Sequence control as a powerful tool for improving the selectivity of antimicrobial polymers

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Abstract

Antimicrobial polymers appear as a promising alternative to tackle the current development of bacterial resistance against conventional antibiotics as they rely on bacterial membrane disruption. This study investigates the effect of segmentation of hydrophobic and cationic functionalities on antimicrobial polymers over their selectivity between bacteria and mammalian cells. Using RAFT technology, statistical, diblock and highly segmented multiblock copolymers were synthesized in a controlled manner. Polymers were analysed by HPLC and the segmentation was found to have a significant influence on their overall hydrophobicity. In addition, the amount of incorporated cationic comonomer was varied to yield a small library of bioactive macromolecules. The antimicrobial properties of these compounds were probed against pathogenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*) and their biocompatibility was tested using hemolysis and erythrocyte aggregation assays, as well as mammalian cell viability assays. In all cases, diblock and multiblock copolymers were found to outperform statistical copolymers, and for polymers with a low content of cationic co-monomer, the multiblock showed a tremendously increased selectivity for *P. aeruginosa* and *S. epidermidis* compared to its statistical and diblock analogue. This work highlights the remarkable effect of segmentation on both, the physical properties of the materials as

well as their interaction with biological systems. Due to the outstanding selectivity of multiblock copolymers towards certain bacteria strains, the presented materials are a promising platform for the treatment of infections and a valuable tool to combat antimicrobial resistance.

Introduction

As an increasing number of studies emphasize the alarming situation concerning life-threatening infectious diseases caused by antibiotic resistant bacteria,[1-3] health organizations urge for the discovery of novel antibiotics.[4-6] The development of antimicrobial resistance (AMR) is partly due to the narrow range of available antibiotics[7] which have reached their limitations in infection treatment because of their high target specificity. In such context, antimicrobial peptides (AMPs) have recently attracted interest as they were shown to target bacterial membranes instead of individual proteins.[8,9] Present in the innate immune systems of various organisms, these peptides have an amphipathic structure which can adopt a facially amphiphilic arrangement with hydrophobic groups on one side and cationic moieties on the other side of the molecule.[8] Although the precise mechanism of bacterial killing is still under investigation, the cationic groups of the AMPs are thought to bind to the negatively charged phospholipids of bacterial membranes *via* electrostatic interactions, upon which the hydrophobic functionalities would induce membrane disruption and lead to cell death.[8] Due to the mostly non-charged surface of mammalian cells, AMPs exhibit good selectivity towards bacterial membranes. However, their isolation or production on a large scale is expensive and their peptidic nature renders them vulnerable *e.g.* to protease based countermeasures.[10] In order to overcome these issues, a wide range of synthetic mimics have been developed in recent years from oligomers to polymers using different methodologies.[11] The key structural parameters which were found to affect antimicrobial activity were the choice and balance of cationic to hydrophobic moieties, the molecular weight, the nature of the charge and the structure of the polymer backbone.[12-23] As the main specifications for antimicrobial potency have been elucidated, current research focuses on reducing the toxicity of synthetic antimicrobial peptides (SAMPs) against mammalian cells, and more interestingly towards red blood cells (RBCs), by investigating new structural parameters.[24-26]

The activity of peptides is highly dependent on their structural organization,[8,27] and mimicking this feature, i.e. by self-assembly into nano-sized objects is the next substantial challenge in improving the performance of SAMPs. The morphology of core-shell nano particles[28] did not affect the antimicrobial activity, whereas the size of PEGylated nanoparticles[29] reduced both the antimicrobial and the hemolytic activity. However, the effect of the shape on the toxicity against mammalian cells was not addressed in those studies. Similarly, flower-like micelles[30],star-shaped polymers[24] and hyperbranched polymers[31] were shown to be efficient at disrupting bacterial membrane whilst maintaining low hemolysis values.

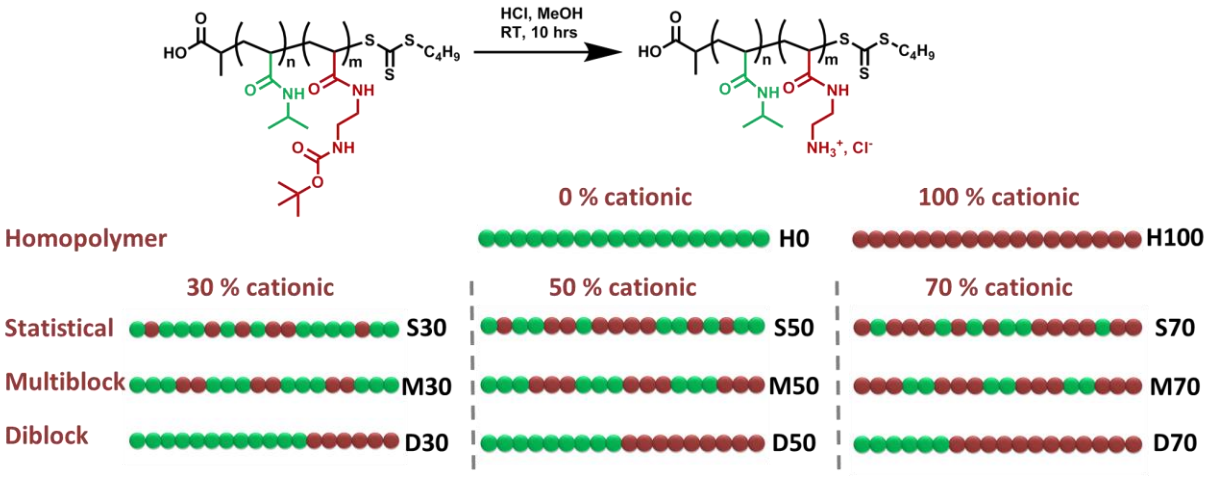
Intramolecular interactions of SAMPs were analyzed to a higher extent, since the helical structure of certain AMPs was thought to be responsible for their activity. However, it was demonstrated that this conformation was not required for SAMPs to exhibit antimicrobial activity. Instead, an amphiphilic structure, able to adapt to the cell surface was sufficient for polymers to induce bacterial membrane penetration, according to Mowery and co-workers.[19] The importance of the conformation of the polymers in the potency of SAMPs was highlighted by Nguyen *et al.*[32] using single-chain nanoparticles. Similarly, unimolecular aggregates with a cationic shell and a hydrophobic core obtained from the folding of amphiphilic copolymers were studied by Oda and co-workers.[33] The diblock copolymers exhibited a lower hemolytic activity than their random counterpart but an increased hemagglutination, demonstrating the effect of the conformation of single polymeric chains on their interactions with cells.

The effect of polymer architecture in SAMPs was investigated by the comparison of diblock copolymers with the statistical[34] and gradient[35] counterparts. The level of hemolytic activity was decreased for diblock copolymers in both cases without observing any effect on antimicrobial properties. However, the self-assembly of these systems was not analysed, therefore, the difference in the hemolytic activity is not necessarily attributed to the sequence but could be related to micelle formation. Indeed, the sequence not only strongly affects the self-assemblies of the polymers, but also the folding of the polymer chains, hence affecting their physico-chemical properties.[36,37] As previously discussed, inter and intramolecular assemblies of SAMPs seem to have an important effect on their antimicrobial activity and hemocompatibility, but the impact of the monomer sequence beyond diblocks and gradient copolymers on these properties has, to the best of our knowledge, yet to be determined for polymers.
By using multiblock copolymers, systems with a controlled monomer sequence can be obtained.[38] By varying the degree of segmentation of functional units within a polymer chain an in-depth structure-activity relationship of the sequence of SAMPs on the antimicrobial activity could be established. Well-defined multiblock copolymers have been synthesized *via* various Living Radical Polymerization (LRP)[39,40] techniques such as Cu(0)-mediated radical polymerization[41,42] or Reversible Addition-Fragmentation chain Transfer (RAFT).[43,44] The latter was proven to be a versatile and robust process,[39] compatible with a wide range of monomers and solvents to obtain polymers with narrow molecular weight distributions.[40] Furthermore, the optimization of the reaction conditions allows for the sequential one-pot synthesis of multiblock copolymers.[45] As the up-scaling of these synthetic processes has recently been explored by using tubular reactors, the availability of SAMPs with a precisely defined structure is not an issue.[46,47]
The current work focuses on the investigation of multiblock copolymers, which represent an intermediate between diblock and statistical copolymers on their performance as SAMPs.

Results and discussion

Design and synthesis of SAMPs. Acrylamides were chosen as a monomer class for this study as their high propagation rate (k_p) allows the synthesis of multiblock copolymers in a straight forward manner and their non-degradable nature.[44,48] The hydrophobic monomer which was selected for the synthesis of SAMPs was *N*-Isopropylacrylamide (NIPAM) as it mimics the structure of Leucine. Furthermore, its hydrophobicity is relatively low compared to monomers which have been used in previous studies,[49,50] and should therefore, lead to a good hemocompatibility.[51] The cationic amino-acid mimic chosen for the design of antimicrobial polymers was an acrylamide-based Lysine mimic: Amino-EthylAcrylamide (AEAM). To avoid potential aminolysis of the trithiocarbonate group of the CTA during the polymerisation process, the Boc-protected equivalent of the monomer (Boc-AEAM)[52] (Fig. SI-1 and SI-2) was polymerised and a post-polymerisation deprotection method was used to obtain functional SAMPs.

As we were interested in the influence of the monomer distribution along the chain on antimicrobial activity and toxicity against RBCs, statistical, diblock and multiblock copolymers (Scheme 1) were synthesised *via* RAFT polymerization using different ratios of hydrophobic to cationic pendant groups. The SAMPs were named according to their degree of segmentation (H, S, M and D for homopolymer, statistical, multiblock and diblock copolymers respectively) and their content of cationic co-monomer in % (0, 30, 50, 70 and 100) with protected polymers labelled BOC.



Scheme 1. Schematic representation of structure, composition and segmentation of synthesized polymers

As the bacterial cell wall might be impermeable to long polymers and hemocompatibility decreased with increasing molecular weight the targeted molecular weight of the polymer had to be chosen

carefully.[53] In this study, the degree of polymerization (DP) was set to 100, as the maximum degree of segmentation achievable using a RAFT-based multiblock approach scales with the length of the polymer chain.

The shortest block length that was targeted was DP 10, since it was shown that for DPs below 6, a significant number of chains would fail to contain the total number of blocks, according to an analysis of the statistical nature of polymer chain growth.[54] By taking this limitation into consideration, multiblock copolymers were designed with the highest number of blocks compatible for each composition. Therefore, seven blocks were targeted for 30 and 70 % BocAEAM content, ($M30^{BOC}$ and $M70^{BOC}$ respectively) and ten blocks for $M50^{BOC}$ which has 50 % of BocAEAM (Table SI-1) each in an alternating fashion. From the design of these polymers, modifying the structure from statistical to multiblock and diblock copolymer will demonstrate the effect of having cationic and hydrophobic domains of variable lengths, and segregations, whilst maintaining the overall DP and chemical composition.

(Propanoic acid)yl butyl trithiocarbonate (PABTC) was chosen as the Chain Transfer Agent (CTA) as it has previously been used to synthesise multiblock copolymers by maintaining a high livingness.[45] Additionally, the choice of a carboxylic acid based end group has been shown previously to favour lower levels of hemotoxicity.[55] Optimization of the reaction conditions allowed to reach full conversion after each block according to ^{1}H NMR spectroscopy (Fig. SI-3 − 8), to achieve a sequential one-pot synthesis of the protected statistical, diblock and multiblock copolymers. The size distribution of the protected polymers as determined by SEC was narrow for all synthesized architectures (Table SI-7, Fig. 1A, Fig. SI-9 − 10). The deprotected polymers could not be analyzed by SEC as they were not soluble in the available eluents. The polymers were then quantitatively deprotected using hydrochloric acid as supported by the disappearance of the signal associated with the BOC-protecting groups in ^{1}H NMR spectra (Fig. 1B, Fig. SI-11 − 12) as well as by the shift of CH_2 protons associated with the amine pendant groups.

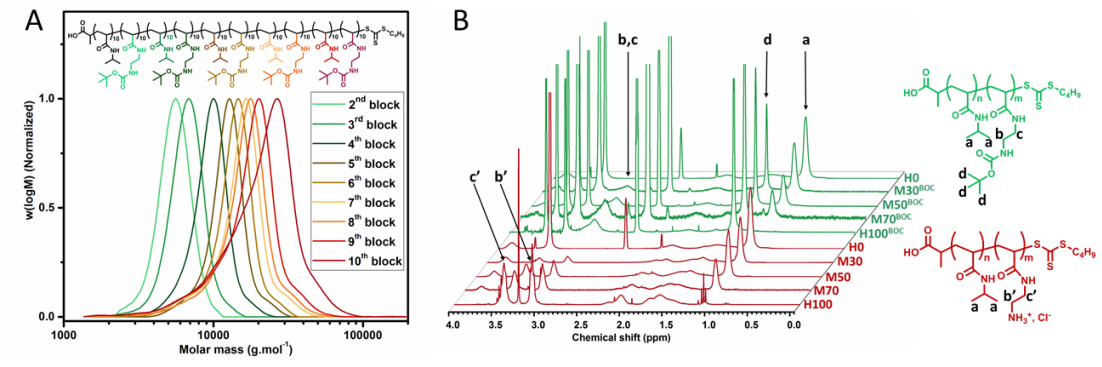


Figure 1. A) DMF-SEC chromatograms for successive chain extensions of $M50^{BOC}$; B) ^{1}H NMR spectra of SAMPs on the example of homopolymers and multiblock copolymers before and after deprotection in $DMSO_d$ and D_2O respectively.

Physico-chemical properties of SAMPs. As electrostatic interactions play a major role in the binding of SAMPs to bacterial membranes, the polymers have to maintain their positive charge in a physiological environment. The cationic properties of the primary amines at physiological pH was confirmed by potentiometric titration. Furthermore, by comparing the behaviour of statistical, diblock and multiblock copolymers of similar composition, the effect of segmentation on the pK_a of the pendant groups can be evaluated. The window of pK_a of the primary amines of H100 (Fig. SI-13) ranges from 8 to 11, which was similar to the pK_a of the amine group of Lysine at 8.9.[56] The range of pK_a observed for the three copolymers was similar to that of the homopolymer. The majority of the primary amines are protonated at physiological pH, rendering the polymers positively charged. However, on the titration curves of the polymers (Fig. SI-13), a lower change in the pH was observed with the statistical copolymer S30 compared to D30 and M30, when the same amount of base was added. Therefore, S30 would have a higher buffering capacity than its diblock and multiblock counterpart. Indeed, the deprotonation of the primary amines could be facilitated when the groups are statistically distributed along the polymer backbone.

While positively charged groups are necessary for the antimicrobial activity of the SAMPs, the balance between hydrophilic and hydrophobic groups has arguably the most significant impact on their selectivity.[11,12] For this reason, the effect of segmentation on this property of SAMPs was analyzed by High Performance Liquid Chromatography (HPLC). Non water-soluble diblock and statistical copolymers have previously been studied by HPLC, showing that the elution volume varied between the two different structures with a similar composition.[57] However, to the best of our knowledge, this remains the only investigation of the influence of copolymer architecture on the amphiphilic balance. A comparison of the amphiphilicity of the various polymers can be established by monitoring their elution characteristics, as a higher elution volume indicates a more hydrophobic polymer in a reversed phase system (Fig. SI-14 and SI-15).

For a set architecture (statistical, diblock or multiblock), content of THF necessary to elute the polymer decreases with the cationic content (Fig. 2). As expected, the hydrophilicity increases with the amount of positive charges along the polymer chain. As NIPAM shows a cloud point behaviour in water[58] measurements at 20 and 60 °C (Fig. SI-16) were carried out and revealed no significant difference in the elution volume. For a fixed composition, the hydrophobicity varies with the architecture of the SAMPs, with diblock copolymers being most hydrophobic, followed by the multiblock and finally, the statistical counterpart. Interestingly, this trend was observed for all three compositions (30, 50 and 70 % cationic). As the multiblock represents an intermediate level of charge segregation between diblock and statistical copolymers, the order at which the polymers elute matches the monomer distribution. This observation demonstrates that the hydrophobicity of the polymers does not only depend on their

composition but also on the monomer sequence and distribution of isopropyl functionalities along the polymer backbone, which could have an impact on their bioactivity.

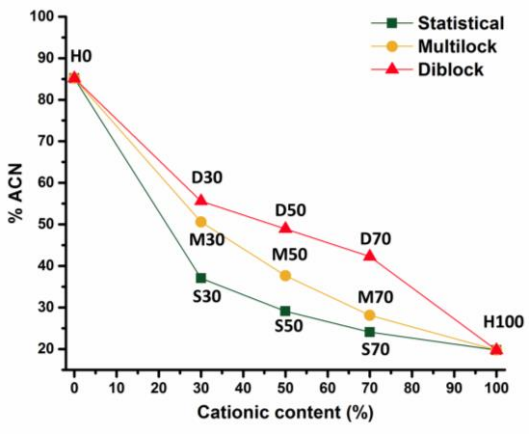


Figure 2. Elution characteristics of the SAMPs depending on the composition and the architecture by HPLC (▲ Diblock copolymers, ● Multiblock copolymers, ■ Statistical copolymers).

As the amphiphilic properties of the polymers might induce self-assembly,[59] Dynamic Light Scattering (DLS) was used to investigate the solutions of polymer at 37 °C in Phosphate Buffer Saline (PBS) (Fig. SI-19, Table SI-8). The measurements revealed no phase segregation for cationic copolymers, whereas particle formation was observed with H0. Therefore, the positively charged primary amine groups appear to prevent any particle formation, even for diblock copolymers, which could be a result of the electrostatic repulsion. This further supports that the variation in hydrophobicity depending on the polymer architecture is not associated with intermolecular interactions.

Dye leakage study. Before investigating the antimicrobial activity, the ability of the SAMPs to disrupt bacterial membranes was examined using a dye leakage assay. Liposomes mimicking Gram-positive and Gram-negative bacterial membranes were loaded with a fluorescent dye which signals membrane disruption. All polymers, except for H0, showed an increase in fluorescence against both Gram-positive (Fig. SI-17) and Gram-negative (Fig. 3 and SI-18) membrane models. Additionally, as the charge content of SAMPs increases, so does the induced leakage in most cases, which demonstrates the importance of electrostatic interactions in this process. Statistical, multiblock and diblock copolymers exhibited similar dye leakage profiles. Although this assay is not quantitative as demonstrated by Tew *et al.*,[53] it shows that the SAMPs are membrane active independent of their architecture.

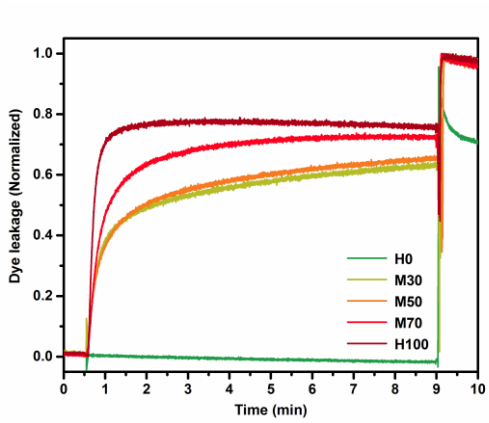


Figure 3. Dye leakage study with multiblock copolymers (M30, M50 and M70) and the homopolymers H0 and H100 on Gram-negative bacteria model. Fluorescence was read at 537 nm (emission) at an excitation wavelength of 492 nm. The sample was added at 30 s measurement time and vesicles were lysed by addition of Triton X at 9 min.

Antibacterial susceptibility assays. Growth inhibition was studied using two strains of Gram-negative bacteria: *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*); and two Gram-positive strains: *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*). For statistical and diblock copolymers, the antimicrobial activity against all four strains increased with the cationic content as shown on Table 1, which is consistent with previous investigations.[15] For multiblocks, no clear trend could be established.

Interestingly, for the set of polymers with 30 % charge, a drastic reduction of the MIC was observed from the statistical copolymer (S30), which was inactive towards most bacterial strains tested (MIC>1024μg mL^{-1}, except against *S. epidermidis* with MIC=32 μg mL^{-1}) , to diblock and heptablock (D30 and M30), showing MIC values as low as 4 ug mL^{-1}. Across the four bacterial strains, Gram-negative bacteria such as *P. aeruginosa* were particularly affected by the polymer architecture, with MICs decreasing from over 1000 (S30) to 32 and 8 μg mL^{-1} for D30 and M30 respectively (Fig. SI-20), which could be due to the difference in the composition[60] of their bacterial cell envelope. Gram-negative bacteria possess an outer membrane (OM), which is a lipid bilayer comprised of an outer leaflet of lipopolysaccharides and of an inner leaflet of phospholipids.[61] As the lipopolysaccharides have long saturated acyl chains (leading to an increased membrane stiffness) combined with hydrophilic saccharides, they offer a protective barrier which renders Gram-negative bacteria difficult targets. The variations in the physico-chemical properties due to the monomer sequence, as monitored by HPLC, could hence alter the disruption of this OM. The presence of hydrophilic domains in the block copolymers, as compared to the broad distributions of the cationic pendant groups in the statistical copolymers might lead to an increased efficiency of the interaction of the macromolecules with the hydrophilic outer layer of the OM. The overall hydrophilicity of the polymer, which was shown to differ

between architectures using HPLC, could also have an influence. However, the improvement of the OM permeabilization of Gram-negative bacteria with segmented systems seems unlikely to be related to the increase in the hydrophobicity of the polymers with the segmentation.

Table 1. Antimicrobial activity, hemolytic activity and hemagglutination of antimicrobial polymers.

Sample	HC_{10} [a] (μg mL^{-1})	c_H [b] (μg mL^{-1})	MIC[c] (μg mL^{-1})				Selectivity[d]			
			E. coli	*P. aeruginosa*	*S. aureus*	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*
H0	> 1024	> 1024	> 1024	> 1024	> 1024	> 1024	> 1	> 1	> 1	> 1
H100	512	16	4	4	4	32	4	4	4	0.5
S30	> 1024	32	> 1024	> 1024	> 1024	32	0.03	0.03	0.03	1
S50	> 1024	32	64	128	8	2	0.5	0.25	4	16
S70	> 1024	32	64	64	4	2	0.5	0.5	8	16
M30	> 1024	> 1024	128	8	64	4	> 8	> 128	> 16	> 256
M50	> 1024	32	1024	64	32	8	0.03	0.5	1	4
M70	> 1024	32	1024	32	4	4	0.03	1	8	8
D30	> 1024	> 1024	512	32	128	32	> 2	> 32	> 8	> 32
D50	> 1024	> 1024	64	64	8	4	> 16	> 16	> 128	> 256
D70	> 1024	> 1024	32	32	8	4	> 32	> 32	> 128	> 256

[a] HC_{10} is the minimum concentration at which at least 10 % of the maximum lysis was observed

[b] c_H is the lowest concentration at which the polymers induce aggregation of RBCs

[c] MIC is the minimum inhibitory concentration at which no visible bacteria growth can be observed.

[d] Selectivity: lowest value between HC_{10} and c_H (hemocompatibility concentration) divided by the MIC of the bacterial strains concerned.

Hemocompatibility of SAMPS. Although the main target is to obtain SAMPs with high potency against bacteria, their toxicity towards mammalian cells has to be minimized to be considered for any human health application. Since blood is the principal vector distributing active compounds to cells, a focus has been drawn to the toxicity of SAMPs towards RBCs. The first assay was directed towards the investigation of the lysis of the RBCs in presence of the polymers, since they were shown to be membrane active according to the dye leakage studies. The hemolytic concentration HC_{10} (concentration to elicit 10 % hemolysis), was determined at concentrations between 2 and 1024 μg mL^{-1} with only H100 disrupting RBCs (Table 1, Fig. SI-21), which is a result of the limited hydrophobicity of NIPAM compared to having pendant alkyl chains of 4 carbons and more.

To obtain a complete picture of the hemocompatibility of the polymers, hemagglutination, which is not necessarily related to hemolysis was studied as well since positively charged polymers can interact with negatively charged sialic acid groups at the surface of RBCs, leading to intercellular binding.[17] The hemagglutination concentration c_H, which is the lowest concentration to induce agglutination of RBCs,[33]

was determined (Table 1,Table SI-9). In line with the study of Locock and co-workers,[17] H100 induced hemagglutination at low concentration, whereas no aggregates were observed for H0, which confirms the hemocompatibility of polyNIPAM. Interestingly, the three diblock copolymers (D30, D50 and D70) as well as M30, had c_H values of over 1000 μg mL^{-1}. Therefore, with similar cationic content, these SAMPs did not induce any hemagglutination, as opposed to their statistical counterparts. These results could be explained by cationic moieties being distributed over the full length of the chain, as for statistical and multiblock copolymers, rendering cross-linking between RBCs more likely as opposed to diblock copolymers for which the charges are concentrated on a single segment of the macromolecule. These results highlight the importance of charge segregation in the interaction with RBCs. Furthermore, according to HPLC data (Fig. 2), the four cationic copolymers which did not induce any hemagglutination, are the most hydrophobic SAMPs, hence a correlation between these parameters is possible.

Using the data obtained by probing the hemocompatibility of the presented polymers, a selectivity value was determined for each bacterial strains using the ratio between the hemocompatibility concentration (which is the lowest value of HC_{10} and c_H, Table SI-10) and the MIC against the specific strains (Table 1, Fig. 4). This value is a powerful tool to assess the potential of antimicrobial substances, as only those with a pronounced activity against bacteria and which do not affect RBCs will have high selectivity values.[18] As none of the SAMPs exhibit distinct hemolytic potency, hemagglutination concentration was used to calculate the selectivity (Equation 1, Table 1).

$$Selectivity = \frac{c_H}{MIC}$$

Equation 1. Determination of the selectivity against RBCs for a specific bacterial strain.

Increasing the cationic content improves the selectivity for statistical and diblock copolymers with all four species of bacteria studied. Fig. 4 illustrates the selectivity of the SAMPs by dividing them into categories with the most inactive and hemotoxic polymers in the bottom-right corner (highlighted in red; IV), most potent and hemocompatible species in the top-left corner (I), and two yellow intermediate zones in the top-right (II) and bottom-left (III) corner being the inactive but hemocompatible polymers and active but hemotoxic ones, respectively. The most selective polymers for the four different bacterial strains appear to be the diblocks (D30, D50 and D70) and the multiblock copolymer M30. The largest variation with segmentation was observed for SAMPs with a charge content of 30%, with S30 being in the red corner (IV) for all the bacterial species, except *S. epidermidis*, as opposed to M30 and D30 which were highly active and hemocompatible. For 50 and 70 % cationic SAMPs, the selectivity also improved from statistical and multiblock to diblock due to an increased hemocompatibility. By altering the polymer sequence, thus charge segregation, the selectivity with RBCs can be drastically modified whilst maintaining the same composition.

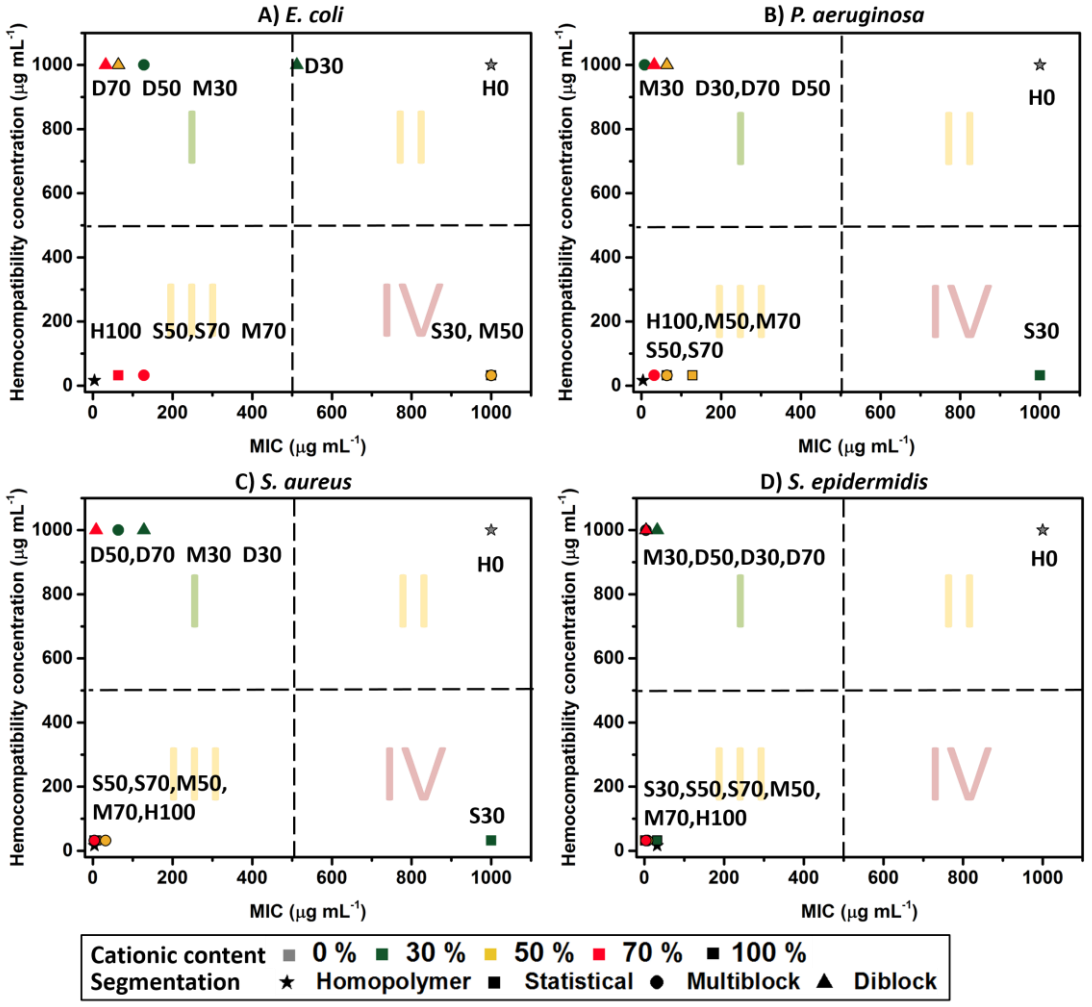


Figure 4. Selectivity of the SAMPs with RBCs against *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C) and *S. epidermidis* (D).

Biocompatibility of SAMPs. Potential applications for the SAMPs include their use as wound dressings or as oral antibiotics. As such, murine embryonic fibroblasts (NIH 3T3) and human colorectal epithelial (Caco2) cells were used to further determine the biocompatibility of the SAMPs. NIH 3T3 are one of the most commonly used fibroblast cell lines, a type of cell that is part of the connective tissue of animals, and is involved in the synthesis of the extracellular matrix thus playing a critical role in wound healing. Additionally, Caco2 cells are well characterised colorectal cells that can be used as a model for intestinal absorption.[62]

To determine the ability of the compounds to inhibit cell proliferation, NIH 3T3 and Caco2 cells were incubated with polymer concentrations ranging from 32 to 1024 μg mL^{-1} for 3 days. As expected, H0 displayed no toxicity at any of the concentrations used, while H100 showed pronounced interference with the cell viability (Fig. SI-22) for both cell lines. The Inhibitory Concentration (IC_{50}) values, which are the polymer concentrations inhibiting growth of 50 % of the cells, were calculated for NIH 3T3 and Caco2 (Table SI-11, Fig. 5) using the toxicity curves from Figure SI-22. As expected, an increasing

content of cationic groups per polymer chain led to an increased toxicity in both cell lines, which is similar to the trend observed with hemotoxicity results. This could be attributed to the membrane activity of the polymers. Additionally, the SAMPs were more toxic on Caco2 cells than NIH 3T3 cells, which might be due to increased uptake by the colorectal cells. According to Fig. 5, for polymers with 30 % of charged co-monomer, the IC_{50} increases from D30 to M30, followed by S30 with Caco2 cells.

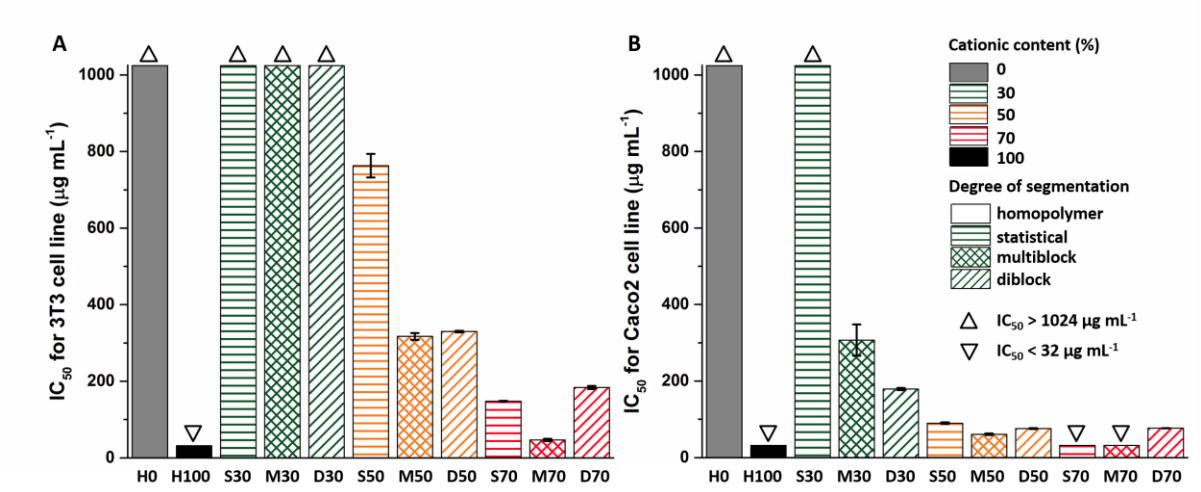


Figure 5. IC_{50} of the SAMPs for A) NIH 3T3 and B) Caco2 cells after incubation at 37˚C for 72 h by XTT assay.

These cytotoxicity results seem to direct potential application of the SAMPs towards skin wound treatment rather than oral use. Therefore, the following discussions relative to cytotoxicity will be based on the results obtained with NIH 3T3 cells. Similarly to the selectivity calculated for the RBCs, the therapeutic index (TI) was obtained from the ratio of the IC_{50} for the mammalian cells to the MIC for each bacteria strain[63] (Equation 2, Table SI-11) and graphs representing the IC_{50} against the MIC illustrated the TI for NIH 3T3 (Fig. SI-23).

$$TI = \frac{IC_{50}}{MIC}$$

Equation 2. Determination of the TI with a cell line for a specific bacteria species.

From Fig. SI-23, M30 appears to be the ideal candidate against the four strains of bacteria when taking into account the toxicity towards NIH 3T3 cells. Indeed, at 30 % charge, the segmentation allowed for the improvement of the selectivity of S30 (being non-toxic but poorly active) to D30 and M30 (exhibiting low toxicity and high potency). Although D50 and D70 were shown to be as selective as M30 with RBCs, their selectivity towards NIH 3T3 was much lower, as displayed in Fig. SI-23.

The overall performance was analysed by illustrating the TI of the SAMPs for NIH 3T3 cells against the selectivity for the different bacterial species over RBCs (Fig. 6 only displays polymers with a value of at least 1 for both parameters, whereas Fig. SI-24 represents the full plot). The most selective

polymers are located in the top-right green corner which represents the SAMPs of selectivity and TI of 10 and above. The highest values regarding the selectivity of the three mammalian cells combined were associated with M30 for both *P. aeruginosa* and *S. epidermidis*.

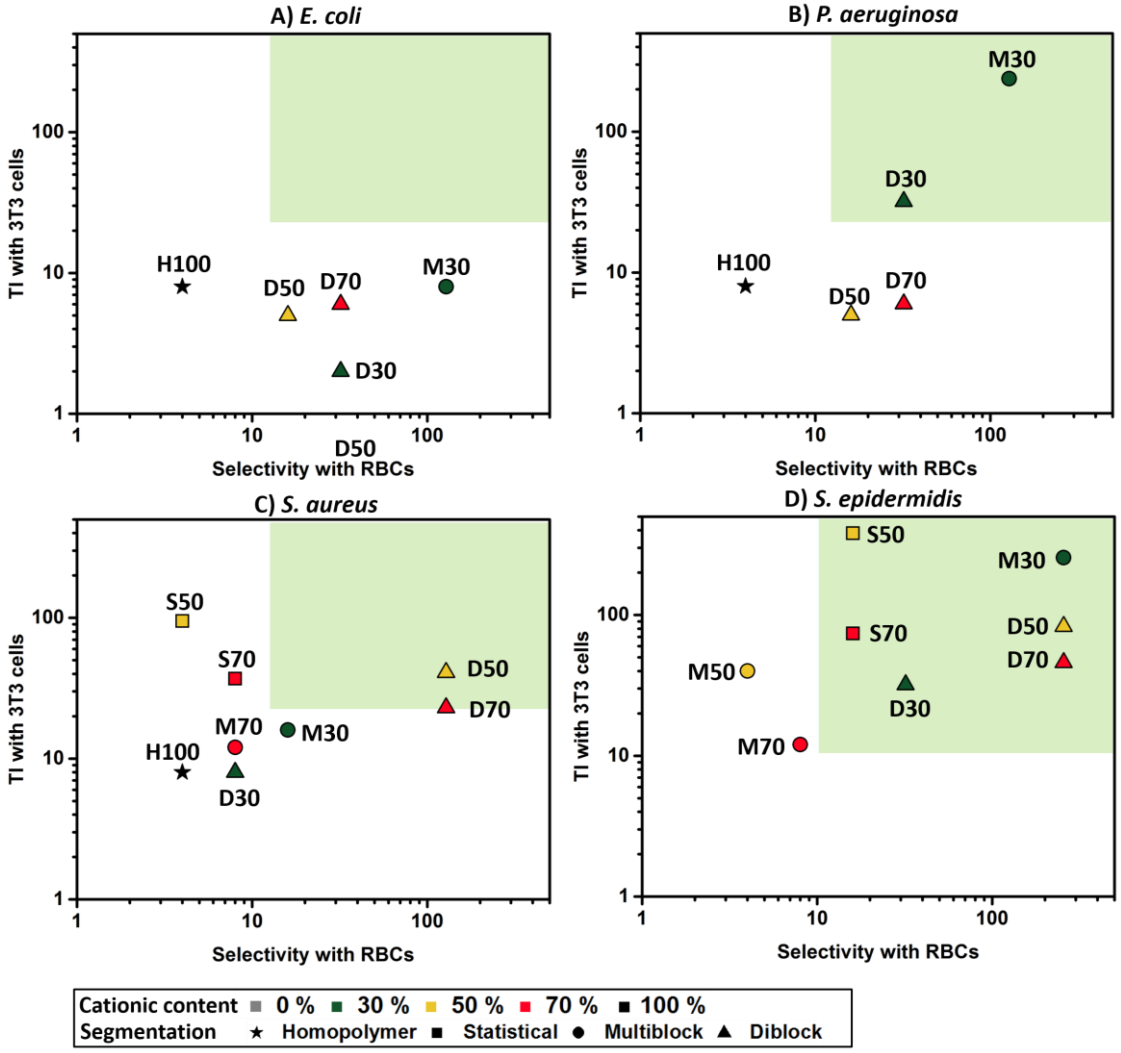


Figure 6. TI of the SAMPs with NIH 3T3 cells against their selectivity with RBCs for *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C) and *S. epidermidis* (D).

This finding highlights the importance of segmentation on the overall performance of these materials as despite similar chemical composition M30 outperforms S30 and D30. The multiblock copolymer shows a low tendency of erythrocyte aggregation and does interfere less with mammalian cell viability as compared to its diblock analogue, while maintaining a high membrane activity against pathogenic bacteria. It needs to be emphasized that presented polymers show a pronounced activity against Gram-negative bacteria despite the presence of an OM which confers a protective barrier to these bacteria. This renders this class of SAMPs a highly promising candidate for the treatment of infections, as the antibiotic pipeline for this type of bacteria is depleting rapidly. Their excellent compatibility with fibroblasts renders them interesting candidates as antimicrobial material in wound healing treatments.

Bacterial resistance. One of the main issues with currently used antibiotics is the ability of bacteria to develop resistance against them, rendering the antibiotics inactive after an extended contact of bacteria with non-lethal doses.[1] However, as previously mentioned, bacteria cannot acquire resistance against SAMPs as easily, since the polymers seem to act directly on the cellular membrane. In order to demonstrate the potential of the SAMPs as an alternative to currently used antibiotics, the development of the MIC was studied for S30, M30 and D30 against a MRSA strain (USA 300) over 4 weeks of exposure at a concentration of a tenth of the MIC (one passage per day). The antibacterial activity did not vary throughout the assay (Fig. SI-25) and no resistant mutants could be detected by incubating the final bacterial suspension. Therefore, the development of resistance against these polymers by bacteria is not easily acquired, which is independent of the segmentation. This indicates a similar, membrane based mechanism for the herein presented SAMPs.

Conclusion.

Synthetic antimicrobial peptides (SAMPs) based on a cationic (AEAM) and a hydrophobic co-monomer (NIPAM) with various degrees of segmentation were synthesised by exploiting RAFT multiblock technology. From our study, the segmentation of the monomer distribution appeared to have an impact on various levels. Firstly, the hydrophobicity of the polymers increased with the length of the blocks, thus introducing an additional handle for tuning of this parameter. Furthermore, antimicrobial activity was dependent on the sequence at low contents of cationic co-monomer, particularly with Gram-negative bacteria, which could be a result of an increased interaction of the cationic moieties with the outer membrane of bacteria, when these functionalities are organised in domains. Additionally, this study has shown that NIPAM is a co-monomer of choice as no hemolytic activity was observed, whilst the antimicrobial potency of the copolymers was maintained.

By cross-examination of the selectivity towards erythrocytes, epithelial cells and fibroblasts, the biocompatibility of the SAMPs was further investigated. In all cases, diblock copolymers were found to outperform statistical copolymers, and at low incorporation of cationic co-monomer, the multiblock showed a tremendously increased selectivity for *P. aeruginosa* and *S. epidermidis* compared to its statistical and diblock analogue. These results are independent to any self-assembling behaviour as, within the tested concentrations, the SAMPs are in their unimolecular form. The multiblock technology allowed us to highlight the remarkable effect of charge segregation on both, the physical properties of the materials as well as their interaction with biological systems. The performance of these SAMPs demonstrates their potential as alternatives to standard antibiotics for wound treatment, as these polymers possess a low toxicity towards mammalian cells and do not seem to generate bacterial resistance.

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Competing interests

The authors declare no competing financial interests.

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