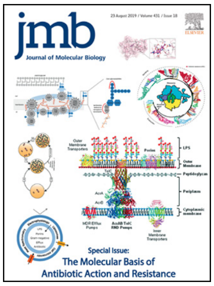

Peptide Design Principles for Antimicrobial Applications

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Abstract

The increased incidence of bacterial resistance to available antibiotics represents a major global health problem and highlights the need for novel anti-infective therapies. Antimicrobial peptides (AMPs) represent promising alternatives to conventional antibiotics. AMPs are versatile, have almost unlimited sequence space, and can be tuned for broad-spectrum or specific activity against microorganisms. However, several obstacles remain to be overcome in order to develop AMPs for medical use, such as toxicity, stability, and bacterial resistance. We lack standard experimental procedures for quantifying AMP activity and do not yet have a clear picture of the mechanisms of action of AMPs. The rational design of AMPs can help solve these issues and enable their use as new antimicrobials. Here we provide an overview of the main physicochemical features that can be engineered to achieve enhanced bioactivity and describe current strategies being used to design AMPs.

Antimicrobial Peptides

The current increase in multidrug-resistant bacteria is an alarming global health problem. In fact, antibiotic-resistant infections [1,2] are expected to cause 10 million deaths annually by the year 2050 if no new antimicrobial approaches are implemented [3]. Antimicrobial peptides (AMPs), produced by virtually all organisms on Earth, offer an alternative to conventional antibiotics.

AMPs are part of the host's defense system. These agents are key components of the innate immune system of a number of organisms, including humans. Their mode of action is diverse and dependent on their structure and biophysical parameters, as described by Nguyen *et al.* [4]. These authors, highlighting well-known structure–activity studies, provide insights into the mechanisms by which AMPs act on membranes. Most AMPs disrupt the membrane of microorganisms in the sub-micromolar or micromolar range [5].

Important mechanisms of action of AMPs include the carpet model [6], in which peptides solubilize the membrane into micellar structures destabilizing it; peptides lying on the membranes reach a threshold concentration and spontaneously insert themselves in the barrel-stave model [6] or form pores with peptide and lipids disposed alternately in the toroidal pore model [6]. In additional to peptides forming pores by interacting with other peptides and lipids that form the bilayer, some peptides and lantibiotics can also mediate pore formation through interaction with the peptidoglycan lipid II from Gram-positive bacterial membranes [7–9]. These peptides interact directly with both glycan and peptide portions of lipid II that act like a mediator facilitating their insertion in the lipid bilayer [8,10]. Other specific or less common mechanisms of action of AMPs have been described, such as membrane thickening/thinning [11], charged lipid clustering [12], nucleic acids targeting [6], anion carriers [13], electroporation [14], non-lytic membrane depolarization [15], and non-bilayer intermediates [16]. Among the specific modes of action of AMPs, their effect on lipid phase behavior is currently a topic of interest [17]. Several peptides may interfere with processes underlying how lipids cluster, especially cationic amphipathic AMPs independently of their conformational tendency [18–20]. AMPs may also act by modulating lipid membrane curvatures [21,22] through direct interactions with lipids that compose the membranes and stressing and disrupting the structure of the bilayers [23].

In addition, AMPs can interfere with cellular processes and metabolic pathways [24,25]. Recently, some AMPs were also reported as immunomodulatory compounds [26,27]. For example, cNK-lysin, the chicken homolog of human granulysin, is a peptide produced by cytotoxic T cells and natural killer cells. cNK-lysin and its synthetic derivatives exhibit antimicrobial activity against apicomplexan parasites via membrane disruption. The immune-modulatory activities of cNK-lysin derivatives involve the mitogen-activated protein kinase-mediated signaling pathway, including p38, extracellular signal-regulated kinase 1, and c-Jun N-terminal kinases, as well as their internalization into cells [26]. This newly recognized ability of AMPs to modulate both innate and adaptive immune responses has led many researchers to refer to these molecules as host defense peptides. Other mechanisms of action include intracellular targets, such as nucleic acids and proteins [28,29], cell wall biosynthesis [30], cell division [31], RNA synthesis [32], inactivation of chaperone proteins necessary for proper folding, and even targeting mitochondria [33].

Regarding their immunomodulatory functions, AMPs generally display specificity toward particular pathways and immune cells [34–37], such as immunostimulatory upregulation of cytokines, which increases anti-tumor immunity [34], controlling lactic acid formation, which causes casecidins to have immunostimulating action [35], stimulating lymphocyte proliferation [36], or inducing macrophages, and reducing expression of lipopolysaccharides, among others [37]. In addition, membrane-active peptides are well known and have been studied for decades. However, the precise mechanisms by which they interact with membranes and cells are still not fully understood. It is generally accepted that peptides initially interact with membranes of microorganisms via electrostatic interactions, which are enabled by the cationicity of AMPs and the net negative charge of bacterial membranes. Hydrophobic interactions play a key role when AMPs come into contact with membranes, thus highlighting amphiphilicity as an important mechanistic feature of this family of peptides.

Subsequent to contacting the membrane, peptides usually fold into the most stable structure possible, according to their sequence or composition and the balance of their intra- and inter-molecular interactions. Most AMPs have helical structures; nevertheless, some sequences tend to adopt beta-like structures. In their stable conformation, these peptides interact with membranes, eventually leading to membrane lysis or incorporation of the peptide into the cell.

Several outstanding challenges have prevented widespread implementation of AMPs in the clinic. For example, AMPs are sometimes hemolytic and cytotoxicity, linear L-amino acid-containing AMPs are susceptible to degradation by host proteases, which requires delivery vehicles to introduce them into the body, and may lose biological function in the presence of high-salt content environments. Here we provide an overview of design tools that may be harnessed for optimizing currently available AMP templates.

Strategies for Determining Structure–Activity Relationships

AMP structure–activity relationship (SAR) studies can be used to address ways to systematically modify naturally occurring molecules or *de novo* designed synthetic peptides and to determine both their structure and their biological activities. The overall aim is to maximize antimicrobial activity and resistance to proteolytic degradation while minimizing toxicity toward the host. The most well-known methodologies that have been used to design new AMPs and guide SAR studies are site-directed mutagenesis, computational design approaches, synthetic libraries, template-assisted methodologies, and mechanism-based strategies. These strategies are conceptually different but share the same goal of designing improved peptides (Fig. 1).

Site-directed mutagenesis

This approach involves re-engineering natural peptides by adding, deleting, or replacing one or a few amino acid residues. The functional effects of single substitutions on the template peptide can be readily understood. Systematic mutation of peptide molecules is needed to cover as much sequence space as possible and to identify peptide variants with improved activity. Alanine [38–40] or lysine [41] scannings are examples of useful approaches [42], as they allow for coverage of all positions within a peptide chain, thus making it possible to analyze the effect of all amino acid side chains on structure and function. C-terminal [43–45] and N-terminal modifications [46–48] are additional engineering approaches that can be used

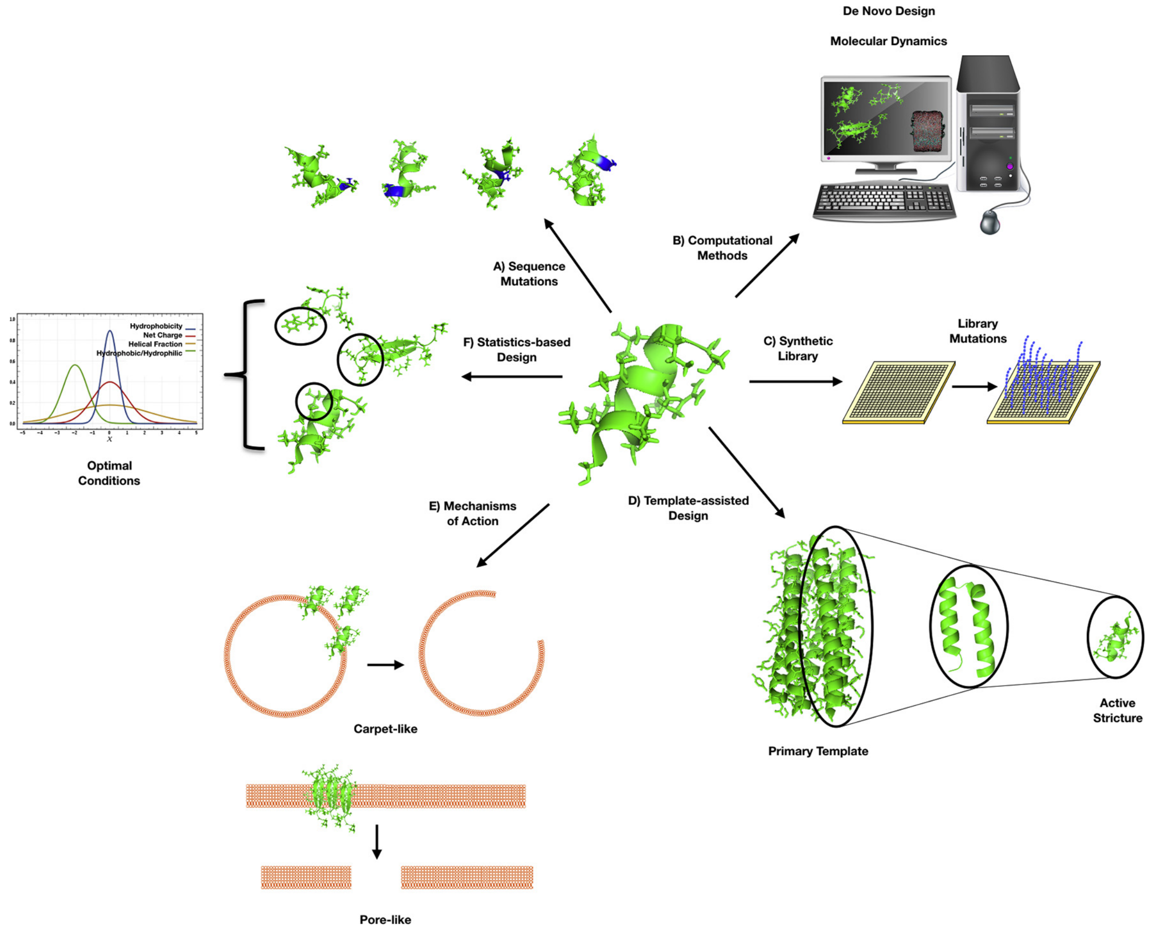

Fig. 1. Schematic showing SAR strategies commonly used for peptide design. (A) Sequence modifications by adding, deleting, or replacing amino acid residues. (B) *De novo* design of amphipathic structures. (C) Synthetic libraries containing simple mutations based on biophysical features. (D) Sequence patterns with relevance to biological activities. (E) Use of biophysical assays or molecular dynamics to predict important properties based on the mechanism of action. (F) Use of databases to extract statistically relevant features for designing biologically improved peptides.

to enhance the tendency of peptides to assume a particular secondary structure via simultaneous modulation of intramolecular interactions and hydrophobicity. Another example is peptide shuffling [49,50], in which residues that are part of a sequence are shuffled, generating sequences with the same composition but with the amino acids in different orders. This method, however, involves more complicated downstream analysis to isolate those sequence requirements associated with the desired function.

De novo design

Generally, *de novo* AMP design involves favoring an amphipathic structure [51–53], which summed to other physicochemical features are optimal for antimicrobial activities. Through this approach, peptides present both basic and hydrophobic residues in a given sequence, so that they form hydrophobic and hydrophilic regions. The disadvantage of this method is that it "misses" subtle properties of AMP sequences that may have been selected for by evolution, and the resulting peptides may lack specificity or have different folding patterns than native AMPs, as well as other undesired functions, such as increased cytotoxicity [54].

Synthetic libraries

Synthetic combinatorial library design is a powerful and widely used tool for rapidly obtaining optimized classes of active peptides [55,56]. One problem with using this technology for the development of AMPs is the relatively large size of these libraries, which makes positional scanning or iterative approaches costly. Furthermore, it becomes difficult to screen for function, although progress has been made in building more cost- and time-effective screening methods [57]. The variance implicit in the combinatorial approach can be reduced by limiting the number of amino acid types used to construct the library based on their biophysical features and size [58]. For example, in screening for the effects of hydrophobicity on activity, representative aromatic and aliphatic residues may be selected as opposed to probing every hydrophobic residue within the peptide sequence. These biased design strategies help reduce the complexity inherent in large peptide libraries.

Template-assisted approach

Template sequences can be obtained by comparing the sequences of naturally occurring peptides and extracting significant patterns important for function [59,60], for example, the type of residues (charged, hydrophobic, etc.), and then using these to design AMPs *de novo* [61]. The advantage of this method with respect to conventional sequence modification methods is that it reduces the number of peptides that need to be synthesized in order to obtain promising results, thus reducing cost while maintaining some sequence-based information. That information may be lost in more minimalist approaches such as sequence mutations and *de novo* design.

Statistically based computational methods

AMP databases are available (Table 1) and can be harnessed to enhance and facilitate AMP design. Various bioinformatics tools have been developed, including simple statistical modeling, SAR studies, neural networks [81], genetic algorithms [82], deep learning [83,84], and machine learning [85–87]. In general, these bioinformatics-based tools require a database of peptides with known antimicrobial activity. An extended overview of AMP databases and data mining was recently described by Porto *et al.* [88]. Using these computational methods, key structural and biophysical features can be extracted from large databases, and this information can be used to predict [89] or further enhance the antimicrobial activity of the peptides [90].

Mechanism-based methods

Computational methods are usually simple but provide little information about the mode of action of AMPs, such as how these agents interact with microbial membranes of high compositional complexity. Therefore, additional methods are needed to more comprehensively evaluate peptide–membrane interactions. Molecular dynamic simulation represents a powerful method that provides information at the atomic level of interactions between AMPs and bacterial membranes. If the timescale of the simulation is long enough (ranging from about 20 to 100 ns, depending on the specific AMP and type of membrane), membrane disruption or pore formation can be directly observed [91]. However, there are specific simulations of events (e.g., peptide–membrane interactions [92]) that require high computational cost and time. This method has been used to elucidate simple modes of action of AMPs and has been applied to design new AMPs and antimicrobial peptidomimetics, which act by pore-forming or disrupting mechanisms [93,94]. Peptidomimetics are molecules generated to mimic natural peptides three-dimensionally in order to preserve their biological activity and prevent common issues with natural peptides, such as stability, resistance to proteolysis, and bioavailability [95].

Important Features for Peptide Design

The physicochemical properties of complex molecules are generally addressed by descriptors, which are computational vectors that provide information about physicochemical parameters of amino acids in a given peptide chain. As peptides are large and as they present secondary, and sometimes tertiary structures, they are considered complex molecules. Slight modifications in the amino acid composition can change the whole geometrical disposition and physicochemical properties of a peptide. Basic features such as sequence and hydrophobic/hydrophilic ratio are as important for designing peptides as complex anisotropic structural properties (Fig. 2).

Sequence motifs

AMPs present a diversified composition and their sequence motifs are a major focus of investigation among peptide researchers. Several authors have proposed that the antimicrobial activity of peptides does not depend on specific amino acid sequences or peptide structures [96–98], but instead on their overall amino acid composition (basic, acidic, aliphatic, or aromatic) and physicochemical features [99]. Indeed, these features are intrinsic to specific amino acids and subtle changes may lead to major functional differences that in turn affect the ability of these peptides to kill microbes and their toxicity toward host cells.

In order to understand and propose the SARs of this class of molecules, it is necessary to dissect the biophysical and biochemical properties of amino acids and the changes in their behavior resulting from specific mutations. Currently, we do not fully understand SARs of AMPs [100,101], and it is for this reason that researchers rely heavily on statistical methods. Several problems arise with such methods. First, two or more peptide molecules with different amino acid compositions may be used as templates for this approach. Second, the origin of the templates is usually not considered, and therefore, the peptides might belong to different peptide families, leading to a design that is not judiciously refined.

Table 1. Functional databases containing antimicrobial, anticancer, and other peptide activities[a]

Database	Content	Entries	Website	References
DADP	Peptides that constitute anuran immune system	2571	http://split4.pmfst.hr/dadp/	[62]
APD3	Peptides active against vary organisms, antioxidant and protease inhibitor peptides	2850	http://aps.unmc.edu/AP/main.php	[63]
CAMP$_{R3}$	AMP, structure, patents, and signatures	8164	http://www.camp3.bicnirrh.res.in/index.php	[64]
SATPdb	Curated from 20 public domain peptide databases and two data sets	Combined entries from the databases	http://crdd.osdd.net/raghava/satpdb/	[65]
NeuroPep	Neuropeptides as potential therapeutic targets for the treatment of some nervous system disorders	5949	http://isyslab.info/NeuroPep/	[66]
PepBank	Database of peptides based on sequence text mining and public peptide data sources	21691	http://pepbank.mgh.harvard.edu/search/basic	[67]
SPdb	Signal sequences of archaea, prokaryotes, and eukaryotes	27433	http://proline.bic.nus.edu.sg/spdb/	[68]
DRAMP	Open-access and manually curated database with sequence, structure, antimicrobial activity, physicochemical, patents, clinical trials, and references information	17508	http://dramp.cpu-bioinfor.org/	[69]
ADAM	A comprehensive AMP database with sequence–structure relationships	7007	http://bioinformatics.cs.ntou.edu.tw/adam/	[70]
Hemolytik	Experimentally validated hemolytic and non-hemolytic peptides	2970	http://crdd.osdd.net/raghava/hemolytik/	[71]
PhytAMP	PlantAMPs	271	http://phytamp.hammamilab.org/main.php	–
Cybase	Tail-to-tail cyclic peptides	2357	http://cybase.org.au/	[72]
MilkAMP	Antimicrobial dairy peptides, including microbiological and physicochemical data	405	http://milkampdb.org/home.php	[73]
Peptaibol	Database of short peptides with non-standard amino acid residues and termini modifications	317	http://peptaibol.cryst.bbk.ac.uk/home.shtml	[74]
Defensin Knowledgebase	Manually curated database of the defensin family of AMPs	363	http://defensins.bii.a-star.edu.sg/	[75]
Bactibase	Sets of bacteriocins with detailed analysis of a number of microbiological and physicochemical data	229	http://bactibase.hammamilab.org/main.php	–
AVPdb	Experimentally verified antiviral peptides	2683	http://crdd.osdd.net/servers/avpdb/	[76]
ConoServer	Sequence and structures of conopeptides	176	http://www.conoserver.org/	[77]
CPPsite 2.0	Cell-penetrating peptides (linear, cyclic modified and with non-natural residues) and wide-range delivery cargos	1700	http://crdd.osdd.net/raghava/cppsite/	[78]
Neuropedia	Information about neuropeptides, their structure and a spectr library	847	http://proteomics.ucsd.edu/Software/NeuroPedia/	[79]
LAMP	AMPs	5547	http://biotechlab.fudan.edu.cn/database/lamp/	–
BaAMPs	AMPs tested specifically against biofilms	219	http://www.baamps.it/	[80]
CancerPPD	Anticancer peptides and proteins verified experimentally	3491	http://crdd.osdd.net/raghava/cancerppd/	–

[a] Last checked on June, 2017.

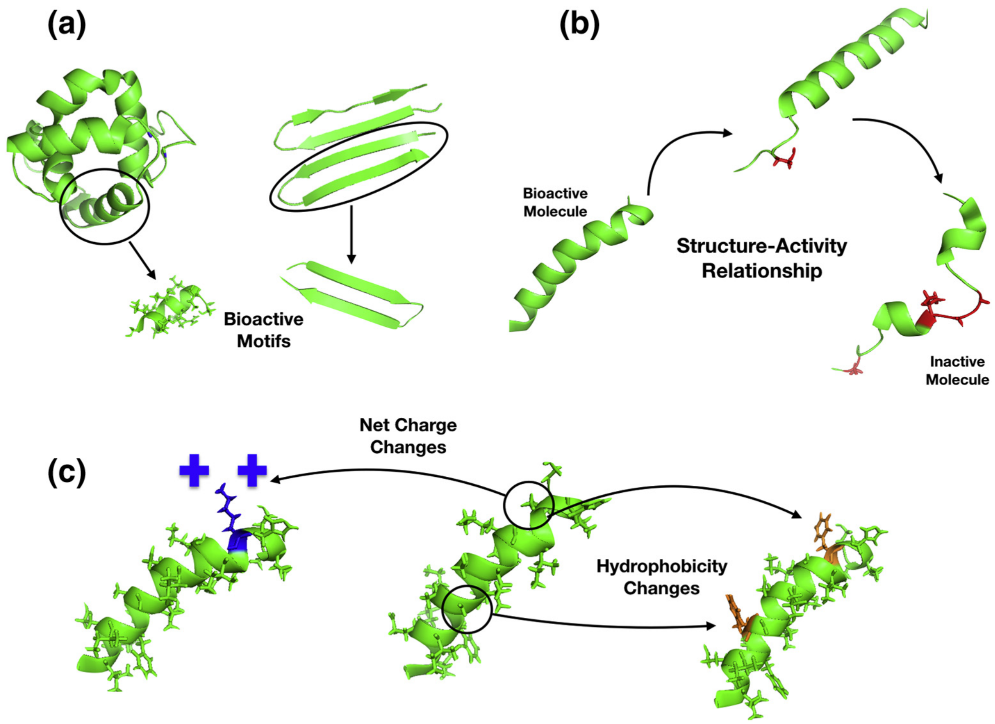

Fig. 2. Summary of biophysical and structural features relevant for enhancing antimicrobial activity. (a) Amino acid sequences and motifs important for designing new templates of active peptides. (b) Changes in structure and amphipathicity may lead to abrupt modification in important biophysical properties and potentially to loss of biological activity. (c) Positive net charge and hydrophobicity are the most common changes made to enhance selectivity and antimicrobial potency without disrupting the amphipathic structure of the designed peptides.

Peptide sequence-based design often ensures the conservation of residues within certain regions of peptide molecules, even if these residues do not have the same length or the same effect on the overall structure of the peptide. The most common features relevant for AMP design are net charge, hydrophobicity, and helix penalty of each residue separately [102]. Penalty is defined as the amount of energy required to transition from an unstructured conformation into a helical structure [103].

Sequence-based design can be executed via insertion or substitution of the first residue of a given peptide by a glycine residue [104]. This is an effective capping residue for helical structures because it confers resistance to aminopeptidases, which cleave peptides at their N-terminus. Another way is amidation of the C-terminal, which confers resistance to carboxypeptidases and displaces the net charge toward more positively charged molecules. Additional modifications that can be performed include the introduction of restrictors such as lactam and disulfide bridges, which are commonly used as conformational inductors (β-like structures) and can provide resistance to degradation by proteases [105,106]. A well-known method that is widely used for identifying the role that specific residues play is mutational scans, in which all residues are substituted one at a time for another residue with a small aliphatic side chain (e. g., alanine or valine, thus called Ala-scan and Val-scan, respectively), and the effect of each side chain on topological, biophysical and functional properties of the peptides is analyzed [38].

The helical wheel proposed by Schiffer and Edmundson [107] is a widely used tool for sequence change design of helical peptides, which represent the majority of AMPs. This tool provides a good approximation of the sequence of residues along the helical structure, facilitating substitutions or insertions in specific positions for modifying important parameters that directly influence the biological activities of these peptides.

Structure

The structures and conformations peptides undergo during the process of interacting with biological membranes have been described as one of the key aspects determining the antimicrobial activity of AMPs [108]. The main structural tendencies adopted by AMPs when in contact with membranes are α-helical and β-like [109]. Most peptides with activity against microorganisms are helical; accordingly, this conformational tendency is the most well studied. Lifson and Roig's helix-coil transition approach [110] is the most accepted theory of how helical structure is preferred over other types of three-dimensional arrangements. This theory proposes that most cationic amphipathic peptides that present antimicrobial activity are unstructured in the presence of polar protic medium, such as water or buffer solutions, where bacteria grow. The net positive charge of these molecules leads to electrostatic interactions between the peptide and the membrane thus enabling the peptide to reach the membrane. After this initial contact, the amphipathic sequence tends to reorganize, while coulombic interactions are interchanged by hydrogen bonds stabilizing both portions of the molecule. One of the portions is hydrophobic, where aliphatic and hydrophobic residues tend to interact with the lipids buried in the membranes, and another hydrophilic, where polar charged and uncharged residues continue to interact with polar protic solvents through hydrogen bonds between side chains and water. This stable helical secondary structure is optimal for peptide interactions with the membrane core destabilizing it and/or penetrating into the cell and reaching the intracellular medium by an entropy-driven process and potential difference, caused by negatively charged headgroups of lipids that compose the internal part of the lipid bilayer [102].

Torres *et al*. [111] observed that Polybia-CP activity was closely tied to its helical activity. By means of a systematic approach, the authors showed that by single substitutions of Polybia-CP original residues, it was possible to predict the helical tendency and that increased helicity of the peptide led to increased activity against Gram-negative, Gram-positive bacteria and fungi *in vitro*, and augmented anti-infective activity in a skin scarification mouse model.

In addition, Fázio *et al.* [112] showed the significance of the β-turn structure to all biological activities of gomesin. The authors designed analogs suppressing and displacing the disulfide bonds of the peptide and observed that increasing β-like tendency of the peptide improved the antimicrobial activity, whereas unrestrained analogs lost their activity.

Important design choices for manipulating peptide structure include the introduction of conformational stabilizers or destabilizers, the variation of the amphiphilicity, and the generation of changes in peptide net charge. Examples of helix-stabilizing residues are leucine, alanine, valine, isoleucine, lysine, and arginine, and prototypical destabilizing residues are proline and glycine [113]. Capping the N-terminus or C-terminus [114] may lead to changes that can be detected as variations in the electronic distribution of peptides. Other peptide-stabilizing effects involve the interaction of charged residues with the helix dipole, salt bridging between residues with opposite charges (usually at $i + 3$ or $i + 4$ positions of the helical step), and the interaction of residues in the hydrophobic face of the amphipathic helix with the acyl chains of phospholipids in the membrane bilayer.

The energy involved in structure formation is also taken into consideration when a peptide is designed [115]. For example, there are estimated differences in free energy ($\Delta\Delta G°$) between residues or even residue conformers, given in kcal/mol per residue in a helical configuration, as described by Pace and Martin Scholtz [103]. The factors that determine helix propensities are not completely understood, but several approaches have been proposed to increase helicity: burying a hydrophobic surface in the helix [113,116], reducing unfavorable steric contacts in the helix [117], screening for electrostatic effects [118], and incorporating favorable side chain-to-helix van der Waals interactions [119]. More positive free energies are less favorable for helical structure because they are less stable. However, significant deviations from predetermined free energy values are possible, depending on neighboring residues, which can interact intramolecularly via hydrogen bonds, van der Waals forces, or hydrophobic interactions, thus stabilizing determined conformers with favorable geometry between side-chain groups of the residues that are sufficiently close to each other.

The insertion of helical peptides into membranes represents an enthalpy-driven process, which is stabilized by helix formation, enabling nonclassical hydrophobic interactions of the hydrophobic side chains with the lipid bilayer, and subsequent rearrangement of the lipid bilayer. Cholesterol plays a significant role in protecting eukaryotic membranes from AMPs; such protection involves complex modulation of hydrophobic interactions and reorganization of the lipids by cholesterol and other sterols that, besides decreasing membrane fluidity and changing the charge distribution on membranes, can directly interact with hydrophobic portions of amphipathic molecules [120]. The energy required to introduce peptide bonds into the bilayer is lowered by hydrogen bond formation, which disperses the electronic density of the polar regions of peptides (even for short peptides), thus leading to helix formation.

β-Like [121] structures are the second most common structure [122] for AMPs and are usually restricted peptides with either disulfide [106,123] or lactam bridges [105,124], or cyclic peptides with N- and C-terminus as cyclization elements [125]. However, sometimes small linear structures can fold into β-turns via water mediation [126]. These molecules often present high resistance to degradation by peptidases [127] and are held by hydrogen bonds, which play a crucial role in regulating three-dimensional structure and function of biological systems. The β-like secondary structures are stabilized both by hydrogen bonding [128] and by hydrophobic interactions of the side chains [129,130], as are α-helical peptides.

Efforts are being expanded to enhance peptide structuring to increase AMP activity. Lee *et al.* [131] presented new molecular architectures that adapt to different solvent environments (water, amphiphilic interface, hydrophobic membrane core) by being metaphilic rather than statically amphiphilic. The authors propose that metaphilic peptides are a molecular analog of engineered omniphilic/omniphobic surfaces, that is, peptides with quasi-liquid surfaces that adapt to environmental change by rearranging their flexible side chains, enabling unusual interactions with membranes.

Net charge

Gram-positive bacteria have a thick cell wall (15–30 nm), which is rich in peptidoglycans and other polymers, such as teichoic acids, teichuronic acids, neutral polysaccharides, lipoteichoic acids, and glycolipids. On the other hand, Gram-negative bacteria are structurally and chemically more complex, consisting of an outer membrane containing lipopolysaccharide, phospholipids, and protein, and a peptidoglycan layer between the outer and the inner membranes, which is composed of phospholipids and proteins. All bacterial membranes, though, have large quantities of negatively charged molecules, which influence their interactions with AMPs [132].

Natural AMPs vary notably in cationicity, from 0 to more than 20 positive charges, but most active peptides fall into an intermediate range, from +3 to +6 net charge. Some studies have shown correlation between charge and potency. However, no optimal structure–function profiles have been determined, likely because additional parameters have to be considered to increase prediction sensitivity. These parameters include secondary interactions, solvation, and amino acid composition, and dictate how these molecules are going to interact and which ones are most likely to have broad-spectrum or specific activity.

For example, Du *et al.* [133] designed AcrAP1 and AcrAP2 analogs derived from scorpion *Androctonus crassicauda* venom peptides with enhanced cationicity. The designed derivatives presented increased potency and broader spectra of antimicrobial activity but, unlike the native peptides, also modulated the growth of a range of human cancer cell lines.

Guo *et al.* [134] designed two linear amidated peptide analogs (TsAP-1 and TsAP-2) based on peptides extracted from the Brazilian yellow scorpion *Tityus serrulatus*. The cationicity of the analogs was enhanced by lysine substitutions on the hydrophilic face, which was neutral with polar uncharged residues in the template molecule. These synthetic peptides presented increased antimicrobial activity and broad-spectrum activity against both gram-positive and gram-negative bacteria.

Jiang *et al.* [135] studied extensively the effects of net charge and the number of positively charged residues within the hydrophilic face on the biological activity and biophysical properties (hydrophobicity, amphipathicity, helicity, and peptide self-association) of the amphipathic helical AMP L-V13K. The net charge of V13K analogs varied between +5 and +10, and the number of positively charged residues ranged from 1 to 10. This study showed that the modifications generated contributed to both antimicrobial (against six *Pseudomonas aeruginosa* strains) and hemolytic activity. The most significant result obtained was the marked increase in hemolytic activity (>32-fold) that occurred after adding an additional positive charge to the polar face of peptide V13K (from +8 to +9).

Hydrophobicity

Besides negatively charged molecules, the bacterial membrane is composed mostly of lipids. Therefore, not surprisingly, hydrophobicity is an important feature for designing peptides with antimicrobial activity. The percentage of hydrophobic residues in naturally occurring peptides also varies, but less markedly so than their net charge, generally fluctuating between 40% and 60% [102], consistent with the requirement for energetically stable amphipathic structures for antimicrobial function.

Amino acid residues are classified according to their side chain groups as hydrophobic, hydrophilic charged, and hydrophilic uncharged. This classification originates from hydrophobicity scales, in which experimental tests or computational simulations classify quantitatively the hydrophobicity of amino acids in determined media. There is a wide range of hydrophobicity scales, which are based on different approaches, but all of them aim to compare side chain hydrophobicity. Keller [136] grouped these approaches according to the similarities of their parameter and value approximations.

These scales are essential for comparing and optimizing peptide templates for specific applications, such as specificity toward a particular class of microorganism or enhanced antimicrobial activity. As an example, Chen *et al.* [137] synthesized a group of analogs with decreased hydrophobicity in order to understand their effects on biological function. The authors found that N-terminal substitutions led to enhanced cell selectivity of the mutants, relative to the control peptide G(IIKK)$_3$I-NH2. In contrast, C-terminal variants exhibited lower anticancer activity and much lower hemolytic activity, except for G(IIKK)$_3$V-NH2. These features correlated with the lower surface pressures and decreased hydrophobicity of these peptides, thus indicating that surface activity governed the anticancer activity of the peptides and hydrophobicity influenced their hemolytic activity.

Khara *et al.* [138] reported a series of short amphipathic helical peptides, comprising the backbone sequence LLKK$_2$, with the ability to kill susceptible and drug-resistant *Mycobacterium tuberculosis*. The authors analyzed the effect of key physicochemical parameters, including hydrophobicity, on anti-mycobacterial activity. The most hydrophobic analog, W(LLKK)$_2$W, displayed selectivity against mycobacteria, whereas peptides with intermediate hydrophobicity were equally active, yet significantly less toxic.

It is important to note that charge and hydrophobicity are not necessarily inversely proportional to each other. Some peptides have a reduced charge but contain a relatively high number of non-charged polar residues or have a balance of positively charged and acidic residues, and their activity depends on how their electronic density is distributed.

Amphipathicity

Amphipathicity, considered the feature of AMPs most relevant to their antimicrobial activity, directly affects their mechanism of action. AMP amphipathicity is commonly described as peptide helicity, but amphipathic structures are not limited to helical molecules as exemplified by β-turn or β-sheets, which can present high amphipathicity depending on their sequence [139]. The common features of these amphipathic molecules are charged, which is generally cationic, and a number of hydrophobic moieties. The cationic moiety is responsible for initial peptide–membrane electrostatic interactions with lipid anionic or zwitterionic headgroups. Subsequently, the hydrophobic moiety interacts directly with the hydrocarbon chains of lipids. Most peptides are unstructured until hydrophobic interactions take place and become prevalent, after which intramolecular interactions increase and the lowest-energy conformations are generated.

It is difficult to define standards for comparing peptides belonging to different families because they have such a great diversity of features and composition. Amphipathicity is closely tied to the hydrophobic moment of the molecule, which depends directly on the secondary structure adopted by the peptide. Amphipathicity was first described as the resultant vector from hydrophobic moment vectors of the individual residues [140,141]. Most recently, amphipathicity has been related to the structural distribution of hydrophobicity and the effects of neighboring residues on the scalar value of these vectors [142].

Eisenberg *et al.* [140,141] described a general behavioral tendency related to the amphiphilicity of peptides that can be used to predict possible mechanisms of action or to design peptides with different selectivity or specificity when these functions are related to structural features. Amphipathicity is closely tied to the hydrophobic moment, which depends directly on the secondary structure adopted by the peptide, and thus correlates directly with the distribution of hydrophobicity along the structure and not just the mean hydrophobicity of each amino acid residue. The Eisenberg plot relates mean residue hydrophobic moment to mean hydrophobicity. Most peptides, independently of their length or composition, show moderate or high values of amphipathicity and can be plotted near the surface and globular regions of Eisenberg's plot [136].

Pillong *et al.* [142] recently introduced “hydrophobic submoments” for overlapping peptide segments as a new descriptor that makes use of a three-dimensional peptide conformation rather than an idealized alpha-helical wheel representation to appropriately include local deviations from an overall amphiphilic distribution of amino acids. This approach is a way to overcome Eisenberg's conceptual limitations and may be a useful tool to more precisely describe the role of amphipathicity in AMP mode of action.

AMPs can have high amphipathicity values with markedly different hydrophobicity scores because they maintain well-defined hydrophilic and hydrophobic sectors, but the sizes of these sectors vary. This defines two important features that are usefully taken into consideration in the designing of amphipathic peptides: hydrophilic and hydrophobic sectors or angles. Some authors try to mainly relate the hydrophobic angle to cytotoxic and hemolytic activities, but it is also important to consider its additional effects on antimicrobial [143,144], antifungal [145], antiparasitic [145], and anticancer [146] activities.

Unnatural modifications

Synthetic AMPs are mostly naturally inspired molecules; thus, they are commonly composed of the 20 naturally available amino acids. Advances in synthetic methodologies with new non-canonical amino acids and additional chemical modifications have increased the combinatorial space, enabling unprecedented modifications in the most important features. These approaches are increasingly popular because they can broaden the functional scope of naturally occurring peptides [147–151]. Modifying amino acids can enhance the stability of peptides [152] by increasing their resistance to proteolytic degradation, as unnatural amino acids are not recognized by cleaving enzymes such as proteases that only catalyze reactions with L-amino acids [153].

The design of synthetic peptides containing non-canonical amino acid residues also requires balancing the cationic and hydrophobic regions, which can be easily fine-tuned via specific chemical modifications, such as halogenation, oxidation, reduction, introduction of aliphatic or electronegative groups and lipid conjugations, or direct insertion of unnatural amino acids. Other features may also need to be taken into consideration.

An example of this was described by González *et al.* [154], who showed that the arylation of Trp-containing microbial peptides resulted in substantially improved their biological activity, while keeping hemolytic activity low. In addition, Dalzini *et al.* [155] synthesized 13 analogs of a peptaibol-inspired peptide Trichogin GA IV, with non-coded α-amino acid residues that confer proteolytic resistance, and further showed that cytotoxicity was tunable by single amino-acid substitutions. Many analogs maintained the same level of nonselective cytotoxicity of Trichogin GA IV, and three analogs were non-toxic. Two of them, which had the positively charged unnatural amino acid aminoacidin introduced into the hydrophobic face of the helix, selectively killed T67 cancer cells without affecting normal cells.

Another advantage of using unnatural modifications is their potential to circumvent susceptibility to conditions of high salt content present in the host. Charged residues of peptides can complex with counterions, making it difficult to stabilize structural conformers with intramolecular interactions, such as salt bridges or hydrogen bonds, which are necessary for a defined structure. The substitution or insertion of unnatural residues, in this case, can provide stabilization; an example of this approach is a strategy that is being developed to increase the salt resistance of AMPs by replacing tryptophan or histidine residues with the bulky amino acid β-naphthylalanine, described by Wang *et al.* [156]. In addition, β-naphthylalanine replacements may help these peptides penetrate cell membranes by increasing the amphiphilicity of their hydrophobic face. However, the exact connections and relationships among the increased hydrophobic surface area of non-natural amino acids, the membrane immersion depth, the orientation, and the salt-resistance of these peptides remain unknown.

Target and Application Design

The design of new synthetic AMPs, apart from increasing antimicrobial activity, aims to reconfigure peptides to achieve increased selectivity, resistance to degradation, and decreased hemolytic activity or cytotoxicity toward healthy cells. The frontiers and challenges for designing and eventually applying AMPs as novel therapies include obtaining precise control over such functions. Specificity is another major property that is increasingly being considered, as next-generation antimicrobials should be designed to kill select pathogens without damaging the organisms that constitute the host microbiome [157] (Fig. 3).

Specificity

Incorporating selectivity into peptide sequences is still challenging and mostly involves tuning primary sequences for specific interactions with determined membrane elements to enable targeted killing of microbes.

Lipids [158–160] and proteins [161] are the most frequent sites of interaction between peptides and microorganisms, although the lack of systematic studies revealing which representative sets of microorganisms are targeted by each AMP limits our understanding of the sequence requirements for selectivity. Interesting studies have been conducted to structurally modify AMPs or to add amino acids side chains, including halogenation of aromatic side chains responsible for modulating antimicrobial activity. Setty *et al*. [162] reported that ortho-fluoro substitutions in phenylalanine residues are responsible for maintaining Temporin L activity against *Escherichia coli* and led to loss of activity against *Staphylococcus aureus* and *P. aeruginosa* strains.

Mishra and Wang [163] used an *ab initio* method based on database filtering to generate peptides with antimicrobial activity against methicillin-resistant *S. aureus*. Another example showed that a protease-resistant cathelicidin LL-37 analog containing three D-amino acids was able to kill *E. coli*, a Gram-negative bacterium, but did not affect the growth of resistant *S. aureus*, a Gram-positive bacterium [164]. This peptide presented a different secondary structure (unstructured) compared to the wild-type peptide (helical). The amphipathic structure, in this case, facilitates hydrophobic interactions with the outer membrane of Gram-negative organisms.

Cytotoxicity

The translation of peptides into the clinic depends on demonstrating lack of toxicity for human cells in pre-clinical studies. Cytotoxicity is a key parameter to consider when designing novel peptides. In fact, a considerable number of naturally occurring peptides are cytotoxic, as they are derived from toxins and other types of natural host defense systems. Thus, the development of AMPs with specificity toward bacterial cells remains an exciting grand challenge in peptide design [165].

In order to tune this feature by modifying a model molecule, one must consider several relevant features that make some of these peptides selective to bacteria. One such property is the electron density (i.e., positively charged surface) of these amphipathic structures, which enables interactions with the negatively charged membranes of microorganisms but restricts interactions with eukaryotic cells, which present zwitterionic lipids in their membrane. In addition, the high levels of cholesterol in the membranes of mammalian cells (20%–30%) stabilize the lipid bilayer by increasing its overall cohesion and mechanical stiffness [166], making it less flexible and, consequently, less permeable to AMPs. Brender *et al.* [167] detail the importance of cholesterol and the conditions in which these sterols modulate the membranes. These authors also found that the effect of cholesterol on AMP activity was about the same regardless of whether cholesterol was in the disordered liquid crystalline lipid phase or the ordered gel phase. Therefore, cholesterol inhibition of this class of molecules may be due to an additional factor, such as dehydration of the lipid headgroups, that directly affects peptide–membrane interactions.

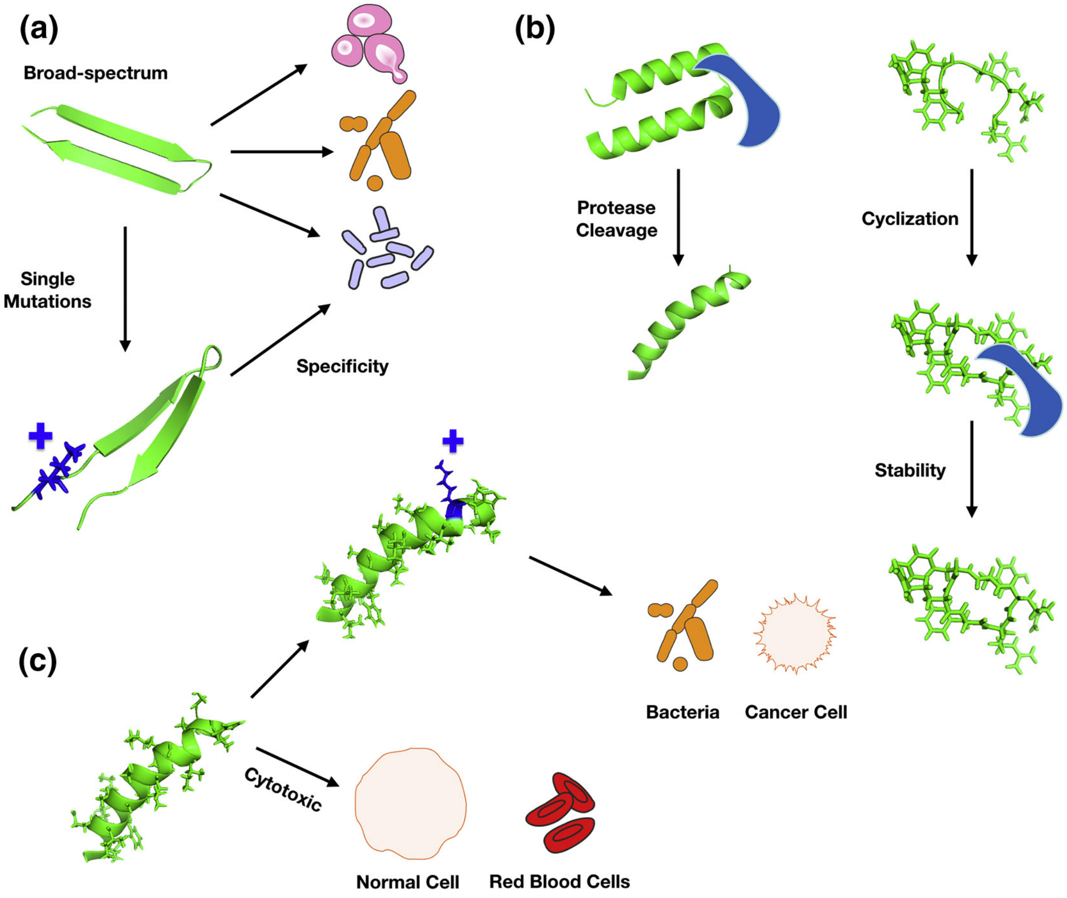

Fig. 3. Peptide design to achieve specific functions. Representation of how designed peptides are enhanced to alter (a) specificity and (b) stability, and to decrease (c) cytotoxicity.

Several studies of the selectivity of peptides toward bacterial cells have reported strategies to design families of peptides that exhibit low cytotoxicity against eukaryotic cells. Singh *et al.* [168], in describing α-MSH, a 13-residue AMP that displayed antimicrobial activity against *S. aureus*, set out to determine whether an increase in net positive charge contributed to improving its anti-staphylococcal potential. They designed analogs by replacing polar uncharged residues with lysine and alanine. The designed peptides preserved their helical conformation in the media studied and showed preferential insertion in the hydrophobic core of anionic membranes. The increased cationic charge resulted in a considerable increase of activity without compromising their cell selectivity.

Another example is the design of GNU derivatives with enhanced stability and cell specificity; these are derived by systematic amino acid arrangement without incorporating non-natural amino acids or peptidomimetics. Following basic principles in peptide design, Kim *et al.* [169] designed peptides to be amphipathic when folded into helical structures, by having the hydrophobic residues converge into one face and the polar residues into the other face of the amphipathic structure. The structural features crucial for effective antimicrobial activity and selectivity of AMPs, such as amphipathicity, net positive charge, and hydrophobicity were maintained, while amino acids were arranged to avoid protease-scissile sites. Amino acids with aliphatic side chains, such as Val, Leu, Ile, and Gln, were used to increase both the hydrophobic and polar face depths, which come into play when the AMPs interact with the bacterial membrane. Two of the peptides they proposed, GNU6 and GNU7, showed potent antimicrobial activity against bacteria, including clinically resistant strains (such as methicillin-resistant *S. aureus* and VRE), and fungi. In addition, the peptides were resistant to degradation by proteases and did not cause human cell cytotoxicity.

Ciobanasu *et al.* [170] investigated the interactions of NKCS, an AMP derived from the cytotoxic peptides NK-lysin and NK-2, with the *E. coli* cytoplasmic membrane. Sequences were designed to elucidate how structural and physicochemical peptide features such as helicity, net positive charge, and amphipathicity enable interactions with the bacterial membrane. Peptide NKCS was generated by replacing the cysteine at position 7 with serine, which yielded a more stable peptide, resistant to oxidation. NKCS exhibited no cytotoxicity against eukaryotic cells.

Stability

Peptides intended for human use must be not only harmless for mammalian cells but also able to withstand the host environment, particularly exposure to proteases (e.g., in the human gut). Human proteases are the main cause of peptide degradation in human serum. Trypsin, which hydrolyzes basic residues, and chymotrypsin, which hydrolyzes aromatic hydrophobic residues, are the most well-known proteases responsible for peptide endogenous cleavage [171–173]. Amino acids can be arranged to avoid protease-scissile sites, and simple mutagenesis may lead to increased resistance to degradation [169]. Kim *et al.* [169] showed that introducing single amino acid substitutions in GNU derivatives yielded AMPs, including linear peptides, resistant to proteases. These substitutions preserved and sometimes enhanced their antimicrobial activity and selectivity against bacteria cells. Although there are many other ways to build peptides that are resistant to degradation by proteolytic enzymes, they are often problematic. The most widely exploited of these are the introduction of unnatural or D-amino acids, which is costly, and restrictions by cyclization, which may deeply alter the physicochemical features of the peptide and structurally compromise its activity when it is not naturally constrained.

The poor physiological stability of peptides in biological systems inspired Qiu *et al.* [174] to synthesize derivatives of protonectin by substituting all the amino acid residues with the corresponding D-amino acids. The analogs exhibited high antimicrobial activity against bacteria and fungi, and one of the analogs showed higher stability against trypsin, chymotrypsin, and human serum than the original peptide, constituted of the L-amino acids.

Zhang *et al.* [175] described DJK-5, a short D-enantiomeric and protease-resistant peptide with broad-spectrum antibiofilm activity. It presented activity at 10 μg mL^{-1} preventing microbial growth in culture media in a time-dependent manner.

In addition, peptides can be designed based on cyclic peptides that are already described as protease-resistant and antimicrobial. As an example, Dahiya and Gautam [176] reported the synthesis of a naturally occurring tetrapeptidecyclo-(isoleucyl-prolyl-leucyl-alanyl) with antifungal and anti-helminth activity. Asfaw *et al.* [177] described the use of the synthetic library approach for designing new analogs of Wollamide B, a cationic antimycobacterial cyclohexapeptide that exhibits activity against *Mycobacterium bovis* (IC_{50} of 3.1 μmol L^{-1}) and that is resistant to degradation by proteases.

Applications of AMPs in materials and devices

Designer AMPs show promise as an alternative to conventional antibiotics. AMPs can be broadly applied in a wide range of materials for applications in biomedical science (Fig. 4). They can be used directly for the treatment of infections [178] and as immunomodulatory peptides [37]. The next step is to achieve AMP stability in various environments in order to apply these molecules as vaccines or ointments [179] or to overcome fast degradation and clearance with effective delivery methods. Different functionalization might alter AMP selectivity and biological activity so that the resulting peptide would not be degraded or cleared as quickly [180–182]. As examples, we highlight the functionalization of peptides with titanium via surface immobilization to create artificial cornea skirts with antimicrobial properties against *S. aureus* and *P. aeruginosa* [183]. Peptides can also be used for dental care as described by Townsend *et al.* [184], who proposed a double coating (peptides attached covalently by electrostatic deposition) of hydroxyapatite surfaces; such coatings prevented infections by gram-positive and gram-negative bacteria for over a year. Implanted surfaces such as those of catheters can also be coated with AMPs to prevent bacterial contamination: Yu *et al.* [185], in order to combat catheter-associated urinary tract infections, coated polyurethane with AMPs by using an anti-adhesive hydrophilic polymer coating with an arginine-rich peptide having a cysteine residue at its C-terminus, designed for this purpose. The surface coating prevented adhesion by up to 99.9% of both gram-positive and gram-negative bacteria and inhibited planktonic bacterial growth by up to 70%.

The use of hydrogels as matrices for AMPs is another way of combining these molecules with useful materials and devices. Mateescu *et al.* [186] described two types of hydrogels containing the peptide cateslytin. The first hydrogel was based on alginate modified with catechol moieties, which adhere strongly to various surfaces, and the second type of gel tested was a mixture of alginate catechol and thiol-terminated pluronic, which is a well-known biocompatible polymer. These two gels offer new possibilities for clinical use, as they can be injected and jellify in a few minutes. These gels strongly adhere to implant surfaces and gingiva, and once gelled, they demonstrate a high level of rheological properties and stability. The cateslytin hydrogels exhibited potent antimicrobial activities against *Porphyromonas gingivalis* and displayed low toxicity for human gingival fibroblasts.

In addition to medical applications, AMPs can be widely used in agriculture and the food industry, especially as long-term and indiscriminate use of pesticides and antibiotics have led to the development of resistance among pathogens and other pests. Many plants and animals have been manipulated with AMP-encoding genes and several pesticides and drugs have been produced based on these peptides.

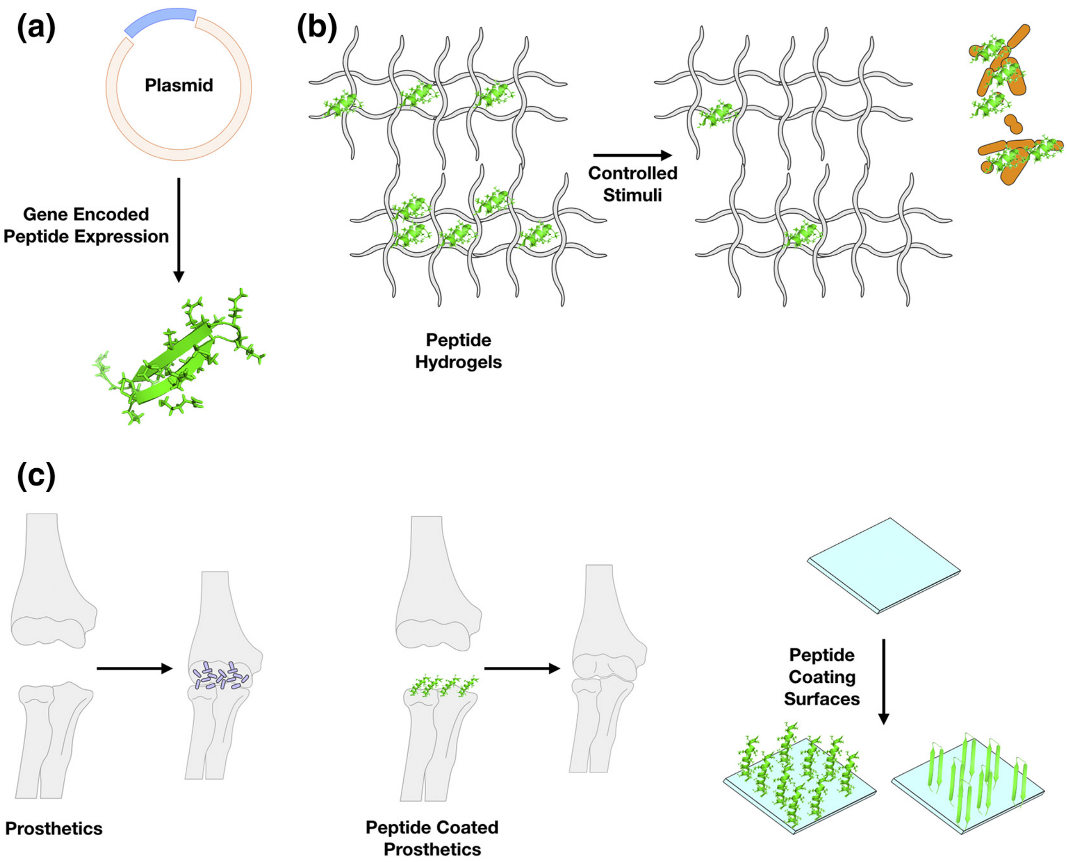

Fig. 4. Applications of AMP-based antimicrobial strategies. (a) Peptides encoded in DNA plasmids for specific targeting of microorganisms. (b) Use of peptide hydrogels as an efficient delivery method of therapeutic payloads. (c) Peptides coating materials such as metallic surfaces and prosthetics for antimicrobial applications.

This research is detailed by Keymanesh *et al*. [187] in their review.

Advances to overcome the high manufacturing cost of large-scale production of AMPs have been extensively studied since these are promising molecules. Engineering living organisms is a useful approach to produce such AMPs in an inexpensive manner. Cao *et al.* [188] reported a genetically modified yeast, *Pichia pastoris*, which produced the prototypical AMP apidaecin-Ia using a fusion protein approach. The methodology used by the authors leveraged the beneficial properties, such as stability, of human serum albumin, which was used as a fusion protein. The AMP produced by the yeast was first isolated from the fusion protein construct, purified, and tested against *E. coli*. The authors also scaled-up production in bioreactors to generate high AMP yields, showing that this approach may be a good alternative to using *E. coli* or cell-free extracts [189].

Future Perspective

All the methods described above are useful depending on the kind of study and aims proposed. Mutagenesis and other traditional methods are still being used for their simplicity and effective results obtained, but as computational power has increased, *in silico* methods have provided the most commonly applied and precise techniques for bioactive peptide design. Additional computational tools such as neural networks, genetic algorithms, and machine learning are being developed, which leverage descriptors (i.e., physicochemical parameters) to build peptides with specific biological activity and selectivity. The learning tasks can be defined and improved later by allowing additional and more sophisticated predictors to be implemented [178]. Machine learning overcomes several limitations encountered with more traditional approaches, such as how to compare totally different peptides (since conditions of their bioactivity assays are not standardized) or how to estimate important physicochemical features that are key for choosing the design method and biological activities desired for the derivatives generated if only approximately 10%–15% of the AMPs have their secondary structure elucidated [63].

Thus, choosing a specific method for peptide design must take into consideration important determinants for the biological activity of peptides. Combined approaches currently represent the optimal way of designing peptides and may provide useful information about such complex systems, making this class of molecules a potentially unlimited source of antibiotics.

Conclusions

Peptides are promising compounds not only for antimicrobial therapeutics but as immunomodulatory agents [190] and anticancer drugs [191]. The versatility and tenability of this class of molecules can be exploited to combat antibiotic resistance. In order to achieve high-quality design of peptides, the appropriate method should line up with the problem being addressed and the available options. Subsequently, important AMP features must be considered and included in the rationale. These compounds and their derivatives can be applied commercially, and examples of peptides on the market include the following: (1) Ziconotide (Prialt®), used to treat chronic pain, is a ω-conotoxin peptide from the cone snail *Conus magus*, and (2) Boceprevir (Victrelis®), used to treat hepatitis caused by hepatitis C virus (HCV), is a protease inhibitor that binds to the HCV nonstructural protein 3 active site [33,192,193].

On the other hand, peptides can be used as templates or inspiration for the design of other molecules. For example, Palermo and Kuroda [194] described antimicrobial polymers, which were designed to mimic the structural features of host defense peptides. The polymers can be modulated by tuning structural parameters such as amphiphilicity, net positive charge, hydrophobicity, sequence, and structure, for use in various environments. More specifically, the authors showed that modifying amphiphilicity, net positive charge, and hydrophobicity modulated antimicrobial activity.

Other examples include cationic amphiphiles inspired by naturally occurring AMPs, which have been described for the treatment of drug-resistant bacterial infections. Such peptides were designed as synthetic mimics to overcome the limitations associated with their predecessors, including high manufacturing cost and low metabolic stability. They presented facial amphiphilicity and were optimized by systematically tuning their hydrophobicity. The optimized molecules exhibited potent broad-spectrum antimicrobial activity but negligible hemolytic activity [195].

Systematic descriptions of the important features of AMPs are so far lacking but may be key to increasing our understanding of structure–function relationships. Comprehensive knowledge of AMPs could truly enable the extraction of rational design principles and their extrapolation to both new AMPs and AMP-like molecules. Based on these principles, next-generation synthetic AMPs having unprecedented functions could be produced, potentially resulting in peptide-based therapies for the treatment of drug resistance.

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Abbreviations used:
AMPs, antimicrobial peptides; SAR, structure–activity relationship.

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