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# Antibacterial Low Molecular Weight Cationic Polymers: Dissecting the Contribution of Hydrophobicity, Chain Length and Charge to Activity

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The balance of cationicity and hydrophobicity can profoundly affect the performance of antimicrobial polymers. To this end a library of 24 cationic polymers with uniquely low degrees of polymerization was synthesized via Cu(0)-mediated polymerization, using three different cationic monomers and two initiators: providing two different hydrocarbon chain tail lengths ($C_2$ and $C_{12}$). The polymers exhibited structure-dependent antibacterial activity when tested against a selection of bacteria, viz, *Staphylococcus aureus* ATCC 29213, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* ATCC 19606, and *Pseudomonas aeruginosa* ATCC 27853 as a representative palette of Gram-positive and Gram-negative ESKAPE pathogens. The five best-performing polymers were identified for additional testing against the polymyxin-resistant *A. baumannii* ATCC 19606R strain. Polymers having the lowest DP and a $C_{12}$ hydrophobic tail were shown to provide the broadest antimicrobial activity against the bacteria panel studied as evidenced by lower minimum inhibitory concentrations (MICs). An optimal polymer composition was identified, and its mechanism of action investigated via membrane permeability testing against *Escherichia coli*. Membrane disruption was identified as the most probable mechanism for bacteria cell killing.

## Introduction

An increase in the number of antibiotic resistant bacteria appearing together with a lack of new antibiotics in the drug-development pipeline presents a critical problem for global heathcare.[1] Bacterial pathogens typically gain resistance to antibiotics by acquiring genetic material from other species, or through evolutionary changes reducing susceptibility to antibiotics.[2, 3] For example, bacteria can evolve an ability to use intracellular efflux pumps for antibiotic removal from the cytosol.[1] As a further example, the resistance to methicillin demonstrated in *Staphylococcus aureus* is caused by the mecA gene coding for an altered penicillin-binding protein.[4] With increased bacterial resistance reducing the efficacy of the current limited library of antibiotics, there is an urgent need for new classes of antibiotic drugs and antimicrobial materials. Further, in addition to the health impacts of antibiotic resistance, there is also a significant economic impact, with the estimated cost to the US health system alone being between USD $21 and $34 billion per year.[5]

One class of materials attracting considerable attention as potential antibiotics are antimicrobial peptides.[6] Antimicrobial peptides are produced by a range of bacteria and fungi, and typically contain a lipophilic segment and a cationic segment. These materials offer considerable advantages in that they have a broad spectrum of activity, are of low toxicity to mammalian cells, and are less susceptible to the development of resistance.[6, 7] However, antimicrobial peptides are costly to produce in large amounts and often have poor pharmacokinetic profiles, exhibiting short half-lives *in vivo* due to degradation by proteases.[8-10] As such, the development of synthetic analogues to antimicrobial peptides is of considerable interest. Compared to antimicrobial peptides, antimicrobial polymers with cationic and hydrophobic moieties can be produced cost-effectively in large quantities, are more adaptable to drug-delivery methods, and provide a flexible framework for systematic chemical pharmacophore modification and adaptation.[11]

The structure of antimicrobial polymers (and therefore the associated antimicrobial activity) can be easily modified through judicious choice of monomer, polymer molecular

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weight (degree of polymerisation (DP)) and chemical structure of any incorporated hydrophobic domain.[12, 13] A recent study by Locock *et al.* indicated a strong correlation between reducing the DP of cationic antimicrobial polymers and minimum inhibitory concentration (MIC) (over a range of DP from 17 to DP 142 for guanidine functional polymers).[9] The same researchers also demonstrated that as the DP was decreased the level of haemolysis induced by the polymer was also reduced. However, to date there has been no comprehensive investigation of whether cationic polymers with lower DP (<20) still retain antimicrobial capacity. Additionally, work by Mowery *et al.* has shown using grafted polymers that when the tail length is increased from 2 to 12 carbons the antimicrobial activity increases, but then decreases with further increase in the length.[14] Michl *et al.* have demonstrated that for polymers synthesized using Reversible Addition-Fragmentation chain Transfer (RAFT), changing the polymer end-group from a $C_2$ to a $C_{12}$ group decreased the MIC value against vancomycin Intermediate *Staphylococcus aureus* (VISA).[15] However, in this study moving from a $C_2$ to a $C_{12}$ tail group also increased the haemolytic activity of the polymer to a small extent (3-4% for the guanidine polymer) at a polymer concentration of 16 μg/mL. Clearly, the hydrophobic tail affects both the antibacterial activity and the haemolytic properties of the polymer, and as such is an important architectural parameter in the design of effective antimicrobial polymers.[6, 9]

As highlighted above there is some indication that the antibacterial activity of cationic polymers could be improved by lowering the DP, however the synthesis of very low DP polymers with well-controlled molecular weight, functionality and low polydispersity via living free radical polymerisation (LFRP) has historically been difficult. The lack of synthetic tools has led to a significant knowledge gap in the structure property relationship of these antibacterial materials: specifically at very low molecular weight. However, we and others have made significant recent progress in the facile synthesis of polymers with low DPs using Cu(0)-mediated polymerization or RAFT methodologies.[16,17] Specifically, Whittaker and co-workers[18, 19, 20] reported that by using Cu(0)-mediated LFRP it is possible to make very low DP polymers with unprecedented control of chain architecture and monomer composition. By using Cu(0)-mediated LFRP the degree of polymerization (and therefore molecular weight) of a wider range of functional polymers can be controlled to a greater degree than by other LFRP techniques; this is especially so when polymers with a low number of repeat units are targeted. Moreover, Cu(0)-mediated polymerization is a very simple technique, being carried out at room temperature with commercially available reagents and without the use of complex equipment.[18,19]

Herein we report the novel synthesis of a library of 24 cationic polymers having varying degrees of polymerization, cationicity and hydrophobic tail length for use in antimicrobial applications. Specifically, polymer chains of less than 27 monomer units were synthesized using Cu(0)-mediated LFRP of 2-(dimethylamino)ethyl acrylate (DMAEA; $pK_a$: 8.41)[21], 2-(diethylamino)ethyl acrylate (DEAEA; $pK_a$: 8.0)[22], and 2-*N*-morpholinoethyl acrylate (MEA; $pK_a$: 6.21)[21]. These monomers were chosen as the corresponding methacrylates have previously been used in the synthesis of high molecular weight (i.e. high DP) antibacterial polymers.[23-26] Moreover, variation of the amine moiety confers a range of cationicities to the materials studied due to their different $pK_a$ values. The polymerizations were initiated using either ethyl α-bromoisobutyrate (EBiB) or dodecyl 2-bromoisobutyrate (DBiB) thereby yielding polymers with ethyl ($C_2$) or dodecyl ($C_{12}$) aliphatic hydrocarbon tails, respectively. The polymers were subsequently quaternized to provide a series of polymers incorporating quaternary ammonium moieties. We then investigated the antimicrobial activity of the library against a selection of multidrug-resistant bacterial pathogens: *S. aureus*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*,[27, 28] and evaluated the haemolytic effect of the polymers. The five polymers that exhibited the broadest activity against the examined bacteria were then tested against HepG2 mammalian cells. Finally, the mechanism of action of one of the most effective compounds was investigated using a fluorescent membrane permeability assay with *Escherichia coli*.

## Materials and methods

### Materials.

2-*N*-morpholinoethyl acrylate (MEA) (Polysciences), 2-(dimethylamino)ethyl acrylate (DMAEA, Sigma-Aldrich) and 2-(diethylamino)ethyl acrylate (DEAEA, Sigma-Aldrich) were de-inhibited by percolating over a column of activated basic alumina. Copper wire (Sigma-Aldrich) was activated by washing in sulfuric acid for 10 min. *Tris*(2-(dimethylamino)ethyl)amine ($Me_6$TREN) was synthesized according to literature procedures.[29] Ethyl α-bromoisobutyrate (EBiB, Sigma-Aldrich) and dodecyl 2-bromoisobutyrate (DBiB, Sigma-Aldrich). Dichloromethane was purchased from Merck Millipore. Dimethyl sulphoxide was purchased from Science Supply. NMR solvent ($CDCl_3$, MeOD, and $D_2O$) were purchased from Sigma-Aldrich and used as received. Milli-Q filtered water was used to prepare solutions, with the aforementioned chemicals, according to their recommended concentration. The four bacteria strains used were *A. baumannii* ATCC 19606, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 13883. The 96-well plates for this assay were purchased from Techno Plas.

### Polymer Synthesis

A typical example of a Cu(0)-mediated polymerization of DMAEA was performed in DMSO at 25°C for 24 h. DMAEA (1.0 mL, 6.59 mmol, 25 eq.), DMSO (1.0 mL), EBiB (0.0387 mL, 0.263 mmol, 1.00 eq.), $Me_6$TREN (0.0112 mL, 0.0422 mmol, 0.16 eq.), $CuBr_2$ (2.9 mg, 0.0132 mmol, 0.05 eq.), and a magnetic stirrer bar were charged to a polymerization flask

fitted with a rubber septum and the mixture deoxygenated via nitrogen sparging for 10 min after which pre-activated copper wire was carefully added under a nitrogen blanket. The polymerization flask was then resealed, deoxygenated for a further five minutes and polymerization was allowed to occur at room temperature for 24 hours (Scheme 1). After 24 hours a sample of the reaction mixture was removed for $^1$H NMR and GPC analysis. The sample for $^1$H NMR was diluted into $CDCl_3$, while the sample for GPC was first diluted with THF then passed over an aluminium oxide column to remove the copper. The THF was removed and the polymer was made up in DMAc to run in the GPC. This polymerization method was then repeated for DEAEA and MEA monomers using similar conditions however DMF was used instead of DMSO for higher DP DEAEA polymers.

**Quaternization of Synthesized Polymers**

Polymers to be quaternized were dissolved in DMF and the methylation reaction was carried out at room temperature using 10 equivalents of methyl iodide per amine group (Scheme 1). Full quaternization was achieved after 4 days of reaction as confirmed by NMR spectroscopy of the resulting polymers. Solvent and excess methyl iodide were initially removed by evaporation under a stream of air followed by drying in a vacuum oven for one week at 40°C. The sample for $^1$H NMR was diluted into $D_2O$.

**Polymer Characterization**

$^1$H Nuclear Magnetic Resonance (NMR) Spectroscopy. All NMR spectra were recorded using on a Bruker Avance III 400 MHz spectrometer using an external lock and referenced to the residual nondeuterated solvent. Chemical shifts ($\delta_H$) are reported in parts per million (ppm).

Size Exclusion Chromatography (SEC). SEC analyses of polymer samples were performed using a Shimadzu modular system comprising a DGU-20A3R degasser unit, an SIL-20A HT autosampler, a 10.0 µm bead-size guard column (50 x 7.8 mm) followed by three KF-805L columns (300 x 8 mm, bead size: 10 µm, pore size maximum: 5000 Å), a SPD-20A UV/Vis detector, and an RID-10A differential refractive-index detector. The temperature of columns was maintained at 40°C using a CTO-20A oven. The eluent was dimethylacetamide (CHROMASOLV Plus for HPLC) and the flow rate was kept at 1.0 mL/min using a LC-20AD pump. A molecular weight calibration curve was produced using commercial narrow molecular weight distribution polystyrene standards with molecular weights ranging from 500 to 2 x $10^6$ g/mol. Polymer solutions at approx. 2 mg/mL were prepared and filtered through 0.45 µm PTFE filters before injection.

**Antibacterial Evaluation**

Three polymers were tested against one bacterium for each 96 well plate over an MIC range of 1500 to 3 µg/mL. A positive control (bacteria and no drug) and a negative control (no bacteria and no drug) were also performed. The polymers were tested against *A. baumannii* ATCC 19606, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 13883. The 96-well plate was then incubated for 20 h at 37°C in a Contherm Biocell 1000 Incubator, and the MIC was determined as the lowest concentration at which no visible growth was observed. The MICs were then pooled and analysed by multiple linear regression to identify to significant ($\alpha$=0.95) physicochemical drivers of antimicrobial activity (as quantified by MIC). The R statistical computing package (3.0.2) was used to perform regressions.

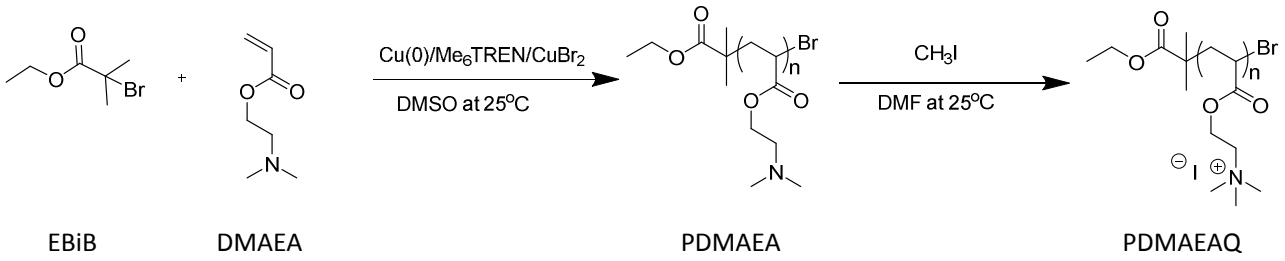
**Membrane Perturbation Assays**

3,3'-Dipropylthiadicarbocyanine iodide (DiSC3-5) assay (Anderson *et al.*[30]) and 1-*N*-phenylnaphthylamine (NPN) uptake assay (Helander *et al.*[31]) were performed as previously described with slightly modifications. In brief, mid-log *E. coli* cells were harvested by centrifugation at 4000 g for 10 min at room temperature and resuspended using assay buffer (5 mM HEPES, 20 mM glucose, pH = 7.4) for NPN uptake assay. The bacteria suspension was then diluted 100-fold in assay buffer with 100 µM EDTA, which was used for diSC3-5 assay. DiSC3-5 (0.4 µM) or NPN (10 µM) was added into the suspension and incubated for 1 hour at room temperature. 90 µL of the cell suspension was added into each well of an Optiplate™-96 well white microplate (Perkin Elmer). After the fluorescence level became stable, 10 µL of a concentration series of polymers was added into each well and the fluorescent intensity (excitation/emission 620/670 for diSC3-5 and excitation/emission 350/420 for NPN) was monitored for 40 min using a Polarstar® Omega plate reader (BMG technologies), 10 µL of PBS was used as a negative control in the assays. All assays were performed at least three times.

**Haemolysis Testing**

Human red blood cells were acquired from the Australian Red Cross Blood Service. In a 96-well plate, 100 µL of the polymer solution was added into each well in the following order with the aid of multichannel reservoirs: PBS only, PBS, 30 µg/mL, 50, 100, 250, 500, 1000, 1500, 2000, 3000 µg/mL, and 2% Triton-X in PBS. The plates were then incubated for 1 hour at 37°C, followed by centrifugation at 1000 g for 5 minutes with the lid off. The supernatant (100 µL) was transferred into a new, sterile flat-bottom 96-well plate, and the absorbance at 450 nm was measured with a Multiskan plate reader.

**Cytotoxicity**




Scheme 1 Synthesis of PDMAEA using Cu(0)-mediated polymerization and subsequent quaternization using methyl iodide

Cytotoxicity was investigated using HepG2 (ATCC® HB-8065™) cell lines under the culture condition with 1% FBS or 10% FBS in DMEM (Life Technologies, Australia, 11995-065) for 24 hours. Stock solutions (10 mg/mL) in 100% DMSO or $H_2O$ were prepared. One single concentration 100 µg/mL was tested for cytotoxicity. After 24 hour incubation, resazurin was added into each well to a final concentration of 10 µM and incubated at 37°C for 2 h. The fluorescence intensity was then read using a Polarstar® Omega plate reader with excitation/emission 560/590 nm. All compounds were tested at 100 µg/mL in four replicates for 24 hours. The cytotoxicity was reported as the cell survival rate (% of cell survival).

## Results and Discussion

### Polymer Synthesis and Characterization

A library of cationic low DP polymers was synthesized by Cu(0)-mediated LFRP polymerization via the methodology shown in Scheme 1.

The monomers chosen for this study were obtained commercially, for subsequent use in Cu(0)-mediated polymerizations; each containing a tertiary amine in their pendant groups amenable to undergo reversible, pH-dependent protonation. The cationicity afforded by the protonation of the tertiary amine is important as the cytoplasmic membrane of Gram-positive bacteria is directly exposed, providing a negatively charged surface for interaction with the cationic polymers. This charge-charge binding mechanism is well established for cationic antibiotics[32] such as paenibacterin and polymyxin. The specific monomers used in this study were 2-(dimethylamino)ethyl acrylate, 2-(diethylamino)ethyl acrylate, and 2-N-morpholinoethyl acrylate (structures are given in SI Figure 1). It has also been demonstrated that the introduction of a pH-independent permanent charge (through quaternization of the tertiary amines) can improve the antibacterial activity for higher molecular weight materials. For example, both quaternized and non-quaternized poly(dimethylaminoethyl) methacrylate (PDMAEMA)[22-25] have been shown to possess antimicrobial activity against both Gram-positive and Gram-negative bacteria (such as *S. aureus and E. coli*).

A typical $^1$H-NMR spectrum and the general chemical structure of the poly(2-(dimethylamino)ethyl acrylate) (PDMAEA) is shown in Figure 1a. The triplet centred at 1.23 ppm corresponds to the $CH_3CH_2$ of the EBiB initiator unit, while the peak at 4.1 ppm corresponds to the O-$CH_2$ of DMAEA. These peaks were used for calculating the degree of polymerization of the polymers (see Table 1). In this specific example the targeted DP was 25, with the actual DP computed from the NMR spectrum being approx. 27. Figure 1b shows the NMR spectrum for PDMAEA of similar DP with a $C_{12}$ tail, while Figure 1c depicts PDMAEA with a DP of approx. 5. The $^1$H NMR in Figure 1d illustrates conversion of a tertiary amino functional polymer to the quaternized analogue, as demonstrated by a downfield shift of the peaks corresponding to the methyl hydrogens surrounding the quaternized nitrogen atom. A similar result has previously been reported by Bütün *et al.*[33] The polymers were also characterised using size exclusion chromatography (SEC) and the data are presented in Table SI1. The use of SEC to analyse extremely low molecular weight polymers is problematic and the results should be treated with caution. This is due to the fact that the molecular weight of the synthesized polymers approaches the lower molecular weight cut-off of the column bank available. Additionally, the results are further complicated by the differing hydrodynamic volume between the analysed polymers and the polystyrene standards used for calibration, as well as differences in chemical structures between each individual polymer set.
Nevertheless, despite these caveats 12 well characterised polymers were synthesized using Cu(0)-mediated polymerization with DPs between DP 4 and DP 27. Quaternization of these 12 polymers further increased the polymer library to 24 (see Table 1).

### Antimicrobial Activity

The MIC values of these polymers were then determined against the Gram-positive bacteria *S. aureus* ATCC 29213, and the Gram-negative bacteria *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 13883. These bacteria were chosen as they are part of the so-called ESKAPE pathogens that are currently problematic in hospital settings worldwide.[28] The resulting MIC values are presented in Table 1. The 24 polymers tested included both quaternized polymers and their non-quaternized counterparts to enable comparison of the effects of adding a permanent charge to the polymer. Examination of the data presented in Table 1 reveals that all materials studied have some broad antimicrobial properties.

Multiple linear regression analysis of the antimicrobial activity screen revealed that $C_{12}$ initiator tails were generally associated with better antimicrobial activity compared to $C_2$ initiator tails. This relationship was stronger in *A. baumannii* (p=0.03) than in *S. aureus* (p=0.1). All polymers had minimal activity against the Gram-negative bacteria, with the possible exceptions of Polymer 4 and Polymer 12 (even then activity was still relatively low). Our finding that $C_{12}$ hydrophobic tail polymers had a greater antibacterial activity than the $C_2$ hydrophobic tail polymers, is in accord with similar observations reported by Mowery *et al.*[14] who showed that polymers containing a $C_{12}$ aliphatic chain demonstrated the best killing results against *E. coli*, *Bacillus subtilis*, *S. aureus*, and *Enterococcus faecium*, although as mentioned above this study used grafted polymers rather than those with a hydrophobic tail. A similar result was also obtained by Michl *et al.* who observed that polymers containing an aliphatic $C_{12}$ chain demonstrated the best killing activity against VISA.[15] It is thought the longer hydrocarbon tail is necessary to facilitate membrane insertion and permeabilization/destabilization of the membrane. A comparison of Polymers 4 and 20 to 3 and 19, respectively, supports the hypothesis that when the DP is decreased the antimicrobial activity of the polymer increases (provided that the hydrophobic tail is $C_{12}$). In contrast, when the hydrophobic tail was $C_2$, either some bacteria specific killing was observed or there was no change in activity.
Taken together, a $C_{12}$ hydrophobic tail and a low DP were generally found to provide the best antimicrobial activity.

Results from the multiple linear regression also showed that the $pK_a$ of the monomer strongly influenced its antimicrobial activity against Gram-negative bacteria ($p < 0.01$). A similar, but not statistically significant correlation between $pK_a$ and activity against *S. aureus* was also observed ($p = 0.055$). The link between higher $pK_a$ and increased activity against Gram-negative bacteria is likely attributable to improved localisation due to electrostatic interactions between the positively-charged polymer and the negatively-charged LPS layer of the bacterial outer membrane. Paradoxically, we observed a trend that quaternization of the polymers worsened the activity of the polymers against Gram-negative bacteria (not statistically significant, $p = 0.093$). The loss of activity observed with a permanent positive charge suggests that the mechanism of antibacterial activity of these polymers occurs in two phases: a localisation step driven by electrostatic interactions followed by an insertion/traversal of the outer membrane, driven by hydrophobic interactions. We hypothesize that the permanent positive charge of the quaternized polymers prevents the polymers from inserting into the hydrophobic core of the inner and outer membranes, limiting their activity. This trend is consistent with results reported by Rawlinson *et al.* who found that for PDMAEMA to effectively permeabilize the outer membrane of a Gram-negative bacteria (*Salmonella ser. Typhimurium*), the polymer needed to be in a relatively less charged/hydrophobic state.[23] This is consistent with the mechanism of action of other quaternary compounds, which first adsorb to the bacterial surface prior to disrupting the cytoplasmic membrane and causing release of intracellular constituents such as $K^+$, DNA and RNA, leading to cell death. [12, 24, 34, 35]

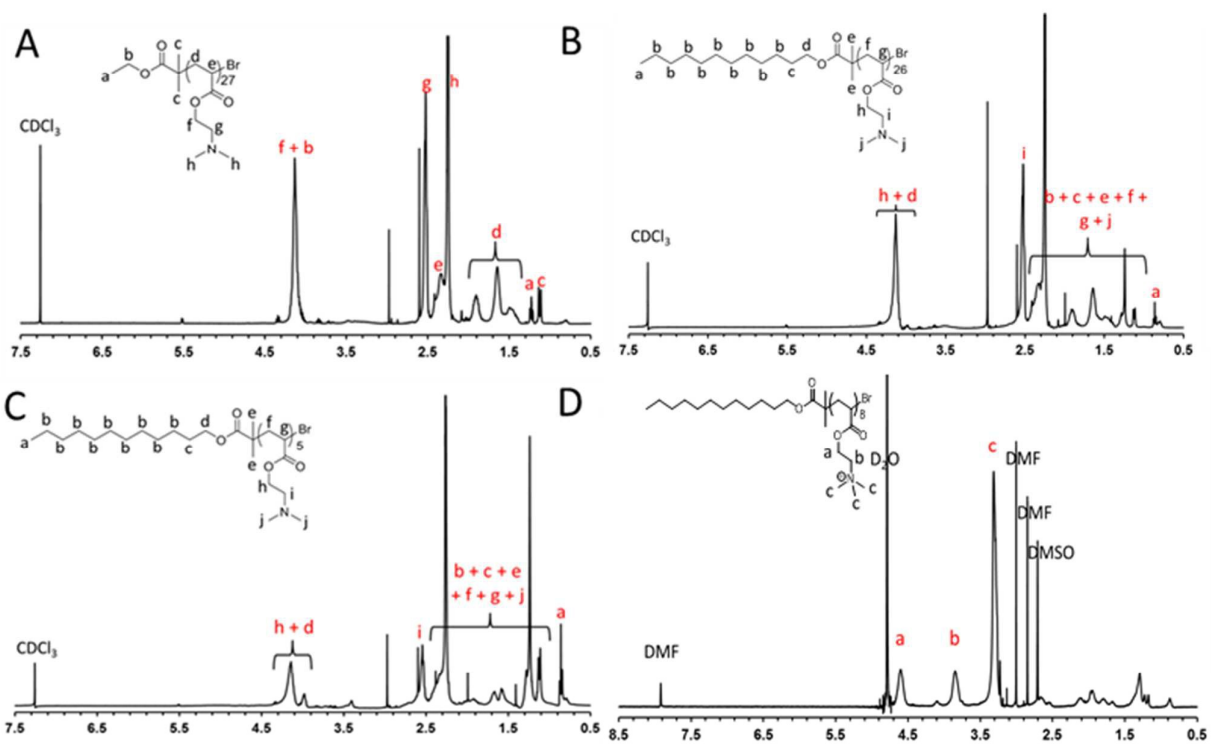



Figure 1 $^1$H NMR spectra of varied DMAEA polymers: **A**: Short tail polymer in $CDCl_3$ with DP 27; **B**: Long tail polymer in $CDCl_3$ with DP 26; **C**: Long tail polymer in $CDCl_3$ with DP 5; **D**: Long tail quaternized polymer in $D_2O$ with DP 8

Table 1 Polymer Characterization and Antibacterial Results

| Polymer N° | Monomer/ Compound | Quaternized (Y/N) | Initiator chain length | DP[a] | MIC[b] *P. aeruginosa* | MIC[b] *A. baumannii* | MIC[b] *K. pneumoniae* | MIC[b] *S. aureus* |
|---|---|---|---|---|---|---|---|---|
| - | Polymyxin B | - | - | - | 1 | 2 | 1 | - |
| - | Ciprofloxacin | - | - | - | - | - | - | 8 |
| 1 | DMAEA | N | 2 | 27 | 500 | 500 | 500 | 750 |
| 2 | DMAEA | N | 2 | 5 | 500 | 250 | 500 | 1000 |
| 3 | DMAEA | N | 12 | 26 | 500 | 250 | 500 | 750 |
| 4 | DMAEA | N | 12 | 5 | 125 | 50 | 125 | 500 |
| 5 | DMAEA | Y | 2 | 27 | 500 | 500 | 500 | 50 |
| 6 | DMAEA | Y | 2 | 5 | 500 | 500 | 500 | 1000 |
| 7 | DMAEA | Y | 12 | 26 | 500 | 250 | 500 | 25 |
| 8 | DMAEA | Y | 12 | 5 | 500 | 250 | 500 | 125 |
| 9 | DEAEA | N | 2 | 16 | 500 | 500 | 750 | 1000 |
| 10 | DEAEA | N | 2 | 5 | 500 | 500 | 500 | 1000 |
| 11 | DEAEA | N | 12 | 15 | 500 | 500 | 750 | 1000 |
| 12 | DEAEA | N | 12 | 5 | 500 | 88 | 125 | 125 |
| 13 | DEAEA | Y | 2 | 16 | 500 | 500 | 750 | 1000 |
| 14 | DEAEA | Y | 2 | 5 | 500 | 500 | 750 | 1000 |
| 15 | DEAEA | Y | 12 | 15 | 500 | 500 | 500 | 188 |
| 16 | DEAEA | Y | 12 | 5 | 500 | 250 | 500 | 50 |
| 17 | MEA | N | 2 | 21 | 750 | >1500 | 750 | 1500 |
| 18 | MEA | N | 2 | 4 | 750 | 500 | 625 | 1000 |
| 19 | MEA | N | 12 | 25 | 750 | >1500 | 750 | 750 |
| 20 | MEA | N | 12 | 4 | 750 | 500 | 750 | >1500 |
| 21 | MEA | Y | 2 | 21 | >1500 | >1500 | >1500 | 250 |
| 22 | MEA | Y | 2 | 4 | 500 | 500 | 500 | 750 |
| 23 | MEA | Y | 12 | 25 | >1500 | >1500 | >1500 | >1500 |
| 24 | MEA | Y | 12 | 4 | 500 | 250 | 500 | 188 |

[a]DP(degree of polymerization) values were determined by $^1$H NMR peak integration analysis. [b]MIC (μg/mL).

Table 2 Antibacterial Testing of lead compounds against polymyxin-susceptible (S) *A. baumannii* ATCC 19606 and polymyxin-resistant (R) 19606R

| Polymer N° | Monomer/ Compound | Quaternized (Y/N) | DP | Initiator chain length | MIC *A. baumannii* S (μg/mL) (Data from Table 1) | MIC *A. baumannii* R (μg/mL) |
|---|---|---|---|---|---|---|
| - | Polymyxin B | - | - | - | 2 | >128 |
| 4 | DMAEA | N | 5 | 12 | 50 | 38 |
| 5 | DMAEA | Y | 27 | 2 | 500 | 375 |
| 7 | DMAEA | Y | 26 | 12 | 250 | 88 |
| 12 | DEAEA | N | 5 | 12 | 88 | 15 |
| 16 | DEAEA | Y | 5 | 12 | 250 | 20 |

**Hit validation**

From the 24 polymers that were tested against the bacteria panel five polymers (Polymers 4, 5, 7, 12, and 16 in Table 1) were identified as the most effective compounds. These demonstrated either the lowest MIC value against at least one of the bacteria and/or had an MIC value below 100 μg/mL. These were subsequently tested against a drug resistant strain of *A. baumannii* (as shown in Table 2). This strain had gained resistance to polymyxins by complete loss of LPS in the outer membrane, this being attributed to inactivation of a gene responsible for biosynthesis responsible of LPS.[37]

The LPS layer is required for the initial polymyxin B binding, (followed by entry into the membrane leading to bacterial cell death), and as such loss of LPS causes resistance to polymyxins and other drugs having a similar mode of action. However, the loss of LPS causes increased susceptibility of the bacteria to other clinically relevant antibiotics, such as the penicillin class and carbapenems.[36] This is because the outer membrane provides a permeability barrier[37], and as such loss of LPS causes the outer membrane to become more permeable to other antibiotics.

The results presented in Table 2 show that in the LPS-deficient strain, the polymers have increased antibacterial activity. This implies that the main obstacle for these polymers in killing Gram-negative bacteria is the outer membrane.

To further investigate the mechanism of action of the lead compounds, quaternized PDEAEA with DP of 5 and $C_{12}$ tail (Q-PDEAEA$_5$-$C_{12}$, Polymer 16) was tested in both an inner membrane depolarization assay and an outer membrane permeabilization assay against Gram-negative *E. coli*.

**Inner Membrane Depolarization Assay**

In order to determine if Q-PDEAEA$_5$-$C_{12}$ causes the depolarization of cytoplasmic membrane, we performed a diSC3-5 assay, which has been widely used for measuring potential changes in bacterial cells. DiSC3-5 accumulates in cells on hyperpolarized membranes where it exhibits self-quenched fluorescence.[28] If the membrane loses its potential or becomes disrupted, the dye is released from the cell and fluorescence is generated. The assay revealed that there is an increase of fluorescence intensity on the addition of the polymer into *E. coli* suspensions, in a dose-dependent manner (Figure 2). This observation indicates that the polymer does indeed interact with the bacterial cytoplasmic membrane and causes inner membrane depolarization/disruption.[39, 40]

**Outer Membrane Permeabilization Assay**

To investigate the interaction of the best-performing polymer (i.e. Q-PDEAEA$_5$-$C_{12}$) with the outer bacterial membrane of *E. coli* NPN was employed as a molecular probe. NPN is a small hydrophobic molecule that fluoresces when in the hydrophobic environment of lipid membranes, but only weakly fluoresces in aqueous environments. NPN is excluded from intact bacterial outer membrane and therefore the observed increase in fluorescence on the addition of the polymer confirms that the polymer permeabilized the outer membrane of the bacteria (Figure 3).[39, 41, 42] The intensity reached a plateau at the concentration of 30 μg/mL, and started to decline at higher concentrations (100 and 300 μg/mL). This could be due to the release of some NPN dye back into the aqueous environment when the outer membrane was disrupted.

**Haemolytic Activity and Cytotoxicity**

The best performing polymers with the greatest antimicrobial activity were subsequently tested against human red blood cells to evaluate their haemolytic activity over a range of concentrations, as shown in Figure 4. Importantly, where low MIC's were recorded for certain bacteria (e.g. <25 μg/mL) there was minimal haemolytic effect at this minimum inhibitory concentration. Moreover, the quaternized polymers generally had a much lower effect on the red blood cells than did the non-quaternized polymers. However, where there was reduced activity (e.g. against the Gram-negative bacteria) the MIC values did exceed the $HC_{50}$ (the value where 50% of the red blood cells are lysed). That said, there were examples (Polymer 5 and Polymer 7) where even the highest concentration tested (1500 μg/mL) did not cause 50% of the red blood cells to be lysed. The ability to lyse human blood cells is likely due to the hydrophobicity of the polymer. Excessively hydrophobic peptides are toxic to both human and bacterial cells because the hydrophobic groups enable binding and insertion into human cell membranes without the aid of electrostatic attraction from the cationic groups. Moreover, Locock *et al.* have demonstrated that polymers with a higher percentage of hydrophobic groups cause increased haemolysis.[9] Tejero *et al.* have also shown that when the hydrophobic group was increased in length from an ethyl group to a hexadecyl group the haemolytic effect of the polymer was increased.[43] Punia *et al.* have also reported that when increasing the cationic and hydrophobic components of an antibacterial copolymer there was a concomitant increase in haemolysis.[44] These results demonstrate that it is critically important for the proportion of cationic and hydrophobic residues in cationic antibacterial polymers to be optimised to allow selective killing of bacteria with minimal effect on mammalian cells.[45]

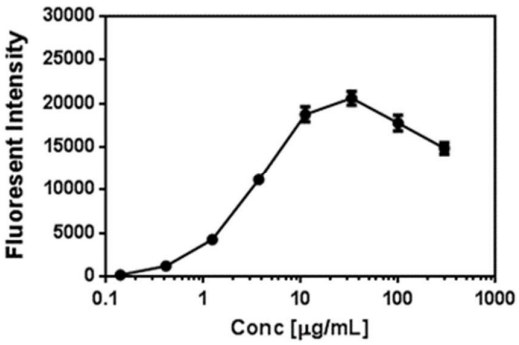



Figure 2 DiSC3-5 assay of Q-PDEAEA$_5$-C$_{12}$. Data are presented as Mean ± Standard deviation

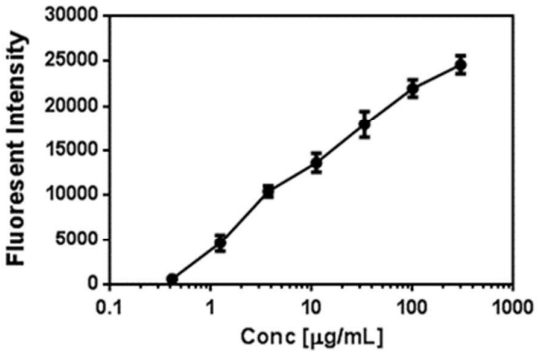



Figure 4 NPN uptake of Q-PDEAEA$_5$-C$_{12}$. Data are presented as Mean ± Standard deviation

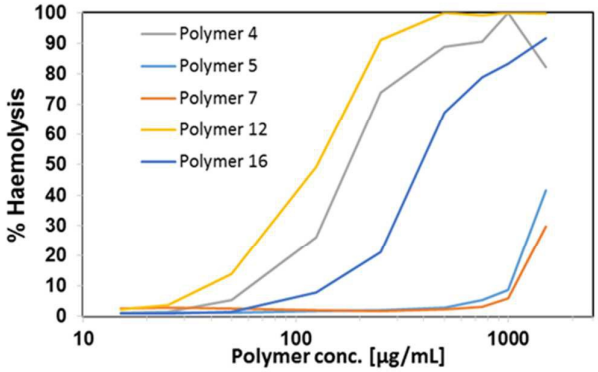



Figure 3 Haemolytic effect of polymers after 1 hour exposure to human red blood cells

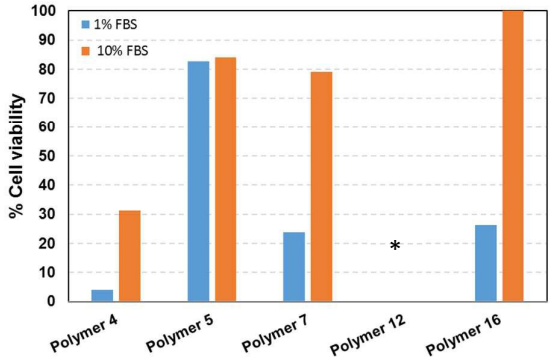



Figure 5 Cell viability for cells against lead compounds (100 µg/mL). *below level of detection

Cytotoxicity to mammalian cells is different to haemolytic activity, as cytotoxicity depends more on the number and arrangement of the cationic charges rather than the structure of any hydrophobic moiety.[46, 47] Generally speaking, as the number of cationic groups increases so does the cytotoxicity.[47] Moreover the cytotoxicity can be reduced by increasing the hydrophobicity of the polymer.[26, 49] The superior compounds were also tested for their cytotoxicity to mammalian cells (HepG2), and the quaternized polymers proved less toxic than the non-quaternized polymers as shown in Figure 5.

Polymers 4, 7, and 12 showed toxicity against HepG2 cell at 100 µg/mL in 1% FBS culture condition resulting in more than 50% cell death. However, in the presence of 10% FBS, only the polymers 4 and 12 induced more than 50% cell death. This observed difference to the 1% FBS outcome is attributed to the higher concentration of growth factors in the 10% FBS which bind with the polymers and reduce the effective concentration of active polymer. This has been observed previously for cationic nanoparticles where the proteins in FBS bind to the nanoparticles lowering the effective cationic charge, and thus reduce the interaction with the cell membrane.[50, 51] Overall, the results for the cell viability test are similar to the results for the haemolysis test. Interestingly, the quaternized polymers showed the least toxicity to human cells compared to the non-quaternized polymers for both haemolysis and cell viability.

**Selectivity: Antimicrobial Activity versus Haemolysis**

Ideally, an antimicrobial compound that is intended for human medical applications should combine both high antimicrobial activity (low MIC) with low haemolytic activity. By comparing the haemolytic effect against the minimum inhibitory concentration for a given bacterium, we can gain an understanding of the selectivity of the lead compounds to bacteria tested compared to human red blood cells. The selectivity index is used routinely to describe the selectivity of the compound towards a particular bacterium.[52, 53] The selectivity index is obtained by dividing the $HC_{50}$ value by the MIC value for a given bacterium; and these values are shown in Table SI 5. From the data presented in Table SI 5, it can be observed that Polymers 5, 7 and 16 show selectivity indices ≥8 for S. aureus, while Polymers 7 and 16 have selectivity indices >17 against the resistant strain of *A. baumannii*. On the other hand Polymers 4 and 12 show limited selectivity towards all bacteria studied compared to the other lead compounds. Interestingly, the higher selectivity of Polymers 5, 7 and 16 agrees well with the cell viability results shown in Figure 5.

## Conclusions

A library of polymers was synthesized using Cu(0)-mediated polymerization with varying length of the hydrophobic tail and

cationic segment in order to evaluate the effectiveness of low DP cationic polymers against clinically significant pathogens. The polymers were quaternized to examine the effect of introducing quaternary ammonium groups on antimicrobial activity. From this study it has been shown that when the DP of a $C_{12}$ tail polymer is decreased, then the antimicrobial activity generally increases. Quaternization of the polymers increased the activity against *S. aureus*, but decreased the activity against the Gram-negative bacteria. This was attributed to the structural differences between Gram-positive and Gram-negative bacteria. By examining the mode of action using a membrane sensitive fluorescence assay it was demonstrated that, for *E. coli*, Polymer 16 caused cell death by disrupting the cell envelope. Quaternization of the polymers was also shown to decrease haemolytic activity against human red blood cells due to the associated decrease in overall hydrophobicity of the polymer. Somewhat surprisingly, quaternization of the polymer also decreased the cytotoxicity of the polymer when tested against mammalian HepG2 cells. These results indicate that low DP cationic polymers may have some utility in treating certain bacterial infections.

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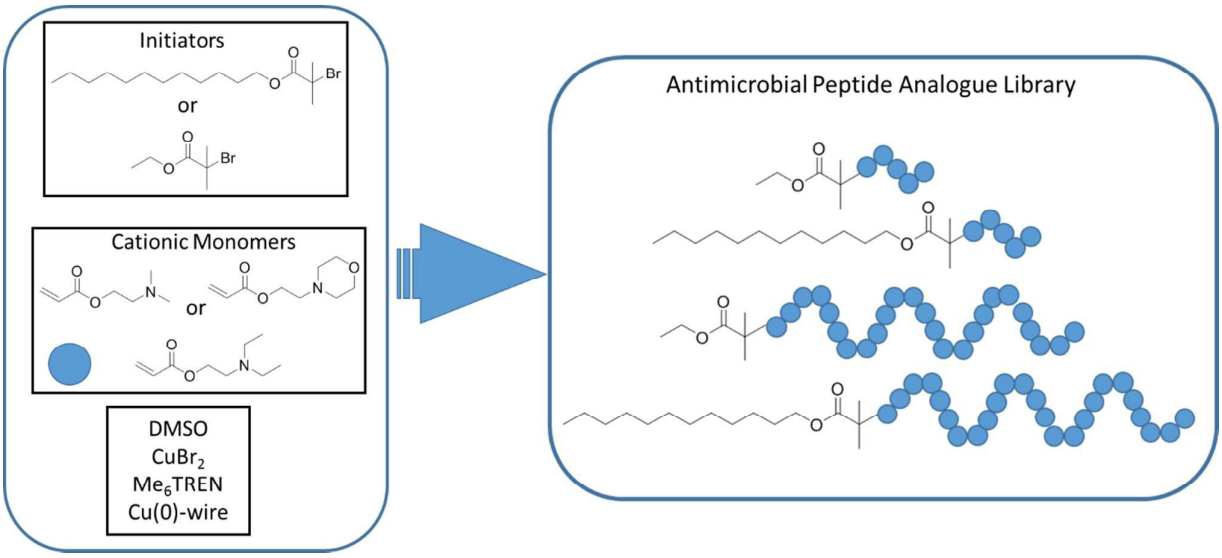


We report the antibacterial activity of a novel class of low molecular weight cationic polymers synthesised using Cu(0) mediated polymerisation.