist environment beneficial for wound healing [119, 120], ii) their soft texture and non-adhesive properties allow for patient-friendly application and removal without interfering the wound bed [121], and iii) their porous three-dimensional structure allows for exchange of oxygen that is highly important for the numerous metabolic processes involved in wound healing [122].

6. Thesis outline

In this thesis multiple strategies were explored to further develop SAAP-148 to its full potential for treatment of infections caused by antimicrobial resistant bacteria. In **chapter 1** the importance of novel agents and strategies to combat clinical bacterial infections that are hard-to-treat with current antibiotics is emphasized. AMPs, like SAAP-148, are promising alternatives to current antibiotics, however the peptides suffer from some limitations regarding their stability and safety. To circumvent some of these limitations, multiple strategies for optimization of AMPs are being considered, i.e. chemical lead-optimization, combination strategies and (nano)formulation technology. **Chapter 2** describes the antimicrobial, cytotoxic and immunomodulatory activities of a library of SAAP-148 peptides chemically modified with PEG-chains. Next, we hypothesized that the therapeutic potential of SAAP-148 could be enhanced when combined with other antimicrobial agents. **Chapter 3** describes favorable antibacterial activities of SAAP-148 when combined with the novel antibiotic halicin. Furthermore, formulation of SAAP-148 into nanogels could be a promising strategy to reduce the cytotoxicity and thus enhance the selectivity towards bacteria. **Chapter 4** is a comprehensive overview of lipid and polymeric delivery systems thus far investigated for encapsulation of AMPs and AMP coatings for prevention and treatment of bacterial infections. Based on these insights, we encapsulated snake cathelicidin Ab-Cath and SAAP-148 into polymeric nanogels for topical treatment of skin wound infections. These results are described in **chapters 5 and 6**, respectively. Snake cathelicidin Ab-Cath was included in this thesis, because this peptide showed improved antimicrobial activity in presence of human plasma and an improved safety profile compared to SAAP-148. Finally, the main findings of this thesis are summarized and discussed in **chapter 7**. A Dutch summary can be found in the **appendix**.

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