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Antimicrobial polymers: Mimicking amino acid functionality through to three-dimensional structure of host-defense peptides

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Abstract: Peptides and proteins control and direct all aspects of cellular function and communication. Having been honed by nature for millions of years, they also typically display an unsurpassed specificity for their biological targets. This underlies the continued focus on peptides as promising drug candidates. However, the development of peptides into viable drugs is hampered by their lack of chemical and pharmacokinetic stability and the cost of large scale production. One method to overcome such hindrances is to develop polymer systems that are able to retain the important structural features of these biologically active peptides, while being cheaper and easier to produce and manipulate chemically.

This review illustrates these principles using examples of polymers designed to mimic antimicrobial host-defence peptides. The host-defence peptides have been identified as some of the most important leads for the next generation of antibiotics as they typically exhibit broad spectrum antimicrobial ability, low toxicity toward human cells and little susceptibility to currently known mechanisms of bacterial resistance. Their movement from the bench to clinic is yet to be realised, however, due to the limitations of these peptides as drugs. The literature provides a number of examples of polymers that have been able to mimic these peptides through all levels of structure, starting from specific amino acid sidechains, through to more global features such as overall charge, molecular weight and three-dimensional structure (e.g. α-helical). The resulting optimised polymers are able retain the activity profile of the peptides, but within a synthetic macromolecular construct that may be better suited to the development of a new generation of antimicrobial therapeutics. Such work has not only produced important new leads to combat the growing threat of antibiotic resistance, but may also open up new ways for polymers to mimic other important classes of biologically active peptides.

Keywords: antimicrobial peptides, polymers, bacteria, peptide mimic, host-defense peptides

1. INTRODUCTION

The discovery of penicillin and its use in treating bacterial infection is hailed as one of the greatest breakthroughs in modern medicine, responsible for saving millions of lives. We are, however, facing a time where infectious diseases that were easily treatable, are now once again life threatening. The increasing prevalence of bacterial resistance, compounded with the slowing rate of novel antibiotic discovery in recent times has created what the World Health Organization has dubbed as a post-antibiotic era [1]. We have not experienced such low levels of effective infection control for more than 75 years [2]. This has placed great pressure on medicinal chemists to identify novel classes of antibiotic, particularly those that have a lessened ability to elicit resistance in the microbes they combat.

A class of molecules that hold great promise are the antimicrobial peptides (AMPs). These are small peptides, usually 10-50 amino acids long, amphipathic in nature, with a high concentration of cationic residues. Many have also been shown to adopt facially amphiphilic structures, with hydrophobic residues aligned on one side of the molecule, cationic groups along the other. AMPs are produced by all forms of life, forming part of innate immune systems in higher organisms, as denoted by their alternative name, host defence peptides [3]. The key aspects of activity for these peptides is that they show broad spectrum antimicrobial effects, a high safety profile against mammalian cells and a reduced susceptibility to resistance. In fact, it is estimated that bacteria have been exposed to AMPs for over half a billion years[4] yet show a low prevalence of resistance compared with conventional antibiotics [5]. This has been in part explained by the unique mechanism of action underlying these peptides.

The cationic groups present on AMPs are thought to drive binding to bacterial membranes which are anionic at their surface (Fig. 1). From here, hydrophobic residues disrupt the integrity of the membrane to cause cell death. As mammalian cells have a net neutral charge at their surface, AMPs are less likely to bind to these cells, accounting for the selectivity observed for microbial membranes. This mechanism contrasts to that of conventional antibiotics such as penicillin, that involve binding to very defined moieties within bacterial proteins. The specificity of this binding, allows only very small genetic adaptions to invoke resistance, whereas resistance to AMPs would more likely require a global change to membrane architecture. It should also be noted there is growing body of evidence for

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additional intracellular bacterial metabolic targets that mediate AMP effects.

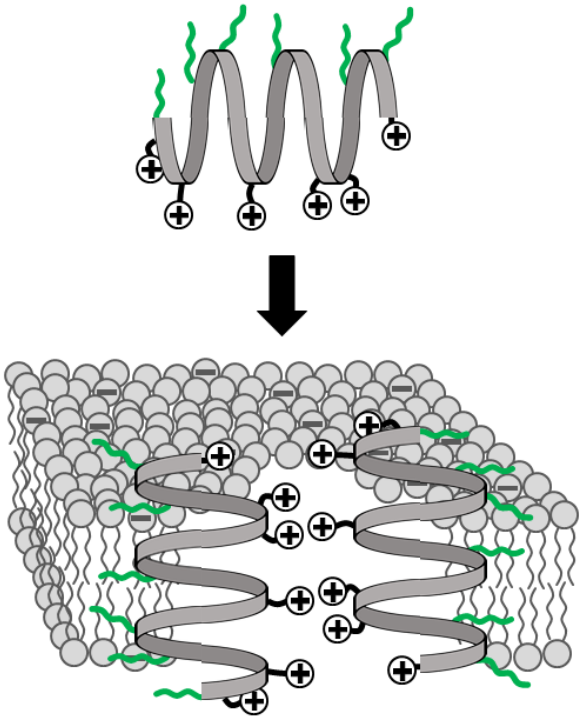


Fig. (1). Schematic representation of the mode of action of AMPs

While the activity profile of AMPs warrants further examination, their transition from the bench to bedside has been slow [6]. Peptides are not typically ideal drug candidates as they are expensive to produce on industrial scale and show limited pharmacokinetic stability. To overcome this, chemists are now examining synthetic mimics of AMPs. With the discovery that a facially amphipathic structure was not essential for AMP activity, came the ability to examine a wider array of synthetic architectures. Perhaps the most promising of these are the AMP mimicking polymers. This review illustrates the principles used to design such polymers, with a key focus on mimicry of different levels of peptide structure, stretching from specific amino acid functionality, mimicry of primary structure with polymer sequence control through to the tertiary level with the three-dimensional structure of polymer nano-particles.

2. STRUCTURE-ACTIVITY RELATIONSHIPS IN AMP MIMICKING POLYMERS

2.1 Amphiphilic Balance

In the context of polymer mimics of AMPs, on many accounts the balance of hydrophobic unit and cationic charge, the amphiphilic balance, is the most investigated parameter. This originates from the proposed mechanism of action of killing bacterial cells and the selectivity between bacterial and mammalian cells.

The antimicrobial activity of AMPs and their mimics is based on the interaction and disruption of the cell membrane. In order to select bacterial membranes over others, the charge plays an important role. The share of negatively charged phospholipids on the outside of bacterial cell membranes (*i.e.* phosphatidylglycerol in gram negative or cardiolipin in gram positive bacteria) is significantly higher as compared to mammalian cells (where usually zwitterionic phosphatidylcholines are present) enabling a targeting of these membranes by charge-charge interaction.[7] If the positive charge potential of a polymer at physiological conditions is high enough, it will preferentially bind to bacterial cell membranes by electrostatic interactions. The hydrophobicity of the material then enables it to to insert into the hydrophobic domain of the membrane, leading to its disruption, which ultimately leads to bacterial cell death. A further difference between mammalian and bacterial cell membranes is the increased presence of sterols, which regulate membrane fluidity and potentially prevent the disruption by AMPs and their mimics.[8] Different mechanisms have been observed for the membrane disruption of AMPs, with the barrel stave model (*i.e.* alamethicin[9]), the toroidal pore model (*i.e.* Magainin[10] or Melittin[11]) and the carpet model (*i.e.* Dermaseptin[12]) being the most accepted mechanisms.[13] Since polymeric mimics of AMPs usually do not possess a facially amphiphilic structure, but rather acquire this conformation when interacting with membranes, their mode of action is more likely to follow the carpet model (Fig. 2).[14]

In order for this process to operate efficiently and in a selective way, a subtle balance of hydrophobicity units and cationic charges has to be matched; such balance also affects solubility of the polymer. Unfortunately, both parameters are interconnected: an increase in positive charge will decrease the overall hydrophobicity of the polymer and *vice versa*. Many studies on different polymeric systems have been published on the systematic variation of this parameter.

2.1.1 Poly(methacrylate)s

Kuroda and coworkers have focussed on methacrylic polymers synthesized by irreversible chain transfer polymerization of mostly binary systems. [15] Amino ethyl methacrylate was usually used as charge generating unit and copolymerized with methacrylates bearing various hydrophobic side chains (methyl,[16] butyl,[17] ethyl,[18] hexyl[18] and phenyl[18]) (Fig. 3). The ratio of both comonomers, was varied in order to achieve the optimal compromise of antimicrobial activity and hemolytic activity (= highest selectivity). If the copolymer was too hydrophobic, selectivity for bacterial cell membranes was lost, leading to increased haemolysis, while an excess of cationic charges resulted in high MIC values, which indicates that the

polymers were not able to destabilize the target bacterial cell membrane.[16] In a direct comparison of different side chain length /architectures it was found that more hydrophobic monomers require a higher amount of cationic comonomer in order to be effective/selective, and for methacrylate based polymers, the methyl side chain was identified to achieve the highest selectivity when the amphiphilic balance is matched,[19] which also holds true when tertiary amines are used as a hydrophobic component (dimethylamine vs. diethylamine).[20] It becomes obvious how fragile that balance is when small changes in the backbone architecture (methacrylamide instead of methacylate) require much higher ratios of hydrophobic comonomer to work.[16] Detailed investigations on quarternized poly (vinyl pyridine)/ methacrylate copolymer systems revealed that one alkyl chain per quarternized amine obtains the best results, and that butyl chain perform best in this type of system.[21]

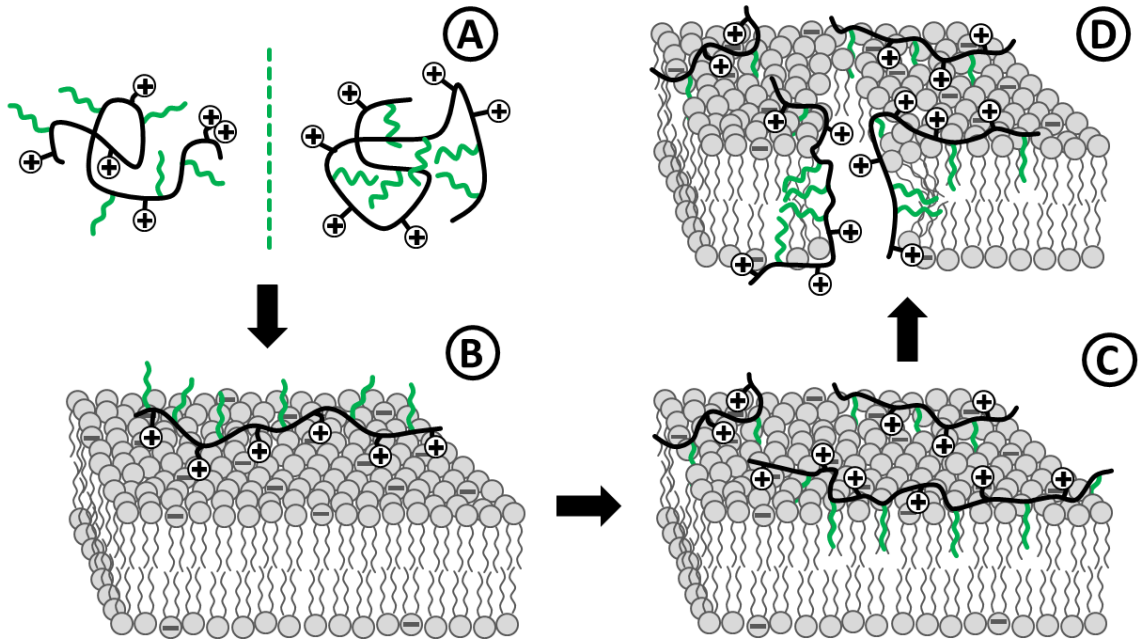


Fig. (2). Schematic representation of the mode of action of AMP mimicking polymers. A) Polymer in solution as either random coil (left) or partially segregated (and possibly aggregated, right). B) Attachment to bacterial membrane by electrostatic interaction. C) Insertion into the hydrophobic domain of the membrane. D) Membrane disruption leading to cell death.

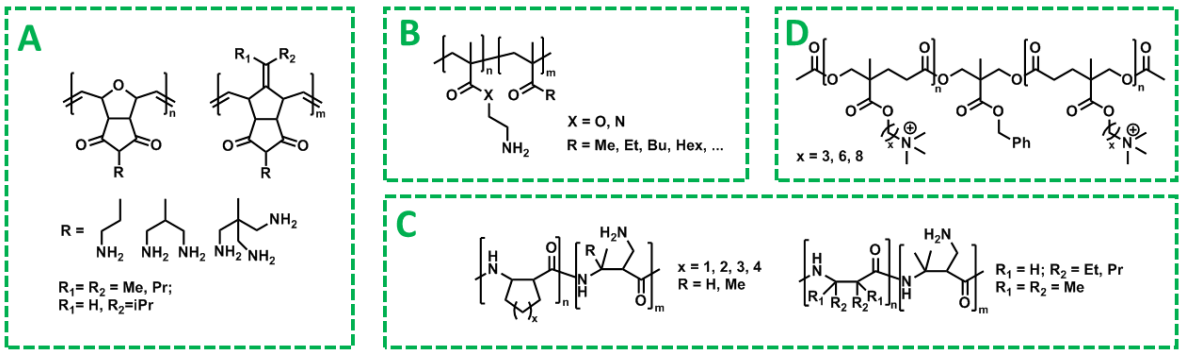


Fig. (3). Typical examples of polymeric mimics of AMPs: A) Poly(norbornene)s, B) Poly(methacylate)/poly(methacylamide), C) Nylon-3 copolymers, D) Poly(carbonate)s.

2.1.2 Poly(norbornene)s

The group of Tew focussed on poly(norbornene) backbone architectures obtained by ring-opening metathesis polymerization (ROMP) to identify the optimal combination of antimicrobial activity and haemocompatibility (Fig. 3). Amphiphilic balance was achieved by homopolymerization of monomers bearing both, hydrophobic and cationic groups with varying alkyl chains,[22] as well as by side chain attached pyridine units, quarternized with various alkyl chains.[23] Also, changes in charge density were addressed by comparing systems having one, two or three primary amine functions per monomer unit.[24] An increase of hydrophilic amine groups diminished the hemolytic activity of the polymers, and introduced a selectivity between gram negative and positive bacteria (*Staphylococcus aureus vs. Escheria Coli*), which could be related to the diverse membrane architectures both types of bacteria possess.[22d] In general, a similar trend to that of methacrylic systems was found: hydrophobicity is needed but leads to undesired hemolytic effects when present in excess. A further study on this system revealed the necessity of a certain charge threshold for antimicrobial polymers[25]. Only above this threshold, polymers will bind effectively (and irreversibly) to bacterial membranes, after which the hydrophobic section will account for the membrane disruption, thus suggesting that additional charges after a specific point will not affect further antimicrobial activity.

2.1.3 Nylons

A third class of polymers, which has been utilized to conduct systematic variation, is the family of nylon-3 copolymers investigated by Gellman and coworkers, composed of a primary amine containing monomeric unit and a cyclohexyl containing monomer as hydrophobic component. In this case also small changes in composition resulted in a changing selectivity with an optimum at about 60% cationic monomer.[26] The monomer architecture was also varied (*i.e.* variation in alkyl ring size[27] or cyclic *vs.* non-cyclic[28]) and it was concluded that an excess of hydrophobicity (*i.e.* by larger alkyl ring sizes) results in increased hemolytic activity and loss of antimicrobial activity (presumably by ineffective membrane attachment).[27] However, the backbone flexibility seems to also play a role as a non-cyclic monomer with the same number of carbon atoms showed improved biological activity.[29]

2.1.4 Beyond cationic and hydrophobic units

The global amphiphilicity of the polymer can also be influenced by the introduction of a third, non-cationic, hydrophilic component. This approach was used for nylon-3 copolymers,[30] with best results obtained when the ratio of cationic to hydrophobic comonomer was kept constant (related to the best performing binary system) and small doses of hydroxyl functionalized monomer were added, which resulted in a minor increase in MIC while hemolytic activity was decreased drastically. Similar results were observed for poly(norbornene) copolymers when extended by either zwitterionic, sugar or PEG moieties.[31] In the case of polyacrylics, a complete exchange of the hydrophobic component (HEMA[32] or PEGMA[33]) still results in antimicrobial polymers. However, it is unclear whether these systems still target the bacterial membrane like other AMP mimics.

A further parameter that can be introduced into AMP mimics are hydrogen bond donors and acceptors. As recently demonstrated using quarternized amine containing polymers the presence of amide bonds in the side chain of the polymer strongly increases the antimicrobial activity as compared to a control polymer equipped with ester units instead.[34] It was also shown that polymers with amide moieties require less hydrophobic units to be efficient antimicrobial agent in comparison to ester containing AMP mimics.[35]

As shown by Yang and coworkers it is also possible to control the antimicrobial activity of a polymer by a stimuli responsive change in amphiphilicity. In this example, the presence of methacrylic acid prevents membrane attachment at alkaline pH level, as the acid function is deprotonated and forms a complex with the cationic part of the polymer. Decreasing the pH value to an acidic level induces antimicrobial activity.[36]

In summary, while the amphiphilic balance seems to be the main parameter to consider for polymeric mimics of AMPs, it is difficult to predict, and it needs to be determined for each new polymeric system/set of monomers considered. HPLC analysis or water/octanol partitioning factor are excellent methods to determine the global amphiphilicty of a given copolymer, although other parameters also influence the performance of antimicrobial polymers.

2.2 Molecular Weight

One major parameter for antimicrobial polymers is the length of the macromolecule. Many studies have considered the structure-property relationship and have come to the conclusion that the DP of an AMP mimic should be low, preferably below 30 (it is noteworthy that AMPs are typically less than 45 amino acids long[13a]), independently of the nature of the polymer, being methacrylates,[17a, 37] nylon-3 polymers, [27] and poly(norbornenes).[22b] For some examples, longer polymeric chains maintain their activity against bacteria, while the ability to destroy red blood cells increases, [27, 38] for other polymeric systems also antimicrobial activity scales with the molecular weight.[22b, 27] This was investigated in detail for norbornene polymers with small variations in molar mass. Indeed, it was shown that while monomeric units did not show any activity, oligomers did kill bacteria. With increasing polymer length antibacterial activity increased, as did the hemolytic potential.[22b] Further studies showed that there is a possible link to a bacterial outer peptidoglycan layer, which is able to sieve large polymers and prevent them from interacting with the membrane.[22d] For systems, where a hydrophilic component instead of a hydrophobic comonomer is incorporated, an opposite trend is reported (increased antimicrobial activity at higher molar mass).[32] For other polymers (poly(carbonates)) biological activity does not seem to scale with length.[39]

It is important in this context to keep in mind that all described systems are polydisperse synthetic polymers. In contrast to AMPs the presence of multiple sizes of polymers can influence the biological performance drastically. This is nicely demonstrated for nylon-3 copolymers where a small change in dispersity (at constant molar mass) shows tremendous increase in haemolysis ($Đ$ = 1.06, HC_{50} = 800 μg mL^{-1}; $Đ$ = 1.15, HC_{50} = 12.5 μg mL^{-1}).[27] It was confirmed by dialysis experiments that the higher molecular weight fractions cause the toxicity. This demonstrates the importance of the molar mass in this context and the necessity for controlled polymerization techniques in order to draw structure property relationships.

2.3 Spacer Length

The length of the alkyl spacer between cationic group and backbone also influences antimicrobial activity. When interacting with cellular membranes, cationic moieties are responsible for the attachment of the polymer to the membrane surface. Length and flexibility of the connection to the polymer could, therefore, influence the efficiency with which the hydrophobic subunits of the polymer can enter the membrane.

This parameter was addressed using methacrylate copolymers synthesised with a primary amine containing monomer containing a spacer of increasing length/flexibility (ethyl/butyl/hexyl/cyclohexyl) using butyl or ethyl methacrylate as a hydrophobic comonomer.[40] It was found that the pK_a values of the amine groups increased with increasing spacer length, which could be assigned to an increased mobility, able to compensate electrostatic repulsion. Also the antimicrobial activity increased for longer spacers. However, it is not clear if this effect originates from an increased mobility of the spacer or the increased hydrophobicity, which accompanies. A difference in MIC and HC_{50} between hexyl and cyclohexyl spacers (similar hydrophobicity but different mobility) suggests that a higher mobility of the cationic charge results in increased antimicrobial but also massively enhanced hemolytic activity. This is further supported by simulations, where an increased spacer enables a better penetration of the hydrophic domain of the membrane by the polymer.[40]

A similar trend was found by Hedrick *et al.* for poly(carbonate)s where an increased spacer length led to lower MIC and HC_{50} values.[39] They also found improved properties when combining long and short spacer arm length resulting in polymers with slightly decreased antimicrobial activity (in comparison to a polymer with only long spacers) but a drastically increased HC_{50} values. This concept was further investigated by Yang and coworkers, combining a C_6 spacer, primary amine monomer with a C_2 spacer, secondary amine monomer in different ratios to obtain optimal selectivity.[33]

2.4 Nature of the Charged Unit

The charged unit is an essential part of an AMP mimic, as it generates affinity to bacterial cell membranes and, consequently, selectivity. In natural AMPs, lysin is the most common source of cationic charge, however also arginine is incorporated frequently. Many studies investigated the influence of the nature the cationic charge on the biological performance of polymeric AMP mimics.

As seen above, quarternary amines require a higher content of hydrophobic subunits as compared to not quarternized amines in order to become antimicrobial.[16, 41] It was found that for similar polymeric architectures, polymers having quarternized amines are more hydrophilic than polymers with the same amount of primary amines at pH 6,[19] which is attributed to the fact that neighbouring amines in a polymer chain influence each other's pK_a values in dependence on their state of protonation. While it is reported that primary amine containing polymers have an increased tendency to induce haemolysis,[19] highest selectivity was achieved using primary amines.[16] Furthermore, investigations on large unimolecular vesicles (LUVs) (which are used as a model for bacterial cell membranes), primary amine containing polymers were able to induce a much higher membrane disruption (as monitored by dye leakage) compared to tertiary and quarternary amines.[19] Studies on

polymers containing both primary and tertiary amines showed a significant decrease in MIC with increased primary amine fraction.[20] A reason for the superior membrane interaction of primary amines could be the reversibility of the charge, enabling local adjustments in terms of amphiphilicity, which is not possible for quarternary amines.

Additional variation is possible by replacing amine groups with guanidine units as an equivalent to arginine. This approach was investigated for methacrylates,[42] and poly(norbornene)s[43] and in both cases a biological activity was found superior to comparable polymers bearing primary amines.

In a different study, guanidine containing monomers (acrylamide-based) were copolymerized with primary amine bearing acrylamides to investigate the influence on antibacterial performance.[44] In this case a decrease of antimicrobial activity and compatibility with mammalian cells with an increasing guanidine content was found, contradicting the previous studies. However, no hydrophobic comonomer was used in these polymers, highlighting the importance of hydrophobicity in the context of arginine mimics.

The ion pairs of guanidine units are kinetically labile enabling a fast exchange of counter ions (hydrophilic vs. hydrophobic) resulting in efficient membrane crossing.[45] However, investigations of artificial membranes (SUVs) revealed no dye leakage, and also live/dead staining of polymer treated bacteria showed no indication of a compromised membrane. These results suggest a different antimicrobial mechanism of these guanidine based polymers, for instance their possible complexation to DNA/RNA, or interaction with other components of the cytosol. It should be noted here that one major advantage of membrane active polymers as antibiotics is the difficulty for bacteria to form resistances against such macromolecules, which was proven experimentally for different polymer types.[37, 46] For guanidine-based antimicrobial polymers, the development of bacterial resistance after repeated treatment was not investigated so far.

2.5 Counter Ion

Only a few studies have evaluated the presence of salt or the variation of the counterion of the cationic charge in the activity of AMP mimics. Investigation of poly(norbornene)s revealed a dramatic decrease in antimicrobial activity when organic counter ions were used, such as tosylate or dodecanoate.[25] Experiments using LUV indicated a decreased membrane destabilization, which was assigned to the tight nature of the ion pair, interfering with membrane attachment of the polymers. The influence of the solvent and the presence of salts was also investigated. It was found for antimicrobial peptides that a high ion strength has a negative influence on the antimicrobial activity,[47] which was ascribed to a masking of cationic and anionic charges by small ions.[48] A similar effect was observed for methacrylate based AMP mimics.[46a] Furthermore, bivalent cations such as Ca^{2+} or Mg^{2+} were found to have a higher inhibiting potential, which was ascribed to the bridging of membrane components, increasing the membrane stability. Also the interaction of anionic peptides from growth media with cationic nylon-3 copolymers was described resulting in a diminished activity.[49]

In conclusion, the various parameters influencing the efficiency of AMP mimics are very complex. While as a main specification, the amphiphilic balance has to be matched, other structural variations lead to changes is either bacterial interaction or blood compatibility (Fig. 4). As many of these parameters are interconnected, it is difficult to define an optimal design standard. However, as seen in this section, a multitude of studies have dealt with single design parameter, and their influence on the overall activity of the resulting polymer, thus giving an excellent basis for the design of new polymeric systems.

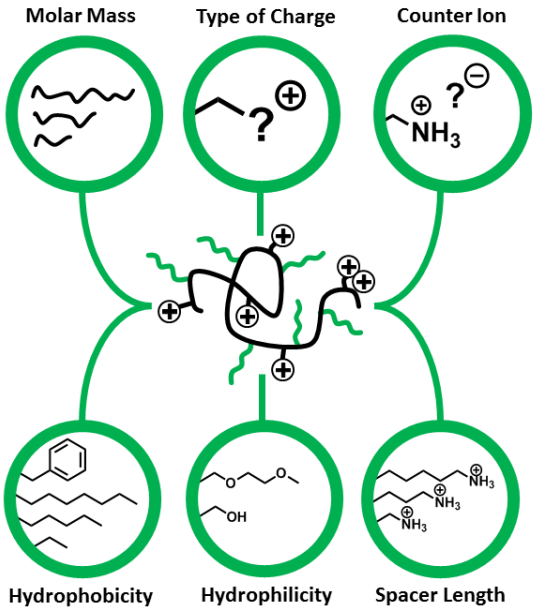


Fig. (4). Parameters reported to influencee the efficiency of polymeric AMP mimics.

3. POLYMER BACKBONE

Several classes of synthetic mimics of antimicrobial peptides (SMAMPs) have been reported, including the poly(norbornene)s, the poly(carbonate)s, poly(carbodiimide)s, nylons, poly(vinylpyrrolidine)s, poly(aniline)s, the poly(methacrylate)s, vinyl ethers, (maleimide-*alt*-isobutylene)_ co-polymers, and copolymers derived from alternating ring-opening metathesis polymerisation.

3.1. Poly(norbornene)s

Poly(norbornene)s represent one of the most studied classes of SMAMPs. Coughlin and Tew employed ring-opening metathesis polymerization (ROMP) for the facile synthesis of poly(norbornene) derivatives to provide an extensive library of amphiphilic polymers. The norbornene monomer enables incorporation of both cationic and hydrophobic functionality within a single monomer, thereby providing dual pendant amphiphilic homopolymers. These poly(norbornene) polymers can be split into two classes – same-faced poly(oxanorbornene)s (Fig 5A and B), and opposite face poly(norbornene)s (Fig 5C).

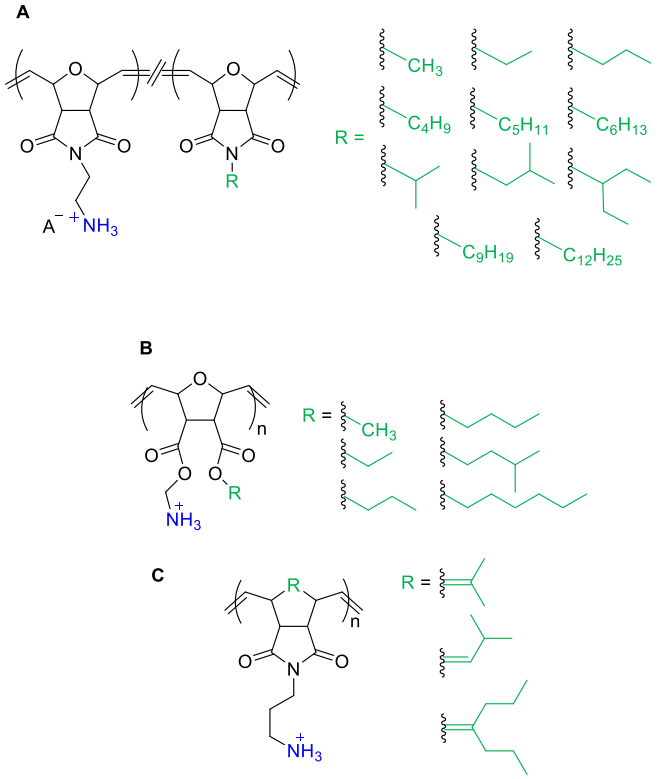


Fig. (5). Same-faced, separate centre (A); same-faced, same centre (B) and opposite-faced (C) poly(norbornene)s

Tew and co-workers reported two classes of same-faced poly(oxanonorbornene)s; the first where cationic and hydrophobic units were contained within separate repeat units (Fig. 5A),[50] and the second where these units were incorporated into a single monomer (Fig. 5B).[51] The polymers comprised of separate repeat unit functionality were synthesised as 50:50 random copolymers in two molecular weight ranges; low molecular weight (2.1-4.2 kDa) and high molecular weight (12.2-15.9 kDa), with the high molecular weight polymer class proving to have no antimicrobial activity with MIC values typically ≥ 400 μg mL^{-1}. The low molecular weight polymers showed more promising activity with MIC values as low as 50 μg mL^{-1} for the C4 and C5 linear and branched alkyl chains. When the ratio of cationic to hydrophobic monomers deviated from 50:50 (for the low molecular weight polymers) no improvement in antimicrobial activity was seen. These polymers displayed lower antimicrobial activity in general, compared to their regularly spaced same-face and opposite-faced counterparts. This is attributed to the random unit sequence of the polymer, leading to pockets of cationic or hydrophobic blocks at the local monomer level, thereby highlighting the importance of the spatial organisation of amphiphilic groups for the poly(norbornene)s.

The polymer class comprising cationic and hydrophobic units in a single oxanonorbornene monomer (Fig. 5B) were prepared as polymers with molecular weights of either 3 kDa or 10 kDa. The lower molecular weight (3 kDa) polymers exhibited much greater antimicrobial activity than their 10 kDa counterparts, in agreement with the segregated poly(oxanonorborene)s (Fig 5A). An optimum alkyl chain length (hydrophobic unit) was identified, with the propyl containing 3 kDa polymer being the most active with MIC_{90} values of 6.25 and 15 μg mL^{-1} for *E. coli* and *S. aureus* respectively.

In 2004 Coughlin reported a series of modular norbornene derivatives for the generation of homopolymers with opposite face amphiphilicity.[52] Figure 5C shows the designed homopolymers with regular spacing of hydrophobic and hydrophilic groups across the facially amphiphilic structure. Coughlin and co-workers generated a library of polymers with a large range of molecular weights from 4.5 kDa to 64 kDa. Their studies found that a minimum molecular weight of 4.5 kDa was required to obtain phospholipid membrane lysis activity, however, no molecular weight dependency on membrane lysis was observed between 4.5 kDa and 64 kDa. Further studies from Coughlin and Tew examined these hompolymers and random copolymers of the following four norbornene repeat units for their antibacterial activity (Fig 6) .[22c]

The polymers displayed narrow dispersities of <1.3, and a large range in molecular weight from oligomers to 137.5 kDa. These polymers were evaluated for their antibacterial activity (MIC value) against *E. Coli* and *B. Subtilis*, and assessed for their hemolytic activity against red blood cells. For the

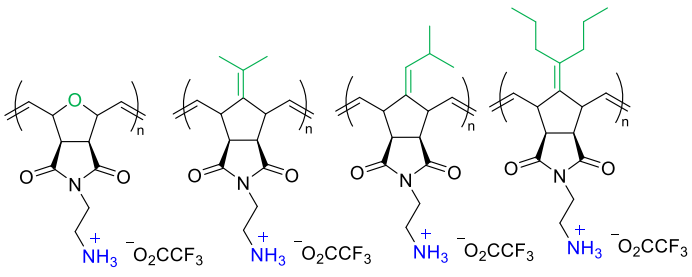


Fig. (6). Four norbornene repeat units used in the study

homopolymers, only the isobutenyl-containing polymer was able to display good antibacterial activity with MIC values of 25 μg mL^{-1} for both *E. coli and B. subtilis* at molecular weights of either 1.6 or 10 kDa. Above this molecular weight MIC values rose to 40 μg mL^{-1} and higher. Whilst the antimicrobial activity of the isobutenyl-containing polymers was high, this polymer series also displayed high hemoylsis. Both hemolytic and antimicrobial activity falls for the isopropylidene and oxanorbornene homopolymers. In general, the homopolymer series of these 4 monomeric units failed to provide any clinical antimicrobial candidates. Random copolymers of these monomers failed to improve antimicrobial activity.

Despite their synthetic versatility, these polymers have limited applicability in antimicrobial treatments such as topical films since they are not biodegradable. One method of ensuring biodegradability is to generate α-peptide linked polymers which can undergo enzymatic degradation.

3.2. Poly(carbonate)s

Initial studies into the poly(carbonate) backbone for synthetic mimics of AMPs were based on maintaining the peptide linkage of naturally occurring AMPs through transition metal catalysed ring-opening polymerization of α-aminoacid-*N*-carboxyanhydrides (NCAs). This method gives rise to α-peptide linked polymers as seen in Figure 7 in one step, compared to traditional solid-phase peptide synthesis. Zhou and co-workers used this method to prepare a series of random copolymers containing primary amines for lysine mimics and alkyl or aryl hydrophobic side chains as alanine, leucine or phenylalanine mimics.[53] The series investigated the optimum hydrophobic content for five different bacterial species (see previous section). The polymers synthesised by this method had narrow dispersities (1.11-1.28) and a range of molecular weights from 5kDa to 35kDa. These polymers displayed broad spectrum antimicrobial activity and represent a promising class of therapeutics due to their low cost of production (<10 $/g).

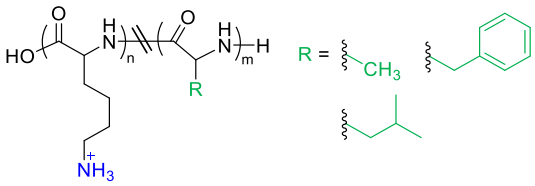
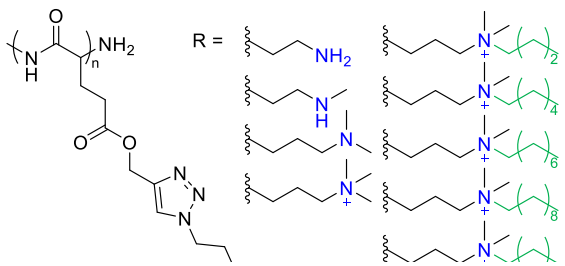


Fig. (7). Poly(carbonate) backbone studied by Zhou and co-workers

Engler and co-workers adopted a similar approach to generate α-peptide backbones bearing pendant azide groups for click reaction to produce primary amine or *N*-alkyl



functionalised homopolymers (Fig. 8). [54] Polymer lengths ranged from 30 to 140 repeat units with varying side group functionality with hydrophobic alkyl groups ranging from 1 to

Fig. (8). Poly(carbonate) backbone studied by Engler and co-workers

12 carbons. Molecular weight was dependent on activity for the gram positive *S. aureus*, with an optimal degree of polymerization at 140. No such dependency was observed when tested against *E. coli*. These polymers displayed minimal antimicrobial activity overall, however, this synthetic approach does enable rapid evaluation of hydrophobic and cationic functional groups.

3.3. Nylon Polymers

Despite their ease of synthesis, α-peptides are readily degraded by enzymes, thus a β-peptide approach was adopted by Gellman and co-workers.[27] Gellman reported the ring-opening polymerization (ROP) of β-lactams to afford random β-peptide, Nylon-3 polymers (Fig. 9) in one step. Several structures were investigated, all with a random backbone structure. Hydrophobic groups were chosen from cyclic, acyclic and geminal dimethyl, whilst a primary amine served as the cationic group. Further hydrophobicity was added with the inclusion of geminal dimethyl groups adjacent to the primary amine group. A detailed analysis of the various side chain groups will be discussed in Section 4.

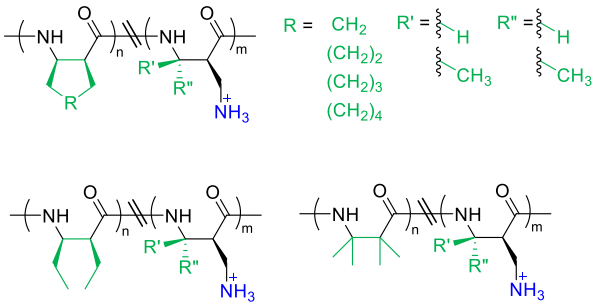


Fig. (9). Nylon-3 backbone studied by Gellman and co-workers

3.4. Poly(carbodiimide)s

Poly(carbodiimide)s have stable polymeric backbones that are resistant to degradation by enzymes. Novak and co-workers generated a series of helical poly(carbodiimide)s through the coordination-insertion polymerization of carbodiimide monomers with a titanium-BINOL catalyst.[55] Adopting CuAAC click chemistry, Novak and co-workers functionalised poly(carbodiimide) polymers with guanidine groups. Two guanidine functionalised series were prepared. Figure 10 depicts the mono-functionalised poly(carbodiimide)s with pendant alkyl chains, generated

from an alkylated carbodiimide monomer (A), and the bis-functionalised poly(carbodiimide)s with two guanidine groups per repeat unit (B), generated from a carbodiimide monomer containing two alkyne functional handles.

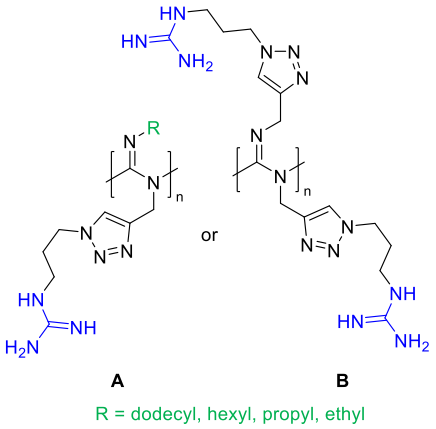


Fig. (10). Poly(carbodiimide)s with alkyl and guanidine side chains (A), or bis-guanidine functionality (B)

Novak and co-workers studied the helical chirality of these polymers and their associated antimicrobial activity and found no influence on antimicrobial activity. These polymers exhibited moderate antimicrobial activities with MIC values >64 μg mL^{-1} and activity was independent of molecular weight, with polymers having a wide molecular weight range from 15 kDa to 35 kDa (note that these molecular weights are for the non-functionalised poly(carbodiimide) backbone - after click reaction the resulting polymers are insoluble in the THF/diethanolamine SEC systems).

3.5. Poly(aniline)s

Ambrose and co-workers studied functionalised poly(aniline)s (fPANI), following reports that PANI-containing PVA/PE films[56], and PANI-coated cotton[57] had antimicrobial properties (Fig. 11). Ambrose reported several different fPANI polymers, incorporating acidic, halogen and hydrophobic groups in the backbone.[58] Unfunctionalised PANI gave MIC values >10 mg/mL when tested against *E. coli, P. aeruginosa, S. aureus* and their associated multi-drug resistant cell lines. Copolymerization of aniline and 2-aminobenzoic acid or 3-aminobenzoic acid (2-ABAPANI and 3-ABAPANI, respectively) had much improved antimicrobial activity when in the emeraldine salt (ES) form with MIC values between 1.25 to 2.5 mg/mL. When crystallised in the emeraldine base (EB) form, antimicrobial activity was reduced to 5 mg/mL. Functionalized poly(aniline) copolymers containing SO_3H, Cl, OMe or CH_3 groups failed to improve antimicrobial activity, with MIC values between 5 to 10 mg/mL.

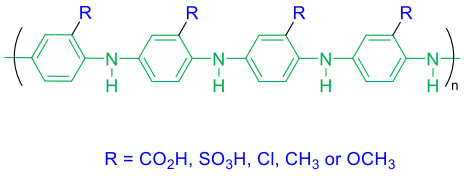


Fig. (11). Poly(aniline) backbone studied by Ambrose

3.6. Poly(vinylpyridine)s

Free radical polymerization is a versatile method to generate random, block and homopolymers with a wide range of functional groups. Sen and co-workers adopted this approach to generate a series of random copolymers of 4-vinylpyridine and methacrylates (Figure 12).[21] Sen prepared three different series via *N*-alkylation of the pyridinyl group: a) alkyl functionalised pyridinium-methyl methacrylate (same centre); b) methyl functionalised pyridinium-alkyl functionalised methacrylate (different centre); and c) alkyl functionalised pyridinium and methacrylate (same centre). This enabled Sen to compare amphiphilic polymers whose hydrophobic and cationic components were either spatially separated (single pendant; Fig. 12a and b) or directly connected to each other (dual pendant; Fig. 12c).

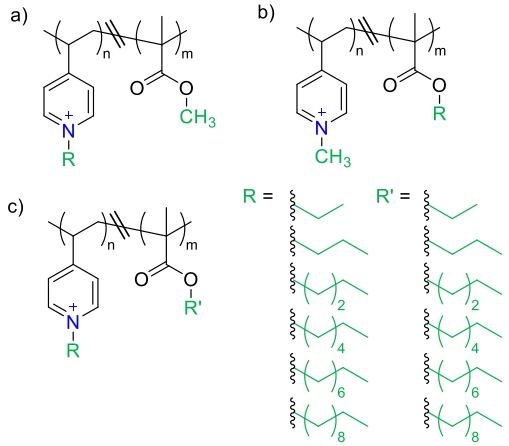


Fig. (12). 4-Vinylpyridine, methacrylate copolymers studied by Sen and co-workers

Sen concluded that while both displayed antimicrobial activities, the incorporation of hydrophobicity within the same centre (Fig. 12a and c) were non-hemolytic, as opposed to their different centre (Fig. 12b) counterparts which were hemolytic. These contrasting trends prove that spatial arrangement of cationic and hydrophobic groups is another factor to be considered in the design of antimicrobials.

3.7. Poly(methacrylate)s

The advent of reversible-deactivation radical polymerization (RDRP) techniques has enabled the controlled synthesis of acrylate and vinyl polymers with pre-determined molecular weights. The Kuroda group reported the synthesis of random copolymers of methyl methacrylate and amino ethyl methacrylate with different hydrophobic to

cationic ratios using mercaptan chain transfer agents (Fig 13).[59] Polymers containing 47% of the hydrophobic methyl methacrylate unit were found to have antimicrobial activity at low micromolar MIC values, and little hemolytic activity.[18] Increasing the hydrophobic balance of the copolymers with longer alkyl chains, such as butyl methacrylate served to increase toxicity, relative to methyl methacrylate-containing copolymers which display the lowest toxicity. Modifying the cationic content from primary to tertiary and quaternary ammonium groups reduced antimicrobial activity significantly, with the quaternary ammonium cationic groups displaying no antimicrobial activity.[18] Antimicrobial activity was independent of molecular weight, however, higher molecular weight polymers displayed much increased hemolytic activities.[17a]

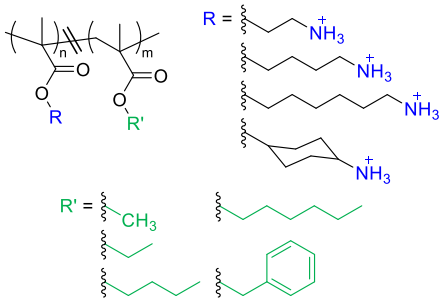


Fig. (13). Kuroda's poly(methacrylate) SMAMPs, employing quaternary ammonium groups as cationic components, and aliphatic and aromatic groups for hydrophobicity.

Kuroda and co-workers further explored the poly(methacrylate) class by incorporation of self-degradable 3-butenyl 2-chloroproprionate into the backbone. This was accomplished using a metal-catalysed simultaneous chain- and step-growth radical polymerisation to provide cationic side chains and main-chain ester linkages. These polymers comprised a random polymer chain, with a post-polymerisation modification of the t-butyl acrylate groups to required amine functionality. Various monomer feed ratios of 1/1, 3/1 and 1/3 were prepared to investigate amphiphilic effects. Amine functionality was present as an acrylic lysine mimic, an acrylamide lysine mimic, or a quaternized acrylic lysine mimic with short alkyl chains (Fig. 14A, B and C, respectively). When these polymers were assessed for their activities against *E. coli*, the acrylic lysine mimicking degradable copolymers provided the lowest MIC values at 16-104 µg/mL, compared to the peptide mgainin-2, which gave an MIC of 125 µg/mL. Lower molecular weight polymers within this discrete class also proved to be more potent, however, they displayed low HC_{50} values, indicating that their hemolytic activity would prevent their development as clinical candidates. Interestingly, a change to the acrylamide back bone resulted in a significant loss of activity, with MIC's observed at > 500 µg/mL, which was attributed to the hydrophilic nature of the acrylamide, preventing the proposed mechanism of insertion of hydrophobic polymer regions into the bacterial cell membrane. Further polymer design must be undertaken to incorporate desired degradability whilst maintaining low MIC values and high bacterial selectivity.[60]

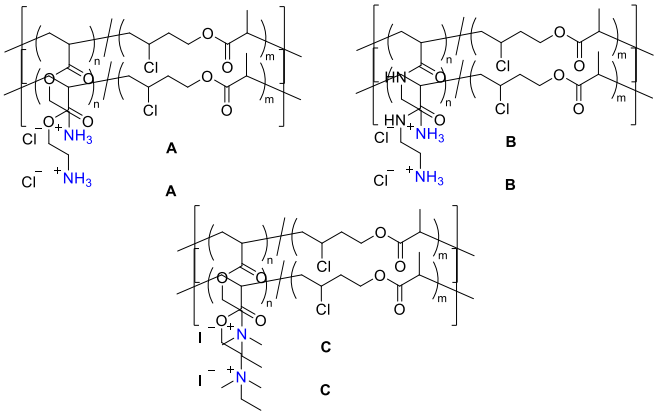


Fig. (14). Kuroda's self-degradable antibacterial polymers made by simultaneous chain- and step-growth radical polymerisation

Locock and co-workers explored the poly(methacrylate) polymer class using Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization for access to controlled molecular weight random copolymers (Fig 15). [42, 61] Polymers were comprised of methyl methacrylate as the hydrophobic unit, and an amine containing methacrylate as the cationic group. Post-polymerization conversion of the amine into a guanidine unit provided a direct comparison of amine and guanidine cation sources for antimicrobial activity and toxicity. The hydrophobic/cationic balance was investigated to identify a lipophilic composition of <40% was required to provide high activity and low toxicity. Molecular weight was dependent on antimicrobial activity, with low molecular weight polymers (3.3 – 4.5 kDa) having significantly lower MIC values than their high molecular weight counterparts (10 -23 kDa).

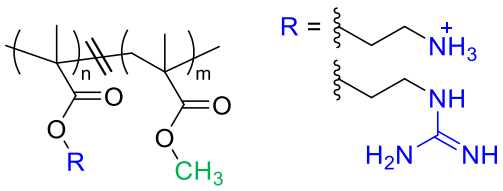


Fig. (15). Lysine and arginine mimicking cationic side groups investigated by Locock in the poly(methacrylate) SMAMPs

3.8. Poly(vinyl ethers)

Building upon their established research into SMAMPS using a poly(methacrylate) back bone, Kuroda and colleagues prepared vinyl ethers containing an hydrophilic amino-ethyl vinyl ether (AEVE) and a hydrophobic isobutyl vinyl ether (IBVE) monomer (Fig. 16). These copolymers were prepared by base-assisting living cationic polymerization, with varied molecular weights from 5 kDa to 25 kDa. Polymers were assessed against *E. coli* for their antibacterial properties in both a random and block copolymer sequence – this will be elaborated upon in Section 5.[62]

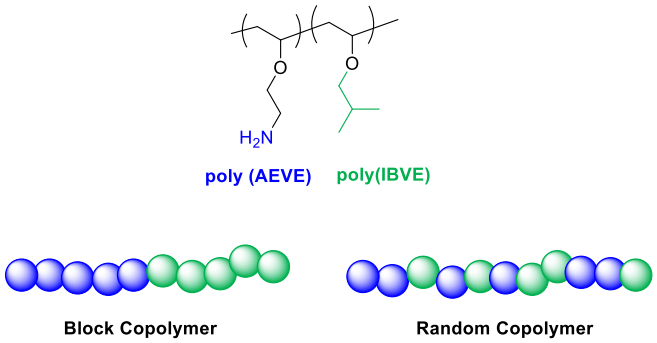


Fig. (16). Poly(vinyl ether)s containing amine and isobutyl functionality in either block or random sequence

Evaluation of antibacterial activity was undertaken using bactericidal concentration ($BC_{99.9}$), characterized as the lowest polymer concentration to cause at least a 3-log reduction (99.9% killing) in the number of viable *E. coli* cells after incubation with the polymers for 4 h at 37 °C. The random and block copolymers gave $B_{99.9}$ values of 1-63 μg/mL, with monomer composition being the influencing factor on bactericidal activity, rather than sequence, when IBVE content was less than 50 mol%.

When comparing hemolytic activity, block copolymers displayed significantly higher HC_{50} values (>1000 μg/mL) compared to random sequences. However, these block copolymers induced hemagglutination, while their random counterparts did not.

This study has focussed on aggregation of hydrophobic vinyl ether polymers to explain trends in decreasing activity with increasing hydrophobic content, yet increased selectivity towards bacterial cells. A relationship between the critical aggregation concentration (CAC) and $BC_{99.9}$ was discovered, whereby block copolymers form intramolecular aggregates to provide a hydrophobic core and outer cationic surface, leading to the increased hemagglutination of RBCs due to electrostatic interaction with surface-bound biopolymers, but reduced hemolysis due to hidden hydrophobicity. The random copolymers, on the other hand, remain in a random-coil structure in the absence of concentrated regions of hydrophobicity. This enables their charged content to interact with bacterial cells walls, but their hydrophobic content is able to interact with both bacterial cells and RBCs, resulting in both bactericidal activity and hemolysis.

3.9. (Maleimide-alt-isobutylene) copolymers

The Haldar group investigated maleimide and isobutylene alternating copolymers to understand the effect of hydrogen bonding and hydrophobicity in concert with a constant cationic charge on the selectivity of antibacterial polymers. This is the first study to include hydrogen bonding as a strategy for improved bacterial selectivity. By creating polymers containing ester- and amide- groups, they aimed to provide a degradable polymer with tunable side chain hydrophobicity (Fig. 17).[63]

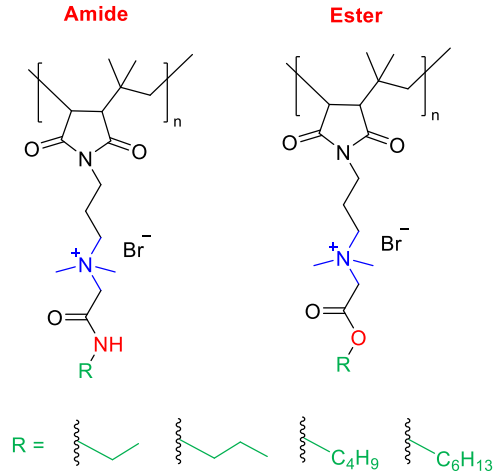


Fig (17). Poly(maleimide-*alt*-isobutylene) SMAMPs

The series probed a molecular weight range between 15-18 kDa, with different length side chains from ethyl to hexyl to study effects of hydrophobicity whilst keeping cationic charge density constant. The amide series (Fig 17, Amide) proved to be effective antibacterial agents, with MIC values as low as 16 μg/mL and 8 μg/mL for *E. coli* and *S. aureus*, respectively when a C6 hexyl group was incorporated. Reduction in hydrophobic content from C6 to C2 resulted in a steady drop off in antibacterial activity, with MIC values of 62 g/mL for both *E. coli* and *S. aureus* for the C2 amide series. Interestingly, the amide polymers are more potent towards *S. aureus* than *E. coli*, particularly for the C4 functionalised amides, however, a hydrophobic group of C3 or less provides low hemolytic activity, and appears to be optimum for activity/selectivity balance.

MIC values for the ester series (Fig 17, Ester) follow a similar selectivity towards *S. aureus*, albeit to a lesser extent. They also show a decrease in activity with increasing hydrophobicity, however, the C6 hexyl group provides the most potent antibacterial effects. In general, the ester series were less effective antibacterial agents than their amide

counterparts, but did display lower toxicity at greater hydrophobic content (C4-C6) than the corresponding amides.

The authors believe that the hydrogen bonding backbone, and, in particular, its alternating sequence, provides a structural strategy and ordered backbone, enabling optimal selectivity towards bacteria over RBCs.

3.10. Alternating ROMP Polymers

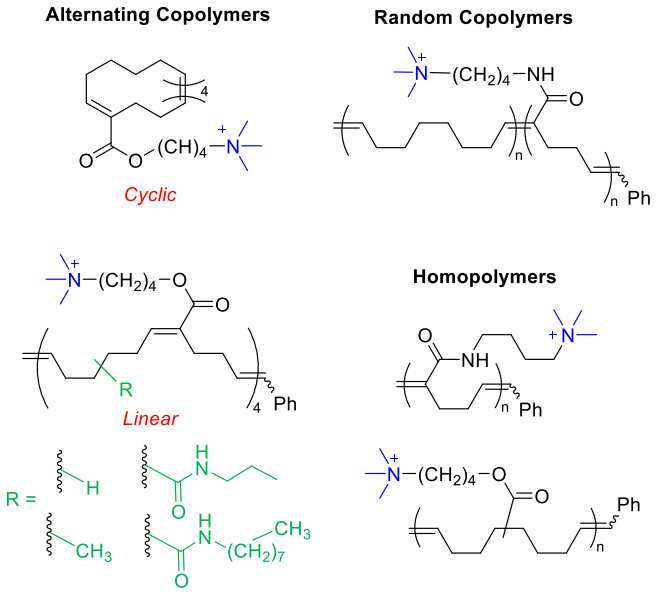


Fig. (18). AROMP copolymers and homopolymers of selected cyclobutene and cyclohexene monomers

The Sampson group have also reported a study on backbone sequence and antibacterial activity. The effects of sequence will be discussed in greater detail in Section 5. Whilst Tew and co-workers employed ROMP to the norbornene monomer to provide rapid access to a wide variety of poly(norbornenes), Sampson has made use of the technology to copolymerise cyclobutenes and cyclohexenes in alternating sequence (AROMP), to provide a carbon backbone bearing cationic and lipophilic groups at regularly spaced intervals or 4, 8, and 10 carbons. Hydrophilicity was tuned by careful choice of substituents on the cyclobutene and/or cyclohexene monomers. A series of random copolymers and homopolymers were also prepared for comparison, to assess the importance of spacing of functionality and the cation source itself.[64]

The optimum AROMP SMAMP was found to be one in which there were no pendant hydrophobic groups on the already sufficiently hydrophobic backbone (Fig. 18; R=H). Of the alternating series, it was found that polymers with higher hydrophobic content were less effective antibacterial agents, and showed a decrease in erythrocyte lysis – contrary to the effects seen in other AMP mimics. Based on the alternating copolymer series, Sampson discovered that polymers with a regular 8-10Å spacing of cationic residues proved to be more potent antibacterials, when screened against a range of Gram-positive and Gram-negative bacteria. An additional requisite is that the spacer must be hydrophobic – addition of a cationic group as a spacer resulted in significant reduction in activity. This finding is consistent with the 5Å cationic spacing found in naturally occurring AMPs. Another feature of the AROMP SMAMPs, which is common to other SMAMP polymer classes is the need for conformational flexibility to afford antimicrobial activity. This was evidenced by Sampson's evaluation of cyclic alternating polymer constructs, which displayed significantly lower antibacterial activities than their acyclic counterparts.

4. MIMICKING AMINO ACIDS

Antimicrobial peptides contain a range of lipophilic and cationic amino acid residues. Commonly found lipophilic groups are short alkyl chains (alanine, valine, leucine and isoleucine) or aromatics (phenylalanine, tyrosine, tryptophan). Lysine (primary amine) and arginine (guanidine) are the two cationic amino acids that are common to most AMPs. Synthetic peptide-mimetic polymers have focussed on mimicking these amino acid pendant groups in order to provide structures that may mimic the facial amphilicity and/or tertiary structure to gain similar activity profiles.

4.1. Hydrophobic Amino Acids

Hydrophobic pendant groups have been more widely studied than their cationic counterparts, with a strong focus on aliphatic chains of varying length and branching. In the poly(norbornene) polymer class, Coughlin and Tew investigated isopropylidene and isobutenyl groups as lipophilic mimics (Fig 19).[22c]

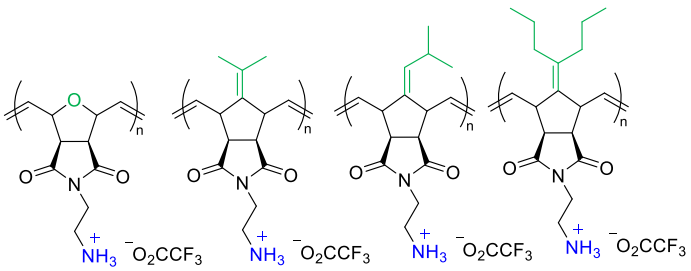


Fig. (19). Hydrophilic side chains studied by Tew and Coughlin

The isopropylidene group provided very little antibacterial activity, but proved to have the lowest hemolytic activity. Incorporation of an extra carbon in the isobutenyl group substantially improved MIC values, but also dramatically increased red blood cell lysis. Further lipophilicity proved to be detrimental to both antibacterial and hemolytic activity. These polymers have inspired a wide

range of modified structures, but whilst they have been outperformed in antibacterial activity, they still represent the most non-hemolytic polymers reported in the literature.

In subsequent studies, Tew and co-workers moved towards the oxanorbornenes with same face amphiphilicity. They reported the study of short (C_1 – C_6) and long (C_6 – C_{12}) linear and branched alkyl chains (Fig 20a and b) as lipophilic amino acid side chain mimics.

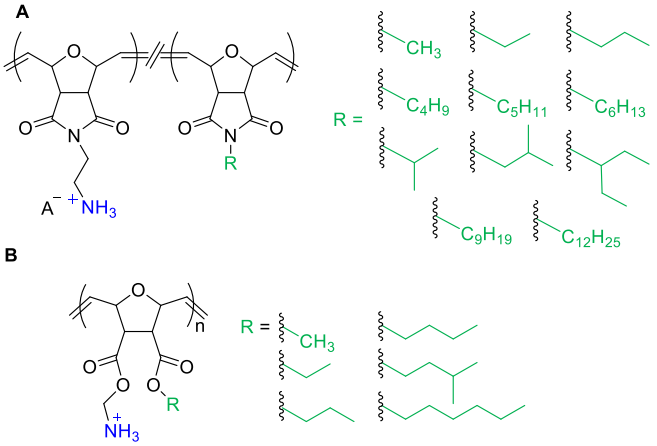


Fig. (20). Same faced, different centre polyoxanorbornes (A), and same faced, same centre polyoxanorbornenes (B) and the hydrophobic pendant groups studied

The same-faced, different centre polyoxanorbornenes (low molecular weight: 2.5-4.5 kDa) displayed the most promising activity for the C3, C4 and C5 linear and branched alkyl chains with MIC values as low as 50 μg/mL. Other alkyl chain lengths showed substantially lower antimicrobial efficacy at MIC values of >100 μg/mL. Hemolytic concentrations of the C3-C5 linear and branched alkyl chains, however, were low, indicating an increased ability to lyse red blood cells. These observations were supported by the same-faced, same centre polyoxanorbornenes with the propyl linear chain having the lowest MIC values (6.25 μg/mL), however at this chain length and above, polymers become increasingly hemolytic, with decreasing antimicrobial activity beyond a C4 alkyl chain length. The ethyl alkyl chain provides moderate activity with MIC values of 50 μg/mL, but shows high selectivity for bacterial cells over mammalian cells.

Zhou and co-workers chose to mimic alanine, leucine and phenylalanine with their choice of methyl, isobutyl and phenyl hydrophobic pendant groups for the poly(carbonate) SMAMPs (Fig. 21). The cationic group was maintained as a lysine mimic for all three hydrophobic series.[53]

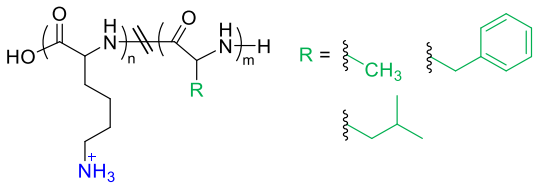


Fig. (21). Alanine, leucine and phenylalanine mimicking hydrophobic groups investigated by Zhou and co-workers

The phenyl series proved to be the most active against all five pathogens (*E. coli*, *S. aureus*, *S. marcescens, C. abicans and P. aeruginosa*), maintaining activity across a wide range of hydrophobic content (20-80% phenyl content). All three hydrophobic series had an optimal hydrophobic content of 60%, but the alanine and leucine mimics displayed no activity against *S. marcescens* and *C. albicans*. The phenylalanine mimic at 60% phenyl content displayed higher hemolytic activities than natural AMPs (e.g. Indolicidin) HC_{50} = 16 μg/ mL). Combination of phenyl and isobutyl groups provided a similarly active SMAMP, again with poor selectivity for bacterial cells.

Within the poly(carbonate) class, Engler's click chemistry approach enabled the study of a range of aliphatic primary, secondary, tertiary and quaternary amines with varying alkyl chain lengths from C4-C12 (Fig 22).[54]

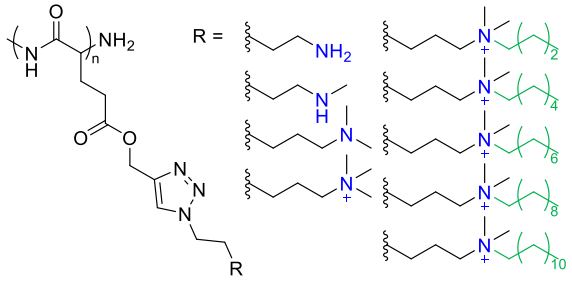


Fig. (22). Primary, secondary, tertiary and quaternary amines employed in Engler's poly(carbonate) SMAMP class

These polymers were evaluated for their activity against *S. aureus* and *E. coli*. The primary amine polypeptides displayed activity against both gram positive and gram negative bacteria, albeit at high MIC values (70-140 μg/mL and 562-1125 μg/mL respectively). Secondary and tertiary amines are inactive against both bacterial classes. Quaternization of the amine proves to be ineffective in improving activity until incorporation of an alkyl chain of >6 carbons.[39] At a carbon chain length of 8, MIC values for *S. aureus* and *E. coli* fall to 78-156 μg/mL and 156-312 μg/mL, respectively. Additional hydrophobicity is detrimental for *E. coli* activity, but enhances activity against *S. aureus* at a C12 chain length (MIC = 39-78 μg/mL). These polymers do exhibit low hemolytic behaviour across the series, but their relatively high MIC values (compared to other SMAMPs) and their ineffectiveness as broad spectrum antimicrobials limits their therapeutic development.

The vinyl pyridine SMAMPs have similarly focussed on comparison of C2-C10 alkyl chains to understand hydrophobic structure activity effects. Two series were designed: same centre – whereby the positive charge and alkyl group were on the pyridyl substituent; and separate

centre – whereby the positive charge was located on the pyridine (methyl pyridinium ion) and the alkyl group incorporated on the acrylate centre.[21] For both series, MIC values decreased to an optimum alkyl tail length of C4 for *E. coli* and C6 for *B. subtilis*. The MIC values then increased with increasing alkyl tail length, as previously observed in other SMAMP polymers, suggesting alkyl lengths from C3-C8 provide sufficient membrane disruption. As expected, hemolytic activity increased with increasing tail length, however, this effect was far more pronounced for the separate centred polymers.

Table 1: Comparison of antibacterial and hemolytic activities for cyclic and acyclic nylon-3 subunits

Polymer	MIC (μg/mL)								
	C. difficile	*B. subtilis*	*E. coli*	VREF	MRSA	*S. enterica*	*P. aeruginosa*	*B. cereus*	HC_{10}
DM:CH	12.5	<1.6	6.3	6.3	6.3	50	25	100	19
DM:TM	12.5	<1.6	13	3.1	3.1	-	-	-	400
DM:βDE	-	6.3	25	25	25	-	-	-	<3.1
DM:HE	-	-	-	-	-	50	50	100	-
DM: βCP	-	3.1	6.3	6.3	6.3	-	-	-	<3.1

The nylon-3 SMAMPs have been the most widely studied synthetic polymer class, with a focus on comparison of ring size and cyclic versus acyclic groups. Figure 23 summaries the different lactam monomers used in these studies.[29]

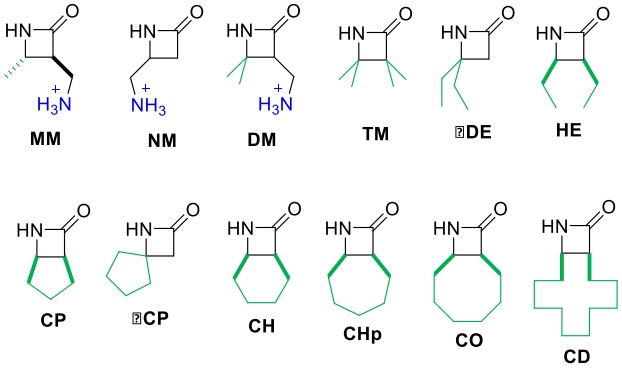


Fig. (23). β-lactam monomers used in study

Initial studies focussed on incorporation of the **MM** subunit with the **CH** cyclic subunit.[27] This random copolymer displayed the most effective MIC against *E. coli*, *B. subtilis*, *S. aureus* and *E. faecium* when the cationic content (**MM**) was ≤50%. Above this level, polymers became highly hemolytic. When the cationic units were compared for their activity against *C. albicans* and other fungal strains (**MM** vs. **NM** vs. **DM**), it was found that the **NM** subunit was intrinsically antifungal[46b]. Homopolymers of **NM** maintained low MIC values against drug-resistant strains of *C. albicans* (3.1 μg/mL for K1 and Gu5 strains). Applying these NM subunit homopolymers to biofilms of *C. albicans* proved their ability to inhibit fungal biofilm growth down to $SMIC_{80}$ (concentration to inhibit 80% of biofilm formation) values of 9.4 μg/mL, whilst displaying very little RBC lysis, even at 2000 μg/mL.[65] When **NM** subunits were supplemented with hydrophobic groups (**CP, CH** and **CO**), antifungal activity was diminished, although the more hydrophobic subunits displayed less dramatic decreases in activity relative to **CP**, attributed to their antifungal effects. Interestingly the **DM** subunit has some intrinsic antifungal behaviour, but also intrinsic hemolytic effects, whereby upon replacement of the **DM** unit with hydrophobic groups, HC_{10} values increase.

Comparison of increasingly hydrophobic cyclic substituents **CP** and **CH** groups generally show lower hemolytic activity and low antifungal activity (for the **NM** and **MM** series). **CHp** and **CO** have similar antifungal activities, but increasing hydrophobicity to CD results in high hemolytic activity. The **CH** group has therefore been selected as the most commonly studied cyclic subunit.

Within the **DM** series, comparison of **CH** cyclic subunit with acylic subunits **TM**, **βDE HE** and **βCP** that **TM** was not only comparably active to **CH**, the subunit also provides a hemolytic effect, with HC_{10} values of >400 μg/mL.[28-29] Table 1 compares the four acyclic groups with **CH** at a 1:1 ratio with **DM**.

The Kuroda group have explored C1-C7 alkyl chains and a phenylalanine mimic in their poly(methacrylate) SMAMPs. The cationic source was maintained as a lysine mimic, using aminoethylmethacrylate (AEMA). Studies of polymer length and hydrophobic content had concluded that a low molecular weight (MW = 1300 – 1900 g/mol) was optimum for antimicrobial activity and toxicity, when combined with a hydrophobic content between 30-40%.[18] When evaluating hydrophobicity (alkyl chain length) for antimicrobial activity, it was found that the C4 alkyl chain gave optimum performance for antibacterial activity at 30% hydrophobic ratio. Shorter alkyl chains required a higher hydrophobic ratio to achieve similar MIC values. The longer the alkyl chain, the more hemolytic the polymer is, and therefore a lower hydrophobic ratio is required to minimise this effect. In determining selectivity between bacterial and human cells (HC_{50}/MIC), Kuroda identified that the C1 (methyl) containing polymers were more appropriate as antimicrobials due to their low MIC values, but higher selectivity towards bacterial cells, whereas polymers

containing C2 (ethyl) and C4 (butyl) had lower or no selectivity and therefore would be appropriate as biocides. The phenylalanine mimic (benzyl group) proved to be less effective as an antimicrobial entity, with MIC values ranging between 20-100 μM compared to <10 μM for its methyl-containing counterpart.

Further exploration of the role of hydrophobic groups in the poly(methacrylate) class was performed by Locock and co-workers, who studied alanine and tryptophan mimicking groups. Tryptophan-rich AMPs such as indolicidin and tritrpticin have been shown to insert into membranes, and partition near the membrane-water interface, enabling the peptide to anchor to the bilayer surface, however this motif has been little investigated in SMAMPs.[66,67] A tryptophan-like indole monomer, 2-(1*H*-indol-3-yl)ethyl methacrylate (IEMA), was incorporated into two tryptophan mimicking series – one with a lysine mimic cation source (aminoethyl methacrylate – AEMA; poly(AEMA-IEMA), and the other with an arginine mimic cation source (2-guanidinoethyl methacrylate – GEMA; poly(GEMA-IEMA.)[68] These polymers were evaluated against *S. epidermidis* and the methicillin-resistant strain of *S. aureus* for their antimicrobial activity. The tryptophan series exhibited lower antimicrobial activity, compared to hompolymers of GEMA, and were found to be more hemolytic. A 5% IEMA content gave the lowest hemolytic activity at 5.6% and 14.0% for poly(AEMA-IEMA) and poly(GEMA-IEMA), respectively. This highlights the importance of hydrophobic/cationic balance, since the alanine mimicking methyl methacrylate (MMA)-containing poly(AEMA-MMA) and poly(GEMA-MMA) copolymers require a hydrophobic content of 30-40% MMA for optimum antimicrobial/toxicity balance. Poly(GEMA-IEMA) at 5% IEMA content was found to be the most potent of the tryptophan mimics, with an MIC of 12 μg/mL and 47 μg/mL for *S. epidermidis* and the methicillin-resistant strain of *S. aureus*, respectively.

When comparing the IEMA hydrophobic source to that of MMA, at a 30% hydrophobic content, the poly(AEMA-IEMA) copolymers provide an MIC of ca. 50 μg/mL compared to 500 μg/mL for its poly(AEMA-MMA) counterpart when tested against *S. epidermidis*. The poly(GEMA-IEMA) copolymers, however, perform at MIC value of ca. 40 μg/mL compared to 10 μg/mL for the poly(GEMA-MMA) copolymers, highlighting the impact of the cation source, which will be discussed in the next section.

4.2. Cationic Amino Acids

Tew and co-workers completed several studies to sequentially investigate different cationic amino acid mimicking side chains. One approach was to generate monomers with multiple primary amines in the single unit (Fig 24a). This multiple amine approach was initially studied using the isobutenyl poly(norbornene)s. Tew had previously reported that the added hydrophobicity of isobutenyl containing poly(norbornene) backbones leads to greater toxicity,[24] however by increasing the hydrophilicity through the doubling and tripling of primary amine groups, the resultant polymers were less hemolytic. Increasing the number of primary amines per unit, however, gave no improvement in antimicrobial activity for *E. coli* or *S. aureus*. A move to the more hydrophilic oxanorbornene systems (Fig 24b) were non-toxic, as expected, due to their hydrophilicity and therefore non interaction with RBCs. These systems also provided no antibacterial activity against *E. coli* bacterial strains, even when the cationic amine content was doubled or tripled. However, when tested against *S. aureus* doubling or tripling the amine content provided improved antimicrobial activity with MIC_{90} values falling from >200 μg mL^{-1} for the single amine polymers to 25 and 15 μg/mL for the double and triple amine polymers, respectively.

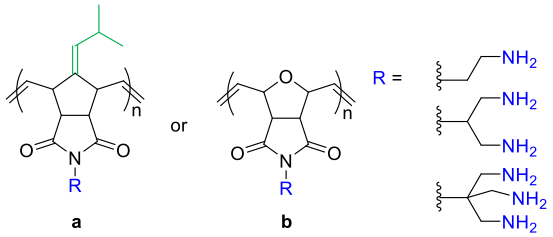


Fig. (24) Investigation of increasing cationic amine content per unit of the isobutenyl poly(norbornene)s (a) and the poly(oxanorbornene)s (b).

The Tew group also compared the activity of lysine and arginine mimicking amine functional groups (Fig 25)[51]. Arginine-rich AMPs have been shown to be highly effective antimicrobials, readily able to translocate cell membranes. This activity is attributed to the multidentate binding capability of the guanidine group for binding to membrane bound phosphate head groups to provide a strong interaction. Tew's studies focussed on the polyoxanorbornene class, which were found to be inactive when containing a lysine mimicking primary amine cation source. However, when the primary amine was converted to a guanidine group (in the oxananorbornene monomer), the resulting polymer was not only highly active against gram positive and gram negative bacteria, it also displayed low hemolytic concentrations against red blood cells. The polyguanidine oxanorbornene (PGON) polymer provided superior activity compared to previous poly(oxa)norbornene polymers and to a modified Margainin AMP, with an MIC value of 6 μg mL^{-1} when tested against *E. coli*.

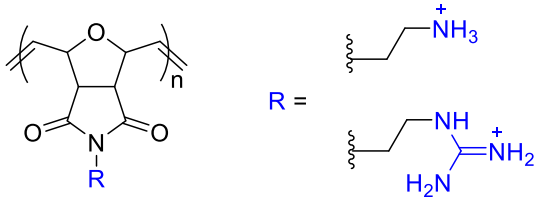


Fig. (25). Lysine and arginine mimicking cationic groups of the poly(oxanorbornene) SMAMP class

Despite the high prevalence of the arginine motif in AMPs, the guanidine group has been little studied compared to primary amine, lysine mimics. Sampson and colleagues investigated the guanidine group for their AROMP SMAMPS, comparing this multidentate group to a trimethylammonium cation. Despite a slight improvement in antibacterial activity in the presence of the guanidine group, the synthetic effort to include this group was deemed an over-riding factor when selecting optimum SMAMP candidates. This could provide a rationale as to why this important cation source is often overlooked. [64]

Locock and co-workers have further built upon the findings of superior antimicrobial activity of guanidine containing SMAMPs by incorporating the functionality into poly(methacrylate)s.[42] Their studies compared the incorporation of aminoethyl methacrylate (AEMA) or 2-guanidinoethyl methacrylate (GEMA) into methyl methacrylate (MMA) copolymers. In both copolymer series, a 30-40% MMA content and a low molecular weight (ca. 3.3-4.5 kDa) provided optimum antimicrobial activities. Molecular weight was strongly dependent on antimicrobial activity for the poly(MMA-GEMA) series, but independent for the poly(MMA-AEMA) series. The arginine mimicking poly(MMA-GEMA) copolymers also exhibited much lower MIC values than their lysine mimicking AEMA counterparts. When evaluated against *S. epidemidis*, *S. aureus*, and *C. albicans* the GEMA series gave MIC values of 10, 94 and 8 μg/mL respectively, compared to the AEMA values of 625, >1500 (inactive) and >128, respectively. Another feature of the guanidine-containing poly(methacrylate)s is their low toxicity when compared to their primary amine counterparts. When comparing hemolytic extent (as a percentage value) via lysis or red blood cells, the guanidine polymers again outperformed the amine functionalised polymers, with low hemolytic activity at their MIC for *S. epidermidis*. Even at a high MMA content, the guanidine polymers exhibited very low hemolytic activity, in contrast to the lysine mimicking polymers, which gave an increase in hemolysis with increased MMA content.

Kuroda also investigated the cationic source in the poly(methacrylate) class, focussing on lysine mimics. The group prepared primary, secondary, tertiary and quaternary ammonium salt cationic groups, and found that the primary group provided the highest antimicrobial activities, whilst quaternary ammonium salts were inactive for the poly(methacrylate) series.[18, 69]

5. SEQUENCE CONTROL IN POLYMERIC AMP MIMICS

As the key requirements for efficient polymeric mimics of AMP are being elucidated, recent work has shown that the distribution of functionalities along the polymer backbone can also affect their activity. As seen earlier, the alpha-helical secondary structure of AMPs is not necessary to exhibit an antimicrobial activity. However, the overall structure of an AMP seems to have a direct impact on the interaction with bacterial membranes, thus, on the antimicrobial activity. In this section, the impact on aggregation and biological activity of the distribution of functionalities along the polymer backbone and in respect to each other is reviewed.

5.1 Structural Control via Monomer Architecture

The usual approach of synthesizing polymeric AMP mimics is the statistical copolymerization of a hydrophobic and a cationic monomer, having both components in undefined proximity in the resulting polymer chain. To investigate the impact on the distance of both functionalities relative to each other, different approaches have been taken.

In order to establish the impact of the relative position of the alkyl chain towards the cationic centre, Sambhy *et al.* designed two types of random copolymers both containing pyridinium and methacrylate units.[21] For one series, methyl methacrylate was copolymerized with cationic pyridinium quaternized by the alkyl chain as the substituent. The other copolymer series contained pyridinium monomer bearing a methyl group and the pendant alkyl chain was attached to the methacrylate units (Fig. 26A). The separation of the alkyl chain from the cationic centre slightly increased the antibacterial and significantly increased the hemolytic activity. Indeed, for similar composition and alkyl chain length, the disruption of cell membranes was boosted by the separation of the charge and the tail, in particular for mammalian cells. Therefore, the selectivity against bacteria was increased by placing the alkyl chain on the cationic centre. A possible explanation for this effect could be found in the mechanism of action of polymeric AMP mimics. When hydrophobicity is spatially separated from the charge it is more likely to insert into the hydrophobic domain of the cell membrane, rendering these polymers more membrane active. This research underlines the importance of spatial control of hydrophobic and cationic units relative to each other.

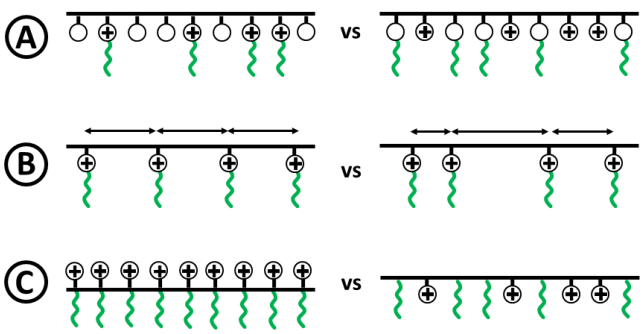


Fig. (26). Different strategies to vary the biological activity of polymer by changes in sequence/monomer distribution. A) Controlled distance of cationic charges. B) Variations in proximity of cationic charge and hydrophobic units along the polymer chain. C) Comparison of facial amphiphilic monomers with random copolymers

Song and co-workers developed alternating copolymers of cationic and hydrophobic monomers obtained by Alternated Ring-Opening Methathesis Polymerization (AROMP).[70] Random counterparts and homopolymers were synthesised for comparison of the antimicrobial activities. Variation of the side chains within alternating copolymers, allowed a control of the spacing between the functional groups. Fine-tuning of this distance was shown to improve the potency of alternating polymers, which exceeded the antimicrobial activity of the random copolymers with similar functionalities in which the spacing between the pendant groups is not controlled (Fig. 26B). The authors furthermore conclude that a distance of 8 - 10 Å between cationic charged units in an alternating backbone result in optimal membrane interaction. However, since the synthesised polymers exhibited dispersities up to 2.4 for the alternating copolymers and 3.5 for the homopolymers at relatively low molecular weights, they were rather undefined, thus making it difficult to reach unequivocal conclusions.

Further investigation of the distribution of functional groups along the polymer backbone was undertaken by Gabriel *et al.*[50] The study was directed towards the comparison of norbornene polymers obtained by ROMP from facially amphiphilic (FA) monomers, which contain a cationic group opposite a hydrophobic alkyl chain within the monomer unit, to those from mono-functional monomers (Fig. 26C). Random amphiphilic polymers were obtained using mono-functional monomers resulting in polymer chains with segregated functional groups, whereas FA monomers with different alkyl chain length were copolymerizsed to balance the overall hydrophobicity of the polymer chains. FA polymers exhibited higher antimicrobial activity and lower toxicity against red blood cells than the segregated counterparts, thus a better selectivity. This could be explained by the mechanism of antimicrobial polymers which undergo phase separation before disrupting the membrane. The FA structure might enhance the process of phase separation, thus boosting the disruption of bacterial membranes.[50] The rigidity of the norbornene backbone might emphasize the effect of the facial amphiphilicity of the monomers. A more flexible backbone could allow the polymer chains to adapt in presence of a membrane, thus rendering the initial distribution of the functionalities along the polymer backbone less important. Another observation was that the change in the ratio of cationic to hydrophobic segregated monomers did not influence the antimicrobial or the hemolytic activity. However, as the length of the alkyl pendant group had a direct impact on the selectivity, it seems that the hydrophilic to hydrophobic ratio is of greater importance at the local rather than overall polymer scale.

In order to mimic the facially amphiphilic structure of AMPs, Tew and co-workers designed oligomers using aromatic rings and other functionalities enabling H-bonding, to rigidify the structure, thus altering the selectivity of these oligomers towards bacteria.[71] Further systems were explored using monomers bearing both a cationic and a hydrophobic pendant group,[22b] or introducing modification in FA monomers affecting the structure of the backbone. [22c] The design of monomers chosen to build the AMP mimics and any modification in the sequence of these monomers seem to have an impact on the local amphiphilicity, therefore, affecting the interaction of the resulting polymers with cell membranes. These FA systems, among other synthetic mimics of AMPs, have been discussed more extensively in a review by Lienkamp *et al.*.[71]

5.2 Impact of the Polymer End Group

As antimicrobial polymers usually possess low DP, variation in the end group of the polymer chains can have a significant influence on the biological activity. This was initially found for nylon-3 copolymers having alkyl chain end groups.[27] An increasing alkyl chain length decreased water solubility of the polymers and increased the general membrane activity (MIC and HC) with a drastic drop in haemocompatibility from C_8 to C_{12} end groups. MIC values did increase again for polymers having a C_{12} alkyl chain or longer end groups, which might be attributed to aggregation. Further investigations on these systems focused on polymers having a bulky hydrophobic group on either N- or C-terminus of the nylon-3 copolymers which, interestingly, had a different influence on the hemolytic activity.[72] While C-terminal functionalized polymers showed a low MIC and a high HC values, placement of the end group at the N terminus of the polymer resulted in increased MIC and strong hemolytic activity. This was assigned to the gradient nature of the copolymer resulting in an increased hydrophobic fraction on the C-terminus, supported by aggregation studies which showed, that polymers with the end group at the C-terminus aggregated at lower concentrations.

Locock and co-workers used the RAFT process to generate varying polymer end groups by changing

substitution on the chain transfer agent.[68] It was found that a carboxylic acid end group significantly reduces haemolysis, which could be attributed to an inter chain complexation. Also, the use of a long alkyl chain on the chain end did increase general membrane activity. No significant change in aggregation behaviour was found for different end groups. Recently, the end group of RAFT polymers varied using modified radical initiators installing a primary amine groups on the chain end of AMP mimics and comparing them to the initial phenylic RAFT end group.[73] The results suggest that eliminating the hydrophobic group on the chain end reduced overall membrane activity.

Similar results were obtained by comparison of poly(acrylate)s synthesised via Cu(0) mediated polymerization with initiators bearing alkyl chains of two and twelve carbons as reported by Grace and co-workers. Furthermore, they observed an increase in the antimicrobial activity when the degree of polymerization was decreased for the polymers which had a twelve-carbon hydrocarbon tail. [74]

These findings are in line with other antimicrobial polymers, although not mimicking AMPs, described by Tiller and co-workers. For these systems the antimicrobial activity was generated only by the two end groups of a biologically neutral poly(2-oxazoline) (POx) chain.[75] While one group was constituted by an amine, quarternized by a C_{12} chain, the second end group was varied (alkyl chains of different lengths) in order to assess changes in antimicrobial activity. Interestingly, the second end group had a huge impact on antimicrobial activity, suggesting a cooperative effect of both groups. This could even be applied to switch off toxicity by enzymatic cleavage of one of the two groups.[76] Also, CMC values directly correlated with the MIC of the polymer, thus suggesting that micellization protects hydrophic end groups from interacting with membranes, thus reducing the toxicity. Unfortunately, no hemolytic activity is stated for this polymeric system. The sum of these studies suggest, that the end group of a polymeric AMP mimic indeed is an important factor, which needs to be considered for the design of efficient systems.

5.3 Structural Control with a well-defined Monomer Sequence

The self-assembly of AMPs has been demonstrated to play an important role in reducing the cytotoxicity and improving the stability of these peptides.[77,78] The self-assembly of antimicrobial polymers might also have an impact on their activity. Extensive work on the modelling of the interactions of antimicrobial polymers with bacterial membrane was carried out by Baul and co-workers.[14] Although specific conformations are not necessary, it was established that the interactions of polymers with bacterial membranes were facilitated by their ability to segregate cationic and hydrophobic groups. Additional simulations showed that the strength of the adsorption of antimicrobial polymers to bacterial membranes depended on the monomer sequence, correlated to the ability to phase segregate.[79] Amphiphilic block copolymers, in comparison to random copolymers, are more likely to undergo phase segregation in an aqueous environment and are, therefore, the subject of recent studies.

An early investigation performed by Sauvet and co-workers[80] compared multiblock poly(siloxane)s bearing quarternized ammonium units with their statistical pendants. No influence of the polymer architecture on the antimicrobial activity was observed. However, the hemolytic activity of the macromolecules was not investigated, preventing a conclusion about their selectivity.

Wang *et al.* performed a similar study on the antimicrobial activity of diblock and random poly(methacrylate)s.[81] Again, the level of activity against microorganisms did not vary with the structure of the polymers. However, the hemolytic activity was highly decreased for diblocks making them more selective against bacteria, in comparison to their random equivalent. Unfortunately, the possibility of self-assembly was not recognised in either report and, therefore, not investigated.

A study of gradient and diblock nylon-3 copolymers was performed by Liu and co-workers.[46b] While hemolytic activity was diminished, the antimicrobial activity was also reported to be lower for the diblocks as compared to gradient copolymers. However, there was no comparison of the self-assemblies of both systems in this report.

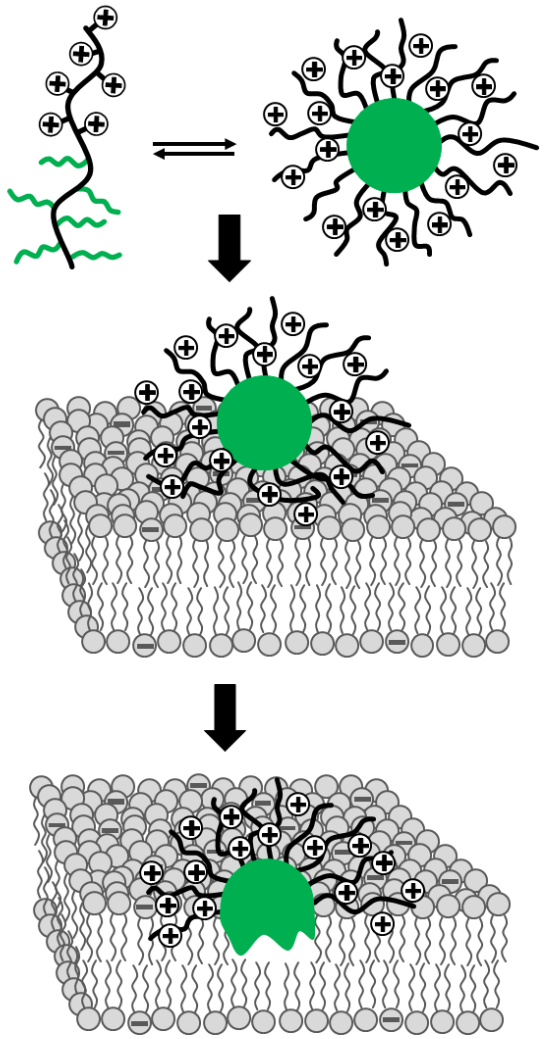
Oda *et al.* designed diblock and random copolymers of hydrophilic and hydrophobic vinyl ethers using cationic polymerization.[62] Although the antimicrobial activity was the same for both types of polymers, the study demonstrated that the block copolymers were far less hemolytic than their random equivalent. Interestingly, block copolymers displayed an antimicrobial activity below the Critical Micelle Concentration (CMC). However, they were considered to form unimolecular aggregates with a cationic shell at low concentration, which allowed a shielding of the hydrophobic domain towards red blood cells, thus reducing the hemolytic activity. This is supported by molecular dynamic investigations on statistical copolymers performed by Taresco and co-workers.[82] A flower-like micelle model was used to fit the structure adopted by the random copolymers in solution indicating the formation of phase segregated systems even without a block or block-like architecture. A further difference with the block copolymers in this study by Oda *et al.* was the increased induction of hemagglutination (RBC aggregation) level in comparison to the random copolymers. This could be a result of the phase separation of block copolymers leading to an increased expression of cationic charges as compared to statistical copolymers. The increased interaction with anionic groups on RBC membranes would consequently result in cross linking and aggregation.

A study by Nederberg and co-workers on triblock poly(carbonate)s by ring-opening polymerization revealed similar observations concerning antimicrobial activity and haemolysis.[83] The polymers self-assemble in aqueous solution into flower-like micelles, able to efficiently disrupt bacterial membranes while maintaining low haemolysis levels. However, in this case MIC values were higher than the reported CMC of the polymers in buffer.

Costanza *et al.* investigated poly(amino acid) based block copolymers consisting of phenyl alanine and lysine.[84]

Poly(ethylene glycol) (PEG) of varied length constituted the second block of the polymers, which formed particles in aqueous solution. It was found that by choosing the length of the PEG shell, the biological active block could be shielded, which reduces the hemolytic activity, as well as the antimicrobial activity.

The influence of the shape of polymeric nano-objects on the killing of bacteria was investigated by Yao and co-workers by synthesis of a diblock with a cationic and a hydrophobic block *via* RAFT polymerization.[85] Phase separated bulk materials were cross-linked and re-dispersed resulting in spherical, cylindrical and sheet-like assemblies. However, the antimicrobial activity was not affected by the morphology, highlighting that increased contact area is not needed to improve antibacterial activity for these polymers. Furthermore, cross-linking of the polymers might interfere with the effective insertion into the hydrophobic membrane domain. Unfortunately, the impact of the shape on the toxicity against mammalian cells was not investigated.



Recently, the impact of cyclization on the activity of AMP mimics was studied.[86] Sequence controlled oligomers were synthesized in a semi-solid phase approach and cyclized yielding defined ring sizes. It was shown that smaller rings possess higher antimicrobial activity and are less sensitive to the substitution of hydrophobic by hydrophilic units, which is important to regulate the hemolysis in this system. Additionally, it demonstrates the impact of the microstructure as identical sequences exhibited different activities depending on the nature of cyclization.

Fig. (27). Schematic representation of the antimicrobial activity of block copolymers.

In summary, although a alpha-helical shape, as found for AMPs, is not necessary for a successful antimicrobial polymer, the distribution of functionalities along the polymer chain certainly has an impact on their biological activity. However, while empirical evidence prove that distance between charges, the proximity between cationic and hydrophobic functionalities, or the existence and placement of an end-group directly influence the membrane activity, no clear correlation for that influence was found so far.

The case of block copolymers and block-like structures is slightly clearer, and micellization seems to be the clear cause of an altered behavior. With the cationic charge exposed for interaction and the hydrophobic domain hidden within the core of the aggregate, interaction with bacterial membranes is favored over RBC attachment. Since the bacterial killing on many accounts did not suffer from this structural feature, it is likely that, once attached, hydrophobic groups can insert into the bacterial membrane in order to disrupt it (Fig. 27).

6. CONCLUSIONS

The antimicrobial activity of AMPs and their polymeric counterparts have demonstrated a high potential as antibiotic agents, since they are less sensitive to the development of bacterial resistances. In particular, polymeric AMP mimics have the key advantages of a low production cost and high stability. The structural requirements of these structures are now relatively well understood. Despite common belief, an α-helical shape is not crucial for their activity; the main parameter to be matched seems to be the amphiphilic balance. This ratio between cationic charges, which enable the polymeric chain to approach the negatively charged membrane of bacteria, and hydrophobic groups, which allow the polymers to penetrate and irreversibly disturb the bacterial membrane, determine the activity and most importantly the selectivity of antimicrobial polymers. Effective systems also require fine-tuning of molecular weight, nature of charge and backbone structure. In addition, as for peptides (including AMPs), which activity highly depends on their primary and secondary structures,

antimicrobial polymers are influenced by their sequence and structure in solution.

The monomer sequence in the polymeric chains, equivalent to the primary structure of peptides, was shown to have an impact on the antimicrobial properties, as demonstrated by variation of the distance between charges, the proximity between charge and hydrophobic units as well as the presence and identity of end groups. However, a clear structure activity relationship is still missing. Timely, recent advances in polymer synthesis now enable the design of sequence-controlled polymers, which will undoubtly be of great importance to elucidate this dependence.

The behaviour of the polymer in solution (e.g. micellization), equaling the secondary and tertiary structure of a peptide, has been shown to also influence the performance of antimicrobial polymers. *Via* phase segregation, it is possible to shield the hydrophobicity of the polymer from unwanted interaction with *e.g.* red blood cells. Upon binding to bacterial membranes, the hydrophobic phase seems to be enabled to interact with the lipid membrane and play a part in its disruption.

The next step in the production of AMP mimics that can compete with current antibiotics is the control of these two structural features, for instance by using multi-block copolymers, which show both controlled sequence of functional blocks as well as amphiphilic and self-assembling properties.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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