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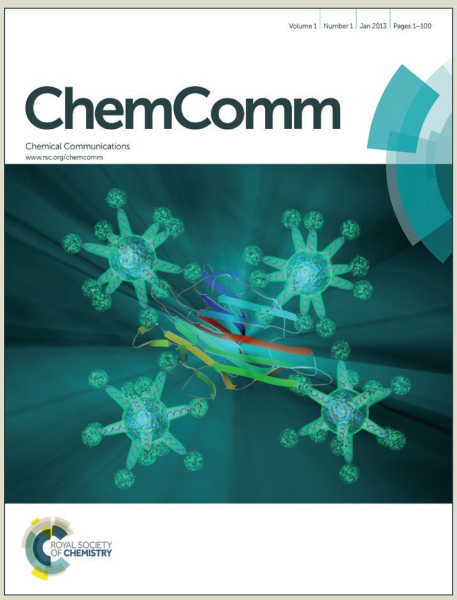

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Cyclization and Unsaturation rather than Isomerisation of Side Chains Governs the Selective Antibacterial Activity of Cationic-amphiphilic Polymers

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Membrane-active agents represent a promising alternative to overcome antibiotic resistance. Here, we report cationic-amphiphilic polymers with variations in side chain architecture such as cyclization, isomerization and unsaturation that resulted in potent antibacterial activity and low mammalian cell toxicity with a membrane-active mode of action.

Antibacterial polymers inspired from natural antimicrobial peptides (AMPs) possess amphiphilicity (cationic/hydrophobic balance) as one of their major structural determinants.[1] The positive charge ensures their selective binding to the negatively charged lipid membranes of bacteria compared to the zwitterionic lipid membranes of mammalian cells whereas the hydrophobic interactions further strengthen membrane disintegration. Optimum amphiphilicity is required to selectively target the bacteria sparing the mammalian cells. The selectivity is tuned by improving the antibacterial activity while reducing the mammalian toxicity which is generally measured from the hemolysis of red blood cells.[1c] However, strategies to further improve this selectivity to target specifically the bacteria remain elusive.

To date, optimization of cationic/hydrophobic balance has been achieved either by varying the cationic charge or side chain hydrophobicity (generally by altering the carbon number) in a variety of polymers.[2] One of the strategies to consider is the spatial/positional variation of cationic charge and hydrophobic side chain. Two approaches are generally used for this spatial variation: the cationic and hydrophobic groups are either on opposite sides (facial) of a homopolymer or spatially segregated from one another in a co-polymer. Another approach of attaching the hydrophobic side chain to the cationic centre in co-polymers (same centered) has also been shown to be effective. An important, yet unexplored, question is how the variation in side chain architecture tunes antibacterial activity and mammalian cell toxicity of cationic-amphiphilic polymers. We address these questions by employing isomerisation (regio- and stereo-), cyclization and unsaturation in hydrophobic side chains of poly(isobutylene-*alt*-*N*-(*N*′,*N*′-dimethyl *N*′-alkyl aminopropyl)-maleimide) (QAPIBMI) based cationic-amphiphilic polymers (Scheme 1). We adopt here a different approach of varying the chemical structure of the side chains appended to the cationic centre by keeping a constant positive charge as well as fixed carbon number. The advantage of this approach lies in keeping the number of carbons fixed in the side chains unlike the conventional strategies that use the concept of varying the number of carbons. The molecular weight of the polymers prepared using the present approach (isomerisation, cyclization and unsaturation at fixed carbon number) remains nearly constant. On the other hand, the conventional concept of changing the length of the carbon chain drastically increases the molecular weight thereby making it difficult to decipher the role of amphiphilicity towards improving the selectivity.

The synthesis of amphiphilic polymers was achieved in a simple two step process of post-functionalization as shown in Scheme 1.[2g,2j] The nucleophilic ring opening and the closure of the highly reactive anhydride of poly (isobutylene-*alt*-maleic anhydride) (average M_w ~6 KDa and PDI ~1.2) (Fig. S1. See ESI† for experimental details and characterization) with 3-aminopropyldimethylamine was achieved in a single step by heating at 120 °C for 48 h (Scheme 1). The complete conversion of the anhydride to form the imide, poly(isobutylene-alt-*N*-(*N*′,*N*′-dimethylaminopropylmaleimide) (PIBMI) (Scheme 1) was investigated by FT-IR and was confirmed by complete disappearance of peaks at 1850 cm^{-1} (C=O asym. str.) and 1785 (C=O sym. str.) for the anhydride ring and appearance of peaks 1767 cm^{-1} (C=O asym. str.), 1696 cm^{-1} (C=O sym. str.) for the imide ring. The second step was the quaternization of the tertiary nitrogen of PIBMI with the activated alkyl amide bromide having different alkyl chains at 75 °C for 96 h to give QAPIBMI (Scheme 1). The degree of quaternization was calculated by ^{1}HNMR analysis of the tertiary nitrogen of PIBMI and quaternized derivative, QAPIBMI (Fig. S2 ESI†). The $NCH_2(CH_3)_2$ protons shift downfield after quaternization and by integrating the protons corresponding to the

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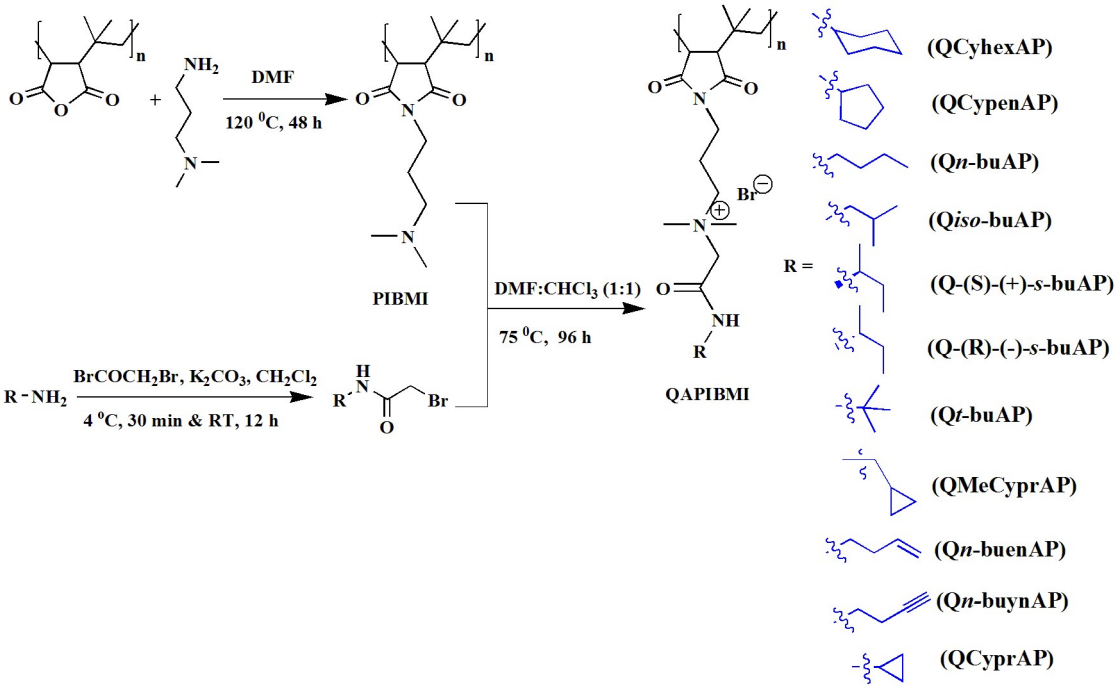
unquaternized $NCH_2(CH_3)_2$, the degree of quaternization was calculated (Fig. S2 ESI†). The cationic charge density given by the degree of quaternization was found to be in the range of 92-95% for all the polymers (Table 1). The molecular weight (M_n) of all the polymers was found to be in the range of 15-18 KDa (Table 1).



Scheme 1. General scheme for the synthesis of cationic-amphiphilic polymers.

Table 1. Characterization, antibacterial activity, mammalian cell toxicity and selectivity of cationic-amphiphilic polymers.

Polymers	DQ[a] (%)	M_n[b] (KDa)	MIC[c] (μg mL^{-1})				MBC[d] (μg mL^{-1})		HC_{50}[e] (μg mL^{-1})	IC_{50}[f] (μg mL^{-1})	Selectivity[g]	
			Ec	*Sa*	MRSA	VRE	*Ec*	*Sa*			*Ec*	*Sa*
QCyhexAP	94	17.3	16	8	8	8	5	2.5	45	65	9	18
QCypenAP	93	16.7	16	4-8	8	8	5	2.5	80	65	16	32
Q*n*-buAP	94	16.4	31	4	8	4	5	2.5	83	65	16	33
Q*iso*-buAP	93	16.3	16	4	8	4-8	5	2.5	80	65	16	33
Q(S)-(+)-*s*-buAP	93	16.3	16	16	31	8	5	2.5	125	77	25	50
Q(R)-(-)-*s*-buAP	92	16.2	16	16	31	16	5	2.5	150	83	30	60
Q*t*-buAP	93	16.3	16	16	31	8	5	2.5	125	65	25	50
QMeCyprAP	95	16.4	31	16	16	8	5	2.5	250	100	51	102
Q*n*-buenAP	93	16.2	31	31	31-62	16	10	2.5	>1000	>100	100	>400
Q*n*-buynAP	92	16.0	31	31	125-250	62	>10	2.5	>1000	>100	N.D.	>400
QCyprAP	93	15.7	16-31	16	125	125	>10	2.5	>1000	>100	N.D.	>400

[a,b]DQ-degree of quaternization and M_n-molecular weight calculated using ^{1}H-NMR analysis; [c]MIC-minimum inhibitory concentration determined in Mueller-Hinton (MH) broth; [d]MBC-minimum bactericidal concentration determined in chemically defined media; Concentration required to cause 50% toxicity to [e]human red blood (hRBC) cells and [f]human endothelial kidney (HEK) cells respectively; [g]Selectivity = HC_{50}/MBC; *Ec-E. coli*, *Sa-S. aureus,* MRSA-methicillin resistant *S. aureus* , VRE- vancomycin resistant *Enterococcus faecium* N.D.-not determined.

At first, to fix the carbon number, we synthesized the polymers with cyclic side chains of variable hydrophobicity (different carbon number) such as QCyhexAP (six carbons), QCypenAP (five carbons), QMeCyprAP (four carbons) and QCyprAP (three carbons) (Scheme 1).For obtaining the polymer with selective toxicity to bacteria, the amphiphilicity had to be tuned by optimizing the hydrophobicity of the cyclic side chains. The higher cyclic side chain polymers, QCyhexAP, QCypenAP and QMeCyprAP displayed minimum inhibitory concentration (MIC) in the range of 4-16 μg mL^{-1} against *E. coli* and *S. aureus* (Table 1). The lower cyclic side chain, QCyprAP had moderate activity against (MIC = 16-31 μg mL^{-1}) against *E. coli* and *S. aureus* (Table 1).

To emphasize the fact that the differences in antibacterial activity are indeed due to the variations in side chain structure and not the culture media conditions, the antibacterial activity has been determined in chemically defined media against *E. coli* and *S. aureus*. It has been found that the animal tissue derived culture media with unknown composition decrease the effectiveness of

antibacterial polymers compared to the chemically defined media.[3] Similarly, we found that QCyhexAP, QCypenAP and QMeCyprAP showed potent antibacterial activity in chemically defined media with minimum bactericidal concentration (MBC) of 5 μg mL^{-1} and 2.5 μg mL^{-1} against *E. coli* and *S. aureus* respectively compared to the animal tissue derived media (Table 1). QCyprAP, although showed good activity against *S. aureus* (MBC = 2.5 μg mL^{-1}), displayed low activity against *E. coli* (MBC >10 μg mL^{-1}) (Table 1). Even against multi-drug resistant bacteria such as MRSA and VRE, QCyhexAP, QCypenAP and QMeCyprAP showed MIC of 4-16 μg mL^{-1} whereas QCyprAP had very low activity (MIC = 125 μg mL^{-1}) (Table 1). These results suggest that differences in antibacterial activity are indeed due to the structural variations.

Though highly effective against bacteria, QCyhexAP and QCypenAP were highly toxic to mammalian cells. As shown in Table 1, the toxicity to human red blood cells (hRBCs, HC_{50} concentration that causes 50% hemolysis) decreased as the carbon number of the cyclic side chains decrease from six to three (Table 1 & Fig. S3A ESI†). The toxicity to human endothelial kidney (HEK) cells, (IC_{50}, concentration that causes 50% release of lactate dehydrogenase (LDH)) also decreased as the hydrophobicity of the cyclic side chain decreases (Table 1 & Fig. S3B ESI†). Both QCyhexAP and QCypenAP showed HC_{50} = 45 μg mL^{-1} and 80 μg mL^{-1} respectively, and IC_{50} = 40 μg mL^{-1} and 75 μg mL^{-1} respectively (Table 1). The smaller ring derivative, QCyprAP showed very low mammalian cell toxicity (HC_{50} and IC_{50} >1000 μg mL^{-1} and 100 μg mL^{-1} respectively). The cyclic four carbon derivative, QMeCyprAP with potent antibacterial activity displayed low toxicity to mammalian cells (HC_{50} and IC_{50} = 256 μg mL^{-1} and 100 μg mL^{-1} respectively). QMeCyprAP had the high selectivity of 50-100 towards bacteria over mammalian cells (Table 1). These results suggest that the four carbon number indicating optimum amphiphilicity is required for selective toxicity to bacteria.

Next, we set forth to study the role of the side chain architecture towards antibacterial activity and toxicity by keeping the four carbon number constant, the optimum side chain as described above. We employed regio- and stereo-isomerization in the acyclic side chains of the polymers to investigate their effect on antibacterial activity and mammalian cell toxicity. The isomeric side chain containing polymers (Scheme 1) such as Q*n*-buAP (*n*-butyl), Q*iso*-buAP (isobutyl), Q-(S)-(+)-*s*-buAP (sec-butyl), Q-(R)-(-)-*s*-buAP (sec-butyl) and Q*t*-buAP (t-butyl) displayed MIC in the range of 4-31 μg mL^{-1} against *E. coli*, *S. aureus*, MRSA and VRE (Table 1) comparable to the corresponding cyclic side chain polymer, QMeCyprAP (methylcyclopropyl). All these four carbon side chain containing polymers also showed the same MBC of 5 μg mL^{-1} and 2.5 μg mL^{-1} against *E. coli* and *S. aureus* respectively (Table 1). Q-(S)-(+)-*s*-buAP, Q-(R)-(-)-*s*-buAP and Q*t*-buAP polymers were slightly less toxic to hRBCs (HC_{50} = 125-150 μg mL^{-1}) than Q*n*-buAP and Q*iso*-buAP (HC_{50} of ~80 μg mL^{-1}). However, all these five isomeric side chain containing polymers displayed more or less similar IC_{50} of 65-83 μg mL^{-1} (Table 1). The Q-(S)-(+)-*s*-buAP, Q-(R)-(-)-*s*-buAP and Q*t*-buAP polymers had higher selectivity in the range of 30-60 than Q*n*-buAP and Q*iso*-buAP (selectivity = 16-33) to bacteria (Table 1). Q*n*-buAP was more toxic to mammalian cells (HC_{50} and IC_{50} = 83 μg mL^{-1} and 75 μg mL^{-1} respectively) compared to the corresponding cyclic derivative, QMeCyprAP (HC_{50} and IC_{50} = 250 μg mL^{-1} and >100 μg mL^{-1} respectively) thus reducing its selectivity. Still, the four carbon cyclic side chain containing polymer, QMeCyprAP was found to be the optimized derivative with potent antibacterial activity and low toxicity to mammalian cells (selectivity of 50-100) (Table 1). This resulted in the understanding that isomerization of hydrophobic side chains do not yield better selectivity.

Furthermore, we studied the role of unsaturation in the side chains to further tune the selective toxicity to bacteria. Both the double and triple bond containing polymers, Q*n*-buenAP (but-3-enyl) and Q*n*-buynAP (but-3-ynyl) respectively showed an MIC of 31 μg mL^{-1} against both *E. coli* and *S. aureus* (Scheme 1 & Table 1). Q*n*-buenAP also showed moderate to high activity of 62 and 16 μg mL^{-1} against MRSA and VRE respectively (Table 1). However, Q*n*-buynAP lost the activity against MRSA and VRE with MIC of 62-125 μg mL^{-1}. Q*n*-buenAP and Q*n*-buynAP also showed MBC of 10 μg mL^{-1} and >10 μg mL^{-1} against *E. coli* and 2.5 μg mL^{-1} against *S. aureus* respectively (Table 1). Nevertheless, both the unsaturated side chain containing polymers were non-hemolytic even up to 1000 μg mL^{-1} (Table 1). But the corresponding Q*n*-buAP (HC_{50} = 83 μg mL^{-1}) and QMeCyprAP (HC_{50} = 250 μg mL^{-1}) were more toxic to red blood cells. The amphiphilic polymers with structural variations in their side chains such as Q*n*-buAP, QMeCyprAP, Q*n*-buenAP and Q*n*-buynAP had different toxicity profiles to HEK cells as shown in Table 1. Q*n*-buAP and QMeCyprAP were found to be more toxic to HEK cells than Q*n*-buenAP and Q*n*-buynAP with LDH release of >50%, 50%, 20% and <5% respectively at 100 μg mL^{-1}. Overall, Q*n*-buenAP and Q*n*-buynAP showed the highest selectivity of >100 and > 400 towards bacteria over mammalian cells amongst all the polymers (Table 1).

These results suggest that the acyclic saturated hydrophobic side chains in these polymers yield less selectivity compared to their cyclic counterparts. Recently, Gellman and co-workers showed that in binary hydrophobic cationic nylon-3 co-polymers, the cyclic and acyclic alternative subunit substitution pattern close to the backbone selectively targets the bacteria compared to both acyclic subunits.[2a,4a] On contrary, Yang and co-workers observed that the shape of the hydrophobic alkyl tail on a six carbon level in 2-aminoethylacrylate co-polymers does not significantly affect their antibacterial and hemolytic activity.[2i] However, our data showed that cyclization of hydrophobic side chains appended to the cationic centre far from the polymeric backbone selectively kills the bacteria compared to their acyclic counterparts. In addition, the unsaturated hydrophobic side chains yield better selectivity compared to the corresponding saturated acyclic and cyclic side chains in these polymers. We have observed that the hydrophobicity profiles of these polymers decrease in the order of Q*n*-buAP, QMeCyprAP, Q*n*-buenAP, Q*n*-buynAP and QCyprAP (Fig. S4 ESI†)[4b,4c]. These results suggested that Qn-buenAP, Qn-buynAP and QCyprAP due to their low hydrophobicity have lower hemolytic activity (high HC_{50}). Also, once carbon difference in QMeCyprAP and QCyprAP led to lower hydrophobicity for QCyprAP resulting in four times higher HC_{50} compared to QMeCyprAP (Fig. S4 ESI†).

After understanding the role of side chain chemical structure in antibacterial activity and mammalian cell toxicity, we set out to understand its influence towards the interaction with the bacterial cell membranes. The membrane-active properties of the optimized polymers, QMeCyprAP, Q*n*-buenAP and Q*n*-buynAP along with the corresponding saturated acyclic side chain polymer, Q*n*-buAP were determined. We assessed the bacterial cell membrane depolarization by the polymers using a membrane potential sensitive dye, $DiSC_3(5)$ (3, 3'-dipropylthiadicarbocyanine iodide). Q*n*-buAP and QMeCyprAP had similar whereas both Q*n*-buenAP and Q*n*-buynAP showed lower effect on membrane depolarization of *S. aureus* (Fig. S5A ESI†). On contrary, against *E. coli*, Q*n*-buAP

showed a very high capability to dissipate the membrane potential compared to QMeCyprAP, Q*n*-buenAP, QCyprAP and Q*n*-buynAP (Fig. 1A). Kinetics of membrane permeabilization was studied by measuring the uptake of a fluorescent probe propidium iodide (PI). The membrane permeabilization of *E. coli* follows an increasing trend with Q*n*-buAP showing the highest permeabilization whereas Q*n*-buynAP had the lowest permeabilization (Fig. 1B). Dissipation of membrane potential affects the energy processes in bacteria

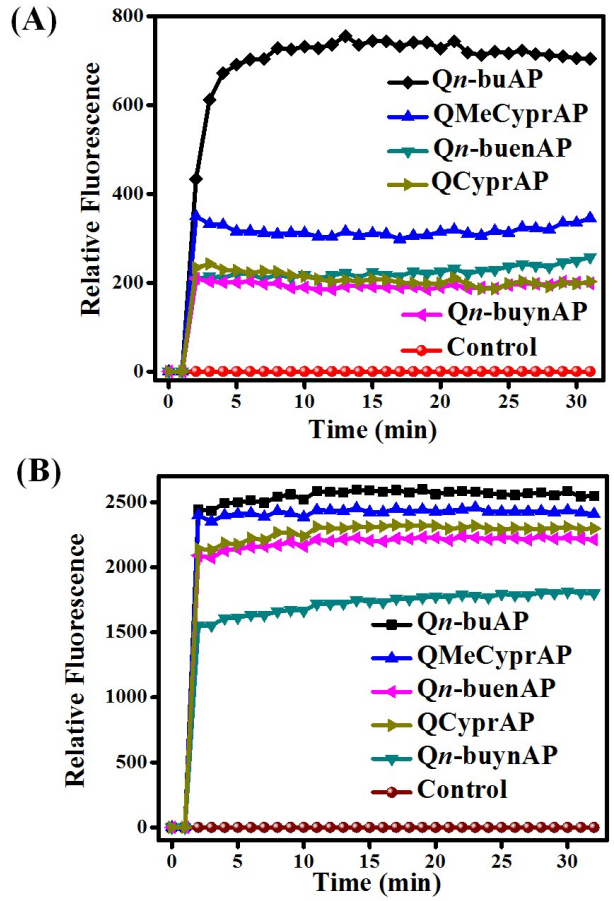


Fig. 1 Membrane depolarization (A) and permeabilization (B) against *E. coli.*

leading to permeabilization (membrane disintegration) and cell death. We analyzed the leakage of intracellular adenosine triphosphate (ATP) levels in bacteria after the treatment with polymers using luceferin/luciferase bio-luminescence assay. Q*n*-buAP had the highest whereas QMeCyprAP, Q*n*-buenAP and Q*n*-buynAP had similar leakage of ATP levels in *E. coli* (Fig. S5B ESI†). Against *S. aureus*, Q*n*-buynAP had lower whereas Q*n*-buenAP, QMeCyprAP and Q*n*-buAP had more or less similar levels of ATP leakage (Fig. S5C ESI†). Q*n*-buAP having the highest interaction with the bacterial cell membranes showed the potent activity whereas QMeCyprAP and Q*n*-buenAP with optimum membrane interactions resulted in optimum antibacterial activity. Q*n*-buynAP and QCyprAP with the lowest membrane-active profiles displayed lower antibacterial activity against all the bacteria. However, Q*n*-buAP was found to be equally toxic to both bacteria as well as mammalian cells. QMeCyprAP and Q*n*-buenAP showed optimum antibacterial activity and minimal mammalian toxicity resulting in selective antibacterial activity. Q*n*-buynAP and QCyprAP was low toxic to bacteria and non-toxic to mammalian cells.

In conclusion, we have demonstrated that cyclization and unsaturation of side chains was found to be better than isomerization in amphiphilic polymers with respect to selective antibacterial activity. We believe that variations in the hydrophobicity profiles (due to polarizability)[5] of these side chain tunable cationic-amphiphilic polymers due to ring strain (cyclization) and π-character (unsaturation) result in selective antibacterial activity but need to be dealt in further detail in future. The data presented here provides an understanding that the side chain chemical structure does play an important role in tuning the selective antibacterial activity of amphiphilic polymers.

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