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Chapter

# Defensin-Like Peptides and Their Antimicrobial Activity in Free-Form and Immobilized on Material Surfaces

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## Abstract

Defensins are naturally occurring antimicrobial peptides secreted in the human body. Mammalian defensins are small, cysteine-rich, cationic peptides, generally consisting of 18–45 amino acids. The antimicrobial activity of defensins arises from their unique amino acid sequence, showing activity against both Gram-positive and Gram-negative bacteria, fungi and enveloped viruses. The use of antimicrobial peptides is rising due to their potential to control biofilm formation and kill microorganisms that are highly tolerant to antibiotics. In free-form, defensins are capable of destroying such microorganisms through numerous mechanisms mainly the *carpet*, the *toroidal* and the *Barrel-Stave* models. However, immobilization of antimicrobial peptides (AMPs) on surfaces with the help of coupling agents and spacers can improve the AMPs' lifespan and stability in the physiological environment leading to applications for medical devices and implants. Fundamental understanding of both free-form and surface-immobilized defensins is important to design more effective antimicrobial peptides and improve their performance in future developments.

**Keywords:** antimicrobial peptides, defensins, mammalian peptides, surface-immobilized antimicrobial peptides, surface-immobilized defensins

## 1. Introduction

The innate immune system is the first line of defence in human body and vertebrates. Defensins are naturally occurring antimicrobial peptides (AMPs) that are a part of the innate immune system, protecting the body against foreign microorganisms. Defensins are produced by the interaction of antigen-presenting microbial cells with pattern recognition receptors, such as toll-like receptors that are present on the membrane of numerous immune cells (i.e., macrophages, neutrophils and leukocytes [1]. Mammalian defensins are small, cysteine-rich, cationic peptides, generally consisting of 18–45 amino acids [2]. Next to being antimicrobial, defensins also serve as immune-stimulating agents.

When synthesized *in vivo*, defensins are initially produced as inactive precursor proteins (i.e., pro-defensins), which consist of the defensin and a pro-peptide. The pro-peptides are present to ensure delivery of defensins through the body without

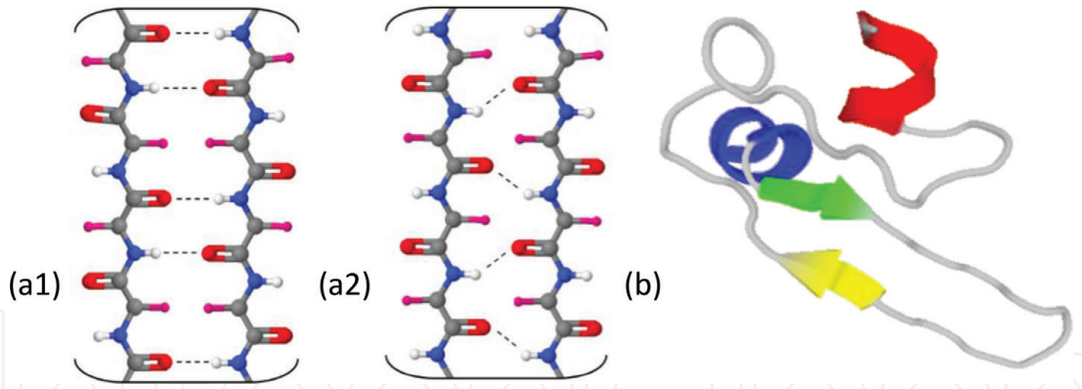



**Figure 1.**
*Schematic of a (a1) parallel and (a2) antiparallel β-sheet structure, made with the use of JSME [12]. (b) Conformation of a β-defensin (hBD3), made with the use of PEP-FOLD3 [13]. Hydrogen bonding represented by dotted lines, hydrogen atoms are white, carbon atoms are grey, oxygen atoms are red, nitrogen atoms are blue and rest-groups are pink; not exact structure.*

premature attachment of defensins to other microorganisms [3]. The pro-peptides inhibit premature attachment to other microorganisms by neutralizing the cationic charge of defensins. Also, the pro-peptides ensure subcellular localization (i.e., the location of where a protein resides in a cell) and folding of defensins into their characteristic conformation. Through proteolytic removal *in vivo* of the pro-peptides, the defensins are activated [4]. The reasoning behind the pro-peptides functioning as a folding assistant is based on the research performed on folding of defensins *in vitro* without the pro-peptide, which is found to be extremely difficult [5, 6].

α-Defensins are expressed by neutrophils and macrophages, that is, a type of white blood cell and cells that can engulf foreign particles, respectively. In general, these tend to have a broader antimicrobial activity, when compared to β-defensins, showing activity against both Gram-positive and Gram-negative bacteria, fungi and enveloped viruses [7]. Paneth cells also produce α-defensins, also known as crypticidins, which are involved in the reduction of bacteria present in the intestinal lumen. β-Defensins are primarily produced and released by epithelial cells and leukocytes, that is, a type of cell that lines the surfaces of your body and a type of blood cell that is made in the bone marrow, respectively. These are mainly active against Gram-negative bacteria and yeast; however, many also show antibacterial activity towards Gram-positive bacteria [8]. The pro-peptides of β-defensins are smaller than those of α-defensins.

Both α- and β-defensins form a triple-stranded antiparallel β-sheet structure that is stabilized by hydrogen and disulphide bonds; bond formations are schematically represented in **Figure 1**.[1] The position of the cysteines and intramolecular disulphide linkages determines the category of the defensin. The consensus of cysteine placement within the amino acid sequence for α-defensins follows C-X-C-$X_4$-C-$X_9$-X-X, and C-$X_6$-C-$X_4$-C-$X_9$-C-$X_6$-C-C for β-defensins [9]. When looking at the position of the disulphide linkages from cysteine in sequential order (denoted by C#), the disulphide bridges are formed between C1-C6, C2-C4 and C3-C5 for α-defensins and C1-C5, C2-C4 and C3-C6 for β-defensins [10], as shown in **Figures 2** and **3**, respectively. The disulphide bridges are important for holding the defensins in their three-dimensional structures. In addition, they contribute to the defensins chemotactic activity (i.e., movement or orientation of an organism or cell towards chemical stimulus) but when altered, only slightly affect their antimicrobial activity [11].

The adopted mechanisms of the interaction between defensins and the invading microorganism are not yet fully understood. However, disruption of

[1] All the figures shown in this chapter have been created by the authors.

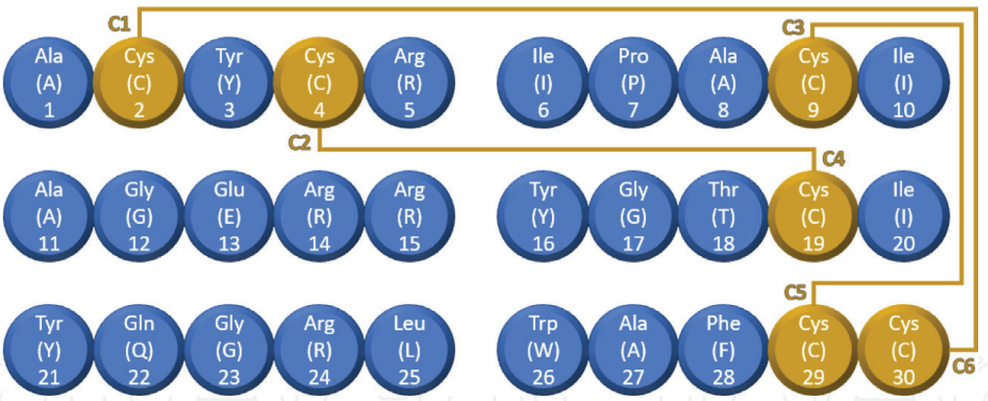



**Figure 2.**
*Schematic of HNP1 (α-defensin) showing amino acid sequence and disulphide bridges.*

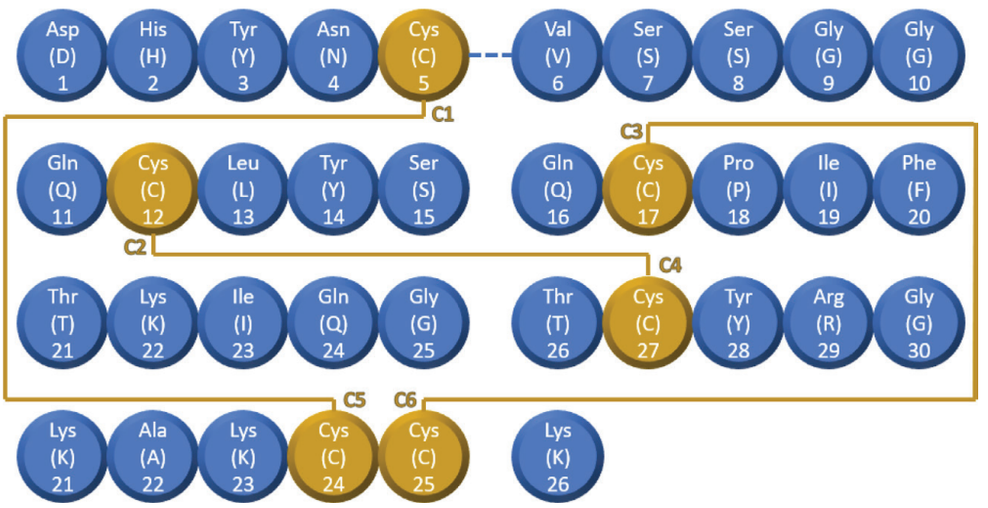



**Figure 3.**
*Schematic of HBD1 (β-defensin) showing amino acid sequence and disulphide bridges.*

the plasma membrane has been shown to be the leading cause of cell death in microbial species. The disruption caused by defensins depends on many factors, such as the polar topology, spatial separating of charges and hydrophobicity. These factors allow the attraction and subsequent interaction of defensins with the lipid bilayer of the bacterial membrane. Conversely, this interaction causes the defensins to insert themselves between the hydrophilic region of the plasma membrane and disrupt the bacterial membrane, utilizing numerous mechanisms. These include the introduction of channel-like pores and carpet-like membrane disruption, resulting in cell lysis. Simultaneously, the introduction of voltage-dependent channels in the bacterial membrane allows the influx of water and results in an increase of osmotic pressure that leads to the rupture of the membrane. On the other hand, some defensins move through bacterial cell walls, bind to target cells and disrupt normal metabolism, which may lead to apoptosis of the targeted cells [14, 15].

## 2. Mammalian defensins

The genomic organization and evolution of defensin genes of several vertebrate species have been studied [16]. The human genome encodes, at least, 35 different defensin peptides [17]. Most of the mammalian defensin genes are divided over three chromosomes, found in four different gene clusters (**Figure 4**). All the genes

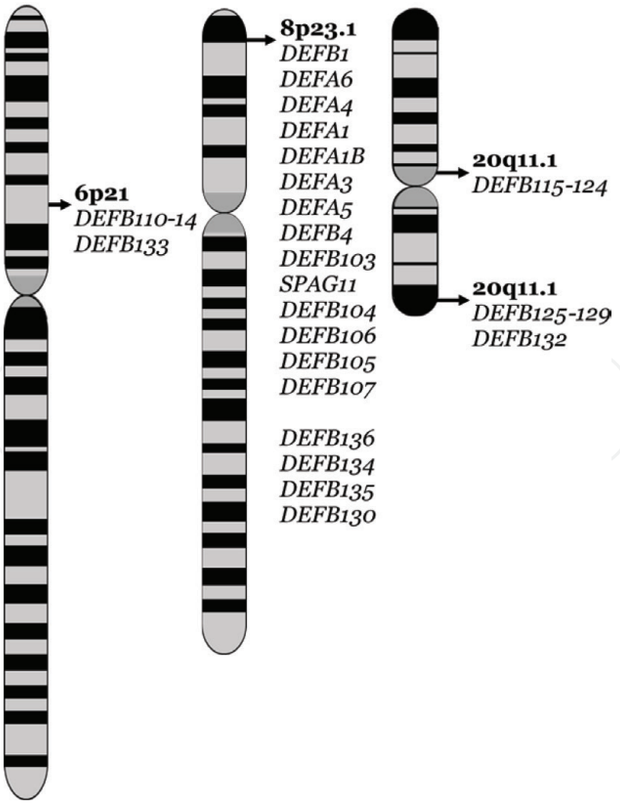



**Figure 4.**
*Schematic of the genomic organization of defensins in three human chromosomes, showing chromosome 6, chromosome 8 and chromosome 20, respectively.*

expressing α-defensins and several β-defensins are found in chromosome 8 (cluster p23.1); genes that express most of the remaining β-defensins are found in chromosome 6 (cluster p21) and chromosome 20 (cluster q11.1 and p13).

Four out of the six human α-defensins are found in neutrophils and other leukocytes, specifically, human neutrophil peptides (HNPs) 1–4 (i.e., DEFA1–4). The remaining two, human α-defensin 5 and 6 (HD5 and HD6, i.e., DEFA5 and DEFA6), are expressed by Paneth cells in the intestinal lumen [18]. Numerous β-defensins are found in the respiratory system, gastrointestinal tract and urogenital system. These are expressed by epithelial cells, namely, human BD1 (hBD1, i.e., DEFB1), hBD2 (DEFB4), hBD3 (DEFB103A) and hBD4 (DEFB104) [19].

Defensins are amphipathic (i.e., having both hydrophobic and hydrophilic groups) and, it has been demonstrated that they show the ability to form dimers and oligomers with toxin molecules [20, 21]. The initial electrostatic interaction is caused by the cationic charge of the peptide and the negatively charged outer membrane of the bacterial cell wall [22]. Bacterial membranes contain many negatively charged phospholipids, lipopolysaccharides or teichoic acid, while eukaryotic membranes contain neutral phospholipids and cholesterol [23]. This explains the destructive ability of defensins towards microbes but not host cells [24].

## 2.1 Antimicrobial mechanisms of defensins

The activity of defensins against microorganisms is determined by the interaction of the cationic molecules with the negatively charged acidic lipopolysaccharide or teichoic acid on Gram-positive and Gram-negative bacterial membranes. The antimicrobial activity of defensins in the body depends on different factors such as salt concentration and serum components [25]. In addition, the configuration of defensins plays an important role on the activity of these molecules. For example,

helical antimicrobial peptides in solution show a typical helical amphipathic characteristic and they are either unstructured until they are in contact with the biological membrane or structured through disulphide bonds. Defensins are among the structured type of antimicrobial peptides and the hydrophilicity of the α- and β-defensins determines the extent of interaction between these molecules and the bacterial membrane [26].

As mentioned before, the antimicrobial mechanisms of defensins have not been fully understood. The formation of membrane pore or channel has been shown to be dependent on the membrane configuration. For example, the abundance of negatively charged phospholipids on the plasma membrane affects the concentration of peptides that are required to form a stretch or curvature on the outer layer of the bacterial membrane, and consequently lead to cell lysis [27]. Other events such as phospholipid reversal and penetration of peptides inside the cytoplasm on the inner side of the membrane leading to the loss of membrane composition and causing cellular inactivation have also been mentioned in the literature [28].

Most studies conducted on defensin mechanisms have been conducted on the α-helical structured peptides. These molecules interact with the membrane of the microorganisms. It is evident that defensins utilize membrane depolarization and permeation, against bacteria and yeast, as their most likely defence mechanisms. Defensins also aid the mobilization of T-cells and immature dendritic cells, which contribute in the activation of acquired immune responses that will trigger a long-lasting cellular response to a potential pathogen [29]. Most defensins provide their antimicrobial activity through interaction with cellular membranes. The pore-forming model describes the interaction between positively charged peptides and the negatively charged head of phospholipid groups of cellular membranes operates once a critical concentration is reached. This will cause the self-aggregation and perpendicular insertion of peptides inside the membrane leading to production of lined transmembrane pores, resulting in the disruption of ionic and proton gradients. The second model causes the formation of channels where the strain of peptide at the critical concentration, induces the inward curving of the membrane, creating dome-shaped channels lined with phospholipid-head groups and peptides [30].

The mechanisms in which the AMPs destroy the bacterial cells have been studied for decades. AMPs that present their secondary structure in the form of α-helix present their destructive effects on a bacterial cell based on three different documented mechanisms. First is the *Barrel-Stave model*, which is the most studied mechanism, where the peptide disrupts the membrane by exposing its hydrophobic site to the lipids in the membrane bilayer. This will force the membrane to undergo conformational changes by forming a pore on the surface of the membrane [31, 32]. The second mechanism is the *toroidal model* where the peptide and the lipid of the membrane bilayer integrate upon interaction and form torus pores leading to the death of bacterial cells. Cell death caused by these mechanisms leads to loss of compositional specificity, leakage of critical metabolites or depolarization of the membrane due to an increased rate of phospholipid reversal [33, 34]. The third mechanism is the *carpet model* where the concentration of AMP is related to the amount of interaction with the bacterial membrane. In this model, the peptides surround the cell membrane by attaching to the phosphide group of the membrane bilayer where they disrupt the curved anionic membrane and dissolve it, killing the bacteria [35, 36].

### 2.2 Antimicrobial activity evaluation methods

The evaluation of antimicrobial activity is widely used in the field of drug discovery as well as epidemiology and therapeutic prediction [37]. Since the low

density of peptides will ultimately result in preservation of membrane integrity and adjustment of the membrane, maintaining a higher concentration of peptides is crucial. This will result in imposed curvature strain on bacterial membrane [38]. Therefore, measurement of the minimum inhibitory concentration (MIC) is an important initial step for the confirmation of antimicrobial activity. MIC refers to the lowest concentration of compound needed for observable inhibition of bacterial cell growth. In addition, other measurements include minimal bactericidal concentration (MBC) which refers to the minimum concentration of the reagent that can cause bacterial death, the haemolytic activity (HC) that refers to the ability to break down red blood cells (i.e.,) to find the inhibitory effect of AMPs on normal mammalian cells and $IC_{50,}$ which refers to the half-maximal inhibitory concentration [39]. Since defensins are found in more diluted concentrations in extracellular environment than in a local environment, the measurement of the interactions between peptides and bacteria *in situ* (e.g., using a mice model) is mandatory [40].

Colorimetric assays are used to determine the concentration of peptides (MIC) where the absorbance is usually measured at 750 nm using a UV-visible spectrophotometer. The assays are performed in 96-well microtiter plates and a series of antimicrobial peptide dilutions are added to the bacterial cells immersed in growth medium. The MIC is measured based on the growth after incubation for a defined period of time (16–20 h) [41]. This methodology measures the colour of the dilutions, which is directly proportional to the number of cells in each well. The absorbance is measured with the help of a microplate reader counting the number of cells killed by the antimicrobial peptide. This methodology provides information about the susceptibility of the microorganism to the peptide [42], but it only applies to aerobic bacteria [43].

Electron microscopy is used to visualize the interaction of peptides with the phospholipid bilayer of bacterial membrane. Simultaneously, fluorescent dyes are used to observe the ability of peptides to permeate and penetrate the membrane of bacterial cells. Fluorescence spectroscopy is used to study the insertion of fluorophores into a membrane as well as provide information about the rate of peptide penetration into the membrane with the help of surface plasmon resonance. Fluorescence quenching can also be used to gain an insight into the depth of peptide penetration inside the cells [2].

In addition, both circular dichroism (CD) spectroscopy [44] and nuclear magnetic resonance (NMR) spectroscopy are used to measure the orientation and secondary structure of an antimicrobial peptide, when bound to a lipid bilayer. However, only NMR is used to measure the penetration of antimicrobial peptides into lipid bilayers in a relevant liquid-crystalline state [45]. The use of CD can distinguish between the randomly coiled, α-helical and β-sheet structures since they show wavelength-dependent differences in the absorption of the right and left circularly polarized light. Using oriented CD can also provide information about the orientation of peptide upon insertion into the membrane. This is dependent on the concentration of these peptides, the nature of the lipid and the extent of hydration [2].

In order to study the peptide configuration, both solution and solid-state NMR can be employed. For solution NMR, a mixture of peptide-detergent micelle is needed for the stabilization of the peptide in water and to overcome insufficient resolution and low (signal/noise) ratio [46]. The most recognized model membrane system used in solution NMR is the dodecylphosphocholine (DPC). This method is used for studying the interaction of peptides with the lipid bilayer since it has the ability to rotate freely in solution and mimic anisotropic environments of lipid membranes [47]. In addition, solid-state NMR is a premium technique to use for the analysis of immobile peptides that are difficult to analyse with crystallography

or solution NMR. They can be used without a need for major peptide modification to determine the structure of membrane proteins in the phospholipid bilayer. This technique offers the examination of the structure and motion for the peptide-lipid interactions in physiologically relevant conditions and produces sharp resonance lines due to macroscopic alignment [48].

In addition, to provide the dynamic interaction between the defensins and the lipid bilayer, using a monolayer mimicking the structure of bacterial membranes can also be used. To observe the interaction between the two, the sum frequency generation spectroscopy (SFG) is employed. This methodology can provide information about gas-liquid, solid-liquid and liquid-liquid interactions and is able to detect the biomolecule orientation and adsorption in sub-micron quantities. This technique uses a pulsed narrow band visible laser beam spatially and temporally overlapped with a broadband infrared laser and measures the incident beam produced from the surface [49]. The vibrational spectrum of C═O groups from the amide backbone group can provide the secondary structure of the peptides. Consequently, vibrations from acyl chains of the lipid bilayer can provide important information about the interaction of peptide with the membrane as well as information about the molecular structure of the peptide, without the use of vesicles and labels to complicate the process of analysis [50].

## 3. Antimicrobial activity of defensins in free-form

There are two types of AMPs: first AMPs that show activity towards both bacterial and mammalian cells; second, AMPs that show only activity towards the bacterial cells. Most linear cationic AMPs are unordered in aqueous solution. The balance between the positively charged and hydrophobic amino acids in cationic AMPs permits the amphipathic adaptation of these molecules in solution. This allows the interaction of AMPs with the negatively charged bacterial membrane and the subsequent penetration of these molecules inside the lipid membrane [51]. The interaction of these peptides with the bacterial membrane is increased due to their high inside-negative transmembrane potential. On the other hand, normal eukaryotic cells have a net neutral charge across their membrane bilayer, and they have reached a zwitterionic (overall neutral) point. This insight can partly explain the attraction of AMPs to prokaryotic cells and the relatively weak attraction of these molecules towards eukaryotic cells [38]. The amphipathic characteristics of defensins aid the adaptation of a folded confirmation for these molecules in both hydrophobic and hydrophilic environments [44]. These molecules are often difficult to stabilize and show poor bioavailability due to their many different cleavage points that provide susceptibility to enzyme degradation and their linear form which leaves their two ends exposed [52].

α-Defensins (DEFA1–4) are produced by endoplasmic reticulum of the bone marrow shown in **Figure 5**, and the highest concentration of defensins is found in granules (i.e., leukocyte storage organelles). Pre-pro-defensins (light purple circles in **Figure 5**), however, consist of 94 amino acids. To produce pro-defensins (dark purple circles in **Figure 5**), 19 amino acids are removed from the N-terminus of pre-pro-defensins. Further proteolysis from the N-terminal side of the sequence of amino acids results in the production of mature defensins (blue circles in **Figure 5**). The prepared defensins are encapsulated into vesicles and fused with phagocytic vacuoles. The introduction of a pathogen into cells will then result in the recognition and engulfing of such molecules by phagocytic leukocytes with the aid of defensins and the subsequent death of the pathogenic organism. Although some α-defensins are produced in the bone marrow, some (DEFA5-6) are also produced

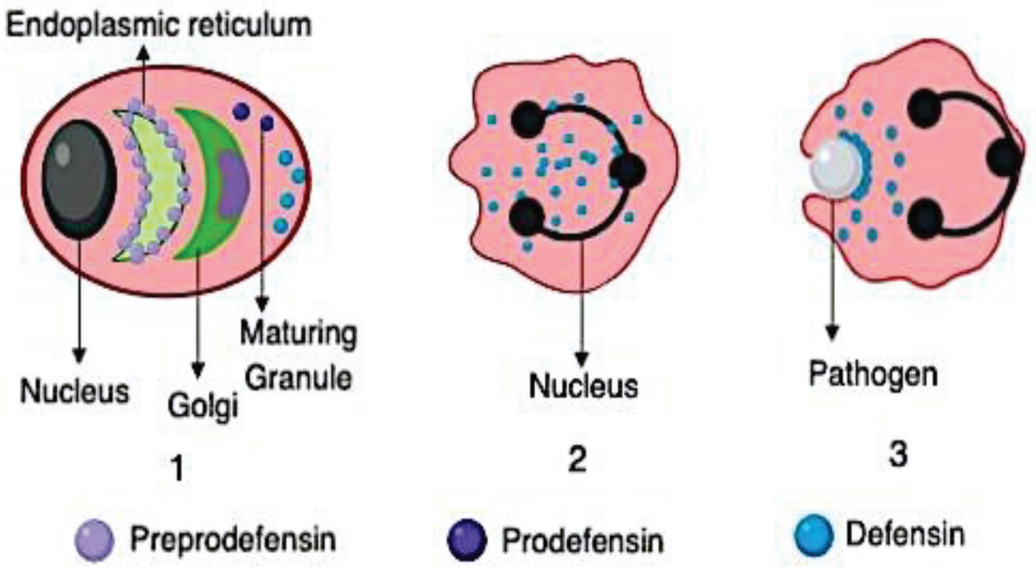



**Figure 5.**
*Schematic diagram of production of α-defensins and the phagocytosis of pathogens with the aid of defensins: (1) bone-marrow promyelocyte; (2) neutrophil; (3) phagocytosis.*

in the Paneth cells of the intestines [53]. β-Defensins (DEFB1–4) on the other hand are mainly produced in the epithelial cells. These regions have the highest concentration of defensins due to the higher susceptibility to a pathogenic attack, which renders them weaker and subsequently in need of the immunity provided by defensins [17].

β-Defensins mature into a secreted peptide after pre-pro-peptide state. This mature peptide has six cystine residues connected with intramolecular disulphide bonds. The connectivity of these disulphide bonds as well as the number of residues are factors differentiating α- and β-defensins [54]. The importance of a balance between the hydrophobicity of the defensins and their net positive charge is highlighted in the activity of these peptides towards the bacterial membrane [55]. The crystal formation of defensins usually results in the production of a dimeric structure. Crystallographic studies of α- and β-defensins show that free-form α-defensins have three intramolecular disulphide bonds (Paneth cell defensins and innate immunity of the small bowel) and they form a dimeric structure with six β-sheets [56]. The monomeric structure of β-defensins consists of three β-sheet folds as well as a helical N-terminus [16]. Nevertheless, defensins undergo conformational changes when introduced to a bacterial membrane. Targeted approaches on a specific protein may render the AMPs useless against the bacteria due to bacterial resistance and genetic changes to its conformation. Therefore, it is important that defensins work as non-specific agents on the membrane of the bacterial cells. Bacteria resistant to antimicrobial peptide activity usually display enzymatic covalent modification on their membrane, which reduces their negative charge.

α- and β-defensins are generally known for their cluster of positively charged amino acid residues. Although the sequence of amino acids is highly variable in defensins, their cysteine residues and their framework are highly conserved [17]. DEFA1-3 and human β-defensins are stored as mature peptides, whereas DEFA5 is stored in its pro-peptide form [57, 58]. The dimerization of β-defensins in solution is shown to be the functional structure for recognition of microorganisms [16]. However, DEFA1 takes a non-dimeric structure in solution, forming a

voltage-dependent channel in the planar lipid bilayer [59]. This specific example also shows that the interaction of DEFA1 with the membrane phospholipid depends on the presence of anionic phospholipids on the membrane. On the other hand, DEFA2, an α-defensin similar to DEFA1 (lack of alanine at position 1), requires the assembly of approximately two dimers for the formation of pores in uni-lamellar vesicles [60]. DEFA1 and DEFA2 are the potent forms of α-defensins and DEFA3, differing by only an additional amino acid at the N terminus, is known to be less active against *C. albicans* [61]. The dome-shaped, three-dimensional structure of amphiphilic defensins has been hypothesized to have the N- and C-termini at the two ends and the hydrophobic section having an amphiphilic structure at the lowest portion. The functional diversity of defensins and their potency mainly depend on the N- and C-terminal residues [26]. A study showed that arginine-rich cationic defensins provide a higher spectrum of antimicrobial activity due to their higher cathodal electrophoretic mobility [62].

The concentration of defensins in epithelial cells averages 10–100 μg $ml^{-1}$ although the uneven distribution of these molecules results in a higher local concentration [17]. Defensins attack Gram-positive and Gram-negative bacteria with the same mechanisms; however, the attack is on the cell wall and the outer membrane of the bacteria, respectively. Accumulation of cationic peptides close to the negatively charged surface in Gram-negative bacteria may lead to binding and crossing of defensins into the cell via a charge-exchange mechanism by competing with $Ca^{2+}$ and $Mg^{2+}$ bound to lipopolysaccharides [63]. Although the porous surface of Gram-positive bacteria allows ease of movement for the defensins [64], safe passage of defensins through the cell wall allows via the same mechanisms to attack the membrane bilayer. Generally, defensins are known to interact with lipopolysaccharides, polysaccharides and phospholipids of Gram-negative, Gram-positive and bacterial membrane bilayer, respectively [65].

Defensins are active against bacteria at a concentration of 1–10 μg in optimal conditions such as low ionic strength conditions, low concentrations of proteins or other substances interfering with this activity. However, cellular conditions are harsh and the salt as well as protein concentrations inhibit defensins' antimicrobial activity depending on the sequence and bacterial target of the defensin [66]. In addition, higher concentrations of defensins have been shown to have toxic effect towards mammalian cells, specifically lung tissue [67]. Permeabilization of defensins renders the production and synthesis of DNA and their subsequent RNA and protein. The extent of interaction of defensins with the bacteria depends on the amino acid backbone and the flexibility of this chain allowing for the presence of potential spatial interactions with the head of phospholipid groups in the membrane [68].

Defensins provide their antimicrobial activity by the creation of pores or membrane disruption, which both lead to the release of cellular contents [69]. Other regulatory factors such as wound closures [70], fibroblast proliferation and chemotaxis of T-cells and dendritic cells can also be mentioned as the activity of defensins. Defensins also have a modulatory effect on the production of cytokines [71]. Other activities of defensins include regulation of immune and inflammatory regions by providing chemotactic activity for monocytes, T-cells and dendritic cells. In addition, nanomolar concentrations of defensins can also activate nifedipine-sensitive calcium channels of mammalian cells, reducing the electrical conduction [72].

Overall, defensins are important molecules for providing a cascade of antimicrobial activity in the human body in defence against pathogens and they are mainly concentrated in regions that are more prone to pathogenic attack.

## 4. Defensin-like peptides immobilized on material surfaces

Bacterial adhesion and colonization resulting in biofilm formation on the surface of biomaterials are responsible for most medical device-associated infections and malfunctions [73, 74]. The magnitude of this problem continues to pose a significant problem to health care providers, often resulting in major negative medical and economic consequences [75, 76]. Many of the currently used medical devices are amenable to modification, either by functionalization or coating of the surface of the device, making it possible to combat inflammation and reduce the risk of infection [77].

In general, device-associated infections have been treated by developing material surfaces containing antibiotics and biocides [78]. The use of this approach comes with the risk of cytotoxicity, raising concern as a potential threat to human and environmental health. An alternative to this is the use of antifouling coatings, making it possible to prevent attachment of bacteria, proteins and other microorganisms. This more passive approach makes it possible to prevent antibiotic resistance and leaching of cytotoxic biocides but is not capable of killing already adherent bacteria and makes it hard to avoid infection completely [79]. Therefore, a combination of antifouling and antimicrobial properties is favourable to combat biofilm formation and further reduce the risk of infection [80, 81].

Defensin-like peptides (i.e., antimicrobial peptides, AMPs) exhibit a combination of antimicrobial and antifouling properties, which is why AMPs have received significant attention as an alternative to conventional biocides and antibiotics, showing the ability to overcome and combat medical device-associated infections. However, so far, there is little success in the development of AMPs for therapeutic applications, with only a few AMPs that have been approved for medical use at their initial introduction. After the unsuccessful introduction of the 'first-generation' AMPs, research has been performed on reducing cytotoxicity. It was found that AMPs were increasingly more toxic when having hydrophobic characteristics, sufficient enough to interact with neutrally charged eukaryotic cell membranes [82]. By replacing or interrupting these hydrophobic regions of AMPs, cytotoxicity was reduced and only showed a slight decrease in antimicrobial activity, providing the amphipathic characteristics were maintained [83]. Also, developing narrow-spectra AMPs would decrease the required concentration needed to combat pathogens and additionally prevent the cytotoxic activity towards eukaryotic cells [84].

In order to further reduce the cytotoxicity, immobilization of AMPs onto material surfaces is a potential approach to reduce the concentration needed when in free-form and will also increase their half-life time. The half-life time of AMPs is found to be based on the rate of protease digestion or related to peptide aggregation [85]. In order to compensate for their relatively short half-life time, increased concentrations of AMPs are used; but, this results in increased cytotoxicity and has limited the use of AMPs. Other efforts to increase the half-life time of peptides have been focused on using substitutes for L-amino acids, because unprotected peptides are more rapidly metabolized (i.e., broken down for nutrition) [86]. By substitution of L-amino acids by D-amino acids, the introduction of unnatural β- and ɣ-peptide bonds and modifications of the N- or C-terminus, it is possible to increase the stability of AMPs [87–89].

Overall, it is of great importance to improve the stability of AMPs against degradative mechanisms *in vivo* and increase the bond stability between the AMPs and materials in order to develop almost non-cytotoxic and long-lasting antimicrobial

surfaces [90, 91]. Therefore, by limiting the necessity of implant removal due to AMP inactivity caused by degradative mechanisms, patient compliance can be increased.

### 4.1 AMP immobilization methods

It is possible to immobilize AMPs through two main pathways, either physically or chemically. A popular physical method is layer-by-layer assembly, in which AMPs are 'sandwiched' between two polyionic polymers, making it possible to integrate a controllable loading of AMPs [92, 93]. However, the interspersed AMPs within the polyionic polymer layers are not able to interact with the surrounding environment, and they will need to diffuse outwards to utilize their antimicrobial activity. Therefore, with the use of physical immobilization, it is difficult to fully utilize the potency of AMPs. Covalent-based immobilization of AMPs has significant advantages, in comparison to physical immobilization, such as the formation of more stable bonds and thus improvement of their relatively short half-life time, while also minimizing the possibility of leaching of AMPs [90, 94].

Furthermore, the material surface is also of great importance to the stability of AMPs. A polymer surface (i.e., dibromomaleimide polymer substrate) prepared by chemical vapour deposition (CVD) and functionalized with AMPs showed a better antimicrobial stability when compared to a self-assembled monolayer (SAM). When exposed to air, this polymer showed slower detachment of bound AMPs when compared to SAM [95]. Additionally, orientation of AMPs was also retained. These results show the importance of using a non-degradable material surface to improve bond stability of AMPs. Next to that, the surface morphology needs to be well defined as an undefined surface could lead to inhibition of attachment of biological molecules. Also, depending on the density of the functional groups that are present or able to be induced, the number of peptides on the material surface can vary significantly. However, even though AMP concentration is of importance, it does not appear to be the most critical criterion for the improvement of antimicrobial activity [96, 97].

The antimicrobial activity of covalent-based surface-immobilized AMPs is seen to be mainly dependent on the used coupling strategy, spacer specifications and peptide orientation and concentration [98]. However, there are certain limits to improving the antimicrobial activity by increasing the AMP concentration, due to factors such as coupling conditions and steric hindrance (i.e., repulsive forces originating from overlapping electron clouds of neighbouring molecules). Next to that, some microorganisms are found to be insensitive to any further increase after reaching a certain limit in AMP surface density or exposure time [99].

It is demonstrated that direct-immobilized AMPs also show antimicrobial activity without the use of a spacer. Nevertheless, most potent developments make use of spacers, the length (i.e., the distance between the material surface and peptide) of which is shown to significantly influence the activity of surface-immobilized AMPs, when compared to the AMP surface density [90]. Conversely, the possibility of chain cleavage of the spacer due to polymer degradation reactions could lead to the release of immobilized AMPs. This could be minimized with the use of stabilized polymer spacers [59]. The increased activity seen with the use of spacers is a result of improved mobility of AMPs, increasing probability of membrane permeabilization and subsequent cell death. However, this would only be a correct hypothesis assuming that AMPs demonstrate a similar mode of action to that of their free-form counterparts. According to the literature, there are also AMPs that will depolarize the cytoplasmic membrane and disrupt the electron transport, which subsequently would lead to partial membrane permeabilization and thus cell death [100, 101].

The difficulty of these developments is the lack of comparable information, since most of the observed reactivity and antimicrobial activity are found to be specific to the reactant environment, type of materials and AMPs. Fully understanding these structure-function relationships is important to clarify and improve the performance of surface-immobilized AMPs in future developments.

### *4.1.1 Orientation of direct immobilized AMPs*

When a peptide is directly bonded to the material surface, as displayed in **Figure 6**, the immobilized terminus determines its orientation. When α-helical peptides are immobilized at their N-terminus, they will point perpendicular to the material surface (i.e., orientate upstanding), but when immobilized at their C-terminus, they will take on a laying-down orientation. In general, when an AMP has its N-terminus bonded to the surface, it has been shown to have a lower MIC when compared to C-terminus and N-side-chain-immobilized AMPs [102]. The relatively high MIC of C-terminus-immobilized AMPs is likely related to the inhibition of membrane interaction [51]. However, according to the literature reporting the antimicrobial activity dependence of the orientation difference between N-terminus and C-terminus-immobilized peptides, there is also a cell-dependent potency, which indicates that the mode of action of AMPs is not only dependent on their own characteristics but also on the characteristics of the targeted microorganism [103]. Another important parameter is the position of the cationic amino acids. When they were closer to the bonding site, an increased antimicrobial activity was observed, while, when they were positioned in the middle or closer to the N-terminus, a decrease of the antimicrobial activity was observed [100].

However, as shown in **Figure 7**, the orientation of AMPs changes after their initial interaction with bacteria (i.e., the immobilized α-helices bind to the anionic lipid bilayer). Since immobilized AMPs cannot follow the *barrel-stave* or *toroidal model* due to limited mobility, it is suggested that the charge-charge interaction plays a dominant role in the elimination of bacteria [104, 105].

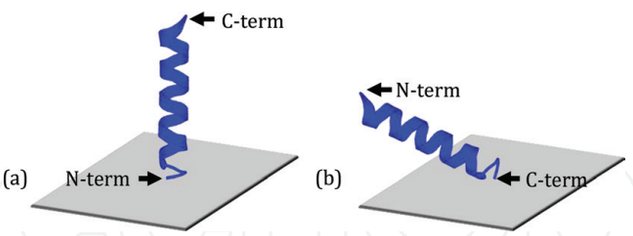



**Figure 6.**
*Schematic of (a) N-terminus and (b) C-terminus surface-immobilized α-helical peptide; not drawn to scale or exact structure.*

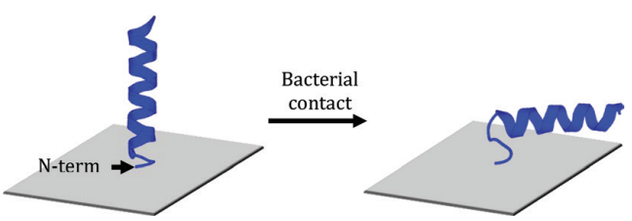



**Figure 7.**
*Schematic of orientation change of N-terminus-immobilized α-helical peptide before (left) and after (right) bacterial contact; not drawn to scale or exact structure.*

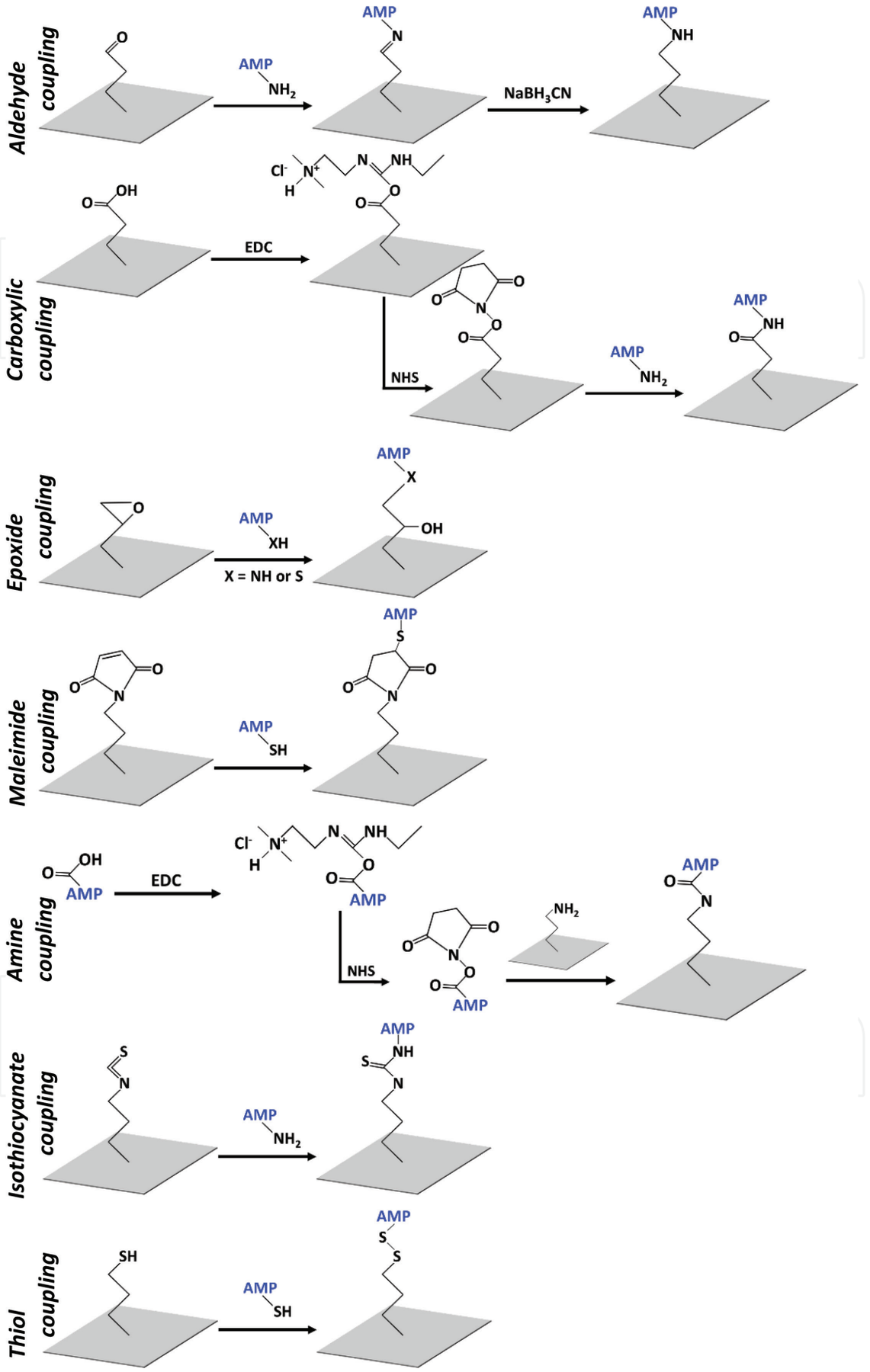



**Figure 8.**
*Immobilization of AMPs onto numerous functional groups, using different coupling methods; not drawn to scale or exact structure.*

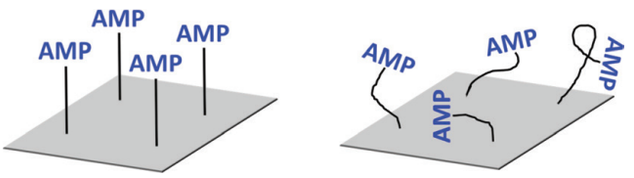



**Figure 9.**
*Schematic of spacer-incorporated immobilized AMPs, showing stiff spacers (left) and flexible spacers (right); not drawn to scale or exact structure.*

Additionally, when functionalizing the material's surface with different reactive groups as seen in **Figure 8**, the orientation of immobilized AMPs can be controlled using chemo-selective (i.e., directed immobilization) coupling reactions. In general, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) are found to be widely used as activating agents for the coupling of peptides to numerous functional groups by forming amides, which inhibit the formation of carboxylic salts with an amine [98, 106].

### *4.1.2 Spacer-incorporated immobilized AMPs*

Although orientation can be partly controlled with the use of chemo-selective coupling agents, they often do not add enough mobility to increase the probability of membrane permeabilization or polarization. In order to improve the mobility and subsequent activity of AMPs, the use of spacers is found to be useful. As shown in **Figure 9**, there are two types of spacers, stiff and flexible spacers. Stiff spacers (e.g., polyvinyl chloride or polymethyl methacrylate) allow the increase of reach and thus might allow membrane permeabilization, but will restrict sideways mobility by keeping the AMPs pointing in a specific orientation [98]. Flexible spacers such as polyethylene glycol (PEG) also allow the increase of reach and are able to allow sideways mobility; however, orientation cannot be determined due to their flexible chains [107].

However, even if the peptide was linked to a PEG spacer, the random orientation of an immobilized AMP through its C-terminus is found to result in the loss of antimicrobial activity [97]. However, the oriented immobilization of the same AMP through its N-terminus is found to restore the antimicrobial activity. It has also been suggested that the water-swelling property of PEG aids in maintaining the activity of immobilized peptides [108]. Additionally, in the absence of the PEG spacer, the AMP did not show antimicrobial activity. Nevertheless, the utilization of PEG as a spacer is found to present numerous advantages, as it can create non-adhesive surfaces due to its non-fouling characteristics (i.e., inhibition of microorganisms binding to the material surface) [108]. Lastly, the solubility and stability of peptides, against protease digestion or peptide aggregation, can be improved with the use of a spacer [107, 109, 110]. These previously mentioned factors show yet again the complexity of the factors on influencing the antimicrobial activity of immobilized AMPs.

## 5. Conclusions

AMPs are demonstrated to show antimicrobial activity at relatively low concentrations, without damaging mammalian cells, being able to utilize several mechanisms against numerous microorganisms similar to defensins in the human body. Immobilization of AMPs improves their lifespan, preserves the mode of action and does not seem to influence the mechanism on the biological level; however, they

do show reduced antimicrobial activity upon immobilization to material surfaces. Nonetheless, this reduced activity can be partly restored with the use of chemo-selective coupling agents and the incorporation of spacers. Whether degradation of the material and coupling agents is desirable or not, understanding the kinetics is of great importance as the decrease of structural integrity and/or release of particles (i.e., molecules, debris, etc.) might result in an adverse biological reaction. Nonetheless, there are significant indications that AMPs are suitable candidates to replace conventional biocides and antibiotics. In addition, they can be utilized to develop the next generation of antimicrobial surfaces as coatings for medical devices and implants.

## Conflict of interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, or other equity interest; and expert testimony or patent-licensing arrangements) in the subject matter or materials discussed in this chapter.

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