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Synthetic Mimics of Antimicrobial Peptides—A Versatile Ring-Opening Metathesis Polymerization Based Platform for the Synthesis of Selective Antibacterial and Cell-Penetrating Polymers

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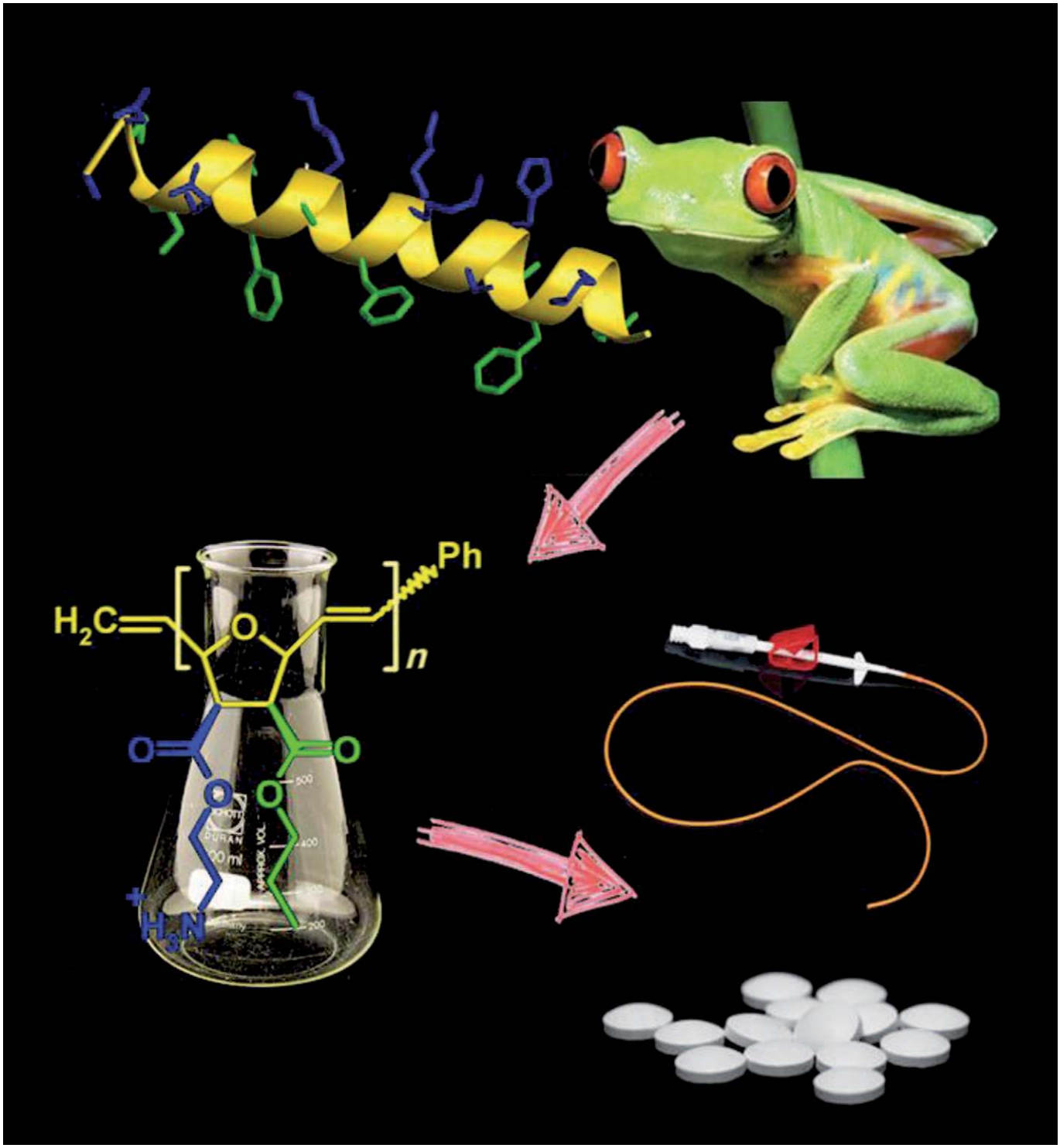

Abstract: Natural macromolecules exhibit an extensive arsenal of properties, many of which have proven difficult to recapitulate in simpler synthetic systems. Over the last couple of years, foldamers have emerged as one important step toward increased functionality in synthetic systems. While the great majority of work in this area has focused on folded structures, hence the name, more recent progress has centered on polymers that mimic protein function. These efforts have resulted in the design of relatively simple macromolecules; one example are the synthetic mimics of antimicrobial peptides (SMAMPs) that capture the central physicochemical features of their natural archetypes irrespective of the specific folded form. Here we present our recent efforts to create polymers which display biological activity similar to natural proteins, including antimicrobial and cell-penetrating peptides.

Keywords: antimicrobial polymers · cell-penetrating peptides · ring-opening metathesis polymerization · peptidomimetics · vesicles

Introduction

Why SMAMPs? Due to the epidemic increase of multiresistant bacteria both in medical facilities[1] and other public institutions,[2] there is an ever-increasing demand for infection-preventing substances and materials. From 1975 to 1999, the percentage of methicillin-resistant *S. aureus* bacteria (MRSA) jumped from 3 to 52%.[3,4] Two million people in the US are infected annually with MRSA during hospitalization, and the follow-up costs of these MRSA infections add five billion dollars annually to the US healthcare budget.[5,6] While these figures indicate that there is an immediate need for new antibacterial substances and materials, they also highlight the fact that traditional antibiotics alone, with their propensity to allow rather rapid resistance development, are an inadequate answer to meet this threat.

In the last few years, significant progress has been made in the development of biocidal molecules and polymers.[7] Those materials are nonspecifically active against many pathogens including bacteria, viruses, and fungi, yet are also toxic for mammalian cells. Therefore, while highly efficient in their potential applications, those polymers cannot be used in settings in which there is intimate and long-term contact with eukaryotic cells, for example, in medical devices, implants, or wound dressings. Synthetic mimics of antimicrobial peptides (SMAMPs), on the other hand, are molecules that are specifically designed to only kill pathogens. SMAMPs were developed to emulate the properties of antimicrobial peptides (AMPs), which are natural molecules produced by many organisms as part of their innate immune system. These peptides have broad-spectrum antimicrobial activity, yet they are benign to mammalian cells.[8] Unlike traditional antibiotic drugs, they are not directed against a precise cellular receptor, but mostly act on the bacterial cell membrane.[8,9] Thus, while resistance development against traditional antibiotics may involve only a few mutations of the receptor site upon exposure to sub-lethal drug doses, resistance to AMPs would require more complex changes including alterations of cell-membrane chemistry. Consequently, resistance build-up against AMPs is slower than against conventional antibiotics.[10] The combined properties of selectivity for pathogens over host cells and low-resistance potential have stimulated intense research in the field of AMPs and SMAMPs during the past few years. Due to their relative ease of synthesis, SMAMPs are promising candidates for both materials and chemotherapeutic applications; at the same time they appear to have the potential to play an important role in the containment of and contagion with MRSA and other multiple-resistant organisms. For example, a "first-in-man Phase I clinical safety study" was just recently reported, in which a SMAMP was used as the therapeutic agent for treatment of pan-*staphylococcal* infections.[11]

AMPs[8,10,12,13] and antimicrobial polymers[7] have been extensively reviewed. In addition, there are a number of new examples of small molecules[14–18] and polymers that imitate AMPs,[19–27] which have also been covered in recent reviews.[6,14] In this concept paper, rather than providing a comprehensive review of the field, we outline how SMAMP design has evolved from structurally rigid, peptide-like molecules towards increasingly less-confined molecular architectures, some of which perform even better than their natural archetypes. We then illustrate, based on our own recent work, how an intelligent, versatile synthetic platform can be combined with lessons from nature to provide easily accessible synthetic molecules that capture the properties of such complex molecules as the AMPs. This case study also shows how polymers can be transformed from being merely the carriers of bioactive cargo, as reviewed, for example, in reference [28], to being encoded such that they are the biologically active molecules. It is this evolution of polymer sophistication that inspired us to pursue this challenge. In addition to the fundamental knowledge gained by trying to teach polymers to act like proteins, it appears that new therapies can also be discovered during the course of this research.[11] It has also led to a series of well-defined model compounds that enabled new insight into the mechanism of action. Although SMAMPs represent an important step toward increased biological activity of synthetic polymers, much work remains to be done.

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In the course of our studies on SMAMPs obtained by ring-opening metathesis polymerization (ROMP), we established a correlation between several structural parameters and biological activity, and gained an understanding of how SMAMPs interact with cell membranes. This provided a background for the design of a new SMAMP with cell-penetrating peptide-like behavior. The examples of protein-like activity in synthetic polymers discussed here merely illustrate the potential of this biomimetic approach; we anticipate that future research toward the evolution of polymer activity will greatly expand the arsenal of protein-like activity and make significant contributions to both fundamental science as well as practical technologies.

From AMPs via foldamers to ROMP-based SMAMPs—an evolution: AMPs are host–defense peptides found in many organisms from invertebrates to humans.[8] Their key feature is that they are facially amphiphilic molecules, as illustrated in Figure 1. They contain a face with hydrophilic, positively charged groups (blue) and a hydrophobic face (green).[29] This segregation of the hydrophilic and hydrophobic side chains onto two opposite sides of the molecule results from the amino acid sequence of the peptide, which also dictates the conformation of the backbone (gold) and thus the secondary structure of the molecule. While most traditional antibiotics have specific cellular targets, AMPs use non-receptor interactions, including direct action against the bacteria's membranes, although intracellular targets have been identified in some cases.[8] Their facial amphiphilicity enables them to insert into cell membranes and locally change the membrane organization, leading to pore formation, membrane rupture, or other interactions that impact the membrane elasticity so that cell viability is compromised. Several mechanisms have been proposed for trans-membrane pore formation by AMPs (carpet, barrel-stave, and toroidal pore model).[8–10,29–31] The careful balance between cationic hydrophilic and hydrophobic groups allows AMPs to differentiate between the neutral phosphatylcholine and cholesterol rich surface of mammalian cells and the negatively charged cell surface of bacteria.

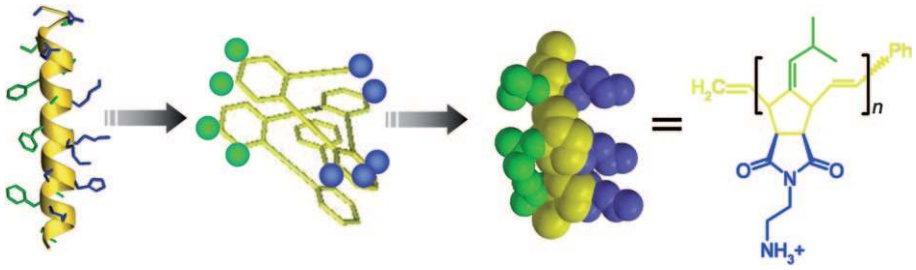

Figure 1. Molecular evolution of synthetic antibacterials: from natural AMPs (left: magainin) via synthetic foldamers to ROMP-based SMAMPs (right: poly**1c** from Figure 4).

As AMP extraction from natural organisms or their production in multistep syntheses is tedious and expensive, SMAMP research strived for a platform that yielded a library of molecules which retained the antibacterial activities of AMPs, but would be obtained in fewer synthetic steps and in larger quantities. As such, many substance classes have been used, including α- and β-amino acids,[32–39] peptoids,[40–42] aromatic oligomers,[27,43,44] steroids,[45–47] and synthetic polymers.[20,22,23,48–50] Initially, it was believed that the helical rigidity of the AMP backbone (Figure 1) was a prerequisite for biological activity. As a result, early work focused on emulating the amphiphilic α-helical arrangement of side chains observed in the natural structures, leading to a large number of potent and selective antimicrobial peptides based on natural amino acids.[37–40] The availability of β-peptides provided another avenue to test and further elucidate the features required for the construction of SMAMPs. β^3-Peptides adopt "14-helices" (14 residues within the repeating hydrogen-bonded rings), which have an approximate three-residue geometric repeat. Thus, if polar and apolar side chains were arranged with precise three-residue periodicity in the sequence of an appropriately designed β-peptide, they would segregate to opposite sides of the helix. Indeed, repeating tripeptides composed of hAla, hLeu and/or hVal (the β-amino acids were β^3-substituted) were found to have antimicrobial activity.[36,38] Gellman and co-workers described a potent and highly selective antimicrobial peptide based on cyclic β-amino acids.[37] These studies, which were subsequently extended to a variety of different helical types formed by β-peptides, showed that charge, facial amphiphilicity, and an appropriate hydrophilic/hydrophobic balance were crucial to obtaining selective, nontoxic compounds.

All of these synthetic derivatives focused on helical secondary structures; however, the critical role of the helix was questioned soon as other folded forms of natural peptides were discovered. Early work on diastereomeric peptides containing D-amino acid substitutions which had little α-helix forming abilities, but potent antibacterial activity, supported this conclusion.[51–53] Further support for the overall amphiphilicity being more important than a specific folded structure came from recent work on scrambled sequence α/β-peptides which are selective antimicrobial agents apparently without the ability to adopt globally amphiphilic helices.[15] Thus, it appears that there is no unique requirement for a rigid conformation so long as the composition of that sequence is conducive for binding to the target membrane.

These results led to the question of whether this general approach could be extended to design much simpler oligomers and polymers that capture the essential biological and physicochemical properties of AMPs. In particular, one can consider two design strategies. On the one hand, the amphi-

philic secondary and tertiary structures of natural AMPs could be mimicked by placing hydrophilic and hydrophobic groups on an appropriate framework. In this case, a pre-organized backbone would help to minimize the unfavorable conformational entropy of binding, leading to good potency. On the other hand, if a rigid conformation is not an absolute requirement for activity, far more flexible (co)polymers might be envisaged, as they would be able to adopt the necessary conformations. In either case, it would be important to optimize the structures for maximal activity by careful consideration of the molecular weight, charge, conformational landscape, and hydrophilic/lipophilic balance.

Probably the most (r)evolutionary step in SMAMP design simplification was to completely dispense with the helical motif in favor of a less complicated but rigid aromatic molecular scaffold. DeGrado and Tew developed aryl–amide-based oligomeric SMAMPs with facially amphiphilic repeat units.[27,43,54] These molecules were still potently antibacterial, and while they did not have the confinement of the helical secondary structure, the rigidity of the aryl–amide backbone and hydrogen bonding between their functional groups ensured that one side of the molecule contained the hydrophobic groups, while the opposite side presented the charged hydrophilic groups. This work clearly demonstrated that a helical backbone was not necessary. By synthesizing molecules with a phenylene–ethynylene backbone, it was possible to test whether the configurational constraints of the backbone could be further relaxed. These molecules still possessed the rigidity of an aromatic backbone, but had no intramolecular hydrogen bonds.[18,23,24,50,55,56] While the aromatic backbone more easily allowed an overall linear conformation, the repeat units were free to rotate around their single bonds. This allowed their functional groups to orient themselves to a facially amphiphilic conformation upon contact with the cell membrane or a similar hydrophilic–hydrophobic interface. The phenylene–ethynylene polymers had the desired antibacterial activities; by small-angle X-ray scattering, it was shown that even phenylene–ethynylene trimers were able to form pores when exposed to model membranes.[9]

The final move in SMAMP design was to get rid of the rigid aromatic backbone altogether and to equip aliphatic synthetic polymers with hydrophobic and hydrophilic repeat units, hoping they would self-orient their functional groups to be facially amphiphilic and membrane active upon contact with cells. Several polymers were synthesized based on this idea,[19,20,48,57,58] and it was demonstrated that, if the functional groups were adequately balanced and positioned, even this last constraint could be removed and the polymers still had superb antibacterial properties.

Antibacterial and hemolytic properties—a comment on definitions and units: The antibacterial potency of AMPs or SMAMPs and their selectivity for bacteria over eukaryotic cells are usually determined by measuring the minimum inhibitory concentration (MIC)[59–61] and the hemolytic activity (HC). To obtain the MIC_{90}, which is the concentration that inhibits 90% of bacterial growth, the percentage of growth upon SMAMP exposure is determined and plotted versus SMAMP concentration (dark squares in Figure 2). These values are usually determined by measuring the optical turbidity, or cell density, of the solution at 600 nm in a UV/Vis spectrophotometer or plate-reader. Other popular MIC values are the MIC_{100} and MIC_{50}, and are defined and determined analogously. While MICs are specific to the given method, when determined properly, they are highly reproducible values that allow reasonable comparisons for the relative potency of SMAMPs, with the only significant disadvantage that they do not differentiate between growth inhibition and killing events. To that end, bacterial growth kinetics (so-called time-kill studies) can be determined, in which the growth reduction of bacteria exposed to different SMAMP concentrations is monitored as a function of time.[62]

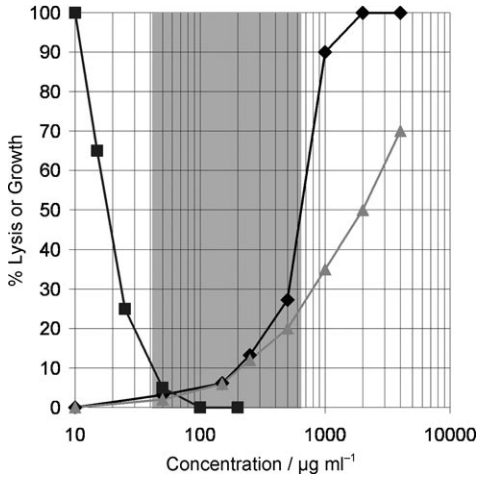

Figure 2. MIC and HC curves. Squares = MIC curve (MIC_{100}, MIC_{90} and MIC_{50} = 100, 50 and 25 µg mL^{-1}, respectively); diamonds = HC curve (HC_{100}, HC_{50} and HC_0 = 2000, 650 and 10 µg mL^{-1}, respectively; triangles = HC curve (HC_{100}, HC_{50} and HC_0 = > 4000, 2000 and 10 µg mL^{-1}, respectively). The two HC curves illustrate that two polymers with identical HC_0 may have drastically different HC_{50}s and HC_{100}s. The shaded region represents the therapeutic width of the compound, that is, the concentration range in which the compound is active yet not too toxic for the host organism.

On the other hand, toxicity is more difficult to define, partly owing 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. Similarly to the MIC curve, a plot of percent lysis versus concentration yields the HC_{50} value, that is, 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 (Figure 2), or, more rigorously, by a fit of the experimental data with the Hill equation.[63] 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 labs determine the HC_{50} value in analogy to the well-known LD_{50} used for in-vivo drug testing. This value can be determined with or without serum, but serum can have a strong influence on the reported value. 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;[64–66] 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,[20] which corresponds to an HC_0 value. These contradictory definitions tend to occur as important fields funnel toward a global minimum, but in the meantime complicate the comparison of hemolysis data between labs. To avoid this confusion, using terms like HC_{100}, HC_{10}, or HC_0, instead of MHC would be helpful. As mentioned below, this also influences the "selectivity" values that are reported.

Although the HC_{50}, HC_{100} and HC_0 values each have a precise definition and are useful parameters to assess hemolytic activity, they do not convey the same amount of information. In the example given in Figure 2, both HC curves have identical HC_0 values, although the compound represented by the curve with diamond symbols is evidently 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. For the sake of interlab comparability of data, we suggest that future researchers should report the complete hemolysis curve of their compounds together with their preferred value. In addition, it is important to remember that significant variation will be present, for example the source of red blood cells has an impact.

The preferential activity of a compound against pathogens over 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 nNP-1[67]) or even >100 (human AMP β-defensin 3[68]) when defined in terms of the HC_{50} and MIC_{90} values. Another parameter to express the same idea is the therapeutic index (= 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}.[69] In our research, we have consistently used the terms MIC_{90}, HC_{50}, and selectivity $= HC_{50}/MIC_{90}$, and will continue to do so in forthcoming publications as this parameter sets a rather rigorous definition for selectivity. As mentioned previously, "toxicity" can be influence by various parameters. Of equal importance is the fact that these hemolysis values provide only general guidelines for fundamental studies. To really understand toxicity, more in-depth studies including in vitro activity against various cell types as well as in vivo activity is essential if one wishes to move these molecules into the clinic for applications.[11]

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 $\mu mol\,mL^{-1}$. 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, although one should note that the purity of the peptide sequences is not always carefully determined or reported, which would influence the molarity reported, leading easily to a 5% error. The polymer SMAMP field seems to prefer the unit $\mu g\,mL^{-1}$ due to the polydisperse nature of synthetic macromolecules. In the case of some polymers, molecular weights are accessible by MALDI-TOF,[58,70] but as soon as the SMAMP structure gets 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 propagate, the interpretation of the 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.

ROMP-based synthetic mimics of antibacterial peptides (ROMP = ring-opening metathesis polymerization): Over the last five years, a large number of ROMP-based SMAMPs were synthesized (Figure 3), and their biological properties and mechanisms of interaction with membranes were studied. The structures of these compounds are summarized in Figure 4. This figure illustrates how, by gradual structural variation, a library of polymers with tunable antibacterial and hemolytic properties was obtained, and how the investigation of structure–property relationships of those polymers helped to elucidate key factors of SMAMP design.

ROMP was chosen as a synthetic platform because it is a living polymerization technique, it yields molecules with low polydispersity over a wide range of molecular weights, and it is highly functional group tolerant.[71–73] The variety of molecules, especially bioactive ones, that were accessible through ROMP has been reviewed elsewhere.[28]

The field of ROMP-based SMAMPs was pioneered by Tew and Coughlin.[48] They reported a series of poly(norbornene) derivatives with facially amphiphilic repeat units (poly**1a**–poly**1d**, series **1** in Figure 4). In this polymer series, the ratio of hydrophobic and hydrophilic moieties per

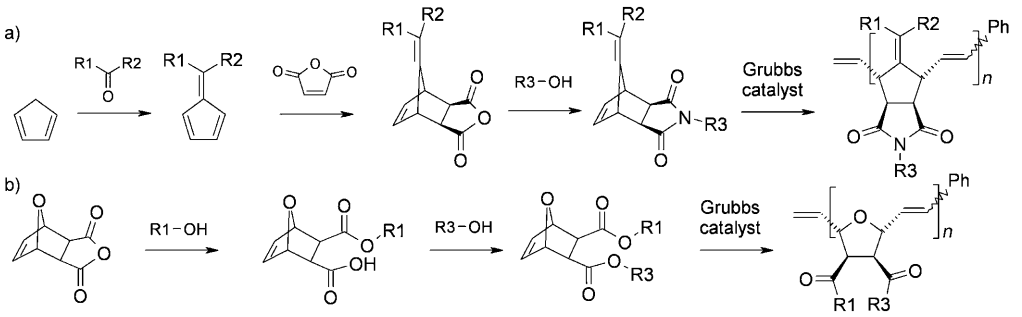

Figure 3. Monomer and polymer synthesis. R1 and R2 denote hydrophobic groups, R3 is the hydrophilic group. a) backbone modification strategy, b) "construction kit" approach.

repeat unit was gradually varied, and the effect of this variation on the antibacterial and hemolytic activities of these polymers was studied.[48] This first SMAMP paper used a backbone-modification strategy to obtain the desired gradient in hydrophobicity across the polymer series, in which the different hydrophobic groups are attached in the first step of monomer synthesis (Figure 3a).

To date, while these polymers are not the most potent any longer, some of them are still the most non-hemolytic SMAMPs in the literature. In addition, they are the largest molecular-weight SMAMP derivatives with good selectivity (>100). Also, this parent series with its norbornene–imide structure inspired many further modifications that allowed property fine tuning or more detailed investigations of certain design parameters, as illustrated in Figure 4.

Another central paper on ROMP-based SMAMPs reports a series of poly(oxonorbornene) ester derivatives from the most versatile synthetic approach reported to date (Figure 3b).[58] Unlike the imide-based series (Figure 3a) where each monomer requires different precursors, the ester-based monomers (**2** in Figure 4) can be obtained from the same precursor. The critical modification, leading to installation of the different hydrophilic or hydrophobic groups, is introduced independently and in any order in the last synthetic steps (Figure 3b). Thus, a large variety of monomers can be obtained from the same precursors, which is why this ester-based platform has been termed a "molecular construction kit".[58]

Within this concept paper, we focus on two areas. In the first part, we will discuss how different kinds of structural modifications (hydrophobicity, molecular weight, charge, or copolymerization approaches) impact the biological properties of these polymers. While this part is mostly a phenomenological description of the trends observed in the hemolytic and antibacterial activities of these polymers, the second section will discuss the physical studies that have been performed to probe the bacteria–membrane interaction, and summarized the mechanistic insight thus obtained.

Structural Modifications and Their Effects on SMAMP Activity

Increasing hydrophobicity in a series of facially amphiphilic homopolymers: Three series of polymers with facially amphiphilic repeat units and gradually increasing hydrophobicity were synthesized.[48,58,70] The already mentioned poly(norbornene)–imide series is shown as **1** in Figure 4.[48] In this series of molecules, the hydrophilic and cationic ammonium group was kept constant, while the hydrophobic group attached to the backbone was modified. The result of this structural modification on the biological properties is summarized in Figure 5a.[48] Eren et al. synthesized a series of poly(oxanorbornene) derivatives similar to poly**1a**, the most hydrophilic polymer of series **1**. They obtained different hydrophobicities by alkylation of the imide with side chains of different length containing quaternary pyridinium groups.[70] This yielded series **3**, with R = ethyl to dodecyl, and R = phenylethyl. The biological data for these molecules are shown in Figure 5b.[70] Using the ester-based platform (series **2** in Figure 4), a systematic change of the hydrophobic group from R = methyl to hexyl led to a third series of hydrophobically modified polymers. The MIC_{90} and HC_{50} values for this series are summarized in Figure 5c. When comparing Figure 5a to 5c, overall the same trends are observed in the antibacterial and hemolytic activities of these polymers. The HC_{50} values are highest for the most hydrophilic polymers and then decrease significantly as the polymers become more hydrophobic, which means that adding hydrophobicity makes the SMAMPs increasingly more hemolytic. The MIC_{90} values start off high for more hydrophilic polymers, meaning that those are inactive, and then go through a minimum for all three series, with poly**1c** (Figure 5a), the octyl imide (Figure 5b), and the propyl ester (Figure 5c) being the most active structures in each series, respectively. At that point, however, the solubility of the SMAMP decreases so much with increasing hydrophobicity that it severely aggregates and consequently becomes unavailable to interact with the bacteria membrane; therefore the MIC_{90} value goes up again. Thus, the diametrically opposed trends of increasing activity and decreasing solubility lead to the observed minimum in the MIC_{90} data for all three series of polymers.

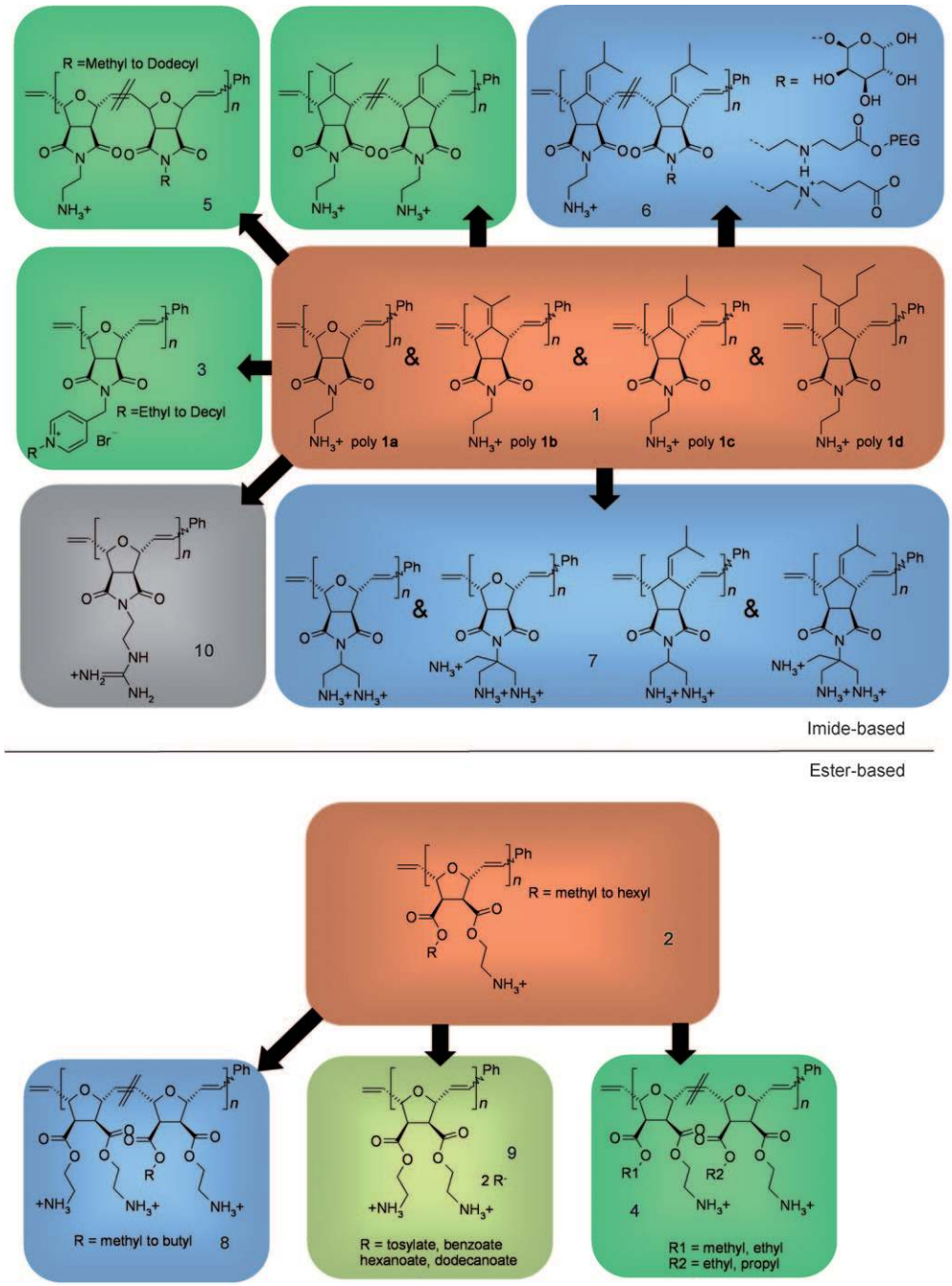

Figure 4. Library of ROMP-based SMAMP polymers. Top: norbornene–imide platform, Bottom: ester platform. The parent series are marked in red and underwent hydrophobic modification (green), hydrophilic or charge related variations (blue), or counterion exchange (light green). One SMAMP was modified with guanidinium groups (grey).

With increasing hydrophobicity, the polymers become toxic to both bacteria and mammalian cells.

Copolymers—facially amphiphilic versus segregated monomers: Since poly**1c** in series **1** had the lowest MIC_{90}, and poly**1b** the highest HC_{50}, Ilker et al. attempted to increase the therapeutic window of these polymers by copolymerizing the corresponding two monomers at different ratios, and tested their activities.[48] The biological data thus obtained is summarized in Figure 6a. As can be seen from this data, for monomer feed ratios from 2:1 to 9:1, the resulting copolymers stayed non-hemolytic, but became active even when the molar ratio of the "antimicrobial" component was only 10%. This led to selectivities as high as 100 against both *E. coli* and *S. aureus* bacteria. Similar results were obtained when monomers of the ester series (**2**) were copolymerized.[58] While the ethyl homopolymers of that series were the ones with the highest selectivity, the methyl polymers

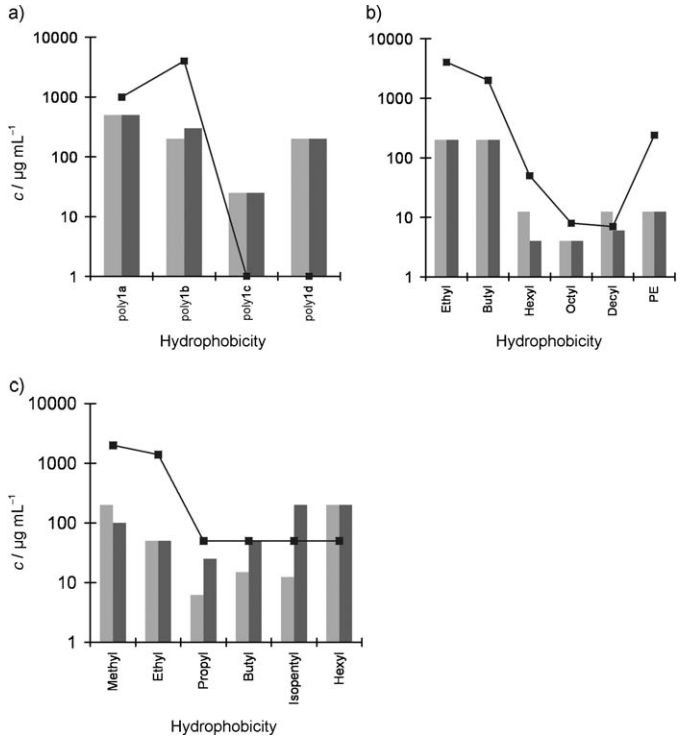

Figure 5. Biological data for three series of SMAMP homopolymers made from facially amphiphilic monomers, plotted as concentration (MIC_{90} or HC_{50}, respectively) versus increasing hydrophobicity; light gray columns = MIC_{90}, *E. coli*, dark gray columns = MIC_{90}, *S. aureus*; black squares = HC_{50}, human erythrocytes; a) series **1**, b) series **3**, in which PE refers to R = phenylethyl and is not strictly a homologue of the series, c) series **2**. Data from references [48,58,70].

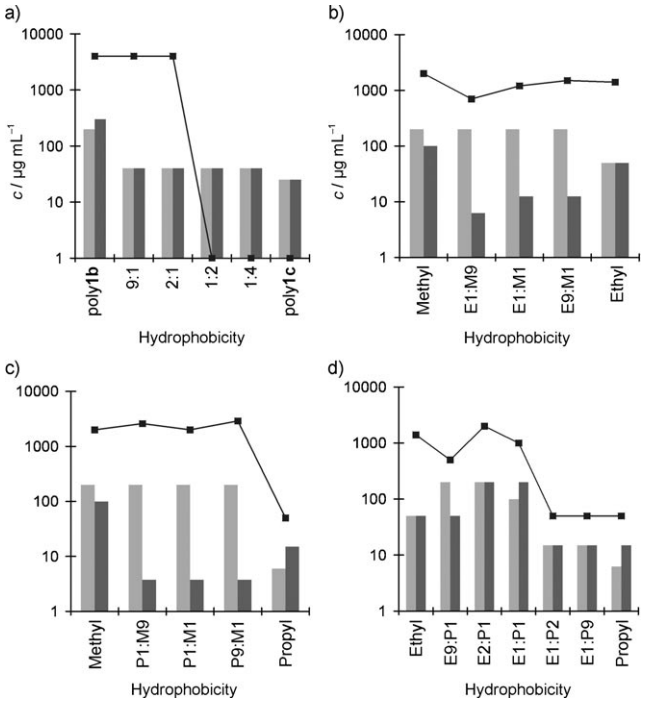

Figure 6. Biological data for for series of SMAMP copolymers made from facially amphiphilic monomers, plotted as concentration (MIC_{90} or HC_{50}, respectively) versus increasing hydrophobicity; light gray columns = MIC_{90}, *E. coli*, dark gray columns = MIC_{90}, *S. aureus*; black squares = HC_{50}, human erythorocytes; a) poly**1b**-*co*-poly**1c**, b) series **4** (Figure 4), R1 = methyl, R2 = ethyl; c) series **4**, R1 = methyl, R2 = propyl, d) series **4**, R1 = ethyl, R2 = propyl. Data from references [48,58].

were the least hemolytic and the propyl polymers the most active. Thus, a systematic property variation was expected by copolymerization of these monomers. Three copolymer series (series **4** in Figure 4, with R^1/R^2 = methyl/ethyl, methyl/propyl and ethyl/propyl) were thus obtained. The biological data from these series are summarized in Figure 6 b–d. Copolymerization of the ethyl with the methyl copolymer gave little improvement in the antimicrobial properties, and no significant difference in the HC_{50} data. Likewise, while incorporation of ethyl into the propyl copolymers made those less toxic, at the same time they lost their antimicrobial activity. The methyl–propyl copolymers, on the other hand, show the same trend as the poly**1b**-*co*-poly**1c** polymers, and can therefore be considered as the direct analogue to that series. From a feed ratio of 1:9 to 9:1, these polymers became more active against *S. aureus* bacteria, and at the same time less hemolytic. The selectivities of those polymers were >533. However, unexpectedly, these polymers lost their activity against *E. coli*.[58] They were termed "doubly selective" SMAMPs as they were active against Gram-positive but not Gram-negative bacteria and did not lyse mammalian cells.

The common structural feature of both the poly**1b**-*co*-poly**1c** copolymers and the ester-based copolymers (**4**) is that both co-monomers were facially amphiphilic, although conformational differences are evident. While successive structural simplification in going from AMPs via foldamers to SMAMPs showed that many design features, such as the rigid nature of the backbone and the presence of aromatic groups, were not essential for obtaining high selectivities for bacteria over mammalian cells, it was found by Gabriel et al. that the facial amphiphilicity on the monomer level was critical.[57] In an attempt to obtain a polymer series with tunable antimicrobial properties without the need to go through the tedious synthetic procedures of the poly**1a**–**1d** series (Figure 4), Gabriel et al. made SMAMP copolymers from one hydrophilic unit (the poly**1a** monomer) and one repeat unit carrying a variable hydrophobic group, with a feed ratio of 1:1 (series **5** in Figure 4).[57] These were termed "segregated" repeat units, meaning the functional groups required for activity (cationic and hydrophobic) lay on two different monomers. Due to the high structural similarity between these polymers and the poly**1a**–**1d** series, it was expected that this approach would lead to polymers with similarly tunable properties. However, while these new polymers (series **5**) 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), the overall selectivities of these polymers remained much lower, with a maximum selectivity of 20 (Figure 7 a). Deviation from the 1:1 monomer feed ratio did not improve the selectivities.

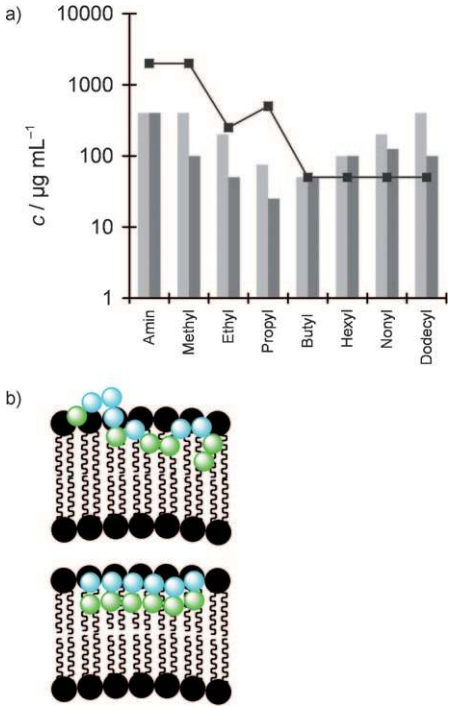

Figure 7. a) Biological data for series **5** (SMAMP copolymers, made from segregated monomers), plotted as concentration (MIC_{90} or HC_{50}, respectively) versus increasing hydrophobicity; light gray columns = MIC_{90}, *E. coli*, dark gray columns = MIC_{90}, *S. aureus*; black squares = HC_{50}, human erythrocytes; b) Illustration of SMAMP-membrane interactions: top: segregated SMAMPs, bottom: facially amphiphilic SMAMPs.[74]

This was thought to be a result of the segregation of the functional groups onto two different repeat units, which leads to runs of hydrophobic and hydrophilic groups in the statistical copolymer (Figure 7b, top). Thus, the local hydrophobicity of these polymers was not uniform, whereas the polymers from facially amphiphilic monomers have a well-defined local hydrophobicity (Figure 7b, bottom). This caused the reduced activity of the segregated SMAMPs when interacting with the bacterial membrane (Figure 7b).[57]

Adding hydrophilicity: The observation that the least hydrophobic polymers of series **1**–**3** were usually also the least hemolytic ones (Figure 5) led to the idea that the hemolysis value of an active, but toxic, polymer could be reduced by copolymerizing the respective monomer with a hydrophilic co-monomer. Colak et al. consequently picked poly**1c**, the most active and hemolytic polymer in series **1**, and modified it by incorporating non-ionic and zwitterionic hydrophilic repeat units.[75] This strategy is different from the above approach, in which hydrophobicity is modified by varying R groups in a series of facially amphiphilic monomers: the hydrophilic moieties used are not facially amphiphilic, each carries a lot of functionalities that impart hydrophilicity, and as a result they are structurally very dissimilar to the previously used inactive co-monomers. Also, these co-monomers all had the same charge, while the hydrophilic repeat units used by Colak et al. were overall neutral.[75] The hydrophilic moieties chosen were a sugar residue, a zwitterionic function, and a short poly(ethylene glycol) chain (series **6** in Figure 4). The activities of these polymers are shown in Figure 8a–c, respectively.[75] The data indeed shows that gradually making poly**1c** more hydrophilic systematically reduced its hemolytic activity; however, the dilution of the active ammonium group also rendered these molecules increasingly inactive, and therefore the selectivities of these polymers remained low.

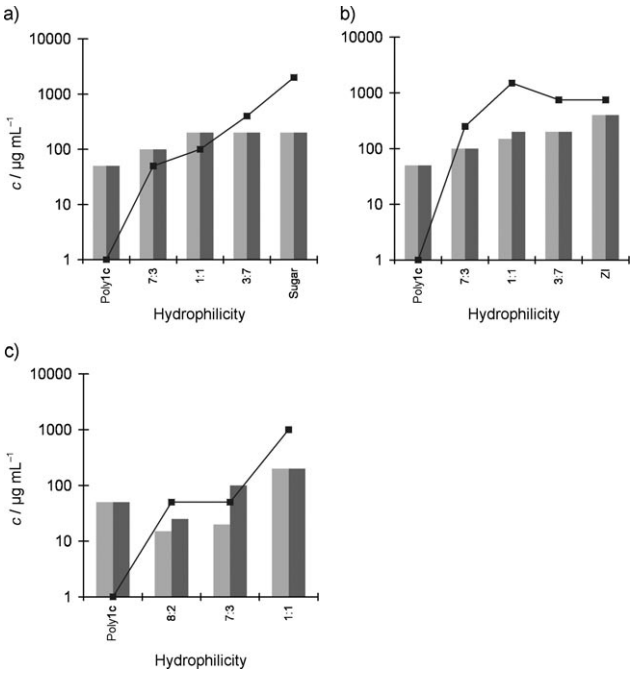

Figure 8. Biological data for three series of SMAMP copolymers, made from facially amphiphilic and hydrophilic moieties, plotted as concentration (MIC_{90} or HC_{50}, respectively) versus increasing hydrophilicity; light gray columns = MIC_{90}, *E. coli*, dark gray columns = MIC_{90}, *S. aureus*; black squares = HC_{50}, human erythrocytes; a) series **6**, R = Sugar, b) series **6**, R = zwitterion, c) series **6**, R = PEG.[75]

The effect of charge: As it is known from the AMP literature that the positively charged group is an important design feature to obtain biological activity, the effect of charge variation on SMAMP properties was investigated. Two series with systematic variation of this parameter exist, one of them imide-based (series **7** in Figure 4),[76] and the other ester-based (series **8** in Figure 4).[58] For the imide-based series, poly**1a** and poly**1c** were taken as a starting point. Structurally alike polymers carrying two and three charges per repeat unit were made (series **7** in Figure 4), and their biological properties were compared to their parent compounds.[76] The data for these polymers is reported in Figure 9. The hydrophobic poly**1c**, which is active and toxic, became drastically less hemolytic and more active against *E. coli* as the charge doubled. However, further addition of charge did not improve the biological properties. On the other hand, the hemolytic activity of the already hydrophilic

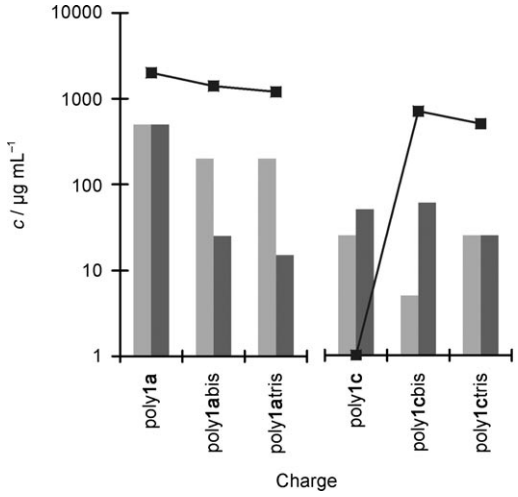

Figure 9. Biological data for imide-based SMAMP polymers with increasing charge per repeat unit (series **7** in Figure 4), plotted as concentration (MIC_{90} or HC_{50}, respectively) versus increasing nominal charge per repeat unit; light gray columns = MIC_{90}, *E. coli*, dark gray columns = MIC_{90}, *S. aureus*; black squares = HC_{50}, human erythrocytes; left: poly**1a** derivatives, right: poly**1c** derivatives.[76]

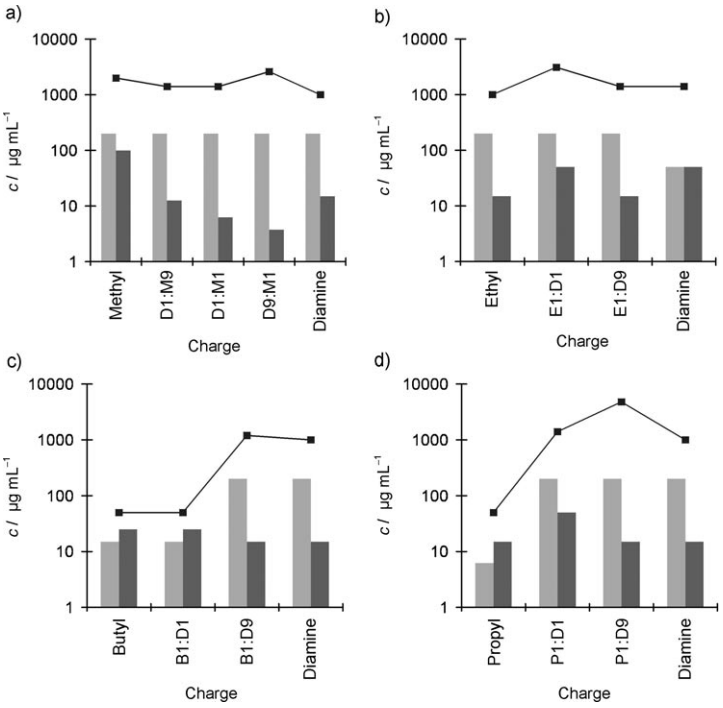

Figure 10. Biological data for ester-based SMAMP copolymers with increasing charge density (series **8** in Figure 4), plotted as concentration (MIC_{90} or HC_{50}, respectively) versus increasing nominal charge; light gray columns = MIC_{90}, *E. coli*, dark gray columns = MIC_{90}, *S. aureus*; black squares = HC_{50}, human erythrocytes; a) methyl copolymers, b) ethyl copolymers, c) propyl copolymers, d) butyl copolymers.[77]

poly**1a** did not improve upon addition of more charge, yet the polymer became more active against *S. aureus* bacteria.

Charge and hydrophilicity are two parameters that are difficult to separate. When increasing the charge across a polymer series, the hydrophilicity is automatically altered also. The effect of this on the biological properties depends on the overall hydrophobicity of the polymer series. In an already hydrophilic polymer like poly**1a**, adding charge does not alter the hydrophilicity dramatically, thus the overall properties of the polymer only change minimally. However, as seen by the drastic decrease in hemolytic activity from poly**1c** to poly**1cbis**, adding an extra charge to a hydrophobic molecule drastically influences its overall hydrophilicity. Thus the poly**1a** series is probably the better model to isolate the effect of charge on the biological properties.

Taking these considerations into account, the ester platform was used to obtain four series of copolymers from a doubly charged and a singly charged repeat unit carrying a variable R group (R = methyl to butyl). The thus obtained polymer series had different overall hydrophobicities between each series. Different monomer feed ratios allowed the charge density to be continuously varied across the series in contrast to the "step-function" (one, two, or three charges per repeat unit) in the previous case.[77] The biological properties of those polymers are summarized in Figure 10.

As these data indicate, all the methyl and ethyl copolymers are non-hemolytic, whereas the propyl and butyl copolymers become more hemolytic with high propyl and butyl co-monomer content, respectively. Thus, the properties of those two series are dominated by the hydrophobicity of those R groups. It was found with complementary methods that the hydrophobicity of the monoamine–methyl homopolymer closely resembled that of the diamine homopolymer, while the ethyl to butyl homopolymers were significantly more hydrophobic.[77] Thus, the methyl copolymers were identified as the most suitable model system of polymers to study the effect of increasing charge density at approximately constant overall hydrophobicity. Indeed, the properties of this polymer series (Figure 10a) are very similar to those of the poly**1a** derivatives (Figure 9): With increasing charge, the hemolytic activity is only slightly affected; however the activity against *S. aureus* dramatically improves. When charge is reduced in the methyl–diamine series [from a copolymer with a molar ratio of methyl/diamine = 9:1 (M9:D1) to monoamine–methyl homopolymer in Figure 10a], there is a sudden jump in the MIC from 4 to 100 µg mL^{-1}. The same is found in the poly**1a** derivatives when going from one to two charges per repeat unit. These findings, together with AMP literature data[78] led to the hypothesis that there is a specific charge threshold that needs to be exceeded to obtain decent activities against *S. aureus*. 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 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.

Counterion effects: To further probe the interaction between charge and hydrophobicity, the effect of counterion exchange on the biological properties of the series **2** polymers was studied.[77] The hydrophilic counterions of the most hydrophilic ester-based polymer (polymer **9** in Figure 4) were exchanged by hydrophobic organic counterions (e.g., hexanoate and tosylate). While the original idea was that exchanging these counterions would impart hydrophobicity onto the polymer and make it more active, it was found that ion-exchanging these polymers completely eliminated their antibacterial activities (Figure 11). Using dye-

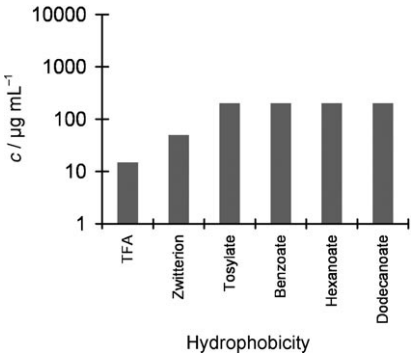

Figure 11. MIC_{90} data (against *S. aureus*) for ion-exchanged SMAMPs (polymer **9** in Figure 4), plotted as concentration versus hydrophobicity. The *x*-axis labels denote the respective counterion, in which TFA = trifluoroacetate.[77]

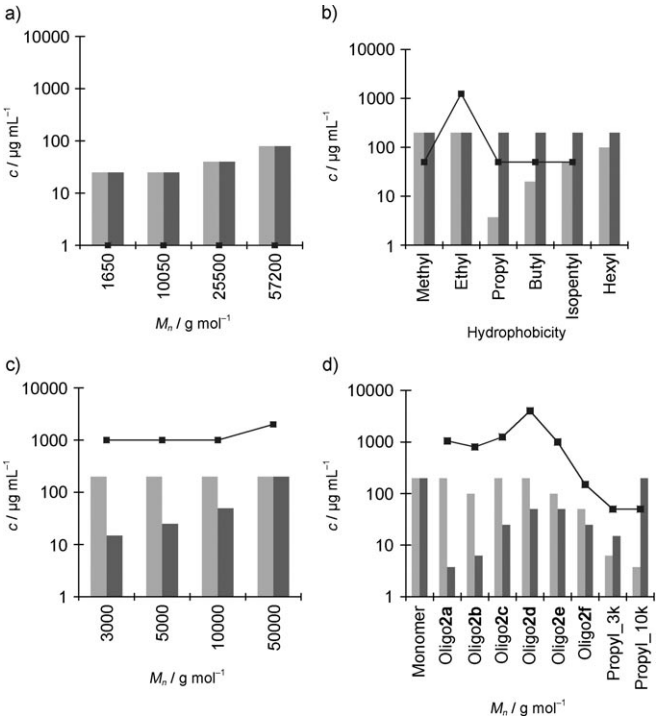

Figure 12. Molecular-weight dependence of biological properties for various SMAMPs, plotted as concentration (MIC_{90} or HC_{50}, respectively) versus increasing molecular mass; light gray columns = MIC_{90}, *E. coli*, dark gray columns = MIC_{90}, *S. aureus*; black squares = HC_{50}, human erythrocytes; a) different molecular weights of poly**1c**, b) 10 000 g mol^{-1} polymers of series **2** (Figure 4), c) different molecular weights of polymer **9**, d) different molecular weights of the propyl polymer from series **2**. Data from references [48, 58, 77].

leakage studies (the principles of which will be discussed in much more detail below), it was found that the ion-exchanged polymers were not membrane active, unlike the parent polymer **9**. This revealed that the ammonium group of these polymers and the organic counterions formed such a tight ion pair that the overall positive charge of the polymer was masked.[77] While this meant that exchange of the counterions does not provide an additional handle to tune the antibacterial activity of SMAMPS, these findings agreed with previous studies using inorganic counterions that correlated the tightness of the ion pair to the antibacterial activities of a polymer.[79]

The effect of molecular weight: The previously presented data all referred to samples with a molecular weight of roughly 3000 g mol^{-1}, although most of the studies mentioned investigated two or more molecular weights of each polymer type. In this section, we summarize how molecular weight affects SMAMP properties.

Ilker et al. found only a weak molecular-weight dependence for their poly**1c** compound, which at that time did not seem significant (Figure 12a).[48] In the case of the poly**1b** and poly**1d** series, no trend was observed as those polymers were in the inactive regime. Eren's low- and high-molecular-weight polymers ($M_n \approx 3000$ g mol^{-1} and 10 000 g mol^{-1}, respectively) all had similar antibacterial and hemolytic activities, whether they were in the active or inactive regime.[70] For the "segregated" copolymers, Gabriel found that the high-molecular-weight polymers ($M_n \approx 10\,000$ g mol^{-1}) were less active than the low-molecular-weight polymers ($M_n \approx 3000$ g mol^{-1}) by a factor of 2–8.[57] Some of them were also slightly more hemolytic. The same general trend was found by Lienkamp et al. for the higher molecular-weight ester-based polymers (series **2** in Figure 4, $M_n \approx 10\,000$ g mol^{-1}, biological data in Figure 12b). Compared to their $M_n \approx 3000$ g mol^{-1} analogues (Figure 5c), these polymers were generally less active against *E. coli*, with the exception of the propyl_10k polymer (propyl_10k is a 10 000 g mol^{-1} diamine–propyl homopolymer) which was surprisingly active against that bacterial type. More notably, they were all inactive against *S. aureus*.[58] Similarly, the diamine homopolymers (polymer **9** with TFA counterions in Figure 4, biological data in Figure 12c) showed a systematic decrease in activity against *S. aureus* with molecular weight, together with inactivity against *E. coli* at all molecular weights. This lead to the hypothesis that, at higher molecular weights, these particular polymers get stuck in the peptido-

glycan layer of Gram-positive bacteria, as will be discussed in more detail below.

To investigate the molecular weight effect in more detail, especially in the low-molecular-weight region, a series of oligomers (oligo**2a**–**2f**) from the propyl polymer series **2** was prepared (Figure 12d). As this data shows, the molecular-weight dependence for both the hemolytic and antibacterial activities is highly nonlinear and different for each bacterial species involved. In the case presented here, oligo**2a** is selective for *S. aureus* over *E. coli*, while propyl_3k shows the opposite tendency. These last data illustrate that it is very difficult to draw general conclusions concerning the dependency of biological activity on molecular weight. In most cases, the general rule seems to hold that, when the molecular weight is above a certain threshold value, the polymers become inactive, as discussed above. Below this threshold, however, it is not possible to predict which molecular weight will give the best activities and selectivities, as this strongly depends on the overall hydrophobicity of the particular polymer studied. While finding that polymers with the same chemical structure, but different molecular weights can differentiate between bacterial types make generalizations and predictions more difficult, it gives the chemist yet another tool to control the properties of SMAMPs such that they target only certain bacteria.

Mechanistic Studies of ROMP-based SMAMPs

Vesicle experiments and fluorescence microscopy: The mechanistic studies of ROMP-based polymeric SMAMPs were inspired by what is known from the fields of AMPs and small foldamer SMAMPs. The general range of techniques used in this field, and the results obtained, have been reviewed previously.[6] In the young field of polymeric SMAMPs, the most popular method to probe the SMAMP–membrane interaction is the dye-leakage experiment. In this experiment, dye-filled lipid vesicles are used as simplified models for bacteria and mammalian cell membranes. When properly chosen, these vesicles capture the key lipid compositions of cellular plasma membranes. Although they lack cell features such as the peptidoglycan cell wall of Gram-positive organisms, the double-membrane structure and lipopolysaccharide (LPS) layer of Gram-negative bacteria, or the many proteins found in cell membranes, they are well-accepted methods to investigate the interaction of membranes with polymers or proteins, and can be used to correlate the membrane-disrupting properties of a compound to its biological activity.[27,38,53] In each case, the lipid composition of these model vesicles is chosen to closely match the cell type they are supposed to mimic in terms of primary lipid(s) and resulting properties like surface charge, fluidity, and lipid curvature. For example, pure cardiolipin vesicles are most commonly used to mimic Gram-positive *S. aureus* bacteria, whereas a mixture of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) is used to mimic Gram-negative *E. coli* bacteria, and phosphatidylcholine (PC) is utilized to mimic human red blood cells.[80] Alternatively, a full lipid extract from the respective bacteria can also be used. Leakage of a self-quenching fluorescent dye (e.g., calcein) from the vesicle upon exposure to the SMAMPs leads to fluorescence, which is plotted as a function of time (Figure 16a). To get more quantitative results, the leakage percentage upon SMAMP exposure can be plotted versus SMAMP concentration to get the EC_{50} value, which corresponds to the SMAMP concentration at the half-maximum amount of leakage (Figure 13a).[80]

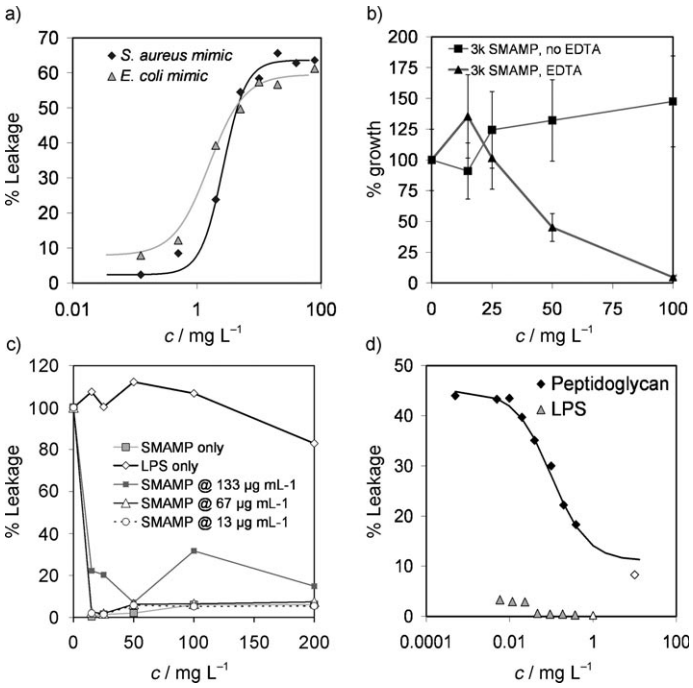

Figure 13. a) Dye-leakage percentage versus SMAMP concentration of *E. coli* and *S. aureus* mimicking vesicles; b) MIC experiment on regular *E. coli* cells (no EDTA) and *E. coli* cells with EDTA-damaged outer membrane; c) MIC experiment on *S. aureus* in the presence of LPS; d) SMAMP–LPS and SMAMP–peptidoglycan binding studies.[83]

Ilker et al. investigated the lysis of neutral cholesterol:PC vesicles (as a mimic for erythrocytes) and of negatively charged phosphatidylserine:PC vesicles.[81] They found that the lysing properties of poly**1b**, poly**1c** and the poly**1b**-*co*-poly**1c** copolymers were in good agreement with their antibacterial activities, poly**1b** being inactive and the other two polymers showing marked dye leakage.[81] A more comprehensive study on polymer series **1** will be discussed below.[82] Eren et al. investigated the effect of exposing polymers from series **3** to PC vesicles (erythrocyte mimics) and *E. coli* extract, and found a good correlation between the membrane disruptive properties of these polymers and their HC_{50} and MIC_{90} data, respectively.[70] However, in this special case, for unknown reaseons the activity towards PE:PG vesicles did not correlate with the MIC_{90} data.[70] Al-Badri et al. also observed good agreement of the leakage from PC vesicles with the hemolysis data of polymer series **7**.[76] Their samples also followed the general trend for both PE:PG and CL vesicles,

that is, that the poly**1c**-derived samples were more membrane disruptive than the poly**1a**-derived ones.[76] For the segregated copolymers (series **5**), overall it was found that their activity against *E. coli* matched the membrane-disruptive potency towards vesicles made from *E. coli* lipid extracts.[57] Lienkamp et al. studied polymers from series **2** on *S. aureus* mimicking CL vesicles. They found a good correlation between the MIC_{90} for *S. aureus* and the vesicle leakage for the methyl to butyl homopolymers.[77] As this body of data shows, overall there is a good correlation between dye-leakage activity of a SMAMP and the corresponding biological activity. This led to the conclusion that the mechanism of antibacterial activity for all those polymers is by disruption of the plasma membrane of the bacteria, which then causes a breakdown of the membrane potential, leakage of the cell content, and, eventually, cell death.

When dye-leakage studies fail to model cell-SMAMP interactions, this can indicate that other components of the cell structure are important, which are not adequately modeled by the simple bilayer membrane, such as the peptidoglycan cell wall of Gram-positive bacteria, and the double membrane structure of Gram-negative bacteria, or that the SMAMPs have other major targets besides the membrane. Lienkamp et al. studied the behavior of their diamine homopolymers (polymer **9** with trifluoroacetate counterions in Figure 4, $M_n \approx 3000\ g\,mol^{-1}$), towards both cardiolipin (*S. aureus* mimic) and PE:PG (*E. coli* mimic) vesicles.[83] These polymers had previously been found to be "doubly selective" first for bacteria over mammalian cells, and for *S. aureus* ($MIC_{90} = 15\ \mu g\,mL^{-1}$) over *E. coli* ($MIC_{90} > 200\ \mu g\,mL^{-1}$).[77] Surprisingly, although the MIC_{90}s of the $3000\ g\,mol^{-1}$ sample of polymer **9** were dramatically different for *E. coli* and *S. aureus*, this polymer had almost identical EC_{50} and maximum leakage values for both vesicle types (Figure 13a). This demonstrated that the differences in lipid composition of the two bacteria were not responsible for the observed differences in MIC_{90}.

In two series of modified MIC experiments, the role of LPS and the double membrane was investigated. *E. coli* cells, the outer membranes of which were damaged by exposition to EDTA, had a lower MIC than regular *E. coli* cells (Figure 13b). On the other hand, the MIC_{90} of *S. aureus* in the presence of added LPS extract was the same as that of *S. aureus* without additional LPS (Figure 13c). Thus, it was concluded that it is the second lipid bilayer rather than the additional LPS layer of Gram-negative bacteria that reduces the SMAMP concentration at the plasma membrane and thus renders them inactive against *E. coli* bacteria.[83] Since it was observed that the $3000\ g\,mol^{-1}$ polymer **9** was active against *S. aureus*, but that the corresponding $50000\ g\,mol^{-1}$ polymer was inactive, it was considered that the peptidoglycan cell wall found in Gram-positive organisms was an impenetrable barrier for large SMAMPs. This could be either due to binding or due to steric hindrance. Dye-leakage experiments on CL vesicles to which peptidoglycan extract had been added led to the conclusion that irreversible binding to peptidoglycan does not occur on the timescale of the experiment (Figure 13d, SMAMP–peptidoglycan binding reduces leakage much less than SMAMP–LPS binding), but that the large SMAMPs have difficulty penetrating the peptidoglycan mesh and thus do not reach the plasma membrane.[83]

In other cases, the cause for discrepancies between dye-leakage experiments and biological data might not have to do with the vesicle experiment at all, but with SMAMP solubility. It was found that the two most hydrophobic polymers of series **5**, with R = dodecyl, and series **2**, with R = hexyl, did not follow the MIC_{90}-dye leakage correlation,[57,77] while the other molecules in both series were perfectly well-behaved. In both cases, a hydrophilic polymer of the same series with the same MIC_{90} as the "odd" hydrophobic polymer caused significantly less dye leakage than the corresponding hydrophobic polymer. The reason for this is the low solubility of these hydrophobic polymers at the comparatively high concentrations of the MIC experiment.[77] The SMAMP concentration in the dye-leakage experiments is usually one or two orders of magnitude lower than in the MIC experiments, as these experiments are understood to be much more sensitive.[84] Thus, if a polymer has poor solubility in aqueous media, it will seem less active in the MIC experiment, but it will still be active in the dye-leakage experiment, causing a discrepancy in the results obtained from the two methods.

Another method to probe the membrane–polymer interaction is the so called "live–dead" stain. This somewhat misleading name refers to an experiment in which the bacteria are incubated with a dye mixture of SYTO9, giving green fluorescence, and propidium iodide, a red fluorescent dye. While SYTO9 can diffuse through the membranes of both intact and membrane-compromised cells, propidium iodide can only enter the cells with damaged plasma membranes and competes with the green dye for binding sites. Thus, a "live" cell will appear green when using the green filter of the fluorescence microscope, and not red under the red filter, whereas a membrane-compromised "dead" cell will appear red when the red filter is applied, and may or may not appear green under the green filter depending on the dye stoichiometry. Using fluorescence microscopy, it was shown that the antibacterially active butyl polymer of series **5** caused red fluorescence and severe aggregation in *E. coli* bacteria, whereas the inactive poly**1a** did neither (Figure 14).[57] This demonstrates again that active SMAMPs compromise the bacteria cell membranes. Using the same method, we recently found when comparing different molecular weights of polymer **9** that there is a correlation between SMAMP concentration and bacteria aggregation, as well as between SMAMP molecular weight and bacteria aggregation. At the same concentration, higher molecular-weight SMAMPs cause more cell aggregation than lower molecular-weight ones. Also, the higher the SMAMP concentration, the more cell aggregation was observed.

Other techniques: In the most detailed physical study on ROMP-based SMAMPs to date, HPLC, dye-leakage studies,

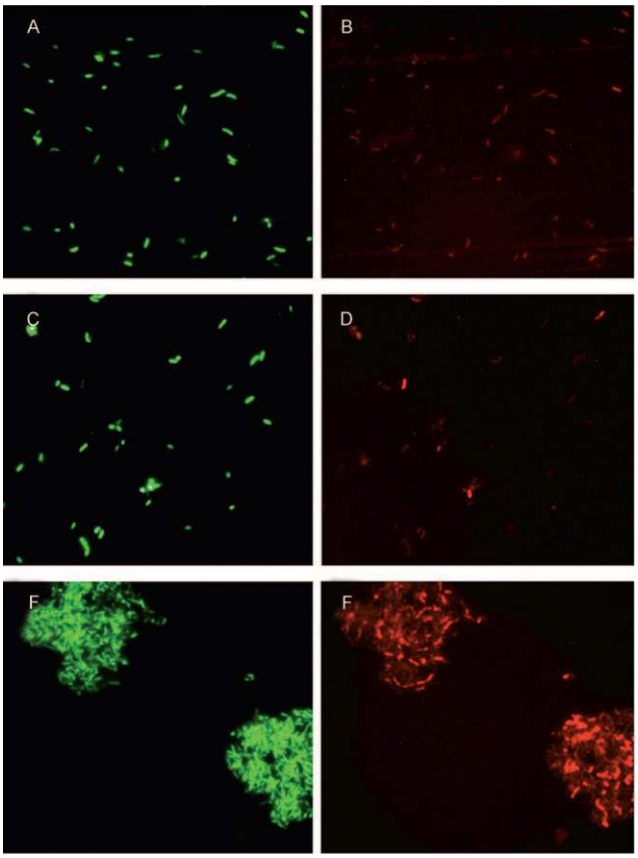

Figure 14. Fluorescence microscopy image of *E. coli* cells treated with live-dead stain. The images of the left column were taken using a green filter (SYTO 9 emission), the ones in the right column were taken using a red filter (propidium iodide emission). A/B: Control, no polymer; C/D: poly**1a**, E/F: butyl polymer from series **5**.[74]

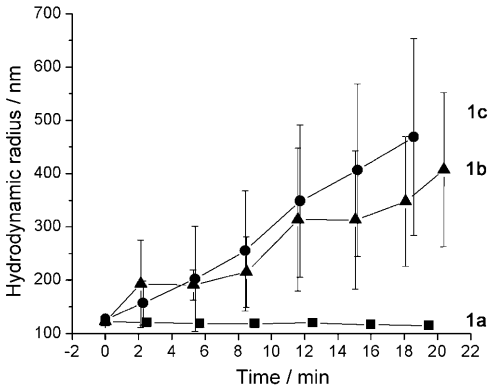

Figure 15. Dynamic light scattering studies on vesicles exposed to polymer series **1** (poly**1a** to poly**1c**). The hydrodynamic radius (R_h) is plotted as a function of time. While R_h did not increase for poly**1a**, the vesicles exposed to poly**1b** and poly**1c** continuously aggregate or fuse.[74]

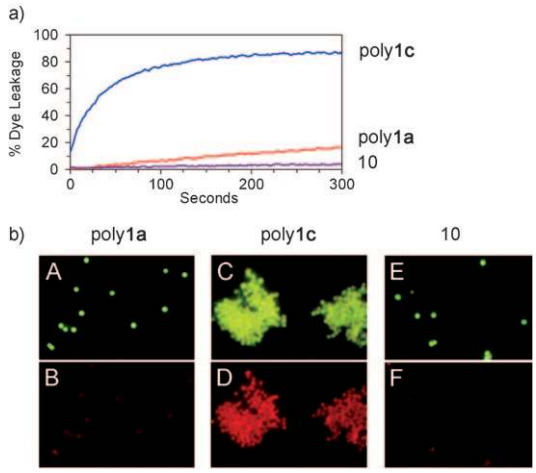

Figure 16. a) Dye-leakage curves for the active poly**1c**, the inactive poly**1a**, and the membrane-permeating polymer **10**; b) Fluorescence microscopy images of *S. aureus* cells treated with live–dead stain. The images of the top row were taken using a green filter (SYTO 9 emission), the ones in the bottom row were taken using a red filter (propidium iodide emission). A/B: poly**1a**; C/D: poly**1c**, E/F: polymer **10**.[82]

light-scattering, isothermal calorimetry, and fluorescence microscopy were combined to investigate the mechanism of polymer–membrane interactions of the poly**1a**–**1d** series.[82] The combination of these methods allowed Gabriel et al. to elucidate the mechanism of SMAMP-bacteria interaction in unprecedented detail. As expected, they found a linear correlation between HPLC elution times and the alkyl side chain length from poly**1a**–**1d**, which proved the intuitive assumption that the hydrophobicity increases across that series. As mentioned above, they also demonstrated that the dye-leakage data of these polymers follows their MIC trend, that is, the inactive poly**1a** does not lyse vesicles, whereas poly**1b** and poly**1c** are increasingly membrane active.[82] Dynamic light scattering was used to monitor the effect of SMAMP addition on the hydrodynamic radius of the vesicles a function of time. While the radii of vesicles exposed to poly**1a** remained unaltered, those exposed to poly**1b** and poly**1c** grew significantly over time (Figure 15).

This is another indication that poly**1a** is not membrane-active, whereas poly**1b** and poly**1c** clearly are, although the light scattering studies do not capture the significant difference in the MIC_{90}s of these polymers (200 vs. 25 $\mu g\,mL^{-1}$). It is also not clear whether the vesicle growth is due to aggregation or vesicle fusion. Consequently, this effect was further studied by fluorescence microscopy on dye-labeled vesicles and stained bacteria cells.[82] When the vesicles that are routinely used for dye-leakage studies, with a diameter of about 200 nm, were exposed to poly**1c**, giant fluorescent vesicles appeared, while the stained *E. coli* bacteria aggregated, as has been observed with other SMAMPs.[57] These aggregation phenomena highlight that SMAMPs are not just very complicated detergents. When added to vesicles, detergents would just dissolve the membranes, instead of causing vesicle fusion or aggregation. Also, detergents do not have the ability to differentiate between cells.

The membrane-SMAMP interactions were further studied with isothermal calorimetry. These studies revealed that, while no binding interaction between the vesicles and poly**1a** or poly**1d**, respectively, was observed, there was a

strong binding event between the vesicles and both poly**1b** and poly**1c**.[82] Fitting the data with modeling software revealed that the binding between the vesicle and these SMAMPs is entropically favorable, and the overall free enthalpy of binding was about the same for both poly**1b** and poly**1c**. However, marked differences were observed in the binding stoichiometry—the ratio of vesicle lipids to ammonium groups of the polymer was 0.4 for poly**1b**, and 1.06 for the more active poly**1c**. From this, the authors drew the conclusion that membrane rupture, and thus antimicrobial activity, necessitates a minimum amount of SMAMP molecules attached to the membrane. Once that threshold of molecules per vesicle is passed, membrane rupture may occur.

Beyond SMAMPs—Cell-Penetrating Peptides

Recently, an unusual SMAMP has been discovered (polymer **10** in Figure 4). While all the previously discussed ROMP-based SMAMPs had ammonium groups as the positively charged moiety, this polymer contained guanidinium groups and had broad-spectrum antibacterial activity against both Gram-negative (*E. coli* and *S. marcescens*, $MIC_{90}=6$ and 50 $\mu g\,mL^{-1}$, respectively) and Gram-positive (*S. aureus* and *B. subtilis*, $MIC_{90}=12\ \mu g\,mL^{-1}$ for both) bacteria. Together with a remarkably low hemolytic activity ($HC_{50}=1500\ \mu g\,mL^{-1}$), this yields a selectivity for *E. coli* over red blood cells of 250, which is the highest selectivity so far observed for a broad-spectrum SMAMP (as the polymers of series **4** and **8** were only active against Gram-positive bacteria). Using bactericidal kinetics studies (also known as time-kill assays), it was shown that this polymer caused a 5 log reduction in less than 60 min at four times the MIC_{90}, meaning that the polymer is indeed bactericidal and not just bacteriostatic.[62] Comparative dye-leakage studies with poly**1a**, poly**1c** and polymer **10** showed that, in spite of its low MIC_{90} value, polymer **10** did not lyse model membranes (Figure 16a). Similarly, while the active poly**1c** caused membrane damage and cell aggregation, as observed in fluorescence microscopy experiments (Figure 16b, panel C and D), the red fluorescence caused by polymer **10** (Figure 16b, panel E and F) was undistinguishable from that of the membrane-inactive poly**1a** (Figure 16 b, panel A and B). These are clear indications that the antimicrobial activity of this polymer is not due to extensive membrane damage, as is the case for the other SMAMPs. The guanidinium groups contained in this SMAMP are also found in poly(arginine) and other cell-penetrating peptides,[85–89] and such cell-penetrating peptides are known to be able to cross membranes, transport cargo into cells, and bind DNA.[85,88,90–95] In the light of this body of literature and the above results, it was postulated that polymer **10** is antibacterially active by first penetrating the cell membrane without causing damage, and then interacting with an intracellular target, potentially the bacteria DNA, which leads to the cell's death.[62] Recent studies confirmed the ability of these polymers to traverse membranes and gave further evidence that this mechanism is reasonable, as these guanidinium-rich polymers behaved remarkably similar to poly(arginine).[96] These polymers represent a new proteomimetic activity which will be reported in detail from our laboratory in the future.

Conclusions

Using ring-opening metathesis polymerization as a synthesis platform, a large variety of synthetic mimics of antimicrobial peptides (SMAMPs) was obtained. By carefully tuning the overall hydrophobicity and charge density of these molecules, polymers with tailor-made properties, from inactive/non-hemolytic through active/non-hemolytic to active/toxic, were obtained. As the biological properties of a SMAMP 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 meanwhile 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 studying macromolecule–membrane interaction would benefit from a more fundamental understanding of such processes.

Nonetheless, from the data available, and by analogy to what is known from the small molecule antimicrobials and AMP literature, it seems reasonable to conclude for that:

- A rigid backbone that dictates the conformation of the molecule is not necessary for antibacterial activity in polymeric SMAMPs.
- Most polymeric SMAMPs are antibacterially active because they disrupt bacterial membranes rather than forming well-defined, discrete pores.
- A minimum amount of charge is necessary for antibacterial activity, otherwise the polymer has limited binding affinity for the bacterial membrane.
- A certain hydrophobicity/hydrophilicity balance is required for the subsequent step of disrupting the cell membrane.
- The polymeric SMAMP–vesicle and polymeric SMAMP–bacteria interactions lead to aggregated structures, which is evidence for significant membrane perturbation.
- One antibacterial polymeric SMAMP carrying guanidinium groups has the ability to nondestructively pass cell membranes.
- Careful polymeric SMAMP design can either lead to broad-spectrum antibacterials or SMAMPs with Gram-selectivity.

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