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Antibacterial Peptidomimetics: Polymeric Synthetic Mimics of Antimicrobial Peptides

Karen Lienkamp, Ahmad E. Madkour, and Gregory N. Tew

Abstract Polymer-based peptidomimetics, or proteinomimetics, are a relatively young and dynamic field of research. The ability to successfully mimic the biochemical activity of antimicrobial peptides (AMPs) has been demonstrated by several groups. This has been accomplished by careful tuning of the molecule's hydrophobicity and charge density. At the same time, many important questions remain to be answered, including the role of backbone rigidity, details of membrane insertion, and the role of curvature in the self-assemblies between these novel peptidemimetics and phospholipids. As the biological properties of polymeric synthetic mimics of AMPs (SMAMPs) result from the interplay of many parameters, it is not yet possible to predict the exact properties of such molecules from their mere chemical structure. However, as demonstrated here, the effect of certain design features such as charge and hydrophobicity on the properties across a polymer series is understood. Compared to the mechanistic specifics that are known about the interactions of AMPs or small antibacterial molecules with membranes and cells, relatively little is known concerning the interaction of polymeric SMAMPs with membranes. Beyond SMAMPs, numerous opportunities exist and protein transduction domain mimics are an active area of research in the Tew laboratory. These two examples, one quite new and the other studied for almost a decade, demonstrate that it is possible to teach synthetic polymers to behave like peptides, despite their lack of sequence specificity and secondary structure.

Keywords Antibacterial polymers · Antimicrobial polymers · Peptide analogs · Peptidomimetics · Polymer–membrane interaction · Synthetic mimics of antimicrobial peptides, SMAMPs

K. Lienkamp, A.E. Madkour, and G.N. Tew (✉)
Department of Polymer Science & Engineering, University of Massachusetts, Amherst, MA 01003, USA
e-mail: tew@mail.pse.umass.edu

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1 Introduction

The term "peptidomimetic" was originally defined as a "compound that, as the ligand of a receptor, can imitate or block the biological effect of a peptide at the receptor level" [1], and referred to molecules that were derived from existing peptides. Another definition refers to a peptidomimetic as "a substance having a secondary structure as well as other features analogous to that of the original peptide" [2]. Today, the term is more generally understood as "a compound that is able to emulate the properties or biologically activity of a peptide." The latter definition emphasizes the importance of similar function rather than similar structure. Indeed, the evolution of these definitions is a mirror image of the development of the field of antibacterial peptidomimetics, which mimic antimicrobial peptides (AMPs), a host defense peptide. In analogy to their parent peptides, these molecules are known as synthetic mimics of antibacterial peptides (SMAMPs). Whereas early SMAMPs closely resembled AMPs both in their chemistry and secondary structure, the most recent polymer-based SMAMPs show no immediate structural similarity to those peptides [3–7]. However, they still capture their essential biophysical properties and thereby are able to imitate their antibacterial activities. In order to design such molecules, scientists first needed to determine what was essential about the target peptide to be able to maintain a similar activity in the peptidomimetic. In this review, we first retrace how the essential features of antibacterial peptidomimetics were identified, and then focus on SMAMPs made from synthetic polymers.

The antibacterial potency of AMPs or SMAMPs and their selectivity for bacteria over mammalian cells, such as erythrocytes, is typically quantified by determining their minimum inhibitory concentration (MIC) and hemolytic activity (HC) [8–10]. MIC_{90} is the concentration of a SMAMP that inhibits 90% of pathogen growth. This value is obtained from a plot of bacterial growth of versus SMAMP concentrations (see dark squares in Fig. 1). Other popular MIC values are the MIC_{100} and MIC_{50}, which are defined and determined analogously. Although MICs are specific to the given method, when determined properly they are highly reproducible values that allow reasonable comparisons of the relative potency of SMAMPs, with the only significant disadvantage being that they do not differentiate between growth inhibition and actual pathogen killing. To distinguish between inhibition and killing, bacterial

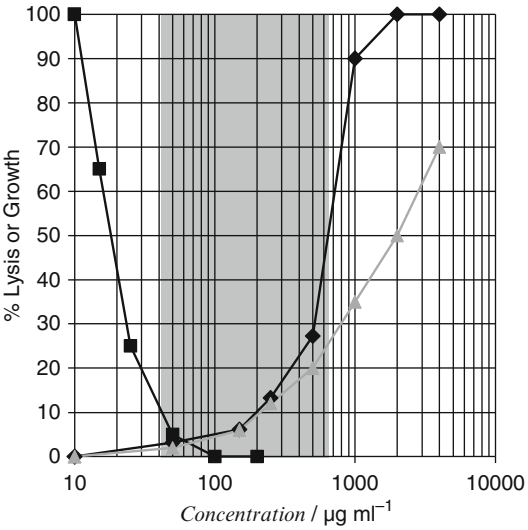


Fig. 1 MIC and HC curves. *Squares* MIC curve (MIC_{100}, MIC_{90} and MIC_{50} = 100, 50 and 25 μg/mL, respectively); *diamonds* HC curve (HC_{100}, HC_{50} and HC_0 = 2000, 650 and 10 μg/mL, respectively; *triangles* HC curve (HC_{100}, HC_{50} and HC_0 = >4000, 2000 and 10 μg/mL, respectively). The two HC curves illustrate that two polymers with identical HC_0 can have drastically different HC_{50} and HC_{100}. values The *shaded region* represents the therapeutic width of the compound, i.e., the concentration range in which the compound is active yet not too toxic for the host organism

growth kinetics are investigated in so-called "time kill studies," in which the growth reduction of bacteria exposed to different SMAMP concentrations is monitored as a function of time [11].

Cell toxicity is more difficult to determine than bacterial activity due to the various types of toxicity that can be measured. Typically, the "toxicity" of SMAMPs is assessed by exposing them to erythrocytes and observing the resulting cell lysis. Analogously to the MIC curve, a plot of percentage lysis versus concentration yields the HC_{50} value, i.e., the value at which 50% of red blood cells are lysed upon exposure to the SMAMP. The HC_{50} value can be obtained directly from the curve by extrapolation (Fig. 1), or by a fit of the experimental data with the Hill equation [12]. However, unlike the MIC values, which are well accepted and broadly applied, there is some variation in the literature with respect to quantification of hemolytic activity. Many laboratories determine the HC_{50} value (in analogy to LD_{50} used for in-vivo drug testing) either with or without serum, which typically has a large effect. Another parameter used is the minimum hemolytic concentration (MHC). However, there are at least two contradictory definitions for this parameter in the literature. Some groups define it as the minimum concentration necessary to obtain complete erythrocyte lysis [13–15]; this makes it the same as the HC_{100} value. More recently, it has been defined as the concentration at which lysis *starts* to be seen [16], which corresponds to an HC_0 value. These contradictory definitions complicate the comparison of hemolysis data between laboratories. To avoid this confusion, using terms like HC_{100}, HC_{10}, or HC_0, instead of MHC would be helpful.

Also, HC_{50}, HC_{100}, and HC_0 values do not convey the same amount of information. In the example given in Fig. 1, both HC curves have identical HC_0 values, although the compound represented by the curve with diamond symbols is obviously more hemolytic. This fact is captured when reporting the HC_{50} or HC_{100} value for these compounds, but not the HC_0 value. On the other hand, the HC_0 value is a very sensitive parameter and is useful when comparing substances with very low hemolytic activity, or when the SMAMPs might become insoluble at high concentrations before the HC_{50} or HC_{100} is even reached. Thus, each of these hemolysis parameters has merits and there can be important reasons for selecting certain terms in any giving report. Overall, the SMAMP field seems to prefer the use of the HC_{50} value.

The preferential activity of a compound against pathogens rather than against host cells is typically expressed by taking the ratio of the HC value and the MIC value, which is termed the selectivity of the compound. As can be seen quite clearly, the selectivity is then strongly influence by the selected HC and MIC values. Common AMPs have selectivities of 10 [for the frog peptide magainin (MSI 78)], >40 (human AMP *n*NP-1 [17]) or even >100 (human AMP β-defensin 3 [18]) when defined in terms up the HC_{50} and MIC_{90} values. Another parameter to express the same idea is the therapeutic index (which is the same as the therapeutic ratio). This pharmacological term is generally defined as the ratio of the toxic dose for 50% of the test species population and the minimum effective dose for 50% of that population (here HC_{50}/MIC_{50}); however, it has also been used to denote the ratio of the HC_{100}/MIC_{90} [19]. Hemolysis values provide only general guidelines for fundamental studies.

To really understand toxicity, more in-depth studies (including both in vitro activity against various cell types as well as in vivo activity) are essential if one wishes to move these molecules into use for clinical applications [20]. As far as units are concerned, both MIC and HC values can be reported in moles per volume, or mass per volume. The AMP community prefers to give MIC and HC values in units of micromoles per milliliter. This is certainly a good choice when dealing with monodisperse, well-defined materials, and when the determination of the molar mass of the compound is easy. However, one should note that the purity of the peptide sequences is not always carefully determined or reported, which would influence the molarity reported and could easily lead to a 5% error. The polymer SMAMP field also seems to prefer the units of micromoles per milliliter because of the polydisperse nature of synthetic macromolecules. In the case of some polymers, molecular weights are accessible by MALDI-TOF [21, 22], but as soon as the SMAMP structure becomes more complicated, or higher molecular weights are considered, polymer characterization techniques (e.g., gel permeation chromatography, osmometry, or static light scattering) have to be used, which often have substantial experimental errors (e.g., 20% for static light scattering). When these errors propagate, the interpretation of biological data is further complicated and subtle trends might be concealed. Also, in the case of polymers, molarity can refer to the number of molecules or repeat units (number of active groups), and by choosing one or the other, a premature opinion about the mode of action of the sample is given.

2 The Natural Archetype: Antimicrobial Peptides

AMPs, a class of natural host defense peptides, served as a starting point for SMAMP design [23, 24]. AMPs are part of the innate immune system and among the first lines of defense against bacterial pathogens in many species, including plants, invertebrates, humans, and other mammals [24]. Unlike antibodies, which are highly specific components of acquired immunity, AMPs have broad-spectrum antimicrobial, antifungal, and antiviral activity [24]. Examples are magainin from the African clawed frog [25] and human defensin [26]. Virtually all natural AMPs have a distinct secondary structure, either an α-helix as in the case of maginin (Fig. 2a), or a β-sheet, as in human defensin (Fig. 2b). This fairly rigid secondary structure forms the "backbone" of the molecule (colored gold in Fig. 2) and dictates a certain spatial arrangement of the pendent amino acid residues. It was found that most AMPs consist of amino acids with cationic hydrophilic groups and hydrophobic groups , which are arranged on opposite faces of the molecule, thus creating an overall facially amphiphilic architecture [23, 24].

Most state-of-the-art antibiotics interact with specific cell structures. They may inhibit RNA replication or prevent cell wall synthesis and thereby kill bacteria (if they are bactericidal), or inhibit bacterial growth (if they are bacteriostatic). However, even slight mutations at the cellular target might render them inactive – this is why resistance build-up against antibiotics is observed, most notably in strains of multiple-resistant *Staphylococcus aureus* (MRSA) that are spreading in hospitals and the community. Unlike conventional antibiotics, AMPs act via non-receptor interactions. In most cases, they cause lysis of the bacterial membrane, although other targets also exist [23, 24]. AMPs can attach to the net negatively charged bacterial membranes via their cationic groups [27, 28]. The hydrophobic groups then help insertion into the membrane, which can locally change the organization of the membrane lipids such that transmembrane pores are formed, or compromise the membrane fluidity, which leads to membrane-disrupting mechanisms including the carpet, barrel-stave, and toroidal pore mechanisms [23, 24, 29]. These interactions then lead to a breakdown of the membrane potential, the leaking of the cytoplasm, and the death of the bacterial cell. Bacterial pathogens

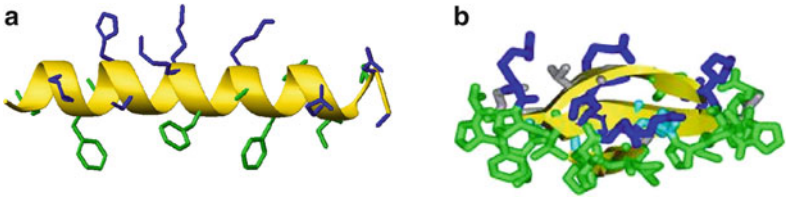


Fig. 2 The host defense peptides magainin and defensin. Magainin (**a**) has an α-helical secondary structure, whereas the amino acids of defensin (**b**) form a β-sheet. In both peptides, the molecule is overall facially amphiphilic, with the hydrophobic (*green*) amino acids on one side, and the hydrophilic (*blue*) amino acids on the other side of the backbone (*gold*)

can only develop resistance towards AMPs acting by such mechanisms if they alter their entire membrane chemistry – thus resistance to AMPs is retarded as compared to other antibiotics [23]. The cells of the host organism, on the other hand, are usually charge-neutral due to different lipid compositions, and are thus less affected as there is no electrostatic driving force for AMPs to attach to their surface. This is the main reason why AMPs act selectively against bacteria and not the host organism. These features – selective antimicrobial action against pathogens only, and a low propensity of resistance build up – make AMPs highly attractive as antibiotics of the future. However, the two alternatives for obtaining AMPs – peptide synthesis or AMP extraction from natural organisms – are expensive and tedious. This has triggered an increased effort in many laboratories to develop new SMAMPs. As we will illustrate, these include the SMAMPs made of α- and β-amino acids, peptoids, aromatic oligomers, and synthetic polymers. Although early peptide-based SMAMPs were only available on the milligram scale, the more recently developed polymeric SMAMPs are easily accessible in a few synthetic steps and can already be obtained in gram batches. This could open up new applications, for example in medical devices and in materials in areas with high infectious risk. The current knowledge on AMPs has been summarized in a number of excellent reviews [23, 24, 30, 31]. Likewise, developments in the field of polymeric antimicrobials and biocides, foldamers, and small molecules have been reviewed and we would like to refer the reader to that literature for complete and detailed coverage of these fields [6, 32–37]. We focus in this review on the most recent developments in the field of antimicrobials and biocides, from small oligomers through polymers; on the evolution of design principles based on the results of biophysical studies; and on polymeric SMAMPs.

3 Amino-Acid-Based SMAMPs

The first SMAMPs that were designed to emulate the properties of AMPs were based on the same repeat units that make up those peptides, i.e., L-α-amino acids. Unnatural amino acid sequences were constructed in such a way that their amino acid sequence would lead to a segregation of the hydrophobic groups and the hydrophilic groups on opposite faces of the molecule, and would induce helix formation. The helix was a primary target because most of the parent AMPs form α-helices when exposed to the cell surface [38], although active AMPs with other structures such as cyclic, turn-forming, and hairpin-forming peptides also exist [39, 40]. This design concept led to a number of potent and selective AMPs based on natural L-amino acids [38, 41–61]. Using the same design principles – potential helicity and facial amphiphilicity – another family of SMAMPs was obtained from β-amino acids. Like the α-peptides, these helix-forming β-peptides were also active and selective [56, 57, 59, 60, 62, 63]. For example, β^3-peptides form "14-helices," in which 14 residues are within the repeating hydrogen-bonded rings and form an approximate three-residue geometric repeat. Thus, their amino acid side chains can arrange with precise three-residue periodicity. The resulting

tripeptides composed of β^3-substituted amino acids (hAla, hLeu, and/or hVal), with the polar and hydrophobic groups segregating to opposite sides of the helix, were found to be antimicrobially active [56, 59]. Based on similar design principles, Gellman and coworkers described a potent and highly selective AMP that was based on cyclic β-amino acids [57]. β-Peptides that formed a different type of helix were subsequently investigated and it was shown that, besides the helical backbone, parameters such as charge, facial amphiphilicity, and an appropriate hydrophilic/hydrophobic balance were crucial for obtaining selective, nontoxic compounds.

Based on this body of data, one of the initial conclusions of SMAMP research was that a rigid helical backbone was indispensable for biological activity, especially as some studies showed that a rearrangement of the amino acid sequence of an active, helical SMAMP to a sequence that prevented helix formation ("scrambled sequences") simultaneously eliminated antimicrobial activity [58, 62]. Other results soon challenged this hypothesis. Oren and Shai incorporated a few diastereomeric amino acids – with D-configuration instead of the naturally occurring L-configuration – into their α-peptides. These nonhelical melittin-like SMAMPs were antimicrobially active and much less toxic than their parent AMP [64]. Further SMAMPs with scrambled D and L repeat units were investigated that had an MIC as low as 1.25 μM against *Bacillus subtilis* and caused only 15% hemolysis in red blood cells at a concentration of 100 μM. In both cases, the active and selective SMAMPs were shown to be strictly nonhelical [48], while vesicle studies and electron micrographs demonstrated their ability to disrupt membranes (Fig. 3) [64].

Further, it was found that scrambled sequences of 15-residue α,β-peptides that were not helical, as confirmed by circular diachroism, were also active and selective [65, 66]. With an MIC of 6.3 μg mL^{-1} and an HC_0 of 50 μg mL^{-1} (Fig. 4), an

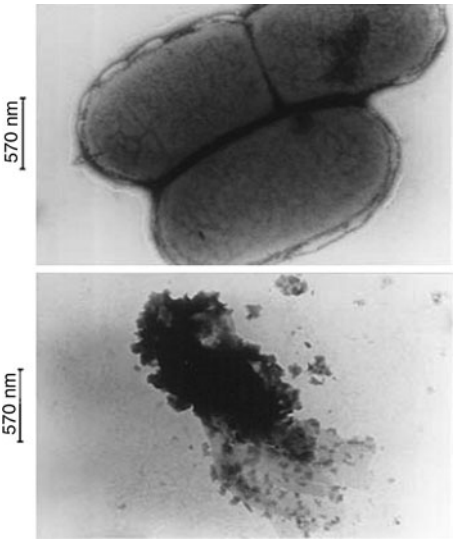


Fig. 3 Electron micrograph of negatively stained *E. coli* cells. *Top*: Intact cell treated with a SMAMP at a concentration below the MIC. *Bottom*: Disrupted cell treated with the same SMAMP at the MIC [64]

a

	E. coli	*B. subtilis*	*E. faecium*	*S. aureus*	max. concn without hemolysis
magainin	12.5	3.1	50	50	25
1	12.5	3.1	3.1–6.3	3.1	3.1
2	>100	6.3	25	50	1.6
3	6.3	6.3	6.3–12.5	12.5	50

b

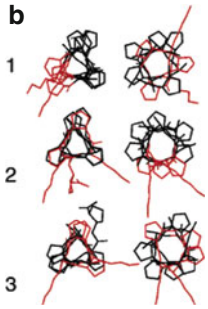


Fig. 4 (**a**) Antimicrobial and hemolytic activity of three α,β-peptides (*1–3*), compared to AMP magainin. (**b**) Axial view of predicted conformations of helical SMAMPs. Cationic residues are in *red*, hydrophobic residues are in *black*. SMAMP *1* is facially amphiphilic as an 11-helix (*left column*), SMAMP *2* is facially amphiphilic as a 14-helix (*right column*), and SMAMP *3* is facially amphiphilic in neither [65]

SMAMP designed not to be facially amphiphilic, either as a 14- or 11-helix, was more selective than its helical counterparts (Fig. 4) [65].

With today's knowledge from the field of polymeric SMAMPs and other model systems, these findings can be rationalized as follows: it is not the helicity of the molecule that is crucial, but the appropriate local amphiphilicity, as well as the ability of the molecule to self-organize into a hydrophobic and a hydrophilic part on the cell interface. In natural AMPs and *active* helical SMAMPs, the local amphiphilicity is appropriately balanced, with a sufficiently high charge density per molecule to attach to the pathogen membrane, and a local hydrophobicity that allows those molecules to insert into bacterial membranes. In the case of the *inactive* scrambled sequences, this balance is wrong, rendering them either locally too hydrophobic (and thus too haemolytic) or not hydrophobic enough (and thus inactive). In the case of the active scrambled sequences of Shai and Gellman, the amphiphilicity was appropriately balanced in the nonhelical conformation, which allowed attachment to the cell membranes. Thus, those molecules were active despite their lack of helicity, and were the first examples of active and selective nonhelical compounds, and stimulated further research to simplify SMAMPs. Another noteworthy amino-acid-based SMAMP family is Mor's oligo-acyl-lysyl oligomers [67–69].

3.1 Antimicrobial Peptoids

Peptoids, or *N*-substituted poly(glycines), are another subclass of peptidomimetics. Structurally, they are closely related to their natural peptide counterparts. Whereas peptides bear their side chains on the α-carbon atom of the amino acids, the side chains of peptoids are attached to the nitrogen atoms, which renders them protease-resistant

[70–72]. Further, the substitution at the nitrogen atoms makes the formation of hydrogen bonds between peptide bonds impossible. Hydrogen bonds are essential for the formation of secondary structures in peptides and proteins, consequently such superstructures are absent and peptoids adopt more flexible molecular conformations. The absence of helicity generally also prevents backbone chirality. However, peptoids can be driven to form helical secondary structures via a periodic incorporation of bulky α-chiral side chains [73, 74]. Despite the absence of a predetermined conformation, Barron and coworkers showed that peptoid-based SMAMPs had antimicrobial activity as low as 9.8 μg mL^{-1} against *Escherichia coli* and 1.5 μg mL^{-1} against *B. subtilis*, with only 1.4% erythrocyte lysis at that concentration [75]. The activities of two enantiomeric antimicrobial peptoids did not depend on overall handedness or on stereospecific interactions with receptors or enzymes [76]. They also showed that the helix stability was not important for the antimicrobial activity. X-ray reflectivity studies indicated that peptoids interact with and insert into membranes, much like natural AMPs. The authors suggested that, similarly to natural AMPs, the antimicrobial activity of peptoids depends on the overall hydrophobicity and net cationic charge of the molecule [76].

3.2 Aromatic Oligomers and Polymers

The findings that nonhelical SMAMPs are nonetheless active against bacterial pathogens encouraged researchers to further simplify SMAMP design and pursue alternative design concepts, such as SMAMPs based on poly(arylamides) [77]. The structure of these molecules is shown in Fig. 5a, b and shows that their backbone design has nothing in common with natural AMPs. These SMAMPs have a rigid backbone made from amide-linked aromatic repeat units, which are further stabilized by hydrogen bonding between a thioester and the hydrogen on an amide group. This bonding situation prevents rotation around the sp^2 C– N bond. Like the

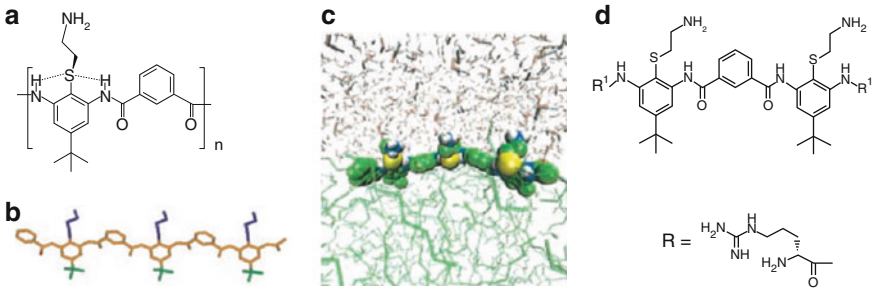


Fig. 5 Arylamide polymers and oligomers: (**a**) chemical structure, $n = 1–3, 8, 60$; (**b**) graphical representation of the facially amphiphilic structure of the trimer; (**c**) MD simulation of the structure of the trimer at the octane–water interface [78]; (**d**) fine-tuning of the hydrophobic–hydrophilic balance in arylamide oligomers [79]

peptide backbone in AMPs, this synthetic backbone dictates a facially amphiphilic conformation of the SMAMP; it locks the pendant hydrophobic *t*-butyl groups and the hydrophilic ammoniums group on opposite sides of the molecule. Unlike peptide-based SMAMPs, whose repeat units are defined sequences of alternating hydrophobic and hydrophilic building blocks, the repeat units of this class of SMAMPs are facially amphiphilic on the repeat unit level, meaning that there is a balanced local amphiphilicity, as well as a global facial amphiphilicity of the whole molecule [78]. Poly(arylamide) foldamers were found to be active against a number of Gram-positive and Gram-negative bacterial strains, and, at an optimum number of repeat units of 8, had MIC_{90} values as low as 7.5 μg mL^{-1} against *E. coli*, and 16 μg mL^{-1} against *B. subtilis* [78]. However, these molecules were also found to be highly haemolytic, most probably due to excess hydrophobicity. To reduce the haemolytic activity, oligomers with only three aromatic rings were synthesized, to which various hydrophilic end-groups were attached (Fig. 5d) [79]. With the guanidinium end-group R shown in Fig. 5d, the MIC_{90} values against *E. coli* and *S. aureus* were 6.25 and 12.5 μg mL^{-1}, respectively; and the hemolysis value HC_{50} was 715, which led to an impressive selectivity of 110 for this oligomer.

Tew and coworkers also synthesize arylurea-based oligomeric SMAMPs in a one-pot synthesis (Fig. 6a, structure 2) [80]. Compared to the arylamide polymers and oligomers, (Fig. 6a, structure 1) [79], these molecules were conformationally even more stable due to additional hydrogen bonding, which constituted further rotational barriers around the C–C bonds of the backbone [80]. The dimer, trimer, and tetramer were obtained, of which the trimer (with an MIC_{90} of 0.7 μg mL^{-1}

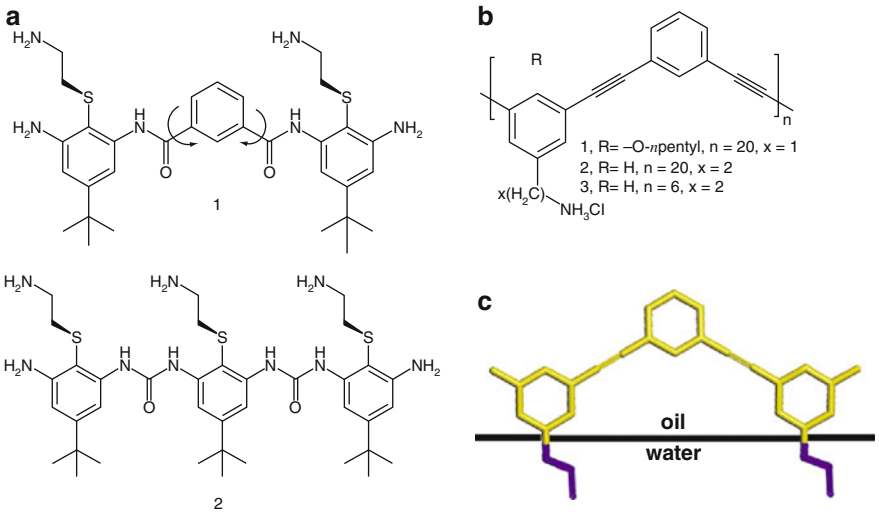


Fig. 6 (**a**) Poly(urea) oligomers (*structure 2*) are conformationally more stable than poly(arylamides) (*structure 1*) [80]. (**b**) Structure of poly(phenylene ethynylene) polymers and oligomers [5]. (**c**) Conformation of poly(phenylene ethynylene) polymers at the oil–water interface [5]

against *E. coli*) was the most active molecule; however, the selectivity of these molecules remained low, with a maximum selectivity of 5 for the trimer [80].

Although the secondary structure of these polymeric and oligomeric SMAMPs is not helical, they nevertheless have internal hydrogen bonds that reduce their conformational freedom (Figs. 5c and 6c), as confirmed by X-ray crystallography, molecular dynamic (MD) simulations, and other methods [78]. This confirmed previous findings from the field of peptide-based SMAMPs, that a helical secondary structure was not necessary to obtain active molecules as long as the backbone dictated an overall facially amphiphilic conformation of the molecule. To test whether it was possible to further relax the SMAMP design constraints, SMAMP molecules with a phenylene ethynylene backbone were synthesized [5, 81–83]. Although poly(phenylene ethynylene) SMAMP molecules possess the rigidity of an aromatic backbone, they have no intramolecular hydrogen bonds. This allowed the repeat units to rotate around the single bonds of the backbone, and enabled them to orient their functional groups to a facially amphiphilic conformation upon contact with the cell membrane or a similar hydrophilic–hydrophobic interface (Fig. 6c). The molecular structure of these SMAMPs is shown in Fig. 6b. Compound 2 (Fig. 6b), with an M_n of 5380 g mol^{-1}, had an MIC_{90} against *E. coli* and *S. aureus* of 25 μg mL^{-1}; however, it was also toxic. Compound 3 (Fig. 6b), with $M_n = 1600$ g mol^{-1}, had MIC_{90} values of 50 and 100 μg mL^{-1} against *E. coli* and *S. aureus*, respectively, and an HC_{50} of 540 μg mL^{-1}, and thus a selectivity of 10.8 for *E. coli* [5]. These phenylene ethynylene polymers were the first polymer-based SMAMPs that had the desired antibacterial activities and selectivities [5]. However, much better activities were obtained from phenylene ethynylene oligomers (Fig. 7a) [84]. By simple variation of the number (n) of carbon atoms in the side chain from one to three (Fig. 7a), the molecular properties could be tuned between inactive/non-haemolytic ($n = 1$), active/selective ($n = 2$), and active/toxic ($n = 3$). The active and selective oligomer had a selectivity of 93 for *E. coli* over erythrocytes (Fig. 7a). This oligomer series nicely illustrates how very small changes in

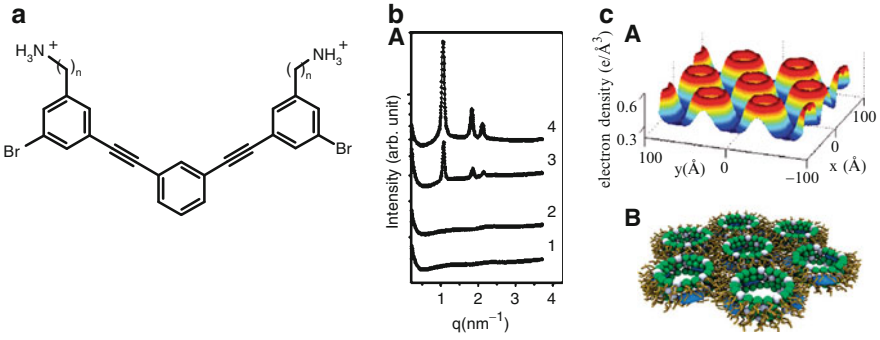


Fig. 7 (**a**) Structure of poly(phenylene ethynylene) oligomers, $n = 1$–3. (**b**) Small angle X-ray scattering data for vesicles only (*curve 1*), and oligomers with $n = 1$–3 (*curves 2–4*). (**c**) Electron density calculation for the lipid membrane (A), and proposed structural model (B) [84]

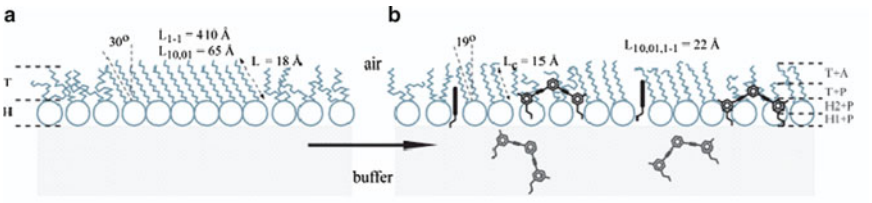


Fig. 8 Model for the insertion of a poly(phenylene ethynylene) oligomer into a lipid monolayer. Ordered domains (**a**) are broken down into small lipid patches as the oligomer penetrates the headgroup region and tilts the lipids (**b**) [85]

the balance of hydrophobic and hydrophilic groups can influence activity and selectivity.

These compounds were also used for a number of model studies on SMAMP activity and on SMAMP interaction with membranes. Using small angle X-ray scattering, it was shown that the active and selective SMAMPs induced an inverted hexagonal phase in the membrane of a unilamellar lipid vesicle that mimicked *E. coli* (Fig. 7b). This lead to pore formation in this model system (Fig. 7b) [84]. Using giant unilamellar vesicles and confocal microscopy, it was shown that small molecules were able to pass through these pores, whereas larger molecules were retained inside the vesicle [84]. For a biological system like *E. coli*, this would lead to a breakdown of the membrane potential, cytoplasm leakage, and cell death. Grazing incidence X-ray diffraction and X-ray reflectivity measurements on lipid monolayers further indicated that these SMAMPs insert into the head-group region of the lipid membrane and change the lipid tilt, thereby disturbing lipid packing in the bilayer (Fig. 8) [85].

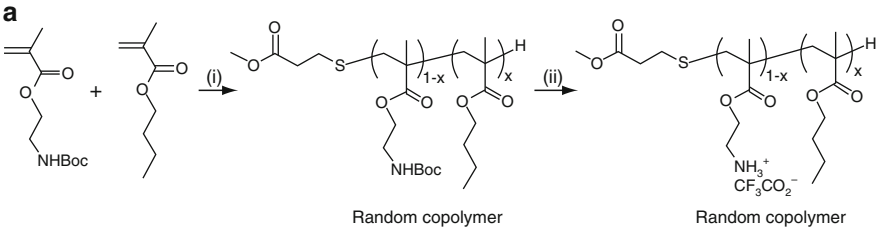
3.3 SMAMPs Based on Synthetic Polymers

Whereas peptide-based oligomers are discrete molecules with one molecular weight per batch, polymeric SMAMPs have a molecular weight distribution. This makes the characterization of their biological activity a little more difficult: besides chemical considerations such as charge and hydrophobicity, polymer-specific parameters such as molecular weight and polydispersity will have an impact on biological properties, as discussed in detail below. The immense advantage of polymeric SMAMPs over the previously described peptide-based and aromatic oligomer-based SMAMPs is that they can be obtained in very few synthetic steps, whereas peptides and other sequence-specific oligomers require tedious step-by-step synthesis and typically cannot be obtained in large scale. So far, this has severely limited the application of SMAMPs as therapeutics [23]. Polymeric SMAMPs represent another important step in the evolution of SMAMP design. Whereas the previously described SMAMP

designs aimed at some kind of secondary structure as a necessary prerequisite for activity and selectivity, the polymer SMAMP community have attempted to teach non-natural macromolecules with no backbone rigidity or otherwise defined secondary structure to behave like AMPs. It was soon found that backbone rigidity was not crucial, as long as the molecule had a properly balanced amphiphilicity and was able to self-organize into an appropriate conformation when exposed to a bacterial membrane.

Kuroda and DeGrado reported an early systematic study of a series of SMAMPs with flexible backbones [4]. Using chain transfer free-radical copolymerization of hydrophilic and hydrophobic methacrylates as a synthetic platform (Fig. 9a), they investigated the structure–property relationship of a series of amphiphilic random copolymers with varying comonomer content. The results are shown in Fig. 9b. Respectable MIC_{90} values were obtained; however, the hydrophobicities of these polymers were significantly higher than those of peptide-based SMAMPs and, consequently, the HC_{50} values were lower (on the order of magnitude of the natural AMP melittin) [4]. Thus, even the best of these molecules only had low selectivity for bacteria over mammalian cells. In a follow-up paper, DeGrado and coworkers systematically varied the hydrophobic groups and the copolymer composition to improve selectivities and succeeded in obtaining MIC_{90} values down to 8 μg mL^{-1} against *E. coli* and improved selectivities (HC_{50}/MIC_{90}) of about 13 for a 3300 g mol^{-1} methyl copolymer with 70% hydrophobic groups [86]. In spite of these modest selectivities (due to too much hydrophobicity), Kuroda and DeGrado correctly realized that, although backbone rigidity was not crucial for these molecules to be active, "preorganized facial amphiphilicity is not necessarily required for antimicrobial activity in polymers, suggesting that the polymer interface can induce an amphiphilic conformation in a large enough population of the polymers to provide a potent antimicrobial effect" [4].

This result was confirmed by Gellman and coworkers, who reported polymeric SMAMPs based on random nylon-3 derived copolymers [16]. The general structure of Gellman's polymers is shown in Fig. 10a [16], and similar polymers were reported in a follow-up paper [87]. A polymer with 60% lactam repeat unit was found to be highly active against bacteria (MIC against *E. coli* was 12.5 μg mL^{-1} and against *B. subtilis* 3.1 μg mL^{-1} though it was not specified whether this data referred to MIC_{90} or MIC_{100}) and slightly less haemolytic than magainin-Ala_3 (HC_0 was 100 μg mL^{-1}compared with 25μg mL^{-1} for the magainin derivative). Increasing the cationic lactam fraction to 63% reduced the haemolytic activity further (HC_0 = 900 μg mL^{-1}; HC_{50}, as estimated from the curve in [87] was 2000 μg mL^{-1}) and led to polymers with impressive selectivities of about 200–400 [87]. MIC (presumably MIC_{100}) and hemolysis (HC_0) data for these polymers are shown in Fig. 10b. In comparison to DeGrado's polymers [4], Gellman concluded that a polar backbone is also important to minimize haemolytic activity [16], as postulated earlier [88], and indeed these polymers are more hydrophilic and, consequently, much more selective than those reported by DeGrado [4]. Gellman also confirmed DeGrado's assumption that a SMAMP does not need to be preorganized by a secondary structure, as long as it has the ability of self-organize in an



[a] Conditions: (i) methyl 3-mercaptopropionate, AIBN, acetonitrile, 60°C, overnight; (ii) neat TFA, rt, 1 h.

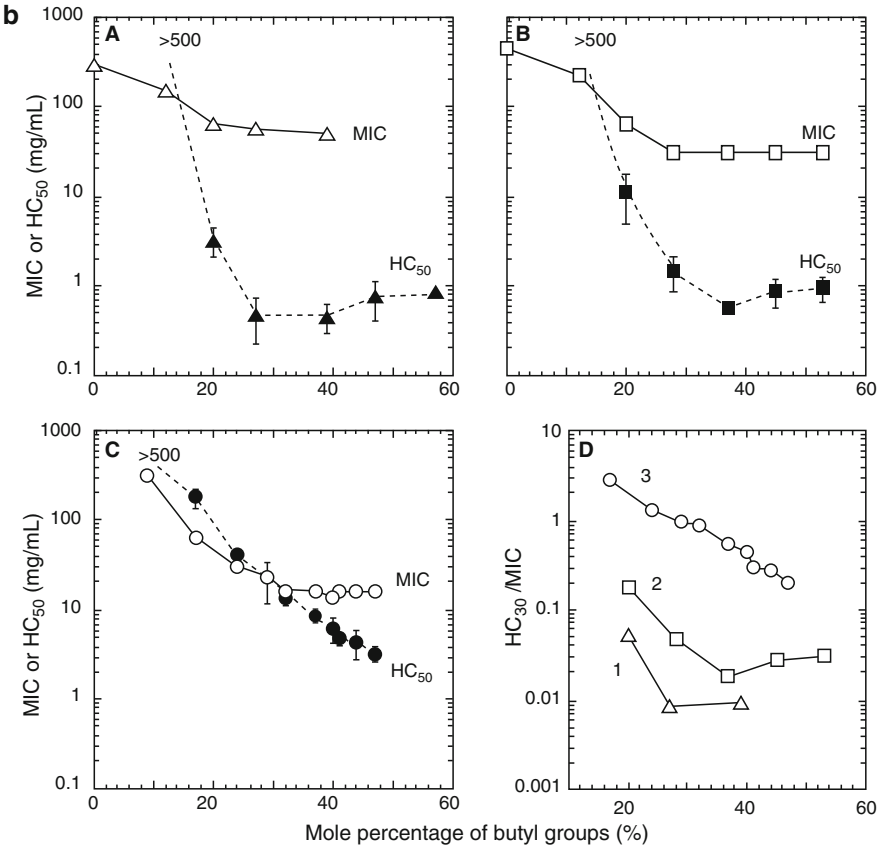


Fig. 9 (**a**) Synthesis and structure of random copolymers from *n*-butyl methacrylate and ethylammonium methacrylate. (**b**) Antimicrobial (MIC) and hemolytic (HC_{50}) activities of (A) polymers *1* with M_n of 8–10 kg mol^{-1}; (B) polymers *2* with M_n of 4.5–6 kg mol^{-1}; (C) polymers *3* with M_n of 1.3–2 kg mol^{-1}; (D) selectivities of polymers *1–3* [4]

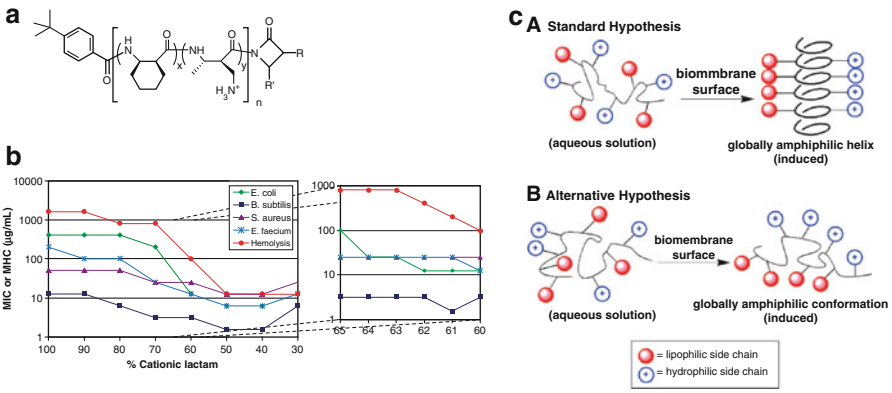


Fig. 10 (**a**) Structure of a SMAMP copolymer based on nylon-3 [16]. (**b**) Antimicrobial (MIC) and hemolytic (HC_0) activities of this polymer as a function of lactam content; the region with the greatest selectivity for bacteria over red blood cells is *shaded* and shown in greater detail on the *right*. (**c**) Self-organization hypothesis: although it was previously thought that AMPs and their synthetic mimics need a defined secondary structure for antibacterial activity (A), SMAMPs are assumed to self-organize at the interface into an appropriate amphiphilic structure (B); however, a defined secondary structure as in proteins is not necessary [16, 87]

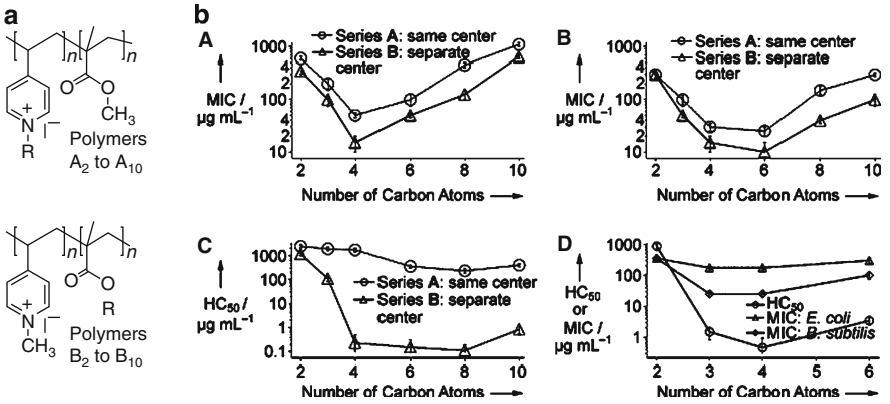


Fig. 11 Poly(pyridinium-*co*-acrylate)s: (**a**) structures of series A and B, where *R* corresponds to an aliphatic side chain with 2–10 carbon atoms, (**b**) MIC and HC_{50} for the various copolymers as a function of the number of carbon atoms [7]

amphiphilic conformation under the influence of an interface (Fig. 10c). Another series of copolymers with a flexible backbone was recently reported by Sen and coworkers [7]. These pyridinium–acrylate copolymers were obtained by free-radical polymerization followed by polymer analogous quarternization of the pyridine. Structures of two of the five polymer series of this work and their biological data are shown in Fig. 11. The polymer with the highest selectivity in

these series is the one containing a four-carbon side chain (polymer A4 in Fig. 11a), with an MIC (not specified whether this is MIC_{90} or MIC_{100}) of 30 μg mL^{-1} and an HC_{50} of 1709 μg mL^{-1}, leading to an HC_{50}/MIC of 56.

The previously described polymer-based SMAMPs by the groups of DeGrado, Gellman, and Sen are all statistical copolymers of a hydrophobic and a hydrophilic comonomer. In contrast, Tew and coworkers developed a series of poly (norbornene) homopolymers. These could be obtained by ring-opening metathesis polymerization of a facially amphiphilic monomer that carried both the hydrophobic and the hydrophilic group. Due to their facially amphiphilic nature, the hydrophobicity is locally balanced, and not just globally balanced over the entire molecule. However, slight structural irregularities still occur in these polymers due to the possible stereoisomers that were formed. The first poly(norbornene) series by Coughlin and Tew used a backbone-modification strategy to tune the hydrophobic/hydrophobic balance of the facially amphiphilic repeat units (Fig. 12a). The effect of these variations on the antibacterial and hemolytic activities are shown in Fig. 12c [89]. Although the selectivity of these homopolymers (HC_{50}/MIC_{90} up to 20) were modest, copolymerization of two facially amphiphilic monomers yielded copolymers with an MIC_{90} of 40 μg mL^{-1} against both *E. coli* and *B. subtilis*, and an HC_{50} of 4000 μg mL^{-1}, leading to a selectivity of 100 against both Gram-positive and Gram-negative bacteria. Although easier to synthesize than peptide-based SMAMPS, this polymer series still required a distinct set of precursors for each monomer and thus considerable synthetic effort. To simplify and optimize the synthesis efficiency of poly(norbornene)-based SMAMPs, a "construction-kit" approach was devised, by which a number of monomers could be obtained from the same set of precursors. The functional groups R1 and R3 (Fig. 12b) were introduced in the last synthetic steps, in either sequence. The homopolymers obtained (Fig. 12b) had selectivities of up to 28 for R1 = ethyl and R3 = ethylammonium [22]. By copolymerization of monomers with R1 = methyl and R1 = propyl, copolymers with an MIC_{90} of 3.75 μg mL^{-1} and an HC_{50} of >2000 μg mL^{-1} were obtained, which overall led to a spectacular selectivity of >533 [22]. Based on the backbone structures shown in Fig. 12a, b, Tew and coworkers synthesized several other series of poly(norbornene) SMAMPs and systematically investigated the hydrophilic/hydrophobic balance [3, 21, 90], charge [90, 91], and hydrophilicity [92]. The effect of counterion exchange was also explored [90]. These results have been summarized in a recent review [37].

In summary, this body of data on poly(norbornene)-based SMAMPs demonstrates that protein-like secondary structure is not required for SMAMP activity, as long as the SMAMP amphiphilicity is appropriately balanced and the molecule can adopt an amphiphilic conformation at the membrane. Polymer-based SMAMPs with antimicrobial activities and selectivities on the same order of magnitude as those of natural AMPs were obtained. In the following sections, we will look in more detail at specific parameters in polymeric SMAMPs and how these influence SMAMP activity.

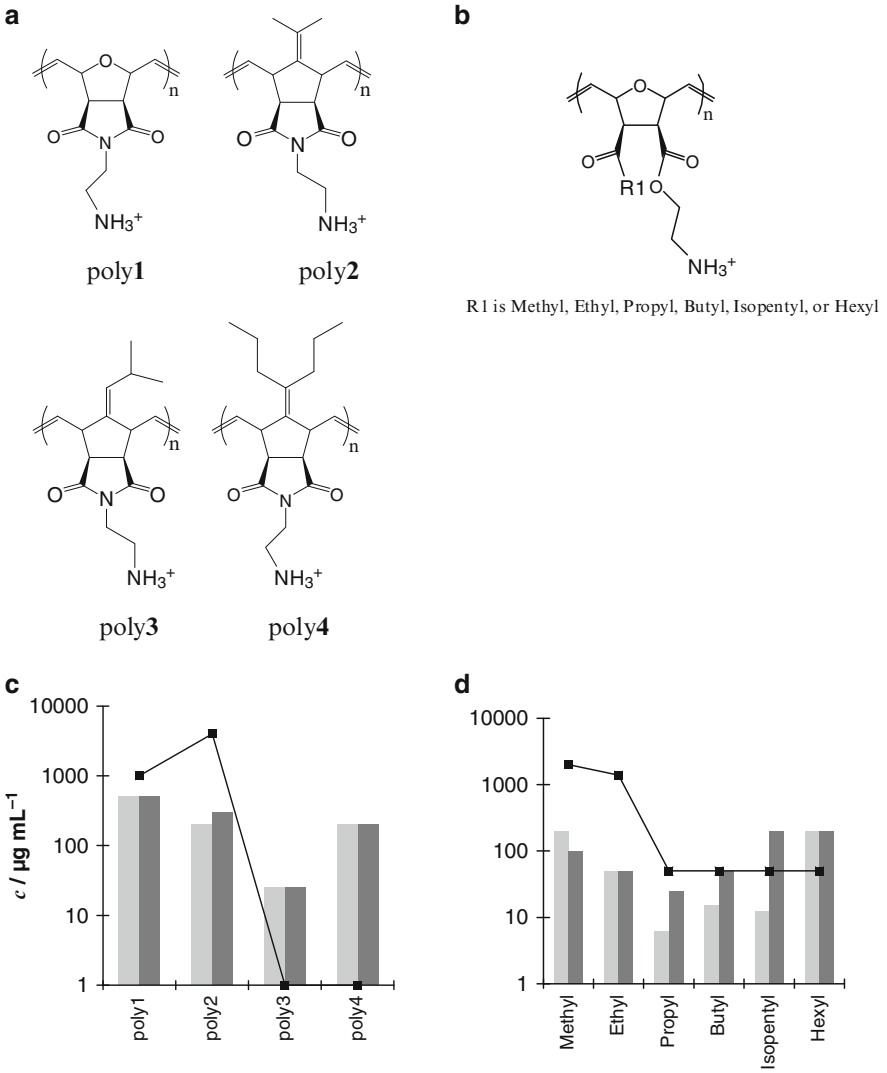


Fig. 12 Poly(norbornene)-based SMAMPs based on(**a**) backbone modification strategy or (**b**) "construction kit" approach. (**c**) Biological data for the polymer series in (**a**). (**d**) Biological data for the polymer series in (b); plotted as concentration (MIC_{90} or HC_{50}, respectively) versus increasing hydrophobicity. In both (**c**) and (**d**), *light gray columns* MIC_{90}, *E. coli*; *dark gray columns* MIC_{90}, *S. aureus*; *black squares* HC_{50}, human erythrocytes

3.3.1 Effect of Molecular Weight

Several recent studies on SMAMP polymers have investigated molecular weight effects. Overall, there seems to be no simple correlation between antimicrobial and haemolytic activity and molecular weight; the body of data is limited as only a few

studies compare more than two or three molecular weights. With his previously mentioned nylon-3 polymers (Fig. 10), Gellman and coworkers observed no significant effect of the molecular weight on the MIC [87]. For a panel of four bacteria and ~8–58 repeat units, the MIC varied one order of magnitude at most, and no trend correlating molecular weight was observed. However, there seems to be a sigmoidal dependence of hemolysis on molecular weight: although these polymers are nontoxic up to ~30 repeat units ($HC_0 \sim 1000$ μg mL^{-1}), above that value, the HC_0 drops more than three orders of magnitude down to <1 μg mL^{-1} at the highest molecular weight (Fig. 13a) [87]. The same tendency was observed by Kuroda and DeGrado for their poly(ethylammonium methacrylate-*co*-methyl methacrylate)-based SMAMPs. These polymers also became more toxic with increasing molecular weight, whereas the MIC values stayed at the same order of magnitude. Thus, overall, their lower molecular weight polymers have better selectivities [4]. For their other poly(methacrylate)-based SMAMPs with different hydrophobic groups (Fig. 13c and supporting information in [86]), the same trends were observed [86].

For poly(norbornene)-based SMAMPs, the picture is less homogeneous. For many of the poly(norbornene) polymers investigated by Tew and coworkers, it was found that low molecular weights (around 3000 g mol^{-1}) can be up to two orders of magnitude more active than higher molecular weights (10,000 g mol^{-1}) [3, 22, 89, 90], whereas the activity of other poly(norbornene)-based SMAMPs did not seem to be affected by molecular weight at all [21, 89]. Naturally, no molecular-weight-dependent trends can be observed if the SMAMPs are inactive or only weakly active ($MIC_{90} = 200$ μg mL^{-1} or above) [89]; but even for some active polymers, like poly3 in [89] ($MIC_{90} = 25$ μg mL^{-1}) or the octyl polymer in [21] ($MIC_{90} = 4$ μg mL^{-1}), the molecular weight effects are rather weak. In general, the higher molecular weight polymers were also more haemolytic, and in at least one case a sigmoidal HC_{50} versus M_n curve was observed (Fig. 13b) [33, 90]. Thus, for this class of polymer, molecular weight effects are not as predictable as in the previously discussed cases. One should note that for poly(norbornenes), molecular weights up to 50,000 g mol^{-1} were tested, whereas most other polymer series had molecular weights below 8000 g mol^{-1}, and it is expected that going to higher molecular weights might eventually render these polymers inactive and toxic. In addition to a molecular weight dependency, Lienkamp et al. found that the antimicrobial activity of some of their poly(oxanorbornene ester)-based polymers depended on the target organism [22]. They synthesized a series of poly(oxanorbornene) oligomers (Fig. 12b, R1 = propyl, R3 = ethylammonium; trimer to $M_n = 10{,}000$ g mol^{-1}) and determined their MIC_{90} against *E. coli* and *S. aureus*. As the data in Fig. 12d shows, the molecular weight dependence is highly nonlinear and different for each species. For *E. coli*, the MIC data shows a sigmoidal behavior that parallels the HC_{50} curve, with the 10,000 g mol^{-1} polymer being the most active. For *S. aureus*, this SMAMP was inactive ($MIC_{90} > 200$ μg mL^{-1}) and the MIC minimum was obtained for the trimer (oligo 1 in Fig. 12b). Finding that polymers with the same chemical structure, but different molecular weights, can differentiate between bacterial types make generalizations and predictions more

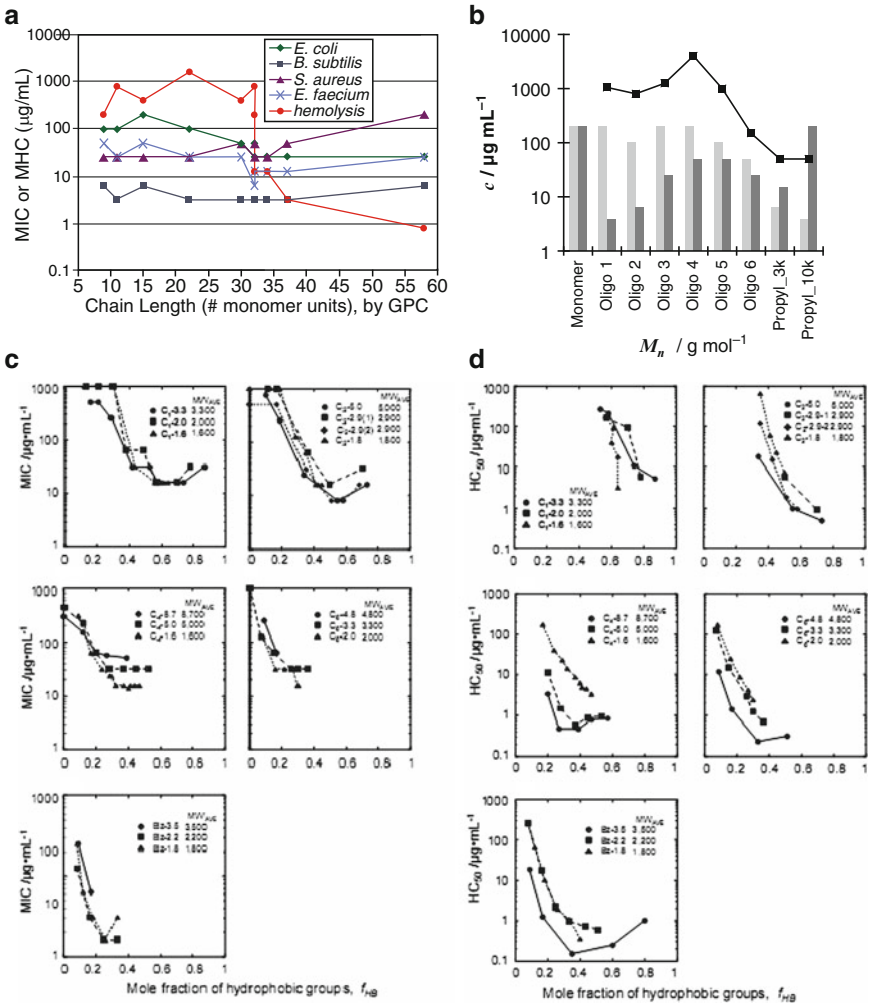


Fig. 13 Effect of molecular weight on hemolytic and antimicrobial polymers. (**a**) For nylon-3 polymer with 63% cationic lactam (see Fig. 10a), plot of MIC against various bacteria and HC_0 values against erythrocytes [87]. (**b**) Poly(norbornene) SMAMPs with R1 = propyl and R2 = ethylammonium (Fig. 11b), *light gray bars* MIC_{90} against *E. coli*; *dark gray bars* MIC_{90} against *S. aureus*; *black squares* HC_{50} against erythrocytes. (**c**) MIC data for cationic random copolymers (Fig. 9a) of various molecular weights. The alkyl group is (*A*) methyl, (*B*) ethyl, (*C*) butyl, as in Fig. 9a, and (*D*) hexyl. (**d**) HC_{50} data for the same polymers as in (**c**) [86]

difficult. However, it also gives the chemist yet another tool for tuning SMAMP properties and making them species-selective.

In summary, there is a general trend for most polymeric SMAMPs that the haemolytic activity increases with molecular weight. This could be due to a cooperative affect of the repeat units of the polymer when attaching via hydrophobic

interactions to the erythrocyte membrane that facilitates membrane disruption. Unlike bacteria, erythrocytes are simple single-membrane cells, i.e., there is no cell wall and no outer membrane. Thus, the correlation between haemolytic activity and molecular weight is much simpler than that for molecular weight and antimicrobial activity, which is also affected by SMAMP–cell wall or SMAMP–double membrane interaction.

3.3.2 Correlation Between Hydrophobicity and Biological Activity

Several research groups have investigated how tuning the hydrophilic and hydrophobic balance of amphiphilic SMAMPs influences their antimicrobial activity and selectivity. Most of that data is already included in previous figures, and we will summarize the findings in this section. For many polymer series, it was found that the SMAMPs were equally active against Gram-negative bacteria and Gram-positive bacteria [3, 22, 89]. In most cases (e.g., DeGrado's methacrylates, Fig. 13c [86]; Gellman's nylon-3 polymers, Fig. 10b [16] and [87], and most of Tew's poly(norbornes) [7]), the following trend is observed: the MIC_{90} values are quite high for the more hydrophilic polymers (i.e., those polymers are inactive), then go through a minimum, after which the MIC goes up again. Thus, with increasing hydrophobicity, the polymers become more active against bacteria. However, their solubility in aqueous media simultaneously decreases, leading to aggregation and/or precipitation. Thus, a considerable fraction of the sample becomes unavailable for interaction with the bacterial membrane, and the MIC_{90} value goes up again. At the minimum of each curve, the optimum balance between hydrophobicity and solubility is obtained. However, with increasing hydrophobicity, the polymers also become more toxic to mammalian cells, and thus the polymer with the minimum MIC value in each series is not necessarily the most selective one. This is nicely illustrated in Figs. 9b and 10b. In order to tune hydrophobicity in a more subtle way, Tew and coworkers copolymerized two facially amphiphilic repeat units with different hydrophobicities at varying ratios [22, 89]. That way, by incorporating an active and toxic repeat unit and an inactive and nontoxic repeat unit in the same polymer, copolymers with superior selectivities were obtained (Fig. 14).

3.3.3 Facially Amphiphilic Versus Segregated Systems

On the basis of studies on peptide-based and aromatic SMAMPs, it was believed that the overall hydrophobic/hydrophilic balance of the whole molecule (i.e., its *global* amphiphilicity) was the most important parameter in determining antimicrobial activity and selectivity. This is true to a certain extent; however, recent studies of polymer-based SMAMPs indicate that a properly balanced *local* amphiphilicity also plays a major role in maximizing SMAMP activity and selectivity. This is best illustrated when comparing copolymers of *facially amphiphilic* repeat units, which have the hydrophobic group and the hydrophilic group on the same

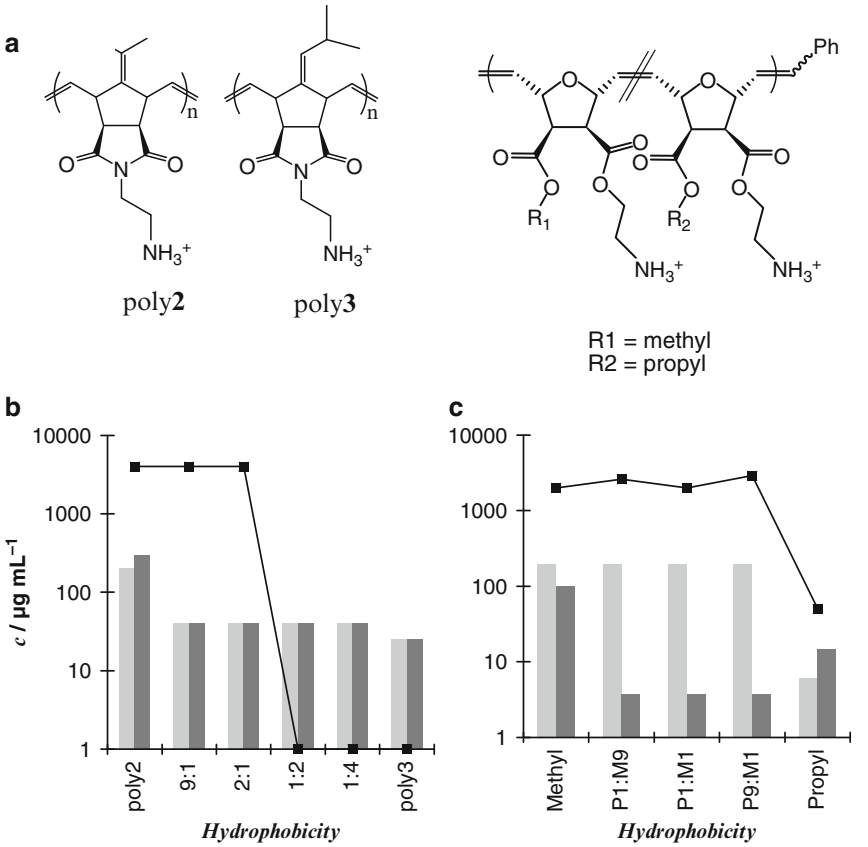


Fig. 14 (**a**) SMAMP copolymers made from facially amphiphilic monomers. Biological data for copolymers made from (**b**) poly2-poly3 and (**c**) methyl-propyl monomers plotted as concentration (MIC_{90} or HC_{50}) versus increasing hydrophobicity: *light gray columns* MIC_{90}, *E. coli*; *dark gray columns* MIC_{90}, *S. aureus*; *black squares* HC_{50}, human erythrocytes. The ratios are the mole fraction of each monomer in the various copolymers

moiety, with *segregated* copolymers, where one repeat unit carries the hydrophobic group and the other carries the hydrophilic group. The copolymers shown in Fig. 14 were obtained from facially amphiphilic monomers. In these systems, the facial amphiphilicity (i.e., local amphiphilicity) is maintained on each repeat unit, while the overall hydrophobic/hydrophilic balance (i.e., global hydrophobicity) is tuned by the ratio of the two hydrophobic groups. This design feature made it easy to optimize the amphiphilicity of the system, and superb selectivities were obtained [22, 89]. On the other hand, the segregated copolymers by Gabriel et al. [3], in which one comonomer carried the hydrophobic and the other the hydrophilic group, were much less active and especially less selective than the all-facially amphiphilic copolymers. Due to the high structural similarity between these polymers and the poly1–4 series shown in Fig. 12, it was expected that this approach would lead to

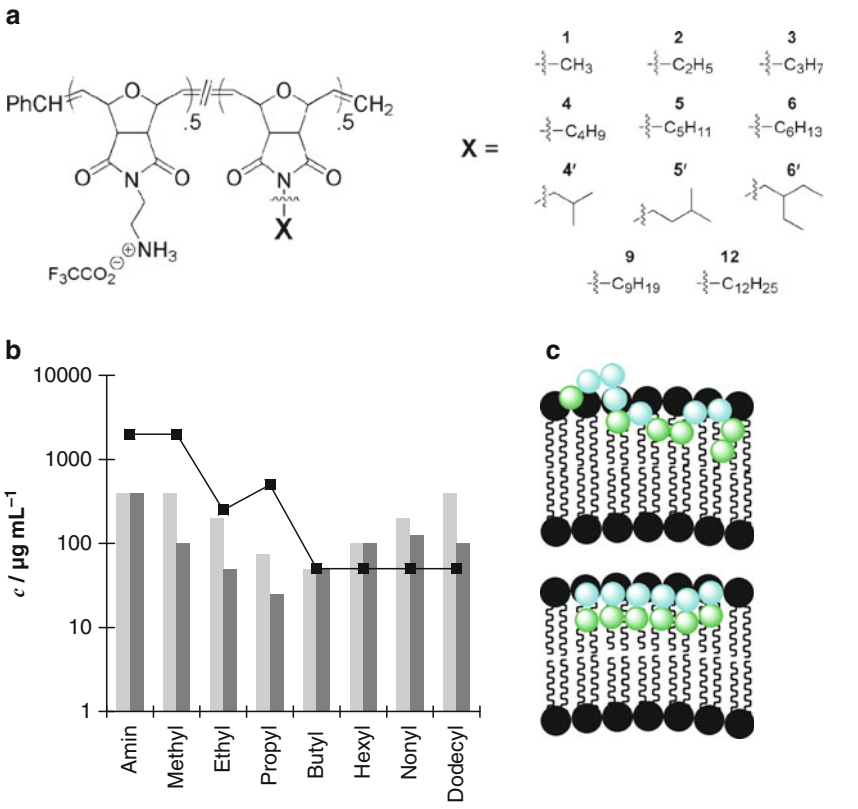


Fig. 15 (**a**) SMAMP copolymers made from segregated monomers. (**b**) Biological data plotted as concentration (MIC_{90} or HC_{50}) versus increasing hydrophobicity: *light gray columns* MIC_{90}, *E. coli*; *dark gray columns* MIC_{90}, *S. aureus*; *black squares* HC_{50}, human erythrocytes. (**c**) Illustration of SMAMP–membrane interactions: *top* segregated SMAMPs; *bottom* facially amphiphilic SMAMPs [74]

polymers with similarly tunable properties. However, although these new SMAMPs from segregated repeat units followed the general trends that had been found before (a minimum value for the MIC_{90}, and HC_{50} values that decreased with increasing hydrophobicity; Fig. 15b), the overall selectivities remained much lower, with a maximum selectivity of 20 [3]. Deviation from the 1:1 monomer feed ratio did not improve the selectivities. The problem with this nonfacially amphiphilic approach, and the reason why the selectivities remained moderate, is that the segregation of the functional groups onto two different repeat units leads to runs of hydrophobic and hydrophilic moieties in the statistical copolymer (Fig. 15c). Thus, while the global amphiphilicity of the molecule is maintained, the local amphiphilicity is disturbed in such a way that over-hydrophobic "blobs" are created, which cause membrane disruption of the erythrocytes and keep HC_{50} values, and thus also the selectivities, low.

Sen's poly(pyridinium-*co*-acrylate) series B (Fig. 11a) [7] can also be considered as segregated copolymers and were found to suffer from the same intrinsic problems as Gabriel's [3]. Those polymers also had the charge and the hydrophobic group on different repeat units and were found to be less potent and more hemolytic than their series A (Fig. 11a), which had the charge and the hydrophobicity on the same repeat unit. The same was found for two other polymer series with similar design concepts.

Another illustration of the detrimental effect of hydrophobic blobs in the molecule is the effect of end groups. Polymer end-group effects on SMAMP activities were recently investigated by DeGrado [79] and Gellman [87]. Whereas DeGrado's group studied the effect of end-groups on an oligomer (Fig. 5d), Gellman and coworkers investigated end-group effects on a polymer with n (degree of polymerization) $\sim$ 30 [87]. This molecular weight is large enough to attribute any effect observed to the end-groups, and not to an overall exchange of the hydrophobic/hydrophilic balance of the whole molecule, as was the intention of the DeGrado study [79]. Gellman increased the number of carbon atoms in the end group gradually from 2 to 18. The results are shown in Fig. 16. The results look somewhat similar to what happens when the hydrophilic/hydrophobic balance is changed across a series. With increasing number of carbon atoms, the molecules become one to two orders of magnitude more active until a minimum is reached at 10–12 carbon atoms. Then, the end-group either compromises solubility or causes aggregation; consequently the MIC value rises again. On the other hand, the haemolytic activity once again shows sigmoidal behavior. Although the extra number of carbon atoms presumably does not have much influence on the overall hydrophobicity of the molecule, the end group seems to causes a dramatic change in the local hydrophilic/hydrophobic balance of the molecule at the chain end and thus makes

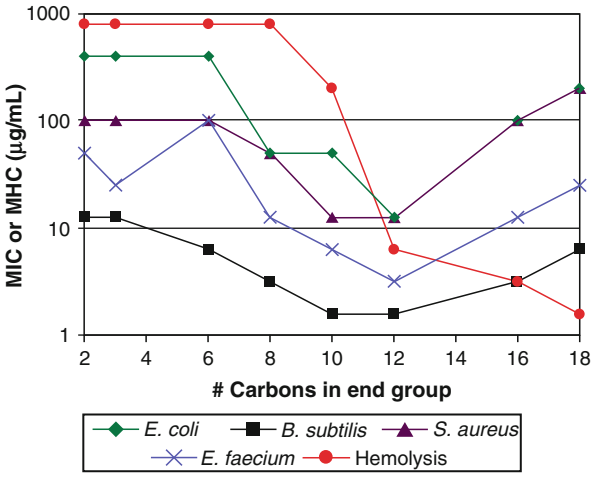


Fig. 16 Examination of how end groups (number of carbon atoms) affect biological activity [87]

it significantly more haemolytic. Thus, the overall SMAMP properties are dominated by the end-group effects, which are otherwise negligible.

3.3.4 Effect of Charge

Since the driving force for interaction between SMAMPs and the bacterial membrane appears to be the electrostatic attraction between the cationic peptidomimetic and the negatively charged membrane, it is intuitive that the charge, or the charge density, of the SMAMP will have an impact on its activity. To investigate this effect, Al-Badri et al. studied the effect of charge variation on two series of poly (norbornenes) (polyA and polyB in Fig. 17) carrying one, two, or three primary amine groups per repeat unit [91]. For the polyA series, which had one hydrophobic isobuteryl group per repeat unit, the single-charged derivative is mildly active but also hemolytic. By doubling the charge, a SMAMP with a drastically increased activity active against *E. coli* and much less hemolytic activity was obtained. Adding a third charge, however, did not improve the biological properties (Fig. 17) [91]. On the other hand, for the polyB series (Fig. 17), which has a hydrophilic backbone, adding more charge did not improve the hemolytic activity, but the polymer became more active against *S. aureus* (Fig. 17).

Charge and hydrophobicity are two closely related parameters, and adding more charge across a polymer will usually affect the relative hydrophobicity of the polymers. Thus, as the biological activity of a polymer is dependent on its

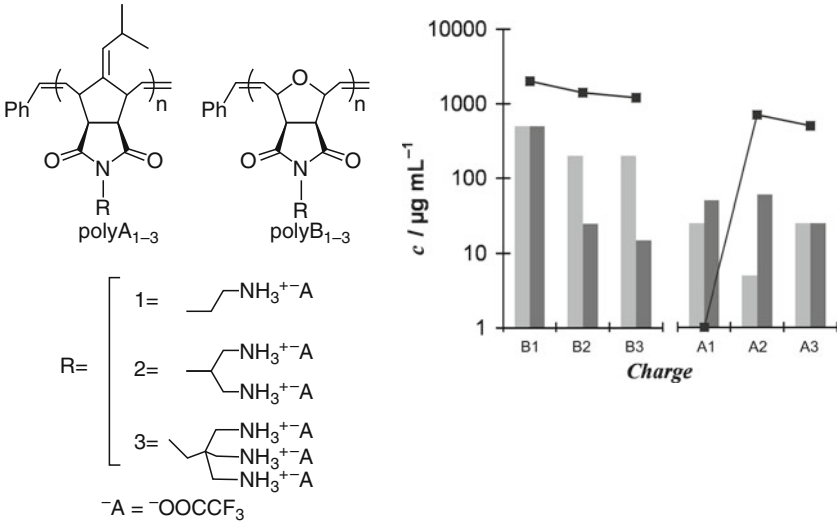


Fig. 17 *Left*: Structures of two series of imid-based SMAMP polymers. *Right*: Biological data for the polymer series, shown with increasing charge per repeat unit plotted as concentration (MIC_{90} or HC_{50}) versus increasing nominal charge per repeat unit: *light gray columns* MIC_{90}, *E. coli*; *dark gray columns* MIC_{90}, *S. aureus*; *black squares*HC_{50}, human erythrocytes

hydrophobicity, one cannot isolate the effect of charge where this is the case. In an already hydrophilic polymer like polyB1 (Fig. 17), adding more charge does not alter the hydrophilicity dramatically, thus the overall properties of the polymer only change minimally. However, for polyA1 (Fig. 17), which has a hydrophobic group, adding an extra charge significantly changed its overall hydrophilicity, which in turn affects the biological properties. Thus, to isolate the effect of charge in a system, it is important to find a system in which changing the polymer charge does not affect the overall hydrophilicity of the molecule. To overcome this problem, Lienkamp et al. studied four series of copolymers from a doubly charged repeat unit (Fig. 12b, R1 = R3 = ethylammonium) and a singly charged repeat unit (Fig. 12b, R1 = ethylammonium, R3 = methyl to butyl) [90]. This polymer design allowed the identification of a polymer series with overall similar hydrophobicity, while the charge could be gradually changed across the series. The hydrophobicities of methyl homopolymer (R1 = ethylammonium, R3 = methyl) and the diamine homopolymer (R1 = R3 = ethylammonium) were found to be similar, whereas the ethyl to butyl analogs were slightly to significantly more hydrophobic. Thus, the methyl copolymers were used to study the effect of increasing the polymer charge density at approximately constant overall hydrophobicity. Figure 18a shows that this polymer series behaves similar to the polyB series of Fig. 17. The hemolytic activity is only slightly affected by charge variation; however, the activity against *S. aureus* dramatically improves with increasing charge. In both polymer series, there is a certain charge density at which there is a sudden jump in the MIC.

These findings, together with AMP literature data, led to the postulation that there is a specific charge threshold that needs to be exceeded to obtain decent activities against *S. aureus* [90]. Rather than a certain number of charges per repeat unit, this charge threshold is to be understood as a minimum charge density, or charge per unit volume, and the exact threshold number of charges per repeat unit will be slightly different for each SMAMP series, depending on the molecular volume of the repeat units. On the molecular level, this postulated charge threshold translates into a minimum charge density that is necessary to trigger successful attachment of the SMAMP to the bacterial membrane. Once enough charge is present to enable this attachment, the overall hydrophobicity of the molecule will determine to what extent the SMAMP is active [37].

3.3.5 Charge Variation by pH

Recently, Palermo and Kuroda studied the role of the nature of the amine functionality on the haemolytic and antimicrobial activities of polymeric SMAMPs. They synthesized poly(methyl methacrylates) with pH-dependent primary amine groups, tertiary amine groups, and permanently charged trimethyl ammonium groups (Fig. 19) [93]. Using potentiometric titration data, it was found that the polymers were completely protonated at pH 6, but a significant fraction of the amine groups were deprotonated at pH 7. They studied the antimicrobial activity of these polymers as a function of pH (Fig. 19). In general, the polymers with the primary amine groups were more active than those with the quaternary ammonium groups.

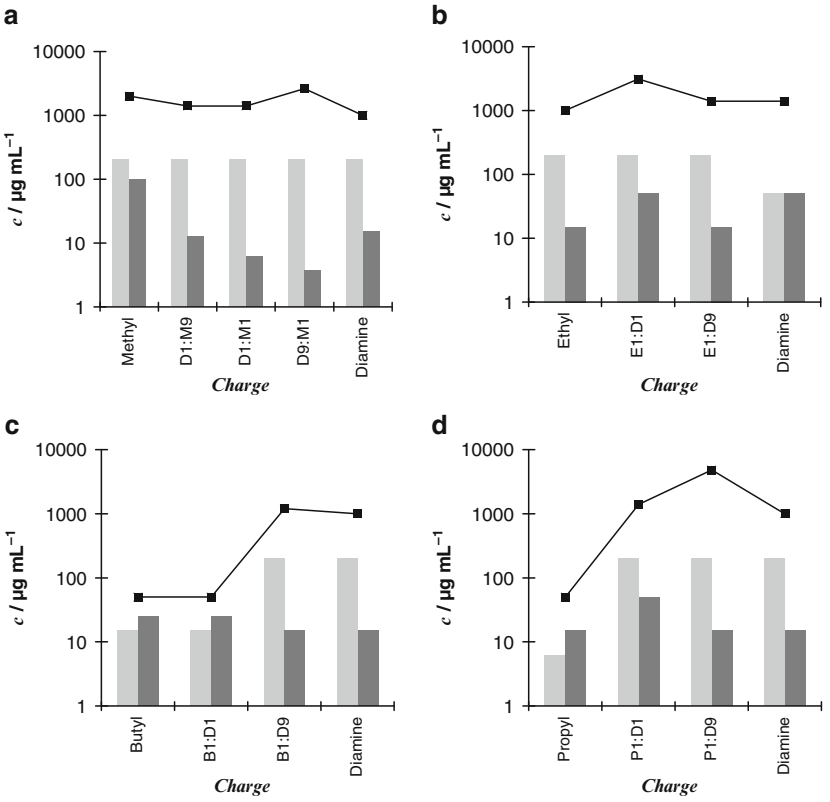


Fig. 18 Biological data for ester-based SMAMP copolymers with increasing charge density (see Fig. 12b), plotted as concentration (MIC_{90} or HC_{50}) versus increasing nominal charge: *light gray columns* MIC_{90}, *E. coli*; *dark gray columns* MIC_{90}, *S. aureus*; *black squares* HC_{50}, human erythrocytes. (**a**) Methyl copolymers, (**b**) ethyl copolymers, (**c**) propyl copolymers, (**d**) butyl copolymers. These polymers are copolymerized with the diamine monomer that contains no hydrophobic groups

The activity of the SMAMPs with a primary and a tertiary amine group was markedly enhanced at more basic pHs (corresponding to a degree of protonation of 0.8), whereas that activity was lost at pH 8, possibly due to polymer aggregation or loss of too much charge to attach to the polymer membrane [93]. The latter explanation would be in line with the above-mentioned charge threshold argument. In general, this data has to be treated with care because different pH values during the MIC experiment might influence the cell growth and viability.

3.3.6 Doubly Selective SMAMPs

How the composition and structure of the bacterial cell membrane (Fig. 20a, b) affects antimicrobial properties was studied recently by Lienkamp et al. [94].

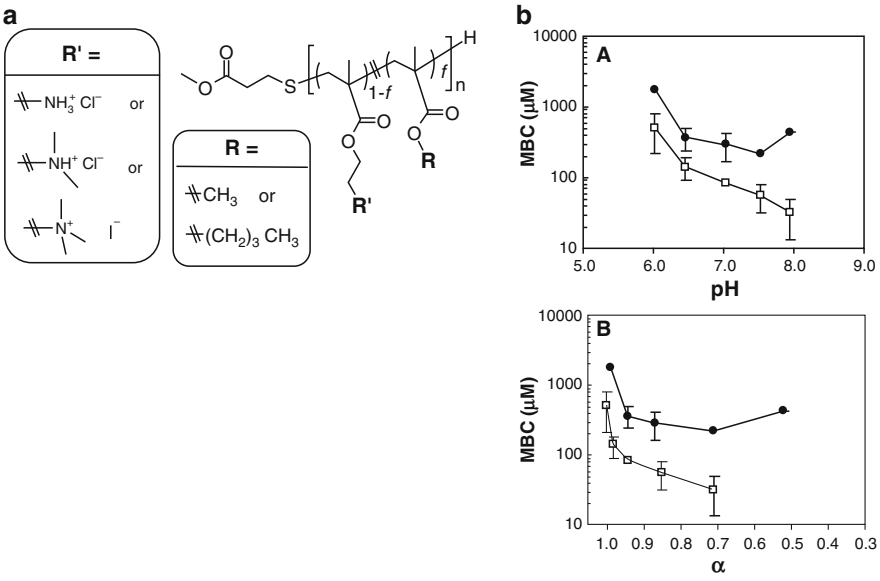


Fig. 19 (**a**) Structure of poly(methylmethacrylate) SMAMPs with different amine groups [93]. (**b**) Antimicrobial activity (here MBC) against *E. coli* as (A) a function of pH and (B) a function of α (*open symbols* primary amine, *closed symbols* tertiary amine). α is the extent of ionization for the polymers at the given pH.

They found that some of their poly(norbornene) SMAMPs had double selectivity – not only for bacteria over mammalian cells, but also for Gram-positive over Gram-negative bacteria [22]. Using a doubly selective model compound (diamine polymer activity is shown in Fig. 18), they were able to correlate SMAMP activity or inactivity with specific cell features [94]. It was first shown using dye-leakage experiments that the difference in lipid composition of the cell membranes of Gram-positive versus Gram-negative bacteria was not responsible for the double selectivity (Fig. 20c). It was also shown that, although lipopolysaccharide can strongly bind the SMAMPs (Fig. 20d), this does not decrease the SMAMP activity in cell experiments (Fig. 20e). However, when the outer membrane of *E. coli* was selectively damaged, although the cell stayed still viable, the doubly selective SMAMP, which had previously been inactive towards Gram-negative *E. coli*, suddenly became active against that bacteria (Fig. 20f), demonstrating that the reason for the previous inactivity was the additional phospholipid membrane of Gram-negative bacteria.

4 Antimicrobial Dendrimers

Dendrimers are a class of macromolecules with a regular three-dimensional branching structure that stems from a central core. This results in a large number of functionalities at their surface, which make dendrimers attractive target structures

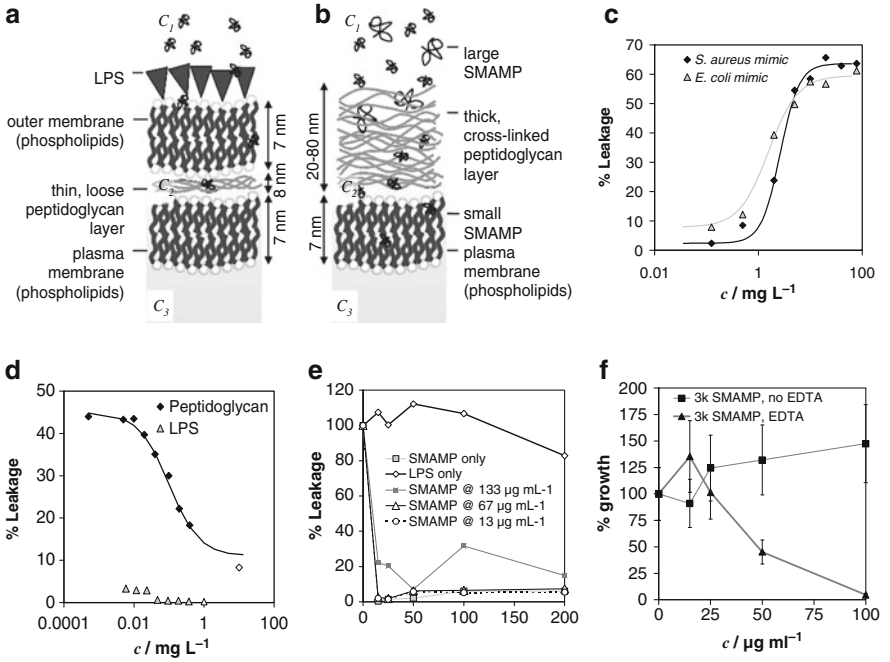


Fig. 20 SMAMPs with double selectivity and molecular-weight-dependent antimicrobial activity. (**a**) Illustration of the cell membrane of Gram-negative bacteria, with lipopolysaccharide (*LPS*) and two phospholipids membranes. (**b**) Illustration of the cell structure of Gram-positive bacteria, with a thick peptidoglycan layer and only one phospholipids membrane. (**c**) Percentage dye leakage versus SMAMP concentration of *E. coli* and *S. aureus* mimicking vesicles;.(**d**) SMAMP-LPS and SMAMP-peptidoglycan binding studies. (**e**) MIC experiment on *S. aureus* in the presence of LPS. (**f**) MIC experiment on regular *E. coli* cells (no EDTA) and on *E. coli* cells with EDTA-damaged outer membrane

as SMAMPs. The antimicrobial properties of dendrimers have been explored by different research groups. Cooper and colleagues [95, 96] studied the antimicrobial activity of a series of quaternary-ammonium-functionalized poly(propylene imine) dendrimers using bioluminescence methods. Their results showed that the activity had a parabolic dependence on molecular weight, with biocidal activities in the order of G5 > G4 > G1 > G2 > G3 (G5 is a fifth generation dendrimer). This behavior was explained as a result of the balance between the number of quaternary ammonium groups and permeability through the cell membrane. The activity was also found to depend on the hydrophobic chain length of the quaternary ammonium groups. Dendritic molecules with C_{10} chains were most effective, followed by C_8 and C_{12}, whereas C_{14} and C_{16} were the least active. Dulger synthesized dendrimers based on a poly(propyleneoxide) amine core [97]. The branching units were constructed from both methacrylates and ethylenediamine. This yielded dendrimers with $–NH_2$ or –COOH functionalities. MIC studies showed that these dendrimers have broad-spectrum biocidal activities [97]. Cai and coworkers investigated the

antimicrobial activity and cytotoxicity of PEGylated poly(amidoamine) (PAMAM) dendrimers [98]. Their results showed that for the unmodified third and fifth PAMAM generation, the MICs against both *Pseudomonas aeruginosa* and *S. aureus* were in the range of 6.6–12.5 μg mL^{-1}. Low degrees of PEGylation of PAMAM (∼6%) greatly reduced the cytotoxicity towards human corneal epithelial cells and resulted in a reduction of the antimicrobial activity against *P. aeruginosa* (MIC ∼ 25 μg mL^{-1}), while the compound became inactive against *S. aureus*.

5 Conclusion

Polymer-based peptidomimietics are a relatively young and dynamic field of research. Various groups have shown that, by carefully tuning the overall hydrophobicity and charge density of synthetic polymers, peptidomimetics with tailor-made properties could be obtained that varied from inactive/non-hemolytic via active/non-hemolytic to active/toxic. Thus, it was shown that it is possible to teach synthetic polymers to behave like peptides, despite their lack of sequence-specificity and secondary structure.

As the biological properties of polymeric SMAMPs result from the interplay of many parameters, it is not yet possible to predict the exact properties of such molecules from their mere chemical structure. However, as demonstrated above, the effect of certain design features such as charge and hydrophobicity on the properties across a polymer series is quite well understood.

Compared to the mechanistic specifics that are known about the interactions of AMPs or small antibacterial molecules with membranes and cells, relatively little is known concerning the interaction of polymeric SMAMPs with membranes. The membrane-disruptive properties of the majority of these molecules have been demonstrated, yet many mechanistic details are still elusive, and further research in this area is highly encouraged due to the importance of this class of substances. In addition, the whole field of macromolecule–membrane interaction would benefit from a more fundamental understanding of such processes.

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