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# Synthetic polypeptide polymers as simplified analogues of antimicrobial peptides

*Pedro Salas-Ambrosio,<sup>a</sup> Antoine Tronnet,<sup>b</sup> Pierre Verhaeghe,<sup>b</sup> Colin Bonduelle.\*<sup>a</sup>*

<sup>a</sup> Univ. Bordeaux, CNRS, Bordeaux INP, LCPO, UMR 5629, F-33600, Pessac, France.

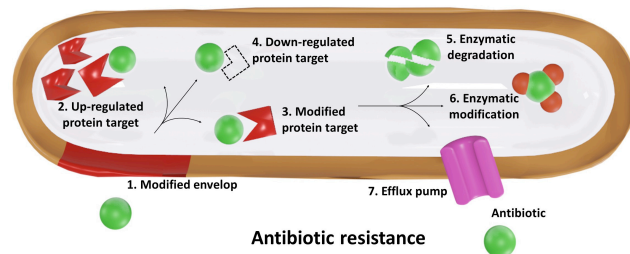
<sup>b</sup> LCC-CNRS Université de Toulouse, CNRS, UPS, Toulouse, France.

**KEYWORDS:** Antimicrobial peptides, synthetic polymers, polypeptides, ring-opening polymerization, *N*-carboxyanhydride, anti-infective agents

**ABSTRACT:** Antimicrobial peptides (AMPs) are naturally occurring macromolecules made of amino acids that are potent broad-spectrum antibiotics with potential as novel therapeutic agents. This review aims to summarize the fundamental principles concerning the structure and mechanism of action of these AMPs, in order to guide the design of polymeric analogues that organic chemistry can generate. Among those simplified analogues, this review particularly focuses on those made of amino acids called polypeptide polymers: they are showing great potential by providing one of the best biomimetic and bioactive structures for further biomaterials science applications.

**Introduction.** Antibiotics are a class of drugs used to prevent and treat bacterial infections. The discovery of antibiotics was a major milestone in medicine that has saved and continues to save millions of lives every year, but their effectiveness is constantly threatened by the capacity

of bacteria to adapt and resist these treatments.<sup>2</sup> The antimicrobial resistance (AMR) is the ability of a microorganism to resist the action of one or more antimicrobial agents.<sup>3-5</sup> AMR is one of the biggest public health challenges of our time. Fighting this threat is a public health priority that requires a collaborative global approach across research sectors.<sup>4</sup> In 2020, the WHO stated that the research of new antibiotics sorely lacks investment and technological developments to address the problem of resistance.<sup>6</sup> The new antibiotics under development do not bring spectacular benefits to existing drugs and only a few of these new treatments target Gram-negative bacteria. A priority pathogen list is clearly defined and highlights pathogens that are posing an increasing risk to human health because they are resistant to most existing treatments.<sup>7</sup> It is important to mention that the mode of action of antibiotics generally involves mechanisms that consist in inhibiting bacterial cell wall synthesis, protein synthesis, or DNA replication.<sup>8</sup> Addressing the problem of resistance to antibiotics first requires a better understanding of its origin (figure 1). As the purpose of this review is not to get into the resistance phenomena, from a microbiological point of view, readers are invited to consult the following reviews for more details.<sup>9-12</sup>



**Figure 1.** Antibiotic resistance. Bacteria develop resistances through mutations, by uptake of external mutated genes or intrinsic mechanism i.e. up and down-regulation causing: 1) modification on the bacterial envelope, 2) amplification, 3) modification, 4) quantitative decrease of the target (or activating enzymes for some prodrugs), 5) enzymatic degradation, 6) enzymatic modification of the antibiotic and 7) pumps that expulse antibiotics.<sup>9-12</sup>

To these considerations, it should be added that three correlated facts impact the rising acceleration of AMR. The first one is the overuse of antibiotics, synthetic drugs, often veterinary

drugs, which degrade very slowly in the environment.<sup>13</sup> Their persistence and presence in our daily food have significantly increased the speed at which resistances develop and spread in bacteria.<sup>14,15</sup> Second, the relation between AMR and healthcare-associated infections (also called hospital-acquired infections, HAIs) have been a serious public health problem since the uncontrolled use and commercialization of antibiotics started.<sup>16,17</sup> Those HAIs occur while receiving health care (i.e. surgical procedures, use of medical devices or long-term treatment of drugs) in a hospital environment which is subjected to a very strong antibacterial pressure. Although this pressure controls the multiplication of bacteria, it also imposes a non-natural selection favouring the emergence of worrying multi-resistant bacteria.<sup>17,18</sup> Finally, the formation of bacteria biofilms represents a third key factor in antimicrobial resistance. Biofilms resemble the tissues formed by eukaryotic cells, in their physiological cooperativity and in the extent to which they are protected from variations in bulk phase conditions by primitive homeostasis provided by the biofilm matrix.<sup>19,20</sup> The antibiotics typically kill the planktonic bacteria, which are released from the biofilm, but the treatment often fails to destroy the biofilm itself.<sup>21</sup> Even worst, the resistance could foster the emergence of resistance in dormant bacteria protected by the biofilm, which later allows the spreading of superbugs with no treatment available.<sup>20,22,23</sup> Overall, despite these growing risks, we lack new chemical entities to challenge these new superbugs and, as a result, we currently face a growing enemy with a largely depleted armoury.<sup>24,12</sup> To face the challenge of AMR in the coming years, there is an urgent need to develop new innovative treatments.<sup>5</sup>

In this context, this review aims to highlight the potential of using polymer chemistry to construct simplified analogues of antimicrobial peptides (AMPs), an amazing group of biomacromolecules displaying potent and selective antimicrobial activities. This review particularly focuses on recent data obtained with polymeric analogues made of amino acids, which seem particularly relevant to bridge the gap between the world of synthetic polymers that are easily accessible but present limited antimicrobial activity, insufficient selectivity or lack of biocompatibility and the world of natural AMPs that are complex structures, difficult or

expensive to reach through organic synthesis but which display a quite interesting antibiotic profile of activity. Even if there is still a long way to go before polymeric structures could be used as antibiotic drugs and not only as antiseptics, polymer chemistry certainly has a major role to play in this difficult context against AMR.

**1. Antimicrobial peptides (AMPs).** The adaptive immune system plays an important role to fight against microbes but it only protects against infections in animals.<sup>25</sup> For many living systems including plants and insects who lack an adaptive immune system, the response to infection involves the secretion of antimicrobial peptides or AMPs, also called host defence peptides.<sup>26,27</sup> They are specific and sequence-controlled macromolecules made of amino acids which form part of the innate immune response against infections.<sup>25,26,28</sup> Historically, the first evidence of AMPs was demonstrated through the growth inhibition of a variety of phytopathogens coming from wheat flour.<sup>29</sup> Further studies proved that a peptide isolated from the wheat endosperm *Triticum aestivum* was responsible for these properties. The peptide was then named purothionin and classified as a member of thionins within the plant kingdom. Since then, AMPs were discovered in all the kingdoms including in humans: lysozyme was the first reported human AMP identified in 1922 from nasal mucus by Alexander Fleming<sup>30</sup> and the discovery of nisin in 1928 established the occurrence of AMPs in humans.<sup>31</sup> The real importance of AMPs was recognized in prokaryotic cells in 1939 when Dubos isolated from *Bacillus brevis* a compound called gramicidin, which demonstrated activity against a wide range of Gram-positive bacteria.<sup>32,33</sup> A complementary study demonstrated that this molecule favoured wound healing on guinea pig skin,<sup>34</sup> which allowed gramicidin to be the first AMP commercially manufactured as antibiotic in 1944.<sup>35,36</sup> Moreover, *Lactococcus lactis* produces an AMP called nisin A which has been used as a food preservative since 1950, date from which no significant development of resistance has been detected.<sup>37</sup> With the rise of antibiotic resistance in the early 1960s, AMPs have been the subject of growing interest from the scientific community:<sup>25</sup> since then, more than 3000 AMPs have been listed and several databases have been created to

document their chemical structures.<sup>25,38</sup> For instance, the Antimicrobial Peptide Database (APD) of the University of Nebraska contains antimicrobial peptides from six kingdoms (343 from bacteria, 5 from archaea, 8 from protists, 20 from fungi, 349 from plants and 2307 from animals).<sup>31,39</sup> In humans, the three most representative groups of AMPs are defensins, cathelicidins and histatins.<sup>30,40-42</sup>

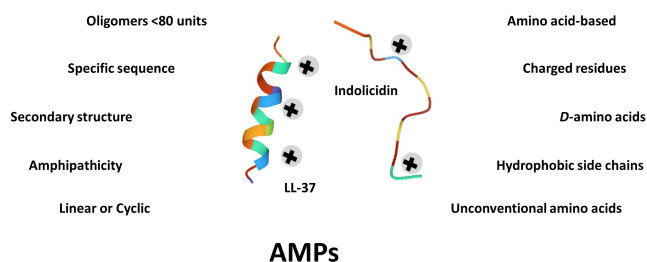
### 1.1. What can be learnt from the chemical structure?

AMPs are macromolecules made of amino acids. Their chemical structures are very diverse and some key structural features are identified as essential to their biological activity:<sup>43</sup> the nature of the amino acids that constitute the peptidic backbone, the overall hydrophobicity and the global charge of the macromolecule.<sup>44,45</sup> To simplify, AMPs can be organized into three main categories:

- *AMPs which are tertiary folded and that are proteins.* They are often enzymes with hydrolytic activities and this class of AMPs will not be developed further in this review. These are the most complex structures and readers are invited to read more specific references on this topic (i.e. lysozyme, human-*beta*-defensin-3, subtilisins, etc.).<sup>46-48</sup>

- *AMPs that are smaller in size than true proteins* (below 80 amino acids) but which still adopt a secondary structure that is associated to antimicrobial activity.<sup>38</sup> Typically, AMPs being part of this group have a minimum length of 22 residues for  $\alpha$ -helical peptides (i.e. magainin 2, LL37, Buforin, dermcidin, etc) or 8 residues for  $\beta$ -sheet peptides (defensins i.e., HNP-1).<sup>40,49</sup>

- *AMPs belonging to a third group corresponding to unstructured peptidic backbones* which can be cyclic structures and are often conjugated with non-peptidic moieties. In this group, activity is usually directly linked to the primary sequence of the macromolecule. (i.e. darobactin, PR39, indolicidin, pyrrolicin, etc.).<sup>50-54</sup>



**Figure 2.** Main chemical features of AMPs such as LL-37 (PDB ID: 5XNG)<sup>55</sup> and indolicidin (PDB ID: 1G89)<sup>56</sup>

The chemical structure of oligomeric AMPs depends largely on six key features including amphipathy, charge, hydrophobicity, specific sequence, size, and secondary structure (figure 2).<sup>44,45</sup> Other parameters that are not always encountered can also play an important role: the presence of amino acids belonging to the D series, the presence of macrocyclic structures and the presence of unconventional amino acids ( $\beta$ -aminobutyric for instance).<sup>57</sup> Despite their great chemical diversity, some specific amino acids are conserved in all the peptide sequences. In particular, lysine and arginine<sup>58</sup> are strongly over-represented in AMPs as well as some hydrophobic residues (valine, phenylalanine, leucine and alanine, etc., see table 1).<sup>44,59</sup> In most AMPs, the antibacterial activity does not depend on a single parameter but rather on a plural contribution including several chemical features within the same macromolecular backbones. This is an interesting observation for polymer chemists as copolymerization processes are remarkably suitable to integrate, in only one step, various chemical elements within one single macromolecules.<sup>60-62</sup> In AMPs, there is no precise rule about the ideal number of hydrophobic residues or charged residues to maximize antimicrobial activity, as this balance varies widely and appears to be associated with some form of selective activity.<sup>45,59,63</sup>

**Table 1.** Representative antimicrobial peptides and key chemical features (source APD).<sup>61</sup>

AMP	Secondary structure	Sequence (No. of amino acid units)	Net charge <sup>a</sup>	Hydrophobic content
<b>Magainin</b> <sup>64</sup>	$\alpha$ -helix	GIGKFLHSAKKFGKAFVGEIMNS (23)	+3	43%
<b>LL37</b> <sup>65,66</sup>	$\alpha$ -helix	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLLVPR TES (37)	+6	35%
<b>Buforin II</b> <sup>67</sup>	$\alpha$ -helix	TRSSRAGLQFPVGRVHRLLRK (21)	+6	33%

<b>θ-Defensin</b> <sup>65,69</sup>	$\beta$ -sheet	GFCRCLCRRGVCRICTR (18)	+5	55%
<b>HNP-1</b> <sup>70</sup>	$\beta$ -sheet	ACYCRIPACIAGERRYGTCTIYQGR LWAFCC (30)	+3	53%
<b>HNP-3</b> <sup>69,71</sup>	$\beta$ -sheet	DCYCRIPACIAGERRYGTCTIYQGR LWAFCC (30)	+2	50%
<b>Nisin A</b> <sup>57</sup>	Coil	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVS K (34)	+3	44%
<b>Indolicidin</b> <sup>63,72</sup>	Coil	ILPWKWPWWPWRR (13)	+4	53%
<b>Mersacidin</b> <sup>73</sup>	Coil	CTFTLPGGGGVCTLTSECIC (20)	-1	45%
<b>Daptomycin</b> <sup>74,75</sup>	Coil	WNDTGKDADGSEY (13)	-3	15%
<b>Darobactin</b> <sup>72</sup>	Coil	WNWSKSF (7)	+1	42%

\* Cationic and hydrophobic amino acids. \*Correspond to the amount of positively charged residues minus the amount of negatively charged residues.

Despite some exceptions where peptide structures are negatively charged or neutral (i.e. mersacidin, dermcidin, etc.),<sup>73,74,76</sup> most AMPs are positively charged (table 1).<sup>44,77</sup> As described later in this article, **the cationic nature of antimicrobial peptides** is used to induce interaction with anionic bacterial membranes, often resulting in membrane destabilization.<sup>78</sup> Several studies have shown correlations between the net charge and its distribution with biological activity or selectivity towards certain bacteria.<sup>63,79-81</sup> When the secondary structure is involved, the presence of an excessive amount of electrostatic charge can negatively affect biological activity by interfering with the structuring.<sup>82</sup> Importantly, the effectiveness of AMPs in killing bacteria also depends very much on pH and salt concentration: antimicrobial activities are often enhanced by a slightly acidic pH because the protonation of certain amino acids is promoted, such as histidine, aspartic acid or glutamic acid.<sup>83-85</sup>

Most AMPs contain around 50% hydrophobic residues in their primary sequences (see table 1).<sup>43</sup> This hydrophobicity can significantly modulate both the antibacterial activity and its specificity (spectrum of action).<sup>45,59,81</sup> This is another important point if one want to design of polymeric analogues: this will be developed in part 2 of this review. Overall, **hydrophobicity is a chemical leverage** to control the efficiency with which a peptide can penetrate bacterial lipid bilayers.<sup>43,81,86</sup> In addition, it was shown that an increase in the hydrophobicity of the peptide at the expense of the amount of net positive charges can significantly modulate the antimicrobial activity.<sup>87</sup> The effects observed are often empirical and the correlations observed are often dependent on the studied bacteria.<sup>86</sup> Above a certain level, hydrophobicity leads to a decreased



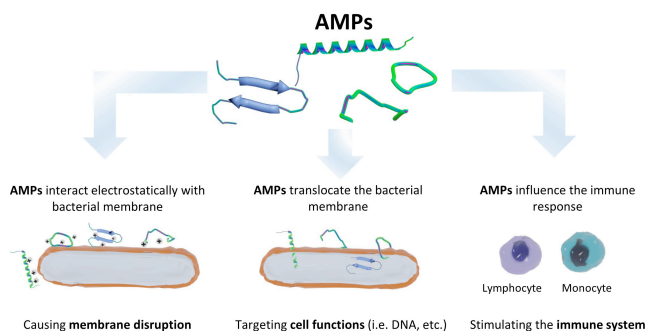
antimicrobial activity and increased toxicity to eukaryotic cells.<sup>86,88</sup> Overall, the hydrophobicity/load ratio is a key determining factor in the design of antimicrobial peptides.<sup>86-88</sup> This ratio between hydrophobic and basic residues often ranges from 1:1 to 2:1.<sup>44</sup>

The length of antimicrobial peptides most often ranges from 6 to about 60 amino acids,<sup>44</sup> but little is known about the correlation between size, charge density and antimicrobial activity. In this direction, peptide polymers would be perfect tools to shed light on these parameters. **The majority of AMPs are amphipathic:** they are both hydrophilic (interaction with the phosphate heads of phospholipids) and hydrophobic (interaction with the lipid bilayer).<sup>89</sup> Quite often, antimicrobial peptides adopt amphipathic conformations when interacting with bacterial membranes (magainin 2, indolicidin, etc.) and are therefore stimuli-responsive backbones.<sup>78,90</sup> They become active only upon membrane interaction, making them less toxic for other cell types for which the interaction is weaker.<sup>76</sup> Finally, it is to note that the incorporation of unconventional amino acids (or *D*-amino acids) or the fact that some AMPs are macrocyclic seem to be key chemical features to minimise the degradation of those peptides by bacteria which secrete proteases to defend themselves.<sup>91,92</sup>

## 1.2. Bactericidal mechanisms of action of AMPs

There are two main modes of action that allow AMPs to kill bacteria (figure 2): they can interact and disrupt the bacterial membrane, they can be internalized and target an important function of bacterial metabolism or stimulate the immune system to kill more effectively bacteria.<sup>38</sup> Among AMPs able to target the inner functions of the bacteria, the frog antimicrobial peptide buforin II, for instance, binds to both DNA and RNA to kill *E. coli*.<sup>67</sup> Similarly, in humans, defensin HNP1, PR-39 and indolicidin are other examples of DNA synthesis inhibitors.<sup>53,70,93</sup> Targeting a more downstream metabolism, mersacidin peptides interfere with transglycosylation of lipid II, a necessary step in the synthesis of peptidoglycan of Gram-positive.<sup>73</sup> Overall, there are dozens, if not hundreds of potentially very selective inner metabolism to be targeted, many of which have not yet been studied. In this framework, the

biological activity of AMPs can be a source of inspiration in medicinal chemistry, with the aim of developing small drugs that are efficient synthetic analogues.<sup>94,95</sup> For such inhibition, the possible contribution of polymer chemistry remains limited although major advances in sequence control could open the way for polymer science to achieve specific targeting in the future.



**Figure 3.** Mechanism of action of antimicrobial peptides: they can disrupt the bacterial membrane, inhibit cellular functions or stimulate the immune system.

Much more interesting for polymer chemists, **the other mechanism implemented by AMPs to kill bacteria involves their ability to interact with bacterial membranes.** As presented above, AMPs exhibit a net positive charge and a high ratio of hydrophobic amino acids (

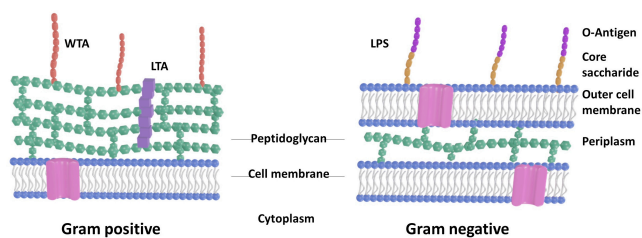
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), allowing them to selectively bind to negatively charged cytoplasmic membranes (see figure 3).<sup>30</sup> As a representative example, the human cathelicidin peptide LL37 is an efficient AMP to bind bacterial membranes: it is cationic,  $\alpha$ -helical and binds to membranes through electrostatic interactions, before inducing lysis of the bacteria by various mechanisms which are presented in

Part 1.3.<sup>66</sup> This membrane destabilization mechanism is extensively developed in the following paragraphs.

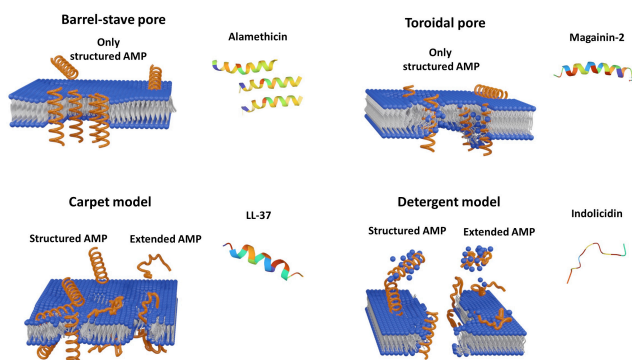
### 1.3 AMPs and membrane destabilization.

The cell wall of bacteria is a complex structure that plays a variety of protective and adaptive functions.<sup>66</sup> The main conserved component in all bacterial cell walls is peptidoglycan, which is essential for stabilizing cell membranes against high internal osmotic pressures (figure 4).<sup>97</sup> Gram-positive bacteria are surrounded by layers of peptidoglycan that are much thicker than those of Gram-negative bacteria.<sup>66</sup> Peptidoglycan alone is not enough to allow bacteria to survive in their environment: in addition to the peptidoglycan, the outer membrane of Gram-negative bacteria, or a dense network of negatively charged polymers incorporated into the peptidoglycan for Gram-positive bacteria, also play important roles in preserving the integrity of the cell wall.<sup>98,99</sup> Typically, AMPs combine cationic and hydrophobic amino acids in a way that provides them with the ability to interact and perturb the cell walls of bacteria: this includes a very large number of different but negatively charged surfaces, rich in lipids i.e. phosphatidylglycerol or cardiolipin, to the outside environment<sup>79</sup>



**Figure 4.** The bacterial envelope of Gram-positive and Gram-negative: WTA, wall teichoic acid; LTA, lipoteichoic acid; LPS, lipopolysaccharide. The cell membrane is the target of AMPs with membrane disruption ability.

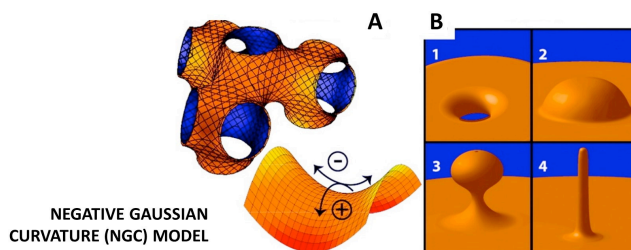
For AMPs capable of being structured in  $\alpha$ -helices or in  $\beta$ -sheets, several physico-chemical mechanisms have been described in order to explain how they can destabilize bacterial membranes (see figure 5), mechanisms that were also comprehensively studied with artificial phospholipid membranes.<sup>100</sup>



**Figure 5.** AMPs are efficient membrane disruptors and induce this destabilization through different mechanisms. Alamethicin (PDB ID: 1AMT),<sup>90</sup> magainin-2 (PDB ID: 2MAG),<sup>92</sup> LL-37 (PDB ID: 5XNG),<sup>55</sup> and indolicidin (PDB ID: 1G89)<sup>96</sup>

The first of these mechanisms, called the **barrel-stave model mechanism**, involves the formation of channels or pores in the membrane through the perpendicular insertion of the AMPs, causing bacterial leakage.<sup>38</sup> Magainins peptides, for instance, fold upon interaction with the anionic lipid membrane, and their secondary structure is stabilized by two to four disulphide bridges allowing the creation of pores in the bacterial membrane that cause lysis of the cell.<sup>90,100</sup> Another model mechanism, called the **toroidal pore mechanism**, involves the insertion and reorganization of the phospholipid bacterial membrane as a result of the interaction of AMPs with phospholipid polar heads (figure 4).<sup>44</sup> In comparison to the barrel-stave mechanism, the hydrophobic and hydrophilic arrangement of the lipids is maintained, whereas in toroidal pores the hydrophobic and hydrophilic arrangement of the bilayer is disrupted.<sup>38</sup> It is important to note that the ability to form toroidal pores in bacteria is particularly linked to the presence of phosphatidylethanolamine, a lipid species that significantly imparts curvature potential.<sup>82,103</sup> Some examples of AMPs involving this mechanism are magainin 2 and melittin.<sup>78</sup> While barrel-stave model and toroidal pores are more likely to occur with structured AMPs,<sup>78</sup> the **carpet model mechanism**, a third possible mechanism, does not require specific secondary structure dependant interactions; it also does not require the peptide to insert into the hydrophobic core to

form transmembrane channels or specific peptide structures.<sup>38</sup> In such carpet model mechanism, AMPs are electrostatically attracted parallelly to the anionic phospholipid head groups. At certain threshold concentrations, they cover the membrane surface forming a carpet which leads to unfavourable interactions on the membrane surface with the consequent loss of the membrane integrity.<sup>100</sup> As a consequence of non-specific interactions, both; structured and unstructured (or extended) AMPs can kill bacteria through this mechanism (figure 5). Examples of AMPs acting *via* the carpet model are the extended indolicidin or  $\alpha$ -helical LL-37.<sup>104</sup> Finally, AMPs also can act as a surfactant-like molecules (**the detergent model mechanism**) due to the strongly charged character disrupting the bacterial membrane and forming micelles; this event can take place as a consequence of higher AMP concentrations in the carpet-like model. Examples of AMPs whose mechanism of action relies on the detergent model are cathelicidin 1 and 2.<sup>105</sup>



**Figure 6.** Generation of negative Gaussian curvature. A) Illustration of the Pn3m cubic phase (lower right: Negative Gaussian curvature requires positive curvature (+) in one direction and negative curvature (-) in the perpendicular direction to locally produce a saddle shape). B) Diagrams of different manifestations of saddle-splay curvature in the interior of a pore (1) and at the base of blebs (2), buds (3), and rod-like projections (4).<sup>106</sup>

Although all these models have had a wide degree of acceptance, they do not sufficiently take into account the importance of the hydrophobic to cationic ratio, a critical key feature of AMPs (see part 1.1 and table 1). In this context, the correlation between this ratio and the lipid membrane composition was more recently demonstrated through a phenomenon presented as an **inductive saddle-splay effect called Negative Gaussian Curvature (NGC** see figure 6).<sup>107</sup> This latter mechanism described more recently, was the subject of numerous experimental and theoretical studies. Importantly, it is also the mechanism that polymers can most effectively

mimic in a view to design antibacterial drug-candidates. Originally, NGC was comprehensively studied by monitoring the interaction created between  $\theta$ -defensins-1 or  $\theta$ -defensins-7 and lipid vesicles through small-angle X-ray scattering technique.<sup>106</sup> The vesicles changed drastically when they interacted with AMPs displaying the formation of a cubic Pn3m “double diamond phase”. This specific phase behaviour generates a positive curvature in one direction and a negative curve in the perpendicular direction, producing a saddle-splay curvature (figure 6). The membrane disruption mechanism can be further explained by AMPs-induced electrostatic wrapping of membranes displaying NGC. From the interior of the pores forming upon interaction with AMPs, it was possible to observe several manifestations such as blebs, buds and rod-like projections (figure 6B).<sup>108</sup> To generate efficiently NGC, the selective character of a given cationic to hydrophobic ratio was fully demonstrated in 2018 through the comparison of human defensin-3 derivative CHR01 (sequence of 14 amino acids) and HBD-3 (sequence of 45 amino acids) a similarly charged peptide but with higher hydrophobic content.<sup>108</sup> This study confirmed that a higher content of hydrophobicity decreased the membrane leakage due to an increase of positive Gaussian curve. This positive Gaussian curve was ultimately drastically decreasing the antibacterial potency.

#### **1.4 Strengths and limitation of AMPs**

From a therapeutic point of view, AMPs display a very good potential to reinforce the antibiotic arsenal, mainly for two reasons. First, since the bacterial cell membrane is often the main target for those peptides, it is difficult for most of bacteria to adapt and develop resistance toward AMPs. Becoming resistant toward AMPs would effectively require that bacteria change significantly the composition of their membrane and, in many cases, these changes are not compatible with preserving the structural and functional integrity of the membrane. Secondly, compared to synthetic antibiotics, AMPs are biodegradable macromolecules that avoid persistence in the environment after elimination of the body, avoiding a long time of contact with bacteria at low concentrations which is one of the main issues that nowadays leads to the

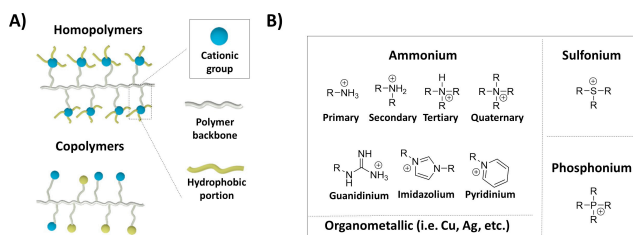
emergence of resistant bacteria worldwide.<sup>109</sup> Nevertheless, AMPs also present three main weaknesses that still need to be corrected in order to extend their use as antibiotics. First, like peptides, AMPs are sensitive either to endo- and/or exopeptidases (and proteases) and are rapidly hydrolyzed into the digestive tract. Then, AMPs are antibiotics that are not administered orally, which is the main limitation to undergo pharmaceutical development.<sup>44</sup> Secondly, it is to note that some AMPs that are currently used in clinics as IV administered antibiotics, such as colistin (also called polymixin E), are highly nephrotoxic, which drastically limits their therapeutic use.<sup>110</sup> This toxicity is related to a lack of selectivity leading to interactions of colistin with the membrane of human kidney proximal tubular cells. Such interactions have recently been characterized, opening the way for the design of novel colistin derivatives that could show a safer profile.<sup>111</sup> Finally, nowadays, commercial AMPs are biomolecules that are exclusively obtained from gene-modified microorganism, a complex and rather expensive production mode.<sup>26,112</sup> For example modified *E. coli* can produce some AMPs but the average cost of production reaches more than 40,000 \$ per kg in the best-case scenario<sup>113</sup> and the low yields at the end of the production are associated to the inherent difficulties to work with living systems. On another hand, AMPs could be synthesized through a solid-phase peptide synthesis (SPPS) approach.<sup>114</sup> But, among other inconveniences, this synthetic approach requires many iterative reaction steps that lead to limited global synthesis yields, affecting scale-up capabilities and restricting the length of AMPs that one could synthesize.<sup>115</sup> In this direction, it is to note the efforts made to prepare synthetic magainin having broad-spectrum activity,<sup>116</sup> or synthetic analogues of gramicidin S with activity against Gram-positive bacteria.<sup>117</sup> Several antimicrobial peptides have been developed thanks to SPPS,<sup>95</sup> taking into account that an average one gram produced by this technique can cost about 10,000 dollars.<sup>118</sup> Thus new synthetic pathways for producing AMPs are awaited.

## **2. Synthetic Antimicrobial Polymers.**

Synthetic polymers are certainly the best candidates to provide an effective response to the limitations posed by the production and use of AMPs. In this direction, access to large amounts

of material is an enormous benefit of using polymer chemistry. Moreover, the growing development of biodegradable polymers makes it increasingly possible to create materials that can avoid persistence into the environment.<sup>119</sup> Similar to most of AMPs, polymers are macromolecules but unlike AMPs, they are constituted of monomer units whose sequence can be poorly controlled and they are polydisperse. Despite these fundamental differences, synthetic polymers have been extensively studied since decades to tackle bacteria as drug carriers, but also as antiseptic polymers.<sup>120-122</sup> Antibiotic-polymer conjugates are outside the scope of this review article and readers are invited to refer to another recent review dealing with the use of polymeric drug carriers improving the formulation of antibiotic drugs.<sup>123</sup>

Perhaps more difficult to conceive but also more challenging, polymers may intrinsically carry antimicrobial activity and become macromolecular antibiotic drug-compounds by themselves. Such antimicrobial polymers, also known as polymeric biocides, usually possess the structure of amphiphilic polycations and display biocidal activities or the ability to inhibit the growth of bacteria.<sup>124,125</sup> As compared to AMPs, they could provide many advantages including low-cost and effective production, stable in long-term usage and storage, and biocidal or broad-spectrum activity against pathogenic microorganism in brief times of contact.<sup>122</sup> Nevertheless, they often exhibit significant toxicity, at least comparable to the toxicity of AMPs, and their use is today restricted to material sciences. It is necessary to note here the possible developments of formulations which enable to limit the toxicity of AMPs or synthetic polymers.<sup>126</sup> Areas that can benefit from the use of antimicrobial polymers are the manufacturing of fibers, textile sector, the design of water filtration systems, food packaging and biomedical and pharmaceutical industries, including the antibacterial coating of medical devices.<sup>127-130</sup>





**Figure 7.** Antimicrobial polymers: essential elements for their chemical design. A) Cationic groups and other chemical functions are supported by either homo- or co-polymers; B) The main cationic groups that were used to develop antimicrobial polymers.

Following key chemical features of AMPs, antibacterial polymers incorporate at least two elements into their chemical structures: cationic groups and hydrophobic groups (figure 7). The biological activity is influenced by the type, amount, location and distribution of these two components. Cationic groups are the essential building blocks that allow antimicrobial polymers to kill bacteria. They mainly include ammoniums<sup>131</sup>, sulfoniums<sup>132</sup> or phosphoniums (see figure 7B).<sup>133</sup> There are two ways of introducing the hydrophobic chemical component, either by carrying out the chemical design of quaternized homopolymers, or by controlling the topology of polymers by preparing copolymers mixing cationic monomer units with hydrophobic monomer units (See figure 7A).<sup>134</sup> We will not describe in this review alternative design such as main-chain cationic polymers or dendritic and hyperbranched cationic polymers: to learn more about it, readers are invited to consult other review articles dedicated to these other types of antibacterial structures.<sup>123,135-137</sup>

Ammonium-based moieties are the most common cationic groups found in antimicrobial polymers.<sup>134</sup> The quaternary ammonium has a permanent positive charge, whereas primary, secondary and tertiary ammonium groups are pH-dependent, i.e. protonation state of polymers depends on the pKa of their constituent amine and the pH of the medium in which they will have to exert their antibacterial activity. Kuroda and co-workers proved that the pH-dependence characteristic is important to obtain less toxic antimicrobial polymers with a series of amphiphilic polymethacrylates.<sup>138</sup> Moreover, they studied a series of polymers with primary, tertiary and quaternary ammonium groups and they showed that primary amine had substantially greater selectivity against *E. coli* than against red blood cells.<sup>139</sup> In addition to ammoniums, pyridinium or guanidinium salts, are also commonly used cationic groups found in antimicrobial polymers because of their high-water solubility and less-toxic properties.<sup>134</sup> In these cations, the charge is delocalized through the  $\pi$  bonds or aromatic conjugated systems and plays a key role

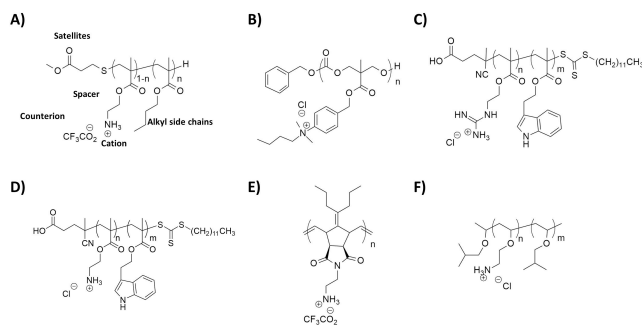
for the adsorption of the polymers on the bacterial membrane. In this direction, Zhang *et al.* synthesized a series of polyhexamethylene guanidine stearate that demonstrated a broad spectrum of action against Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*P. aeruginosa*).<sup>140</sup> Apart from chemical functions involving nitrogen atoms, antimicrobial cationic polymers carrying other cationic groups were also found effective against several bacteria: this mainly includes side chains bearing sulphur<sup>132</sup> or phosphorus atoms.<sup>133,141</sup> For instance, Endo *et al.* studied both *S*- and *P*-containing polymers. The first was poly(*p*-vinylbenzyl-tetramethylenesulfonium tetrafluoroborate) and demonstrated antibacterial activity against *S. aureus* rather than *E. coli* whereas the second was poly(tributyl(4-vinylbenzyl)phosponium and demonstrated activity against *S. aureus* that was enhanced with a mixture of the analogous ammonium salt, highlighting the importance of the ammonium cation toward bactericidal activity.<sup>133</sup>

Overall, quaternization is an important chemical characteristic that greatly enhances the antimicrobial performance of antimicrobial polymers.<sup>142</sup> This optimization is based on the simple inclusion of hydrophobicity on each side chain of the macromolecules. For instance, this characteristic can be easily modulated by the nature and the length of the alkyl lateral chains that are linked to the nitrogen atom in quaternized polymers.<sup>139,143</sup> In this direction, Tiller and co-workers studied series of poly(4-vinyl-*N*-alkylpyridinium bromide) with different linear alkyl chains from propyl to hexadecyl that killed *S. aureus* with the best activity for small alkyl chains.<sup>144</sup> Another study by Lu *et al.* evaluated the activity of dimethylaminoethylmethacrylate quaternized with benzyl, butyl, dodecyl or hexadecyl bromide and determined that benzyl and butyl chains had the best activities.<sup>145</sup> Although homopolymer synthesis is the simplest and most efficient way to produce antimicrobial polymer candidates, it is still nearly impossible to tune their activity, toxicity or to induce antimicrobial selectivity. Closer to AMPs in which cationic and hydrophobic units are carried by different monomer units, synthetic copolymers aim to solve these limitations. The emergence of free-radical polymerization and controlled polymerization reactions has greatly advanced the preparation of such copolymers and many approaches are now

studied to generate these structures and better reproduce the membrane disruption properties of AMPs, regarding both effectiveness and selectivity.<sup>146</sup>

## **2.1 Polymeric analogues of AMPs as membrane disrupters: structure-activity relationships.**

To destabilize the bacterial membrane, the key chemical design of AMPs is to finely control the hydrophobic to cationic ratio of the macromolecule.<sup>134</sup> While this control is often coarse when using homopolymers, topology is a key mean of controlling how two different monomers locate and distribute on polymeric backbones. Different topological distributions will generate backbones with different ratios that will exhibit different antimicrobial activities.<sup>147,148</sup> Many polymerization methods are available and now make it possible to control topology, using copolymerization reactions.<sup>149</sup> Polymer mixtures containing cationic and hydrophobic monomers were found particularly critical with the mechanism involving negative Gaussian curvature, a mechanism that currently explains the membrane destabilization ability of more than 1,000 cationic AMPs (see figure 8).<sup>103</sup> Indeed, the progress achieved over the last few decades in copolymerization reactions allows obtaining perfectly controlled hydrophobic/cationic ratios.<sup>150,151</sup> Moreover, the wide variety of monomers and monomeric side-chains makes it possible to obtain a large pool of polymeric structures<sup>152</sup> and has enabled the development of stimuli-responsive antimicrobial polymers.<sup>153</sup> For instance, the use of the radical polymerization process afforded copolymers of methacrylate and aminoethylmethacrylate that were able, at precise hydrophobic to cationic ratios, to promote specific NGC, membrane pore formation, micellization, blebbing, and budding observed with AMPs.<sup>154</sup> Specific NGC induced membrane disruption was also observed using quaternized poly(2-(dimethylaminoethyl)methacrylate initiated with bis(2-hydroxyethyl)disulfide.<sup>155</sup>



**Figure 8.** Key antimicrobial synthetic polymers: a) poly(aminoethylmethacrylate-co-butylmethacrylate) that involves NGC mechanism<sup>154</sup>, b) quaternary ammonium poly(carbonates),<sup>119</sup> c) and d) poly(methacrylates) bearing aminoacid analogues,<sup>152</sup> e) poly(norbornenes)<sup>156</sup>, and f) poly(vinylethers).<sup>157</sup>

The hydrophobic to cationic ratio, also called the amphiphilic balance, influences both the antibacterial activity and selectivity.<sup>125,158</sup> For instance, Chin *et al.* synthesized, through ring-opening polymerization, a set of cationic polycarbonates with quaternized ammonium groups, which showed antibacterial activity and selectivity that was modulated by the chemical nature of the alkyl group.<sup>119</sup> Related to this amphiphilic balance, the cationic group is one of the elements that critically tune the antibacterial activity. In this direction, Palermo *et al.* compared the biocidal behaviour of ammonium cationic (poly(2-aminoethylmethacrylate), PAEMA), tertiary ammonium (PDMAEMA), and quaternary ammonium groups (alkylated PDMAEMA).<sup>139</sup> It was found that polymers with primary and tertiary ammonium groups exhibited a higher biocidal effect and a better selectivity than the quaternized counterpart, which required higher hydrophobicity to reach similar antibacterial performance. Related to the hydrophobic content, spacers and satellite groups have an important function since they could modify this content. In this direction, a “snorkelling effect” which directly relates the spacer length and the biocidal activity was observed by Palermo *et al.* using poly(aminomethacrylate)-co-poly(ethylmethacrylate) having different alkyl chain spacers (2 to 8 carbons).<sup>159</sup> Satellite groups refer to the termini groups and probably assist the cationic group in disrupting the membrane by co-insertion. Waschinski *et al.* suggested, after having varied the satellite groups of polyoxazolines, that the best one was *N-N*-dimethyl-dodecylammonium end groups.<sup>160</sup> Finally, the control of the topology through copolymerizations also allowed the control of the

microstructure. Generally, statistical copolymers are reported in the literature but the control towards other microstructures could be important, for instance to promote selectivity toward hemolysis.<sup>161</sup> In this direction, Oda *et al.* showed no significant difference in antimicrobial activity between block and statistical copolymers of poly(2-aminoethyl-vinylether) and poly(isobutyl-vinylether) but the statistical copolymer presented much higher selectivity for *E. coli* over human red blood cells.<sup>157</sup>

Another important property to be modulated when designing synthetic antibacterial polymers is the **molecular weight** of the polymer. To inhibit the biofilm formation of *S. aureus*, Ikeda *et al.* found an optimal molecular weight ranging from 50 to 500 kDa using copolymers of acrylamide and acrylates bearing biguanide side-chain groups.<sup>162</sup> In the same way, Kuroda, DeGrado and co-workers showed that polymethacrylates were active against *E. coli* depending on their molecular weight and their hydrophobic to cationic ratio.<sup>163</sup> However, for therapeutic applications, a high molecular weight results in a potential decrease of aqueous solubility and could decrease antimicrobial activity.<sup>156</sup> Moreover, molecular weight can affect the biocidal activities of the polymers, because it can affect the size and the net charge of the polymer.<sup>125</sup> Much less considered, the effect of the **counter-ions** paired to the cationic charges influences both the solubility and the activity: strong affinity to the cationic charge can reduce the solubility i.e. tetrafluoroborate in poly(tributyl(4-vinylbenzyl)-phosphonium) when they were used against *S. aureus*.<sup>164</sup> The counter-ion paired to the cationic charge also tailored the overall hydrophobicity as evidenced with dodecanoate or hexadecanoate in poly(oxanorbornene).<sup>165</sup> However, no difference was found using Cl-, Br- and I- in copolymers incorporating quaternized vinylamines or aminoalkylmethacrylates.<sup>166</sup>

The last important parameter, the chemical nature of the **alkyl side chain** beared by the monomer units plays a crucial role during the interaction with the bacterial membrane as it was demonstrated by Lienkamp *et al.* through the activity against *E. coli* of oxanorbornene polymers.<sup>165</sup> They provided activity when side chains were propyl or butyl and depleted activity

with methyl and ethyl substitutions. In another study, it was demonstrated activity and selectivity with a length of 4 carbons, when the *N*-side chain was varied from 1 to 8 carbons in quaternized polycarbonates.<sup>119</sup> The biocidal activity decreased when increasing the carbon chain length, presumably, due to a decrease in water solubility. Also, the effect of the structure between hexyl, cyclohexyl and benzyl groups was studied and the best activity corresponded to hexyl, the less hydrophobic group as confirmed by water-octanol test. Through the tuning of the polynorbornene derivatives, the influence of the length of the alkyl chain, as well as the amphiphilic balance, was also demonstrated by Ilker and coworkers.<sup>156</sup>

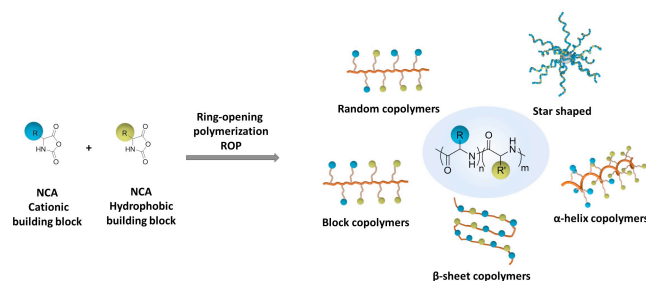
Despite numerous rationalization efforts to better understand and optimize their antimicrobial activity, synthetic polymers remain much simpler than AMPs and do not allow to finely reproduce their characteristics such as the selectivity toward the type of membrane that is disrupted. Although many polymer backbones including polymethacrylates, polycarbonates, polynorbornene, polypyridinium and polyarylamide have shown impressive antimicrobial activity, most of these macromolecules are not suitable for the development of topical or systemic treatments.<sup>134</sup> Very often, the macromolecules are very large and thus may not act as fast as oligomers such as AMPs or small molecules.<sup>121</sup> Indeed they require contact times on the order of hours to provide substantial reductions in pathogens and really have no practical value as therapeutics. They fall into the category of antiseptic agents that can be useful for medical devices and they are generally used as disinfectant additives in coatings, hand washing, detergents, filters, etc. To better optimize polymer candidates for therapy, more efforts must be done to optimize the chemical properties that can be modulated with the polymer chemistry.

## **2.2 Polypeptide analogues of AMPs.**

As presented in Part 1 of this article, AMPs are generally constituted of 10 to 60 amino acid residues and the only synthetic methodology for accurately reproducing their primary sequence is the solid-phase peptide synthesis (SPPS). Although this technique offers great flexibility in the precise control of peptide composition, it is time-consuming and costly. This article does not

cover the use of SPPS methodology to fully reproduce the structure of AMPs and readers are invited to read the following references for more information.<sup>95,167</sup> Alternatively, the most economical and efficient process to prepare synthetic polypeptides is a one-step polymerization process, the ring-opening polymerization (ROP) of *N*-carboxyanhydride monomers (ROP, figure 7). This controlled polymerization process involves the simplest reagents and allows the preparation of polymers made of amino acids in both good yields and large quantities.<sup>168,169</sup> Synthetic polypeptide polymers are simple macromolecules compared to natural proteins. In their structure, an amino acid is repeated many times, retaining the tendency to adopt ordered secondary conformations such as  $\alpha$ -helices or  $\beta$ -sheets, a property that is rare in polymer science.<sup>170</sup> Moreover, as compared to natural proteins, polypeptide polymers can easily undergo secondary structure transitions that can be easily implemented and tuned by tailoring amino acid side chains.<sup>168,171</sup>

To approach AMPs and design more selective molecules with antibiotic potential, polypeptide polymers are certainly one of the best scaffolds to consider.<sup>172</sup> First, they present the same macromolecular backbone as AMPs. Second, they can be obtained in a one-step synthesis and large quantities by using a controlled methodology (figure 9).<sup>61,168,169</sup> Third, they can fold into different structures in the same way as some class of AMPs do,<sup>170</sup> an important feature opening new ways toward antimicrobial polymers.<sup>134</sup> Very early, polypeptide copolymers have been used to induce membrane destabilization.<sup>173,174</sup> The first fundamental studies on this subject showed that cationic polypeptide copolymers were able to bind to phospholipid membranes despite large distributions in chain lengths and a lack of control over the primary sequence of amino acids. Later, controlled polymerization was a decisive contribution to better study the macromolecular parameters involved in such membrane destabilization (amino acid composition, molecular weight etc.).<sup>175,176</sup>



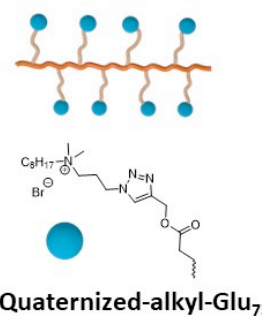
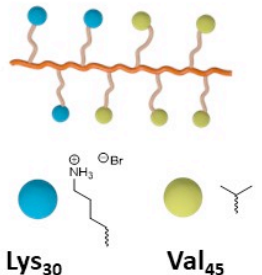
**Figure 9.** Ring-opening polymerization of *N*-carboxyanhydrides affords an efficient methodology to design antimicrobial polypeptides.

Aiming at better reproducing the topological design of AMPs, very interesting designs have emerged in the polypeptide field (see table 2). The first important structure/activity relationship of polypeptide polymers was the amino acid composition, showing sequences mixing positive charges with hydrophobic residues to be particularly active in membranes disruption.<sup>169,175</sup> In this direction, an interesting work published by Zhou *et al.*<sup>118</sup> presented polypeptide polymers with broad anti-infective spectrum using copolymers composed of *L*-lysine, *L*-phenylalanine and *L*-leucine. Against *C. albicans*, *E. coli*, *P. aeruginosa*, *S. marcescens* and *S. aureus*, the activities were found higher than the ones obtained with 3 well known AMPs that are often used as antibacterial positive controls in the literature: defensins LL-37, indolicidin and magainin I.

**Table 2.** Topological studies of linear polypeptides

Key biomimetic feature	Length (monomer units)	Activity against	MIC	Ref
<p><b>Hydrophobic/Cationic ratio</b></p> <p><b>Lys<sub>10</sub></b>      <b>Phe<sub>7.5</sub></b>      <b>Val<sub>7.5</sub></b></p>	25	<i>E. coli</i>	31 $\mu$ g/mL	118
		<i>P. aeruginosa</i>	31 $\mu$ g/mL	
		<i>S. marcescens</i>	250 $\mu$ g/mL	
		<i>S. aureus</i>	31 $\mu$ g/mL	
		<i>C. albicans</i>	125 $\mu$ g/mL	



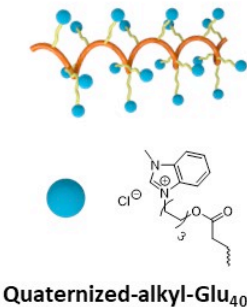
Cation				
 <p>Quaternized-alkyl-Glu<sub>75</sub></p>	75	<i>S. aureus</i>	156-312	177
		<i>E. coli</i>	μg/mL	
Microstructure				
 <p>Lys<sub>30</sub> Val<sub>45</sub></p>	75	<i>E. coli</i>	8 μg/mL	178
		<i>S. aureus</i>	2 μg/mL	
		<i>P. aeruginosa</i>	8 μg/mL	
		<i>S. marcescens</i>	16 μg/mL	

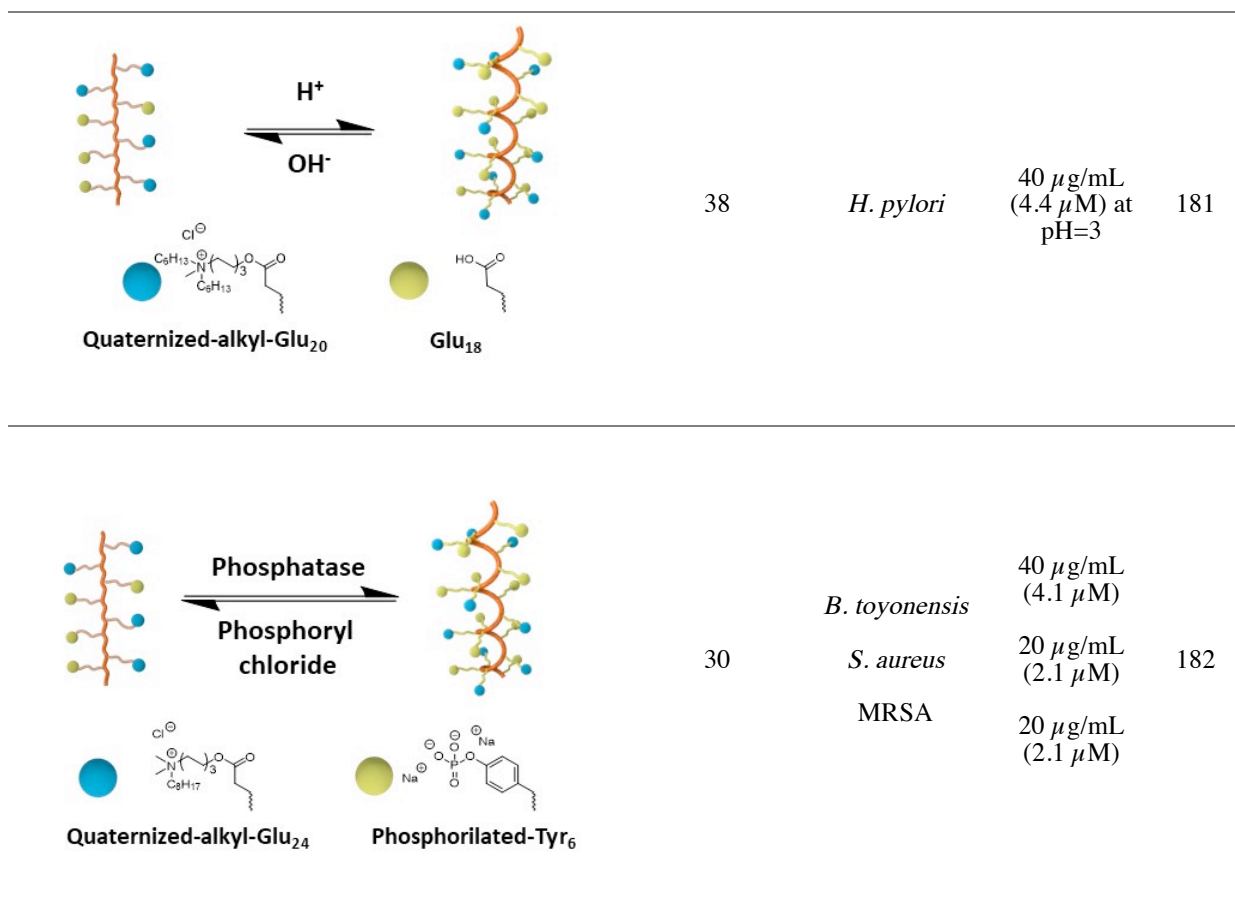
· Minimum inhibitory concentration

As with synthetic polymers presented in Part 2.1, the positive charges carried by the side chains of the polypeptides appear to be crucial. A complete study exploring this parameter was published by Engler *et al.* and reported poly( $\gamma$ -propargyl-*L*-glutamate) backbones modified by click chemistry to design a variety of hydrophobic length with primary, secondary, tertiary and quaternary amine pendant groups.<sup>177</sup> All the polypeptides bearing quaternary amine groups were active against *S. aureus*. On another hand, the chemical nature of the hydrophobic side chain also played an important role to optimize the antibacterial activity as it was demonstrated by the higher effect obtained with phenylalanine as compared to leucine, tackling both Gram-positive and negative bacteria.<sup>179</sup> The microstructure and the hydrophobic to cationic ratio clearly appeared to be other important parameters to promote more active and selective polymers. In this direction, Su *et al* compared the efficacy of block copolypeptides and random copolymers based on lysine and phenylalanine, varying the hydrophobicity from 30% to 60%.<sup>178</sup> All the copolymers evaluated presented activity against *E. coli*, *S. aureus*, *P. aeruginosa* and *S. marcescens*. This

activity was found higher with block copolymers and when the hydrophobic content was increased. The cytotoxicity revealed that the copolymers were safe up to 1000  $\mu\text{g/mL}$  (tested in L02 cell line) even with 60% of hydrophobic side chains and, similarly to AMPs, indirect enzymatic degradation was demonstrated upon 3 h. Polypeptides, therefore, enable the key features of synthetic copolymers to be reproduced. They can also take advantage of their unique features: impressive reports show for instance that the secondary structures of polypeptide polymers have a significant impact on antimicrobial activity (see table 3). Cheng and coworkers showed that helical AMPs can be mimicked with polypeptide polymers using poly(glutamate) bearing benzimidazole cations in the hexyl termini side chain. These polypeptides adopt a water-soluble helical structure with radial amphiphilicity and showed strong antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. toyonensis*, and *H. pylori*.<sup>180</sup>

**Table 3.**  $\alpha$ -Helical antimicrobial polypeptides

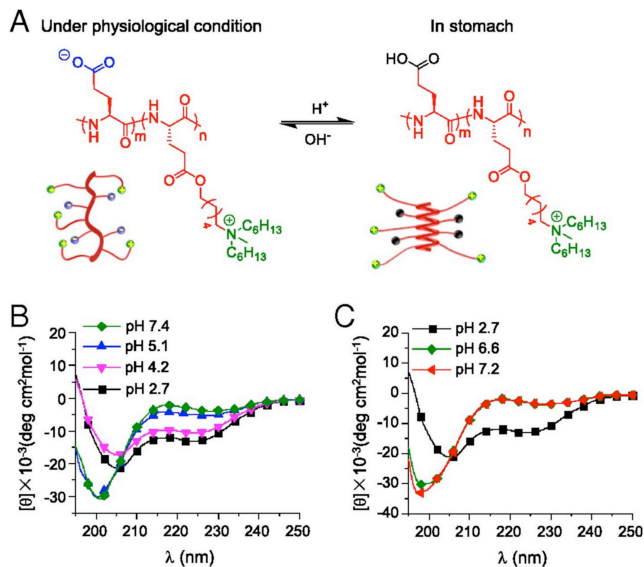
Polymer	Length (Monomer units)	Activity against	MIC <sup>a</sup>	Ref
 <p>Quaternized-alkyl-Glu<sub>40</sub></p>	40	<i>E. coli</i>	3-26 $\mu\text{g/mL}$	180
		<i>P. aeruginosa</i>	13 $\mu\text{g/mL}$	
		<i>S. aureus</i>	13 $\mu\text{g/mL}$	
		<i>B. toyonensis</i>	1.5-6.6 $\mu\text{g/mL}$	
		<i>H. pylori</i>	1.5 $\mu\text{g/mL}$	
		MRSA <sup>b</sup>	1.5 $\mu\text{g/mL}$	



<sup>a</sup> Minimum inhibitory concentration; <sup>b</sup> Methicillin-resistant *Staphylococcus aureus*

Later, the same group developed a copolymer composed of glutamic acid units and positively charged  $\gamma$ -6-*N*-(methyl-dihexyl-ammonium)hexyl-*L*-glutamate units (see figure 10).<sup>181</sup> The copolymer presented a pH-sensitive  $\alpha$ -helical structure which provided a remarkable activity against *H. pylori* at acidic pH, making this copolymer active, selectively in the stomach, after oral administration.<sup>181</sup> Furthermore, the full hydrolysis of the copolymer upon 24 h was demonstrated by labelled resonance imaging. The same research group introduced more recently another important milestone regarding secondary structures by designing bioresponsive copolymers made of phosphorylated tyrosine and ((*N,N*-dihexyl-*N*-methyl)- $\gamma$ -hexyl-ammonium)glutamate.<sup>182</sup> These copolymers adopt an  $\alpha$ -helical structure when dephosphorylated by bacteria (*B. cereus* ATCC11778, *S. aureus* ATCC12608 and *S. aureus* NRS384), increasing the disrupting of bacterial membranes and introducing the concept of zwitterionic antimicrobial

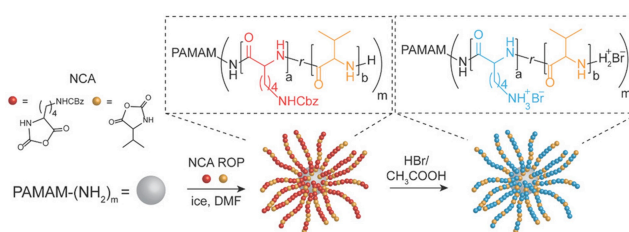
polymers. The fact that water-soluble  $\beta$ -sheets can also be obtained by following similar chemical designs undoubtedly opens up interesting prospects for mimicking antimicrobial peptides that present this secondary structure.<sup>183</sup>



**Figure 10.** Copolyptide displaying pH-sensitive secondary structuring into  $\alpha$ -helix. A) Scheme of the pH transition of the copolymer  $(PGA)_n$ - $r$ - $(PHLG-MHH)_m$ . The random coiled conformation at physiological pH presents low cytotoxicity while helical transition under acidic conditions in the stomach induces potent antimicrobial activity against *H. pylori*. Circular dichroism spectra of the copolymer at different pH values from B) neutral to acidic pH and C) acidic to neutral pH. Reprinted with permission from ref 81. Copyright 2017, PNAS.

Among the important elements that influence the antibacterial activity of polypeptides, macromolecular engineering was also reported (see table 4). Indeed, using a polymerization reaction opens the way to various architectures that would be difficult to implement with simple SPPS. In this direction, a comprehensive study involving copolymers of benzylglutamate and lysine, having a polymerization degree of 30, provided good antibacterial activity against *E. coli* and *B. subtilis* with a remarkable low haemolysis  $HC_{50} > 4000 \mu\text{g/mL}$ .<sup>184</sup> In this direction, the star-shaped architecture was more specifically studied and gave spectacular results. The design of this type of architecture from a dendrimer core was first proposed by Lam *et al.*<sup>185</sup> who synthesized copolymers composed of lysine and valine over poly(amidoamine)-dendrimers. The number of

arms of the polymers was either 16 or 32, keeping the ratio of lysine over valine  $\sim 2:1$ . Efficacy of those star-shaped polypeptides was demonstrated against Gram-negative and positive bacteria including multidrug-resistant *P. aeruginosa* and *A. baumannii*. As compared to linear AMPs such as ovispirin, magainin II or melittin, the copolymers demonstrated superior activity, even *in vivo* against multidrug-resistant bacteria. Membrane disruption was corroborated by fluorescence but no pore formation was confirmed by dye released. This suggested that membrane disruption by remarkable cell depolarization was a consequence of the efflux pump-like interaction with the polypeptides which was investigated later by fluorescence and ion exchange.<sup>186</sup>



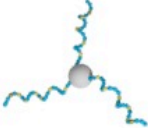
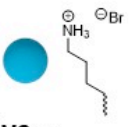
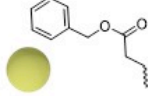
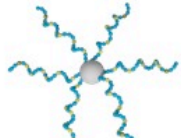
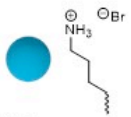
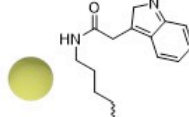
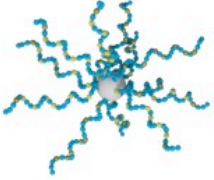
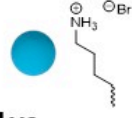

**Figure 11.** Star-shaped antimicrobial copolypeptides of lysine and valine prepared from different dendritic structures (number of arms from 4 to 16). Reprinted with permission from ref 187. Copyright 2018, WILEY VCH Verlag GmbH & Co. KGaA, Weinheim.

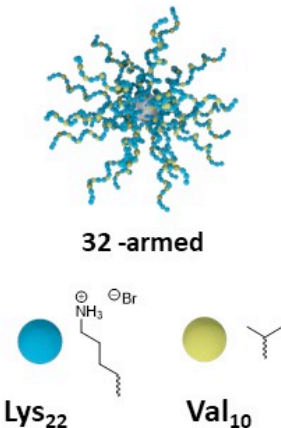
In 2018, Steven *et al.* reported another study involving similar star-shaped copolymers (figure 11) and varying arm numbers (4-16) and arm length (5-30).<sup>187</sup> The study revealed that increasing arm number and length enhanced the antimicrobial activity, which can be related to higher local concentrations of polypeptide arms and increase of  $\alpha$ -helical content. However, the increase in antimicrobial activity was accompanied by an increase in cytotoxicity. In addition, another star polymer based on lysine and post-functionalized with indole (20% mol) was also developed and demonstrated modest cell viability in hepatic cells H1299 (<20%) at 20  $\mu$ M as compared to its linear counterpart which presented better cell viability (40%) at the same concentration.<sup>188</sup> A higher antimicrobial effect of star polymers was nevertheless corroborated for Zhang *et al.*, by modelling and comparing the activity of star polymers with 10 lysine and 5 phenylalanine residues to cecropin A and melittin.<sup>189</sup> It should be noted here that self-assembly is possibly another way of designing nanoingereed architectures based on polypeptide: this approach not

only mimicked AMPs activity but also reduced their cytotoxicity toward red blood cells. For instance, Constanza *et al.* performed the synthesis of PEG-*b*-polylysine conjugated to antimicrobial polypeptides made of lysine and phenylalanine.<sup>190</sup> The resulting nanoassemblies demonstrated reduction in haemolytic activity due to controlled hydrophobicity through pegylation, a strategy that was found effective against Gram-positive and negative bacteria including methicillin-resistant *S. aureus*. More recently, Chen *et al.* developed a similar strategy based on dextran and copolymers of lysine and phenylalanine.<sup>191</sup> Broad-spectrum antibacterial and antifungal activity were shown for the copolymers, including against multidrug-resistant bacteria. This design decreased the haemolytic activity and exhibited biocompatibility in murine myoblast (C21C12) cells.

Overall, polypeptides are certainly important polymers for promoting antibacterial properties, but it should be noted that their activity has been quite exclusively studied *in vitro*. So far few articles reported encouraging results *in vivo*. In this direction, one key contribution was to propose star-shaped polypeptides that demonstrated *in vivo* efficacy against colistin multidrug-resistant *A baumannii* in mice with an 8.3 mg/kg dose.<sup>185</sup> Star-shaped polypeptides were also used to kill enterohaemorrhagic *E. coli* in mice after administration of a 20 mg/kg dose and reducing inflammatory cytokine expression.<sup>188</sup> Regarding the *in vivo* toxicity, star-shaped polypeptides did not disturb two specific organ damage markers (alanine transaminase and creatinine phosphokinase) after intraperitoneal administration of the antimicrobial dose 4 mg/kg.<sup>187</sup> Linear copolymers such as poly(lysine-*Cbz*-glutamate) were also found active *in vivo* at lower concentration against multidrug-resistance *Pseudomonas aeruginosa* after topical administration of the dose 250  $\mu$ g/kg in rats.<sup>192</sup> In comparison, oral administration of secondary structured polypeptides showed selective activity against *H. pylori* when inoculated in mice but at much higher concentration (administrated with  $\sim$ 24 mg/kg, 2.6  $\mu$ mol/kg)<sup>181</sup> a concentration at which they were not found toxic.<sup>181</sup>

**Table 4.** Start-shape antimicrobial polypeptides

Polymer	Length (Monomer units)	Activity against	MIC	Cytotoxicity	Ref
 <b>3-armed</b>  <b>Lys<sub>24</sub></b>  <b>Cbz-Glu<sub>6</sub></b>	30	<i>E. coli</i>  <i>B. subtilis</i>	2 $\mu\text{g/mL}$ (1.6 $\mu\text{M}$ )  4 $\mu\text{g/mL}$ (3.2 $\mu\text{M}$ )	HC <sub>50</sub> >270 $\mu\text{M}$ (4000 $\mu\text{g/mL}$ )	184
 <b>6-armed</b>  <b>Lys<sub>16</sub></b>  <b>Indol-Lys<sub>4</sub></b>	20	<i>K. pneumonia</i>  <i>E. coli</i>  <i>P. aeruginosa</i>  <i>S. sonnei</i>  <i>S. typhimurium</i>  <i>S. aureus</i>	1600 $\mu\text{g/mL}$ (70 $\mu\text{M}$ )  170 $\mu\text{g/mL}$ (7.5 $\mu\text{M}$ )  1600 $\mu\text{g/mL}$ (70 $\mu\text{M}$ )  1600 $\mu\text{g/mL}$ (70 $\mu\text{M}$ )  200 $\mu\text{g/mL}$ (8.7 $\mu\text{M}$ )  2050 $\mu\text{g/mL}$ (90 $\mu\text{M}$ )	HC <sub>50</sub> >20 $\mu\text{M}$	188
 <b>16-armed</b>  <b>Lys<sub>12.4</sub></b>  <b>Val<sub>5.6</sub></b>	18	<i>E. coli</i>	4.4 $\mu\text{g/mL}$	IC <sub>50</sub> (H4IIE) = 0.614 $\mu\text{M}$	187

 <p><b>32-armed</b></p> <p><b>Lys<sub>22</sub></b>      <b>Val<sub>10</sub></b></p>	32	<i>E. coli</i>	4.4 $\mu\text{g/mL}$ (0.05 $\mu\text{M}$ ) <sup>a</sup>		
		<i>P. aeruginosa</i>	1.7 $\mu\text{g/mL}$ (0.02 $\mu\text{M}$ ) <sup>a</sup>		
		<i>K. pneumoniae</i>	7.0 $\mu\text{g/mL}$ (0.08 $\mu\text{M}$ ) <sup>a</sup>		
		<i>A. baumannii</i>	1.7 $\mu\text{g/mL}$ (0.02 $\mu\text{M}$ ) <sup>a</sup>		HC <sub>50</sub> >45 $\mu\text{M}$ <sup>c</sup>
		<i>A. baumannii</i>	2.6 $\mu\text{g/mL}$ (0.03 $\mu\text{M}$ ) <sup>a</sup>		
		<i>P. aeruginosa</i>	2.6 $\mu\text{g/mL}$ (0.03 $\mu\text{M}$ ) <sup>a</sup>		

<sup>a</sup> Minimum inhibitory concentration; <sup>b</sup> multidrug-resistant bacteria including colistin resistance; the values are given according to the minimum bactericidal concentration (MBC); <sup>c</sup> low hemolytic activity; <sup>d</sup> half-maximal inhibitory concentration.

In conclusion, the use of polypeptides enables the design of macromolecules with antibacterial properties similar to those of AMPs, sometimes even superior, and their unique chemical design combines the benefits of polymer chemistry while retaining the unique properties of chemical backbones made of amino acids. For the moment, selectivities obtained with amino acid copolymers resemble selectivities obtained with other synthetic polymers,<sup>118,179,193</sup> but responsive secondary structuring<sup>194</sup> offers additional chemical leverage that promises significant progress opening the way to possible therapeutic applications as oral treatments.<sup>181</sup>

### 2.3 Current prospect and future perspectives

The question of mimicking the secondary structures of AMPs with other chemical backbones coming from polymer chemistry is open and much work is being done on new design choices that could impact the field of antibacterial polymers in the future.<sup>183,195,196</sup> On the other hand, while the polymerization of NCAs has long been confined to a demanding chemistry, recent impressive advances making this chemistry simpler will significantly impact the field of antimicrobial



polypeptides.<sup>60,62</sup> A significant example is the use of LiHMDS-mediated polymerization,<sup>61</sup> which allows open reactor polymerizations in a few minutes and facilitates the preparation of antimicrobial polypeptide libraries. Although peptidic backbones are polypeptides classified among biodegradable polymers, their very high susceptibility to enzymatic hydrolysis can also result in a lack of effectiveness *in vivo*, since they can be quickly degraded by proteases found for instance along the digestive tube, such as trypsin or pepsin.<sup>178</sup> At that stage, these limitations are potential drawbacks and are the future challenges of polypeptides in the field. With respect to polymer degradation, it should be noted that significant efforts have been made in ring-opening polymerization processes, using other monomers, to afford synthetic polymers with antimicrobial properties. In this field, the development of polycarbonates made by Hedrick and coworkers paves the way to particularly efficient antimicrobial polymers.<sup>143</sup> Generally, biodegradable polymers, including polypeptides, are passively degraded and polymers that can degrade in a specifically triggered manner may confer some interesting perspectives to antimicrobial properties.<sup>197</sup> For instance, the control of the degradation process imparts significantly the selectivity towards hemolytic activity and, in this direction, the design of self-immolating polymers is promising.<sup>198</sup> Integrating such new chemical behavior in polypeptide chains is still a challenge but it might be an important feature to better optimize the selectivity. Finally, the design of other classes of macromolecular peptidomimetics using a polymerization reaction is not yet enough developed compared to the large number of peptidomimetics that can be obtained from iterative coupling reactions.<sup>199-201</sup> In this direction, polymers such as polyoxazolines<sup>202</sup> or poly- $\beta$ -peptides<sup>202</sup> could be serious candidates to improve the existing limitations of synthetic polymers. Their chemical designs, including amino acid-based backbones, bring them closer to AMPs and many advances are to be expected.

## Conclusion

This review describes the fundamental principles that should guide the design of antibacterial polymers made of amino acids, based on what we know about the chemical structures and

mechanisms of action of AMPs. The design of these polymers may include the know-how and knowledge developed with other synthetic polymers, but it also allows the integration of specific chemical features coming from the use of amino acids and that are important in AMPs such as secondary structuring and biomimetic stimuli-responsiveness. From a material point of view, the excellent control in polymer synthesis that is available today provides new tools for the synthesis of antimicrobial peptides. Cross-fertilization between advanced polymer synthesis and efficient organic coupling also offers promising prospects for the creation of macromolecular peptidomimetics. Indeed, chemistry today offers several methodologies to access synthetic pseudo-peptides, simplified analogues of antimicrobial peptides. These polymeric AMPs pave the way for the development of innovative therapies that could be an important mean in the fight against bacterial resistance. In addition, synthetic polypeptides or pseudopeptides are innovative materials that should bring important breakthroughs in applications belonging to fields that merge materials science and infectology.

## AUTHOR INFORMATION

### **Corresponding Author**

\*colin.bonduelle@enscbp.fr

### **Author Contributions**

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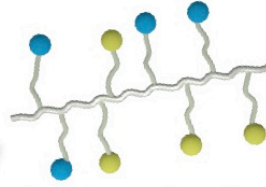
## SYNOPSIS

**Antimicrobial peptide (AMPs)**



*Amino-acid based*

**Synthetic Polymers**



*Simple molecular design*

