

Antimicrobial Polymers Prepared by Ring-Opening Metathesis Polymerization: Manipulating Antimicrobial Properties by Organic Counterion and Charge Density Variation

Karen Lienkamp,^[a] Ahmad E. Madkour,^[a] Kushi-Nidhi Kumar,^[b] Klaus Nüsslein,^[b] and Gregory N. Tew*^[a]

Abstract: The synthesis and characterization of a series of poly(oxanorbornene)-based synthetic mimics of antimicrobial peptides (SMAMPs) is presented. In the first part, the effect of different organic counterions on the antimicrobial properties of the SMAMPs was investigated. Unexpectedly, adding hydrophobicity by complete anion exchange did not increase the SMAMPs' antimicrobial activity. It was found by dye-leakage studies that

this was due to the loss of membrane activity of these polymers caused by the formation of tight ion pairs between the organic counterions and the polymer backbone. In the second part, the effect of molecular charge density

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on the biological properties of a SMAMP was investigated. The results suggest that, above a certain charge threshold, neither minimum inhibitory concentration (MIC₉₀) nor hemolytic activity (HC₅₀) is greatly affected by adding more cationic groups to the molecule. A SMAMP with an MIC₉₀ of 4 µg mL⁻¹ against *Staphylococcus aureus* and a selectivity (=HC₅₀/MIC₉₀) of 650 was discovered, the most selective SMAMP to date.

Introduction

With antibiotic resistant bacteria like MRSA spreading in medical facilities^[1] and the community,^[2] antibacterial polymers are increasingly important, as they can help to eradicate these pathogens.^[3] They are used in high-infectious-risk areas, with applications such as self-sterilizing catheter tubes, coatings of medicinal products, surgical implants, and wound dressings. The antibacterial polymers presented in this work are synthetic mimics of antimicrobial peptides (SMAMPs) and can be considered a subgroup of biocidal polymers. Whereas the latter nonspecifically kill cells, SMAMPs are designed to only attack pathogens and not the cells of the host organism. Both biocidal polymers and anti-

microbial peptides (AMPs), the natural analogues of SMAMPs, have been extensively reviewed.^[4-6]

Some important details of the mode of action of AMPs have been elucidated. In general, they contain a positively charged hydrophilic surface that promotes attachment to the negatively charged bacterial cell membrane, while their hydrophobic groups trigger membrane permeation and disruption.^[4] This facial amphiphilicity of the peptide does not require a specific receptor-like cell target, and leads to broad-spectrum activity against pathogens. At the same time, due to differences in the membrane composition, AMPs are significantly less toxic towards mammalian cells. The nonspecific peptide-membrane interaction leads to less bacterial resistance build-up, as compared to traditional antibiotics.^[7]

The aim in SMAMP design has been to discover polymers with similar properties to their natural parent peptides, meaning a high antibacterial activity (MIC₉₀¹) and limited toxicity to mammalian cells. The latter is commonly estimated by measuring the polymer's HC₅₀² value. The combined

[a] Dr. K. Lienkamp, Dr. A. E. Madkour, Prof. G. N. Tew
Department of Polymer Science & Engineering
University of Massachusetts, Amherst, MA 01003 (USA)
Fax: (+1) 413-545-0082
E-mail: tew@mail.pse.umass.edu

[b] K.-N. Kumar, Prof. K. Nüsslein
Department of Microbiology
University of Massachusetts, Amherst, MA 01003 (USA)

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¹ MIC₉₀ = minimum inhibitory concentration that will reduce the growth of a certain bacteria by 90% as compared to an untreated control; the lower the MIC value, the more active the polymer.

² HC₅₀ = hemolytic concentration at which 50% of human red blood cells are lysed; the higher the HC₅₀, the less hemolytic the polymer.

properties of a high HC_{50} value and a low MIC_{90} lead to the desired high selectivity.³ Magainin (MSI 78), a frog host defense peptide, has a selectivity of 10,^[8] and human defensins are even more selective (>40 for nNP-1,^[9] >100 for human β -defensin 3^[10]). Several studies investigating structure–property relationships of SMAMPs have highlighted the importance of an appropriate amphiphilicity:^[8,11–14] a polymer with too much hydrophobicity will be toxic to mammalian cells, yet too little hydrophobicity will render it inactive.^[8,13,15–17] To complement these reports, the aim of this paper is: 1) to first study the effect of different organic counterions of a model SMAMP on its antimicrobial properties, and 2) to subsequently investigate the influence of molecular charge density on the antimicrobial properties.

While the importance of the hydrophilic–hydrophobic balance in a SMAMP is relatively well understood and can be generalized for many systems,^[11,13,15] the effect of different counterions on the antimicrobial properties of a polymer has been essentially neglected in the SMAMP literature. The few studies published so far investigate the effect of different inorganic counterions on polymer properties. Kanazawa et al. postulated an inverse correlation between the tightness of the ion pair of a polymer–counterion system and its antimicrobial properties ($Cl^- > BF_4^- > ClO_4^- > PF_6^-$).^[18] Chen et al. saw higher activities for bromide than for chloride counterions with their polycationic dendrimers,^[19] while Panarin et al. did not see any significant differences between chloride, bromide, and iodide.^[20] The ranking of “activity” in these studies was based on relative bacterial survival rates in time-kill studies, a legitimate approach. However, none of these studies quantified antimicrobial activity in terms of MIC data. This makes it difficult to assess how much the antimicrobial activity is actually affected by the counterion exchange, and makes comparison between these studies even more difficult. While the previous examples focused on inorganic counterions, the effect of different organic counterions, such as tosylate or hexanoate, to the best of our knowledge, has not been studied. The first aim of this work was to test whether ion exchange using counterions of varying hydrophobicity would provide an easy and versatile tool to tune the hydrophobicity and thus the antimicrobial properties of the polymer. This approach of tuning the hydrophobicity requires much less synthetic effort than the covalent attachment of hydrophobic groups, both on the monomer and the polymer synthesis level. We here report how replacing the trifluoroacetate counterions of a hydrophilic model SMAMP by hydrophobic organic counterions influences the polymer’s antimicrobial properties and its membrane activity, which we studied by dye-leakage experiments.

Another parameter in the SMAMP/AMP literature that is not fully understood is the effect of molecular charge density on the antimicrobial properties. It is well accepted that the ability of an AMP to attach to the negatively charged cell surface is a crucial step for the peptide–cell interaction, and this step is believed to be charge-driven.^[4] However,

few SMAMP studies have managed to isolate the effect of the molecular charge density on the antimicrobial properties. Systematic charge variation without changing the overall amphiphilicity of the system at the same time can only be achieved with difficulty, as these parameters are closely interconnected. Sen et al. have recently published an elegant study that investigates the effect of molecular charge distribution on antimicrobial properties.^[21] Their series of molecules had locally different charge distributions, but the same overall number of charges per molecule, and the same global hydrophobicity.^[21] This work beautifully demonstrates that facially amphiphilic⁴ polymers are more active than random copolymers made from one hydrophobic and one hydrophilic, cationic monomer, which is in agreement with our own research.^[13,15] Two other studies investigated molecules in which the molecular charge and the hydrophobicity were varied simultaneously: Kuroda and DeGrado investigated charge variation by adjusting the comonomer ratio of a charged and a noncharged hydrophobic comonomer.^[12] They thus discovered polymers that were highly active, but only slightly selective for bacteria over mammalian cells. Kanazawa et al. have varied the charge density in their poly-(phosphonium salt) polymers using alkyl spacers of various lengths.^[22] They found that the antibacterial activity increased with decreasing charge density; however the charge per molecule and the hydrophobicity were changed simultaneously and thus the effect of charge variation alone could not be isolated. Very recently, Al-Badri et al. published a study with one to three amine groups per repeat unit, leading to polymers with moderate to good selectivities.^[17] In an effort to more clearly isolate the effect of charge variation from the effect of changes in hydrophobicity, in this work, we study a series of polymers with gradually varied cationic charge, but with only minimal changes to the overall polymer structure and hydrophobicity. We gradually reduced the charge density from two to one charges per repeat unit by copolymerization, and investigated the correlation of the thus obtained molecular charge dilution with the biological properties of the resulting polymers. In the course of this study, a copolymer with a selectivity as high as 650 for *Staphylococcus aureus* over mammalian cells was discovered. As discussed in our forthcoming review on SMAMPs,^[23] there are various ways to report selectivities in the SMAMP literature, but most of the differences focus on hemolysis values. Some groups report MHC/MIC_{90} [which should, due to the different definitions of “minimum hemolytic concentration” (MHC) in the literature better be called HC_0/MIC_{90} if indeed the authors mean the concentration at 0% lysis], while others, including us, have reported the ratio of HC_{50}/MIC_{90} . While HC_{50} can be determined with more precision, HC_0 , which probes the onset of hemolysis, is the more sensitive parameter, so each ratio has its merits. Depending on which ratio is chosen, polymer A may give “better” selectivities than polymer B with one ratio, while

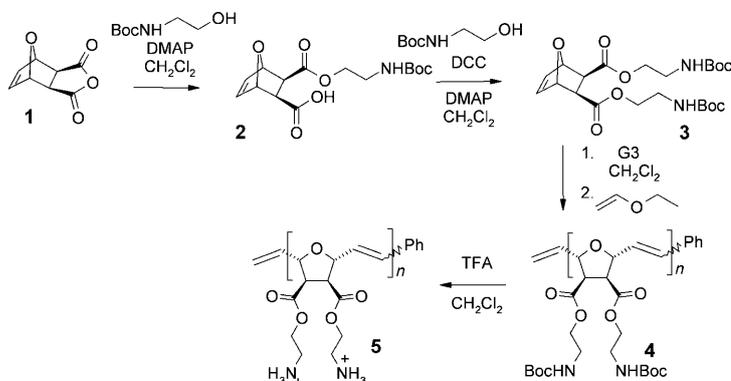
³ Selectivity = HC_{50}/MIC_{90} .

⁴ Facially amphiphilic polymers contain a hydrophobic and a hydrophilic, charged group on the same repeat unit

polymer B may look more selective using the other one. This has led to a somewhat unfortunate discussion about “who has the better SMAMPs”. We have always used HC_{50} and have never focused on precise HC_0 measurement, thus assigning arbitrary HC_0 values to these samples is certainly not helpful. HC_0 determination must be done carefully as it is a “slightly above baseline” value.^[24] Looking at the raw data, what is clear from literature is that many molecules have been designed with selectivities reminiscent of the natural AMPs, which has been the benchmark to mimic.

Results and Discussion

Diamine homopolymer synthesis: To investigate the effect of different organic counterions on the antimicrobial properties of a SMAMP, we designed a predominantly hydrophilic model polymer with two charges per repeat unit (Scheme 1). Base-catalyzed ring-opening of the anhydride **1** with a Boc-protected amino ethanol gave the monoester **2**. DCC coupling of **2** with a second equivalent of the alcohol yielded the diamine monomer **3**, which was polymerized by ring-opening metathesis polymerization (ROMP) using Grubbs’ third-generation catalyst.^[25] The obtained Boc-protected precursor polymer **4** was deprotected with trifluoroacetic acid (TFA) to yield the cationic SMAMP **5** (Scheme 1). To determine the optimum activity of polymer **5**, a range of molecular weights was synthesized. The protected polymer **4** was characterized by gel permeation chromatography (GPC).



Scheme 1. Synthesis and polymerization of the diamine monomer to yield SMAMP **5**.

matography (GPC). All polymers had narrow polydispersities (1.07–1.15, Table 1); however, as observed previously,^[13] polystyrene-calibrated GPC in DMF severely overestimated their molecular weights. This was confirmed by MALDI-TOF mass spectrometry, which showed that the molecular weights M_n of those polymers were close to the targeted ones. When plotting $M_{n,Target}$ versus the molecular weights obtained from GPC, a straight line is obtained, indicating that the polymerization was controlled (Figure 1).

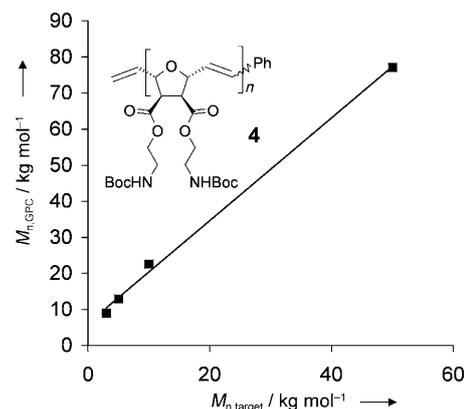


Figure 1. GPC data for the SMAMP homopolymer **4** series. Target molecular weight is plotted versus number average molecular weight from gel permeation chromatography (in DMF/0.01 M LiCl, calibrated with polystyrene standards).

The biological activities, MIC_{90} and HC_{50} , of the deprotected SMAMPs (**5**) were tested using standard procedures.^[26] The results are summarized in Table 1, in which diamine_3k refers to a sample of polymer **5** with a molecular weight of 3000 g mol^{-1} . The data shows that, while all polymers were inactive against *E. coli*, the antimicrobial activity against *S. aureus* was highest for the 3000 g mol^{-1} polymer, with an MIC_{90} of $15 \mu\text{g mL}^{-1}$. Upon increasing the molecular weight, the antimicrobial activity was lost, while the hemolytic activity HC_{50} was about the same for all molecular weights. This molecular weight dependence has been previously observed^[13] and a recent study from our group suggests that this is related to a sieving effect of the outer cell wall of Gram-positive organisms.^[27]

Effect of the counterions: As it was found that diamine_3k was the most active polymer of the series, the ion exchange studies were performed with this sample. As a result of the

Table 1. Characterization of SMAMP homopolymers.

Sample	$M_{n,Target}$ ^[a] [g mol^{-1}]	GPC M_n ^[a] [g mol^{-1}]	M_w/M_n ^[a]	MIC_{90} ^[b] [$\mu\text{g mL}^{-1}$]		HC_{50} ^[b] [$\mu\text{g mL}^{-1}$]	Selectivity ^[b]	
				<i>E. coli</i>	<i>S. aureus</i>		<i>E. coli</i>	<i>S. aureus</i>
diamine_3k	3000	8900	1.15	>200	15	1000	<5	67
diamine_5k	5000	12900	1.08	>200	25	1000	<5	40
diamine_10k	10000	22500	1.08	>200	50	1000	<5	20
diamine_50k	50000	77000	1.07	>200	200	2000	<10	10

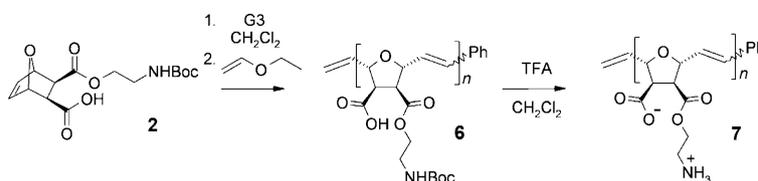
[a] GPC analysis was performed on the protected polymers **4** (in DMF, 0.01 M LiCl, calibrated with polystyrene standards). [b] Biological properties (minimum inhibitory concentration, hemolytic activity and selectivity) were determined for the corresponding deprotected polymers **5**, with TFA counterions.

deprotection conditions, the initial counterion of the polymer was TFA. By passing the polymer solution through an ion exchange column, TFA was exchanged for hydroxide. Immediately after the ion exchange, excess of the acid form of the target counterion (HX) was added. This neutralized the hydroxide before side reactions such as transamidation or base-catalyzed ester hydrolysis of the side chains could occur, and left X^- as a counterion. Excess acid was removed by dialysis. Using this method, the hydrophobic organic counterions X^- = hexanoate, dodecanoate, benzoate, and tosylate were introduced. $^1\text{H NMR}$ spectra of the freeze-dried polymers revealed that the counterion exchange was successful. These hydrophobic organic counterions were chosen because we expected that their distribution coefficient⁵ would be high. Thus, unlike more hydrophilic counterions, such as TFA, they would not diffuse away from the polymer backbone and the effect of the counterion on the antimicrobial properties would be observed in the MIC experiment. The MIC₉₀ results for this series of ion-exchanged polymers are summarized in Table 2. To investigate the effect of a co-

Table 2. Minimum inhibitory concentration of the zwitterionic and ion-exchanged polymers, determined for the deprotected polymers; ion-exchanged polymers are referred to by their counterion, for example, tosylate_3k is the 3000 g mol^{-1} diamine polymer with a tosylate counterion. Diamine_3k has the original TFA counterion.

Sample	MIC ₉₀ [$\mu\text{g mL}^{-1}$]	
	<i>S. aureus</i>	<i>E. coli</i>
diamine_3k	15	>200
tosylate_3k	>200	>200
benzoate_3k	>200	>200
hexanoate_3k	>200	>200
dodecanoate_3k	>200	>200
zwitterion_3k	50	>200
zwitterion_10k	>200	>200

valently attached “intramolecular counterion”, which certainly would not be able to diffuse away from the polymer backbone, the monoester **2** was also polymerized, yielding polymer **6**. After deprotection with TFA, the zwitterionic polymer **7** was obtained (Scheme 2). That MIC data is also included in Table 2. In the following, the 3000 g mol^{-1}



Scheme 2. Synthesis of the zwitterionic polymer **7**.

⁵ In this context, we define the distribution coefficient as the counterion concentration near the nonpolar polymer backbone divided by the counterion concentration in the bulk polar buffer medium of the MIC experiment.

sample of polymer **7** will be referred to by zwitterion_3k for the reader's convenience.

Table 2 shows that the presence of the organic counterions does indeed influence the antimicrobial activity of the polymers, although not in the expected direction. From the results of previous studies,^[13] we expected that adding hydrophobicity to the very hydrophilic diamine_3k through the counterions would increase its antimicrobial activity against both *E. coli* and *S. aureus*. Instead, the results show that all polymers with hydrophobic counterions stayed inactive against *E. coli* and were significantly less active against *S. aureus* than diamine_3k, although unfortunately no discrimination between their relative activities within the range below 200 $\mu\text{g mL}^{-1}$ could be made. Not only does this data confirm that the hydrophobic counterions stay close to the polymer backbone, but in fact they bind so strongly that they eliminate the antimicrobial activity.

There are two possible interpretations for this. First, the hydrophobic counterions could reduce the polymer solubility to such an extent that the SMAMP is unable to interact with the bacteria membrane and thus appears inactive in the MIC experiment. Second, the counterions could form such a tight ion pair with the ammonium groups that the overall positive charge of the polymer is effectively masked and the SMAMP is rendered inactive. One way to differentiate between these two possibilities is to perform dye-leakage experiments.^[28] These experiments are designed to probe the membrane activity of a compound. A polymer that is only poorly soluble will still be active in dye-leakage experiments, because these studies are much more sensitive than the MIC experiment. An example of a polymer that is poorly soluble and therefore inactive in the MIC experiment, but retains significant membrane activity in the dye leakage experiment, is the previously reported hexyl_3k.^[13] However, if the counterions of the ion-exchanged polymers mask the positive charge of the polymer backbone, these polymers will be inactive both in the MIC and the dye-leakage experiments. Using standard procedures,^[28] dye-loaded unilamellar vesicles made from cardiolipin, the predominant component of the plasma membrane of *S. aureus* bacteria, were obtained. Leakage of the self-quenching dye from the vesicle led to fluorescence, which was monitored as a function of time.^[28] The vesicles were exposed to a 20 $\mu\text{g mL}^{-1}$

solution of the ion-exchanged polymers at $t=100$ s.⁶ The resulting dye-leakage curves are plotted in Figure 2 as % Leakage, normalized to the standard Triton X-100, versus time. The dye-leakage results from the *S. aureus* mimicking vesicles close-

⁶ The polymer concentration in the dye-leakage experiments is 20 $\mu\text{g mL}^{-1}$. This makes the ratio of polymer to vesicles much higher than the ratio of polymer to bacteria in the MIC experiment. Thus, although this polymer concentration is lower than in the MIC experiments, the much higher ratio of polymer to vesicle concentration would be expected to show leakage if the molecules are membrane disruptive.

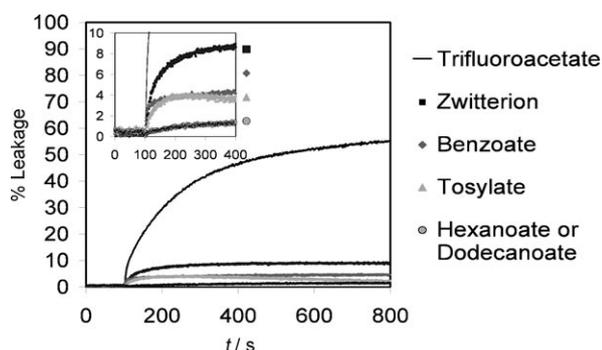


Figure 2. Dye-leakage curves obtained upon adding SMAMP solutions to cardiolipin model vesicles (*S. aureus* mimics). The polymer solutions were added at $t=100$ s ($c_{\text{final}}=20 \mu\text{g mL}^{-1}$). All leakage curves were standardized using Triton X-100 (added at $t=800$ s), which gave 100% leakage.

ly follow the MIC trend against those bacteria: the polymers with hydrophobic counterions were membrane-inactive ($<5\%$ leakage), whereas the parent polymer diamine_3k showed membrane activity ($>50\%$ leakage).

It is instructive to compare this data, especially that of the hexanoate_3k polymer, with the previously mentioned hexyl_3k polymer. As illustrated in Figure 3, the polymers

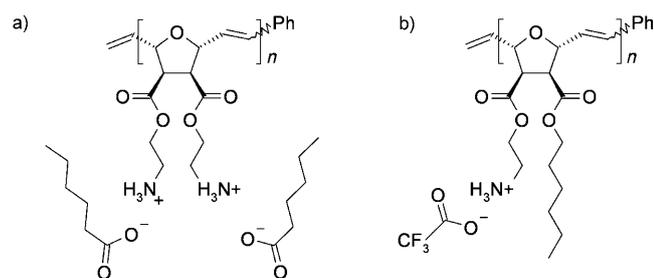


Figure 3. Structural comparison between a) hexanoate_3k and b) hexyl_3k.

are structurally similar, as each contains one hexyl chain per amine group; however hexyl_3k ($\text{MIC}_{90} > 200 \mu\text{g mL}^{-1}$) causes 38% leakage at $20 \mu\text{g mL}^{-1}$ (see the Supporting Information), whereas hexanoate_3k only causes $<5\%$ leakage. This supports the second interpretation, that the hydrophobic counterions form tight ion pairs and thereby dramatically reduce the positive charge of the polymer, resulting in a loss of membrane activity and, consequently, of the antimicrobial activity. While this means that exchange of the counterions does not provide an additional handle to tune the antimicrobial activity, our dye-leakage studies support Kanazawa's reasoning^[22]—the tighter the polymer-counterion pair, the lower the antimicrobial activity, because the polymer is no longer cationic enough.

Interestingly, the zwitterionic sample (zwitterion_3k), with an MIC of $50 \mu\text{g mL}^{-1}$, was more membrane active than the ion-exchanged samples (Figure 2), but less active than the diamine_3k polymer. We assume that this is also due to the formation of ion pairs. Whereas the other anions could diffuse freely to the positive charges on the polymer

backbone, this is not possible for the built-in counterions of the zwitterionic sample. However, because the zwitterionic polymers are structurally irregular,⁷ this leads to an overall irregular charge distribution along the backbone, leading to local cationic and anionic patches, as illustrated in Figure 4. Due to these residual charges, neither the antimicrobial activity nor membrane activity of zwitterion_3k vanishes completely.

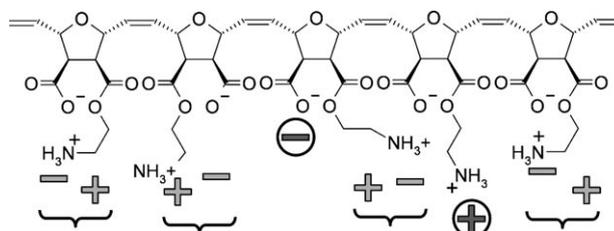


Figure 4. Cartoon representation of local charge surplus for zwitterion_3k. Parentheses show the neutral ion pairs.

Charge variation by copolymerization: Al-Badri et al. recently investigated a ROMP-based polymer system with one to three primary amines per repeat unit and their impact on the antimicrobial properties.^[17] While it was clearly observed that the hemolytic activity decreased significantly with increasing amine content, the effect of charge on the MIC_{90} remained inconclusive, possibly because the changes in the repeat unit structure of this polymer series not only influenced the charge distribution, but also the overall hydrophobicity of each polymer. In the system presented here, we diluted the charge per repeat unit gradually from two to one by copolymerizing the diamine monomer 3 with structurally similar monoamine-alkyl monomers (Figure 5).^[13]

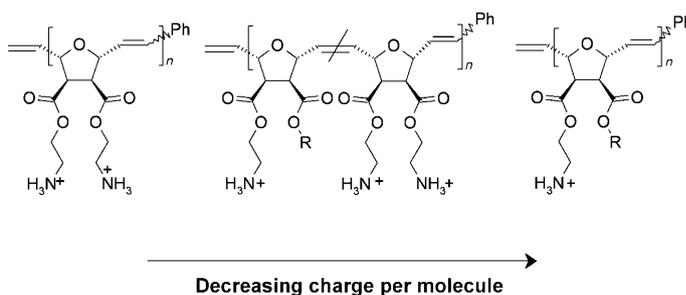


Figure 5. Structure of model polymers with gradually decreasing molecular charge; R = methyl to butyl. For R = methyl, these polymers have constant hydrophobicity.

We synthesized four series of copolymers, with R varying from methyl to butyl on the monoamine-alkyl monomer. The polymer characterization data of the protected copoly-

⁷ The asymmetry of the zwitterionic monomer leads to two stereoisomers during monomer synthesis. This, in addition to the random configuration of the double bond (*cis* or *trans*), yields an irregular tacticity of the repeat units in the polymer.

Table 3. Copolymer characterization.

Sample	Co-monomer	M_n^{Target} ^[a] [g mol ⁻¹]	GPC M_n^{a} [g mol ⁻¹]	M_w/M_n^{a}	MIC ₉₀ ^[b] [μg mL ⁻¹]		HC ₅₀ ^[b] [μg mL ⁻¹]	Selectivity ^[b]	
					<i>E. coli</i>	<i>S. aureus</i>		<i>E. coli</i>	<i>S. aureus</i>
diamine_3k	–	3000	8900	1.15	>200	15	1000	5	66
M1:D9	methyl	3500	17355	1.03	>200	12.5	1400	<7	112
M1:D1	methyl	4100	15400	1.07	>200	6.25	1400	<7	224
M9:D1	methyl	4600	17400	1.07	>200	4	2600	<13	650
methyl	–	3000	11200	1.08	200	100	2000	<10	20
E1:D9	ethyl	4600	13100	1.06	>200	50	3100	<15.5	62.0
E1:D1	ethyl	4100	14700	1.08	>200	15	1400	<7.0	93.3
ethyl	–	3000	9200	1.10	50	50	1400	28	28
P1:D9	propyl	4600	13700	1.06	>200	15	4800	<24.0	320.0
P1:D1	propyl	4200	13900	1.05	>200	50	1400	<7.0	28.0
propyl	–	3000	9200	1.10	6.25	25	50	8.3	3.3
B1:D9	butyl	4600	14200	1.06	>200	15	1200	<6.0	80.0
B1:D1	butyl	4300	14700	1.07	<15	25	<50	>3.3	<2.0
butyl	–	3000	11500	1.08	15	25	<50	<3.3	<2.0

[a] GPC analysis was performed on the protected polymers (DMF, 0.01 M LiCl, polystyrene standards). [b] Biological properties (minimum inhibitory concentration, hemolysis and selectivity) were determined for the corresponding deprotected polymers.

mers, as well as the biological data of the deprotected copolymers, are summarized in Table 3 and Figure 6. The data for the previously published homopolymers is included for comparison.^[13] M9:D1 refers to a copolymer with a molar ratio of methyl/diamine = 9:1.

As these data indicate, all the methyl and ethyl copolymers are nonhemolytic (Figure 6a and b) and inactive against *E. coli* bacteria, whereas the propyl and butyl copolymers become very hemolytic, but at the same time

more active against *E. coli* with increasing alkyl comonomer content (Figure 6c and d). This suggests that the properties of these polymers are dominated by the hydrophobicity of those R groups. Meanwhile, the methyl copolymers are evidently the least hydrophobic as observed by their limited activity against *E. coli* even for the methyl homopolymer, and their high HC₅₀ values. This lack of activity due to limited hydrophobicity is consistent with reverse phase thin-layer chromatography results, and with the polymer solubilities in water and DMF, which showed that the polarity of the monoamine–methyl homopolymer closely resembled that of the diamine homopolymer, while the ethyl to butyl derivatives were significantly more hydrophobic.

These studies confirm that the methyl copolymers are the most appropriate model system to investigate the effect of charge density on biological activity, and we will restrict ourselves to the discussion of the monoamine–methyl homopolymer series for the rest of the paper.

Starting with diamine_3k, which has an HC₅₀ of 1000 μg mL⁻¹, the hemolytic activity decreases across the series to M9:D1 with an HC₅₀ of 2600 μg mL⁻¹. This was paralleled by an increase in the antimicrobial activity from 15 to 4 μg mL⁻¹ against *S. aureus* for the same polymers (see Table 3). When going further down in charge (M9:D1 to the monoamine–methyl homopolymer), there is a sudden jump in the MIC from 4 to 100 μg mL⁻¹, indicating that the optimum value for both the HC₅₀ and the MIC₉₀ against *S. aureus* is obtained for M9:D1.

The sudden jump in the MIC data when decreasing the charge from on average 1.1 to 1.0 per repeat unit is similar to data published by Al-Badri et al.^[17] They observed a distinct improvement of the hemolytic properties when increasing the number of amines per repeat unit from one to two in their polyB series, while a further increase to three amines did not improve the HC₅₀ value.^[17] Apparently, this system had a minimum charge threshold (>1.0) that needed to be crossed to obtain decent selectivities. A similar charge threshold for activity against *S. aureus*, combined with ap-

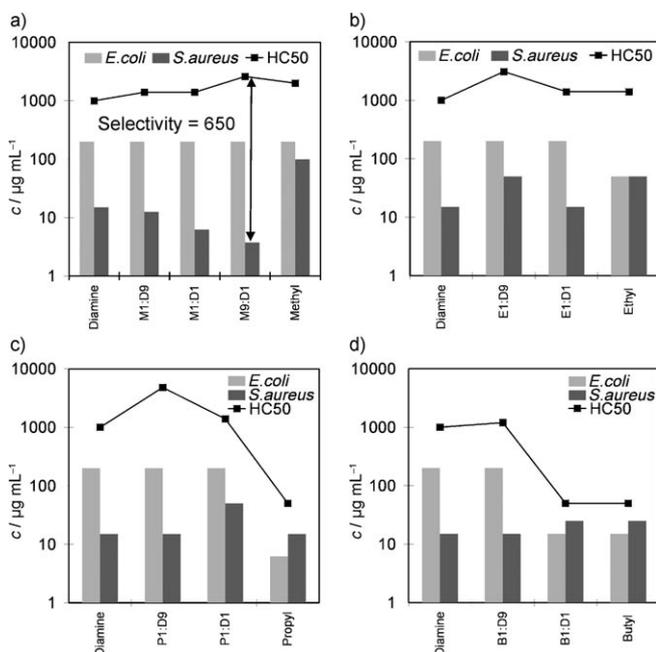


Figure 6. Antimicrobial and hemolytic properties (MIC₉₀ in μg mL⁻¹ against *Escherichia coli* and *Staphylococcus aureus* and HC₅₀ in μg mL⁻¹ of human red blood cells) of diamine–monoamine copolymers: a) diamine–methyl, b) diamine–ethyl, c) diamine–propyl, and d) diamine–butyl. M9:D1 refers to a copolymer with a molar ratio of methyl:diamine = 9:1. The 3000 g mol⁻¹ monoamine homopolymers of each monomer are included for comparison.

propriate amphiphilicity, was observed in a comparative study of an AMP library.^[30] Assuming that such a minimum charge threshold also exists for our system, the methyl copolymers presented here seem to be all above the threshold, as their properties are likewise not much affected by increasing the charge from 1.1 to 2 charges per repeat unit, while the monoamine–methyl homopolymer (1.0 charges per repeat unit) is much less active against *S. aureus*; because it is below this critical threshold. On the molecular level, this postulated charge threshold translates into a minimum charge that is necessary to trigger successful attachment of the SMAMP to the bacterial membrane. Once enough charge is present to enable this attachment, the hydrophobicity of the molecule will then determine to what extent the SMAMP is active. This is clearly observed for the diamine_3k to monoamine–methyl homopolymer series. As more monoamine–methyl monomer is added, the MIC₉₀ steadily decreases because these copolymers are above the charge threshold and slightly more hydrophobic; however, the monoamine–methyl homopolymer is less antimicrobial despite being the most “hydrophobic” because it is below the charge threshold.

An intriguing aside of this study is the finding that the methyl copolymers and diamine_3k are not only selective for bacterial over mammalian cells, but also for *S. aureus* over *E. coli* (Figure 6a). Further MIC experiments with M9:D1 and other bacteria revealed that this polymer has a Gram selectivity: while inactive against Gram-negative *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (MIC₉₀ = 100 µg mL⁻¹ for the last two organisms), it was found to be active against Gram-positive *S. aureus*, *S. epidermis* and even the multiresistant MRSA (MIC₉₀ = 12.5 µg mL⁻¹).

Conclusions

In this study, several important parameters that influence the antimicrobial and hemolytic activity of SMAMPs were investigated. We demonstrated that exchanging the hydrophilic counterions of a ROMP-derived diamine polymer by hydrophobic organic counterions drastically reduced the antimicrobial properties of that polymer. It was shown by dye-leakage studies on model vesicles that this loss in antimicrobial activity was due to the loss of the positive charge of the polymer backbone by ion-pair formation with the counterions. It is expected that by a partial exchange of the counterions, which would add hydrophobicity but at the same time retain the required minimum molecular charge, polymers with tunable properties—between those of the active diamine_3k polymer and the inactive hexanoate_3k polymer—could be obtained; however, this is difficult to realize and quantify experimentally.

Copolymerization of two structurally similar monomers with one and two charges, respectively, led to a series of polymers with gradually decreased charge density but more or less constant hydrophobicity. When compared to litera-

ture data, these results seem to indicate that, for a system of a given amphiphilicity, a minimum threshold of charge is required for obtaining favorable HC₅₀ values and activities. Above that threshold, the biological properties of the polymer are not affected by further charge increase. For the particular diamine–methyl copolymer system investigated here, this threshold is reached for M9:D1, with an average charge of 1.1 per repeat unit. In the process of optimizing the charge density of the copolymer system, a polymer with an impressive selectivity of 650 for *S. aureus* over human red blood cells was obtained. Additionally, this polymer was shown to be Gram-selective for all bacterial strains tested, including MRSA, which makes this SMAMP a promising candidate for materials applications. The reason for the intriguing double selectivity has been studied and published separately.^[27]

Experimental Section

All experimental details are given in the Supporting Information.

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