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RAFT-derived antimicrobial polymethacrylates: Elucidating the impact of end-groups on activity and cytotoxicity

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Antimicrobial polymers as mimics of natural antimicrobial peptides are emerging as an alternative to classic antibiotics due to their potency, selectivity and lower susceptibility to resistance. The key chemical aspects necessary to confer high activity and selectivity to the polymer chain composition are largely known. However, little attention has been paid to how end-groups affect the overall biological activity. Here we report the use of RAFT polymerization to obtain eight well-defined cationic methacrylate polymers which bear either amine (PA1-4) or guanidine (PG1-4) pendant groups, while systematically varying the R- and Z-RAFT end-groups. These polymers were assessed in haemotoxicity assays as well as antimicrobial testing against clinically relevant pathogens; such as a vigorously biofilm forming strain of *Staphylococcus epidermidis* (*S. epidermidis*) and a vancomycin and methicillin resistant strain of *Staphylococcus aureus* (VISA) as well as the opportunistic fungus *Candida albicans (C. albicans)*. The R-group was found to dominate the measured toxicity of polymers. Replacement of the anionic cyanovaleric acid R-group (PA1) with the neutral isobutyronitrile (PA3) led to over a 20 fold increase in the haemolytic activity of the polymers. The Z-group, however, was found to have more influence on the antimicrobial activity of the polymers against both VISA and *C. albicans*, whereby polymers with a long, lipophilic dodecylsulfanyl Z-group (PA1) were found to be more potent than those with either an ethylsulfanyl or no ZCS_2-group. These results indicate that chemical control over the end-groups is a key element for achieving the desired high biological activity and selectivity, particularly when low molecular weights are required for maximum antibacterial activity.

Introduction

Infections caused by drug resistant bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA), are on the rise. The 2014 World Health Organisation (WHO) report confirms the increase in numbers of pathogens which exhibit resistance to common antibiotics.[1] For instance, 5 out of 6 WHO regions have reported 50% or more resistance of MRSA. This increased prevalence brings with it elevated patient morbidity, preventable deaths and an amplified burden on the healthcare system. Hence, there is an urgent need to identify new classes of antimicrobial agents which are in principle not subject to the buildup of tolerance and can be manufactured simply and inexpensively.

Natural antimicrobial peptides (AMPs) have recently gained considerable scientific attention as potential new leads in the fight against bacterial resistance.[2-4] Their mode of action is to bind to negatively charged phosphate head-groups present on the bacterial membrane, followed by induction of membrane permeabilisation and cell death. On the other hand, mammalian cells normally have a net neutral charge at their surface, thus explaining the high selectivity that AMPs display for bacterial cell membranes over those of mammalian cells. For bacteria to develop resistance to an AMP, this would involve altering their entire membrane composition, an outcome which is unlikely to occur given the complexity of the evolutionary changes that would be required.[5] In comparison, classic antibiotics mediate their effects through binding to specific protein target sites inside the bacteria, the genes for which can mutate rapidly and thus render the drug ineffective.

While AMPs show potential as antimicrobial agents, the expense of large scale peptide synthesis and their susceptibility towards proteases renders them commercially problematic. For these reasons, researchers are now trying to imitate the structure and thus antimicrobial activity and selectivity using synthetic polymer mimics. This has been achieved with various classes of polymers such as polymethacrylates[6], polyethers[7], polycarbonates[8], poly-β-lactams[9] and polynorbornenes[10].

The focus of much of this published work has been on establishing the relationship between the composition of the polymer chain and antimicrobial efficacy and selectivity. For example, it has been shown that changes in polymer length[11], hydrophobic character[12] and the nature of the chemical moiety[13] carrying the cationic charge can all have dramatic effects on polymer activity profiles.[14-17] In general, antimicrobial agents, including antimicrobial polymers, whether they be for topical or systemic delivery are developed to meet the common criteria of being highly active against clinically relevant pathogens, while maintaining a low level of human cell toxicity. The antimicrobial activity of agents is typically measured using minimum inhibitory concentration assays which denote the lowest concentration of a given agent to completely inhibit microbe growth. Human cell toxicity is commonly assessed using human red blood cell assays that measure the relative ability of agents to lyse cells (haemolysis) or cause cell agglutination (haemagglutination).

For example, Kuroda, Palermo and co-workers studied the impact of changing the hydrophobic character of cationic methacrylate copolymers by the systematic variation of monomer ratios, nature of the hydrophobic moiety and the spacer length of pendant cationic groups. Their key findings

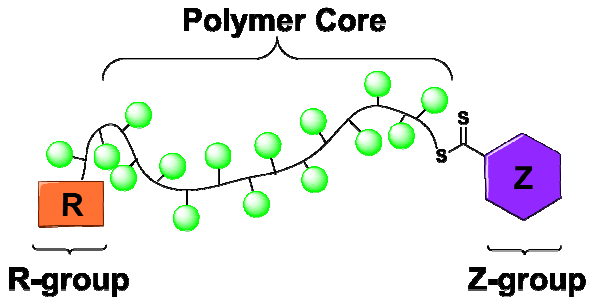


Figure 1: Schematic of the structure of a RAFT-derived polymer

were that the use of higher molar ratios of hydrophobic monomer, bulkier hydrophobic monomers or increases in the length of the hydrophobic spacer arm of the cationic moiety led to a decrease in the selectivity profile of the polymers tested.[18-20] Furthermore, Kuroda and co-workers as well as Gellman et al. have shown that increasing the polymer length lead to an increase in the haemolytic activity.[11, 12] The chemical nature of the cationic pendant groups also plays an important role as Tew and co-workers observed a dramatic increase of activity and specificity in one of their polymers by replacing the pendant amine groups with guanidine moieties.[21] There has, however, been little attention devoted to the impact of polymer end-groups on the activity and selectivity of antimicrobial polymers to date. To the best of our knowledge, only two such studies exist. Mowery and co-workers reported that the hydrophobic nature of polymer end-groups could have an impact on both haemolytic and antibacterial activity in the case of poly-β-lactams.[11] Utilizing the same polymer chain type, Zhang et al. showed that even small alterations to end-group chemistry such as reversing their positions could lead to a dramatic change in haemolytic activity.[22] No such studies have been performed with any of the other polymer classes. If we are to more fully understand the structure-activity relationships (SARs) governing antimicrobial polymers, we need to consider the structure of polymers as a whole, encompassing both the chain composition and the end-groups, as it appears that both are relevant to deriving molecules which are both potent antimicrobials and non-toxic to mammalian cells.

In previous work we have reported the use of Reversible Addition-Fragmentation chain Transfer (RAFT) polymerisation methods to generate series of well-defined antimicrobial polymethacrylates bearing either amine or guanidine pendant groups.[23-25] The general structure of these polymers is made up of three components (Figure 1), the polymer chain, constructed

from the sequential addition of monomer units and the two end-groups labelled R and Z which stem from the RAFT agent used in the synthesis. This study established a number of SARs for the polymethacrylate polymer chain; whereby the guanylated polymers showed superior activity and selectivity when directly compared to their amine analogues. Furthermore, our investigation revealed that lower molecular weight polymers with a hydrophobic component of approximately 30% were the most active and of the lowest toxicity.

In the current study, we aimed to extend our SAR investigations to cover the impact of the RAFT end-groups for the amine and guanidine based polymethacrylates. Thus, we have designed a series of RAFT-derived polymers that systematically differed in both Z- (Z = $S(CH_2)_{11}CH_3$, SCH_2CH_3 or no ZCS_2-group) and R-groups (the tertiary R-group consisted of 4-cyanovaleric acid or isobutyronitrile). In each case, the polymer chain was kept constant and corresponded to the 'optimal' composition and chain length based on previous findings, namely low molecular weight and a 30 % hydrophobic content. This allowed for the clear definition of the role that RAFT end-groups may play in the polymer activity and selectivity (bacterial/fungal versus mammalian cells).

Therefore, to establish relevant SARs governing the role of RAFT end-groups, the polymers synthesised were challenged with a number of clinically relevant pathogens including methicillin and vancomycin resistant *S. aureus* (Vancomycin intermediate *S. aureus* (VISA)) and *Staphylococcus epidermidis* (*S. epidermidis*) using Müller-Hinton Broth (MHB) growth media. Furthermore, biological efficacy against the fungus *Candida albicans* (*C. albicans*), which is often observed as a co-pathogen in *S. aureus* infections[26], was also determined. To evaluate the toxicity of the polymers, their haemolytic and haemagglutination potential was determined using human red blood cells. The sum of these biological tests allowed for comparison of activity and selectivity of the polymers and the elucidation of the SARs governing RAFT end-groups.

Experimental

Materials

N,*N*'-azobisisobutyronitrile (AIBN), diethyl ether, dimethyl sulfoxide (DMSO), methanol (MeOH), ethanol (EtOH), 1*H*-pyrazole-1-carboxamidine hydrochloride, *N*,*N*-diisopropylethylamine (DIEA), 1-ethylpiperidine hypophosphite (EPHP), 1,1′-azobis(cyclohexanecarbonitrile) (Vazo-88) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. 2-Aminoethylmethacrylate hydrochloride (AEMA) was purchased from Polysciences (Warrington, PA). The RAFT agents (CTAs), 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid, 2-cyanopropan-2-yl dodecyl carbonotrithioate and 4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid were synthesized according to literature methods.[27, 28] Oxoid™ Nutrient Agar (CM0003), Oxoid ™ Müller-Hinton Broth (MHB) (CM0405) and 96 well plates (NUNC ™) were sourced from Thermo Fisher Scientific. Concanavalin A, RPMI-1640, Triton X (100) and phosphate buffered saline (PBS) (SLBB6584) tablets were obtained from Sigma-Aldrich. All chemicals were used as received and according to their recommended concentration. Milli-Q™ filtered water was used to prepare solutions and these were autoclaved before their usage. The utilized bacterial strains were *S. epidermidis* ATCC® 35984 ™ and vancomycin intermediate *S. aureus* (VISA) A8094 (JH9). The fungal strain was *Candida albicans* Day185.

Polymer synthesis

Synthesis of Amine Polymers PA1-PA3

Reversible addition−fragmentation chain transfer (RAFT) polymerization of 2-AEMA and MMA was performed in DMSO at 70 °C for 18 h using 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CTA1), 2-cyanopropan-2-yl dodecyl carbonotrithioate (CTA2) or 4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CTA3) as the RAFT agent and AIBN as the radical initiator. A representative procedure for CTA1 to give PA1 is given below. 2-AEMA (4.64 g, 28 mmol), MMA (1.20 g, 12 mmol), AIBN (98 mg 0.6 mmol), and chain transfer agent (807 mg, 2 mmol) were dissolved in DMSO (6 mL) in a 50 mL Schlenk flask. The reaction was subject to three freeze−evacuate−thaw cycles under high vacuum (10^{-3} Torr) before being heated to 70 °C for 16 h. The crude product was first diluted with MeOH before being precipitated three times into ether, collected each time by centrifugation. All traces of solvent were removed under high vacuum to give PA1 as a yellow powder (5.66 g, 85% yield).

Synthesis of Guanidine Polymers PG1-PG3

A post polymerization guanylation method was used to convert amine polymers PA1, PA2 and PA3 to the corresponding guanidine functionalized polymers PG1, PG2 and PG3.[24] A representative procedure is given below.

To a solution of PA1 (4 g, 1 mmol) in anhydrous methanol (50 mL), was added 1*H*-pyrazole-1-carboxamidine hydrochloride (3.78 g, 26 mmol) and *N,N*-diisopropylethylamine base (6.34 g, 49 mmol), which equated to 1.5 and 3 equivalents to the number of amine units per polymer chain (see polymer characterization for details). The reaction was heated at 55 °C overnight under nitrogen positive pressure. Solvent was removed in vacuo and the polymer purified by precipitation from methanol−acetone three times to obtain PG1 as a slight yellow powder in quantitative yield.

Radical Reduction Removal of RAFT End-Groups to give PA4 and PG4

A radical induced reduction method was used to convert PA1 and PG1 into the corresponding proton terminated PA4 and PG4. A representative procedure is given below.

To a solution of PA1 (600 mg, 0.15 mmol) in DMSO (5 mL) was added Vazo-88 (13 mg, 0.075 mmol, 0.5 eq) and EPHP (367 mg, 1.5 mmol, 10 eq) in a 50 mL Schlenk flask. The reaction underwent three high vacuum (10^{-3} Torr) freeze-evacuation-thaw cycles before being heated to 100°C for 16 h. The product was isolated as the hypophosphite salt via three precipitations from methanol-acetone followed by high vacuum to remove trace solvent. This gave PA4 as a white powder (421 mg, 76% yield). The complete removal of RAFT end-groups was confirmed using UV-Vis and ^{1}H NMR analysis (see supporting information for relevant spectra).

Polymer Characterization

Size exclusion chromatography (SEC) was performed on a Shimadzu system with a CMB-20A controller system, a SIL-20A HT autosampler, a LC-20AT tandem pump system, a DGU-20A degasser unit, a CTO-20AC column oven, a RDI-10A refractive index detector, and four Waters Styragel columns (HT2, HT3, HT4, and HT5). Columns were 300 × 7.8 mm^2, providing an effective molar mass range of 100 to 4 × 10^6. *N,N*-Dimethylacetamide (DMAc; with 2.1 g L^{-1} of lithium chloride (LiCl)) was used as an eluent with a flow rate of 1 mL/min at 80 °C. Calibration curves were measured from low dispersity poly(methyl methacrylate) standards purchased from Polymer Laboratories. Dispersity ($Đ$) values were calculated using the Shimadzu software package. UV-visible absorption spectra were recorded on a Cary 5E spectrophotometer. NMR solvent (CD_3OD) was purchased from Cambridge Isotope Laboratories and used as received. ^{1}H NMR spectra were recorded at 400 MHz using a Bruker Avance 400 MHz NMR spectrometer (Billerica, MA). Chemical shifts (δH) are reported in parts per million (ppm). M_n (number average molecular weight), % conversion (% monomer conversion during polymerization), MP_{methyl} (mole percentage of methyl side chains), and DP (degree of polymerization) values were determined by ^{1}H NMR peak integration analysis as previously described.[23-25, 29]

Dynamic light scattering (DLS) measurements were conducted on a Malvern Zetasizer Nano ZS® utilizing the Mark-Houwink equation. For this purpose, the polymers were diluted to a concentration of 128, 64, 32 and 16 µg/mL in PBS and the light scattering was measured at 37°C with a backscattering angle of 173°. Parameters for the refractive index and absorption were chosen to be 1.45 and 0.001 respectively. The measured values were plotted as count % versus size. Concentrations at which no particle size could be measured were omitted.

Antibacterial susceptibility tests

The Minimum inhibitory concentrations (MICs) of single end-product for planktonic cells at mid-log phase were determined by standard CLSI broth microdilution.[30] One hundred microliter volumes of bacterial suspensions (10^6 CFU/mL) were added to microwells preloaded with same volumes of twofold serial dilutions of end-product. MHB was used as growth media for the susceptibility tests respectively. Plates were incubated at 35°C aerobically for 18 h. MICs were defined as the lowest concentration of antibiotics that prevented the establishment of visible turbidity after overnight exposure. The tests were conducted a minimum in triplicates and the reported MIC was determined as the median value and its standard deviation.

Antifungal susceptibility tests

MICs were determined using the broth microdilution method according to CLSI guidelines M27-A3[31]. One hundred microlitre of two-fold serial dilutions of the compounds prepared in RPMI-1640 were added into wells of 96 well plates. Exponentially grown cultures were diluted in RPMI-1640 to a density of ~1-5 × 10^3 CFU/mL and 100 µL of culture were added to each well. Plates were incubated for 48 h at 37°C. Fungal growth was examined visually with the aid of a mirror reader. The MIC was defined as the concentration resulting in complete growth inhibition. The experiments were performed on two separate occasions, with three independent biological repeats included on each occasion. The geometric means of MICs were calculated from the six determinations for each of the compounds, taken to the closest double dilution. The reported MIC was determined as the median value and its standard deviation.

Haemolysis and Hemagglutination Testing

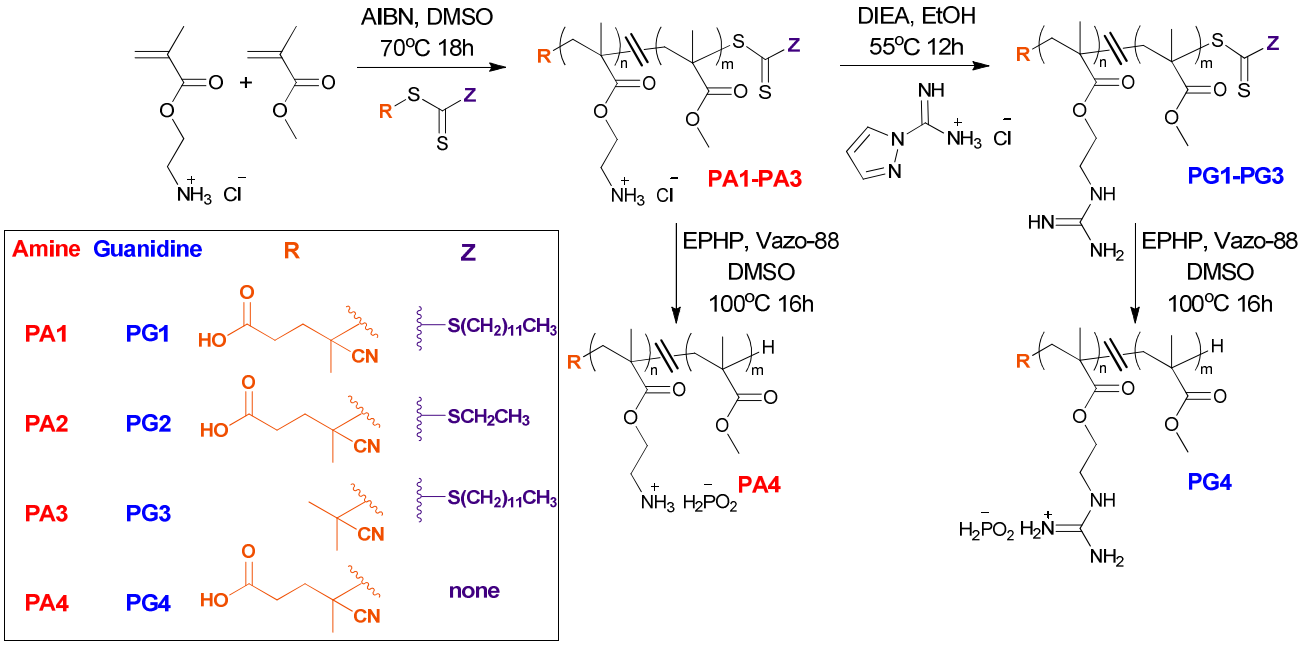
Rinsed human red blood cells (RBC's) were prepared from 2 mL human blood freshly collected in EDTA tubes (Greiner Bio-One) and subjected to four washes with 10 mL endotoxin-free PBS via centrifugation (10 min at 10,000 rpm; 20°C).

Polymers were dissolved in PBS (1500 µg/mL) and twofold dilutions (50 µL) were performed in round bottom plates (BD Biosciences) to a minimum concentration of 1.47 µg/mL. Positive controls (50 µg/mL Concanavalin A for haemagglutination and 0.2% Triton X-100 for haemolysis) and negative control wells (PBS alone) were included on each plate. A suspension of washed RBC (2% v/v, 50 µL) was added to each well and the contents were mixed before 2 hours incubation (37 °C). Macroscopic appearance of the wells was recorded following incubation. Hemagglutination was indicated by noticeable turbidity from the presence of a wide layer of agglutinated cells and the presence of a transparent red solution was indicative of marked haemolysis. A tight central cell button was interpreted as negative for haemagglutination.

Microplates were centrifuged (5 min, 3000 g, 20 °C) and supernatant (60 µL) was subsequently transferred to a clean 96-well flat-bottom microplate for measurement of absorbance of free haemoglobin (570 nm, BIO-TEK Instruments). Percent haemolysis was calculated relative to the positive haemolysis control (Triton-X-100) as follows:

$$\%\text{haemolysis} = \frac{\text{Abs of sample} - \text{Abs of negative control}}{\text{Abs of positive control} - \text{Abs of negative control}} \times 100$$

The well contents in the original microplates were resuspended by repeat pipetting with PBS (60 µL per well) and scored for haemagglutination as strong ++++, moderate +++, mild ++, weak +, or none 0. The presence of visible clumps despite resuspension was representative of haemagglutination whereas wells that were easily resuspended were indicative of an absence of haemagglutination. Data shown are representative of experiments performed in quadruplicate.



Scheme 1: Synthesis of random copolymers bearing amine (PA1-PA4) or guanidine (PG1-PG4) pendant groups that vary in both RAFT Z- and R-groups

Results & Discussion

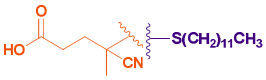
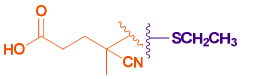
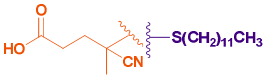
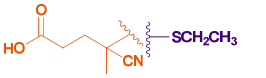
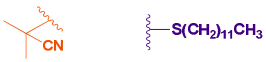
Polymer Design and Synthesis

In previous work, we investigated the potential for RAFT-derived cationic polymethacrylates to act as antimicrobial agents.[23-25] Through systematic optimisation of aspects relating to the polymer chain composition and length we were able to identify a number of candidates that showed highly potent effects against both bacteria and fungi, concordant with low human cell toxicity. These corresponded to those with guanidines as a cation source, a low molecular weight and a low ratio of lipophilic to cationic monomers. These polymers were all synthesised using the RAFT agent (CTA), 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid, a trithiocarbonate (CTA1, Figure 2). This CTA was chosen based on suitable R- (the tertiary 4-cyanovaleric acid) and Z-groups (dodecylsulfanyl, deriving a trithiocarbonate RAFT agent) to provide suitable solvent compatibility and give adequate control over the polymerization of methacrylate monomers. Owing to the short length of polymers within this study, down to an average of only 20 monomer units, the influence stemming from the presence of these RAFT end-groups needs also to be considered. For this reason we prepared a series of cationic polymethacrylates with similar polymer chain composition to that previously described but where both R and Z RAFT end-groups were varied. This was achieved partially through the use of various RAFT agents, either commercially available or synthetically accessible via various chemistries.[32-34] This allowed systematic investigation of the impact of both the R- and Z-groups on the antimicrobial/antifungal activity in a systematic manner. First, to examine the relative importance of the carboxylic acid for the R-group, a comparison was to be made between polymers derived using CTA1 and CTA2. CTA2 maintained the tertiary R-group important for good RAFT control of methacrylates but lacked the terminal carboxylic acid. Holding the Z-group constant between these CTAs allowed for the independent examination of the role of the carboxylic acid group in observed biological activity for derived polymers. To examine the effect of the long lipophilic dodecylsulfanyl Z-group of CTA1, a comparison was made to the ethylsulfanyl CTA3. Extrapolating this further, we also

aimed to investigate the activity profile of polymers that contained no ZCS_2-group, thus providing a test set of three groups, a Z-group that donated a large lipophilic bulk (CTA1), a small lipophilic bulk (CTA2) and no lipophilic group. A number of groups have identified means of RAFT Z-group modification or removal including aminolysis to derive thiols and either thermolysis or radical removal to derive sulphur-free end-groups. For comprehensive reviews see Willcock et al.[35] and Moad et al.[36] The major drawback with the use of aminolysis is that the presence of the free thiol groups can lead to polymer dimers through formation of a disulfide bond, thus convoluting SAR investigations.[37, 38]

Thermolysis, while having the advantage of requiring no addition of chemical reagents, does require the polymers to be stable at temperatures in the range of 120-200 °C.[39] RAFT end-group removal involves the use of free radicals, that react with the C=S bond of the RAFT group to form an intermediate radical which fragments to give new thicarbonylthio compound and a propagating radical on the polymer chain.[27, 40] If this occurs in the presence of an efficient hydrogen atom donor (H-donor) such as ethylpiperidine hypophosphite (EPHP), the propagating radical reacts the H-donor to give the desired product in which the RAFT group is effectively replaced with a hydrogen atom.

Table 1. Polymer nomenclature and characterization

Polymer	R	Z	MP_{methyl} (%)[a]	DP[a]	M_n[a] ^{1}H NMR	M_n[b] SEC	$Đ$[c]
PA1			31	24	4000	9190	1.16
PA2			32	24	3700	9240	1.13
PA3			31	24	3900	10900	1.14
PA4			28	24	4000	n/a[d]	n/a[d]
PG1			29	24	4000	14500	1.17
PG2			30	24	3900	15200	1.16
PG3			32	25	3700	18700	1.16
PG4			29	24	3700	n/a[d]	n/a[d]

[a] M_n (number average molecular weight), MP_{methyl} (mole percentage of methyl side chains) and DP (degree of polymerization) values were determined by ^{1}H NMR peak integration analysis as described in the supporting information; [b] M_n as determined by GPC [c] dispersity ($Đ$) was determined by GPC analysis in DMAc against PMMA standards. [d] $Đ$ could not be determined as polymer was insoluble in DMAc.

This method typically maintains relatively mild conditions and tolerates a wide range of functional groups. Radical removal appeared to be the most applicable method for our purposes. A series of random copolymers of 2-AEMA and MMA were prepared using CTA1-CTA3 to give PA1-PA3 with methods described previously.[23-25] The targeted monomer ratio and molecular weight were closely matched to those previously identified as the 'optimal range' to maximise antimicrobial activity and minimise human cell toxicity. This gave polymers with relatively low molecular weight (degree of polymerization (DP) = 24), a low level of lipophilicity (MP_{methyl} = 31-32%) and low dispersity ($Đ$ = 1.13-1.16). Hence, varying the CTA agent did not appear to dramatically alter the associated RAFT kinetics during polymerization, giving polymers that were well matched in relation to these properties. Note that ^{1}H NMR analysis was found to more accurately estimate number average molecular weights (M_n) values, that those derived using SEC and was hence used in all further analysis. This deviation can be attributed to the difference in the hydrodynamic volume of the cationic polymers of interest to those of the polymethacrylates standards used in SEC analysis. This phenomena has been reported previously by various authors[41-43] and is discussed further in Locock et al. 2014.[29] The corresponding guanidine polymers, PG1-PG3, were synthesised from PA1-PA3 using a base-catalysed post-polymerization guanylation reaction described previously.[23-25] This gave polymers with similarly matched molecular weight (DP = 24-25), lipophilicity (MP_{methyl} = 29-32%) and dispersity ($Đ$ = 1.16-1.17). PA1 and PG1 were treated with radical initiator Vazo-88 in the presence of the EPHP, a hydrogen atom donor, at 100 °C for 16 h to derive polymers PA4 and PG4. The complete removal of trithiocarbonate groups within these polymers was confirmed by UV-Vis spectroscopy, ^{1}H NMR analysis and the disappearance of the yellow colour associated with trithiocarbonate RAFT polymers (see supporting information for relevant spectra). NMR results also indicated that these polymers were isolated as phosphite salts, stemming from ion exchange with EPHP, of minimal consequence given that all biological testing was performed in the presence of phosphate buffers.

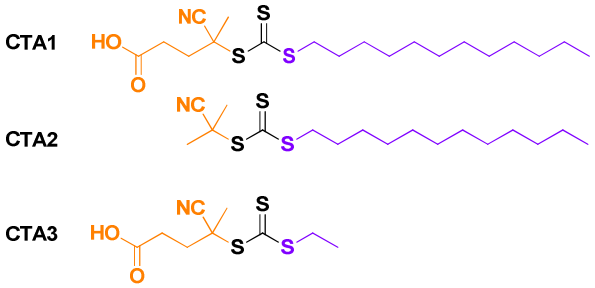


Figure 2: Structures of the three RAFT CTAs used in this study

The only drawback with this approach, however, was the limited solubility of the phosphite salt polymers in DMAc, the solvent used for GPC dispersity analysis. ^{1}H NMR spectra did indicate that both the monomer ratio (MP_{methyl}, PA4 = 28 and

PG4 = 29) and molecular weight (DP, PA4 = 24 and PG4 = 24) matched closely with those of PA1 ($MP_{methyl,}$ = 31 and DP = 24) and PG1 ($MP_{methyl,}$ = 29 and DP = 24). In total, four amine (PA1-PA4) and four guanidine polymers (PG1-PG4) were derived that matched closely for polymer chain composition but systematically varied both RAFT R- and Z-groups, thus allowing for a controlled investigation of how these structural variations influenced the measured antimicrobial activity and human cell toxicity.

General Antimicrobial and Haemotoxicity Trends

In line with previous reports[23-25], all RAFT derived cationic polymethacrylates tested in this study displayed relatively potent antimicrobial activity and in most cases low haemotoxicity (see supporting information). In general, both the amine and guanidine polymers were very active against *S. epidermidis* (16-31 µg/mL) and against VISA (16-128 µg/mL). In addition good to moderate activity was observed against *C. albicans* (32-256 µg/mL). For example, an MIC of 16 µg/mL was observed for PG1 against the Gram-positive vancomycin and methicillin resistant *S. aureus* (*VISA*), an MIC value of 16 µg/mL against *S. epidermidis* and an MIC of 32 µg/mL against *C. albicans*. A relatively low level of haemotoxicity was also observed for PG1, with a value of 13 % haemolysis and moderate haemagglutination when tested at the *S. epidermidis* MIC concentration. These results accord with our previous findings that the pathogens' antibiotic resistances do not lower the potency of the antimicrobial polymers.[24, 44]

The antimicrobial activity of the polymers against VISA signifies a major advantage over conventional antibiotics, as the dual-resistant VISA poses a major threat in modern healthcare. This is due to the various ailments caused by it such as urinary tract infection, chronically infected surgical wounds and pelvic abscesses; it is an infectious agent with potentially fatal outcomes and treatment costs of up to 100'000 USD per patient.[45, 46] Owing to the resistant nature of this pathogen it is essential that new agents are identified that are able to adequately control VISA related infections. The potent action of our cationic polymethacrylates against this bacterial strain creates a new lead in this critical area.

Strong activity of the polymers against *S .epidermidis* also shows promise as this is a pathogen, which together with other coagulase negative staphylococci, is associated with up to 70% of implant related infections.[47] Furthermore our synthesized polymers present a lead for the development of novel antibiotics which are simultaneously active against bacterial as well as fungal infections; such as with *C. albicans* which often occurs in conjunction.[48]

Across the three species tested, it appears that the lower MIC values were obtained for the guanidine based polymers (PG1-PG4) when compared to their equivalent amine polymers (PA1-PA4). The only exception was PG3 which was found to be slightly less effective against *C. albicans* than PA3 (PA3 MIC = 32 µg/mL, PG3 MIC = 128 µg/mL).

Toxicity testing was performed using human red blood cells (RBCs) and measured both the ability of polymers to lyse as well as agglutinate cells at an equivalent concentration to their MIC for *S. epidermidis*. Relatively low levels of toxicity were observed for the majority of polymers (PA1 = 1.2%, PA2 = 1.2%, PA4 = 3.3%, PG1 = 13%, PG2 = 10%, PG4 = 13%), except for PA3 (26%) and PG3 (21%), values which were indicative of a potential end-group mediated effect. All polymers were also found to have a low propensity for haemagglutination, giving low to moderate values for this property.

R-Group Influence on Antimicrobial Activity and Haemotoxicity

Presented in Figures 3 are comparisons between the antimicrobial activity of either amine-based (Figure 3A) or guanidine-based (Figure 3B) polymers synthesised using CTA1 (R = cyanovaleric acid) or CTA2 (R = isobutyronitrile).

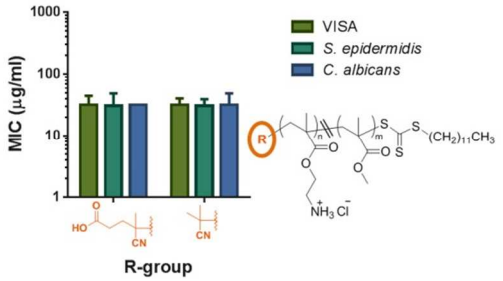


Figure 3A: Antimicrobial activity correlated to structural changes in the R end-group of the amine polymers

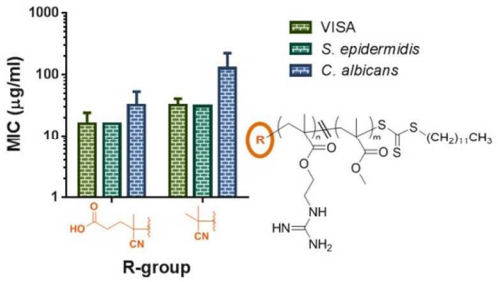


Figure 3B: Antimicrobial activity correlated to structural changes in the R end-group of the guanidine polymers

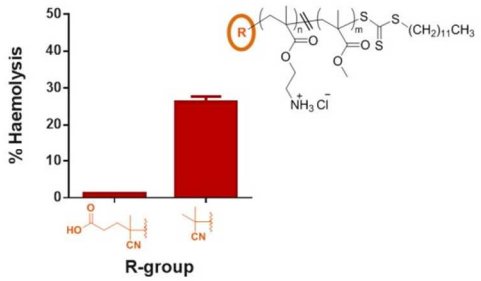


Figure 4A: Haemolytic activity correlated to structural changes in the R end-group of the amine polymers

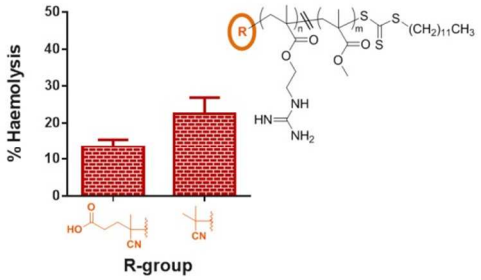


Figure 4B: Haemolytic activity correlated to structural changes in the R end-group of the guanidine polymers

For the amine polymers, changes in the R-group structure did not seem to influence bioactivity, whereas a difference was seen for the guanidine analogues in case of *C. albicans*. Here, PG1 which contained a cyanovaleric acid end-group was found to be four times more potent than PG3 which did not contain a carboxylic acid end group. Thus for antimicrobial activity, it appears that it may be beneficial to select RAFT agents that incorporate a terminal carboxylic acid functionality in the R-group.

Results from haemotoxicity testing, however, revealed a more marked R-group effect. Both for the amine (Figure 4A) and guanidine (Figure 4B) polymers, a significant increase in haemolytic behaviour was observed for polymers without a terminal carboxylic acid. When comparing PA1 and PA3, this difference was more than 20 fold. Such dramatic changes are surprising given that this corresponds to a relatively minor structural change, i.e. each polymer chain contains on average approximately 17 pendant cationic groups. It appears that the presence of a single terminal anionic group may potentially alter how polymer chains interact with RBCs. The mechanism underlying this is the focus of current work but may lie in the ability of these anionic end groups to influence polymer chain aggregation in solution or through specific interactions with RBC membrane-bound proteins or carbohydrate moieties.

These results suggest that the choice of the RAFT R-group may have a role in the activity profile of antimicrobial polymers. It also appears that a cyanovaleric acid based RAFT agent may favour the synthesis of highly potent agents that show minimal haemotoxicity.

Z-Group Influence on Antimicrobial Activity and Haemotoxicity

Controlling the hydrophobic character of the Z-group, by incorporating either a long (dodecylsulfanyl), a short alkyl (ethylsulfanyl) chain or cleaving it altogether, had an influence effect on the antimicrobial activity of both the amine and guanidine polymers (Figures 5A and B respectively).

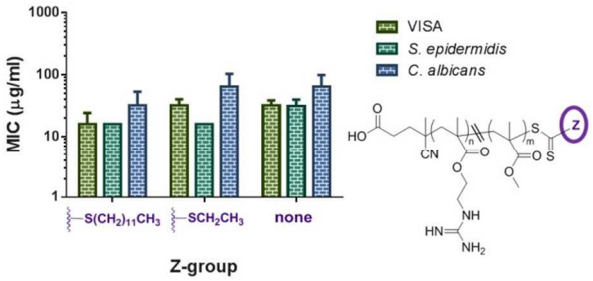


Figure 5A: Antimicrobial activity correlated to structural changes in the Z end-group of the amine polymers

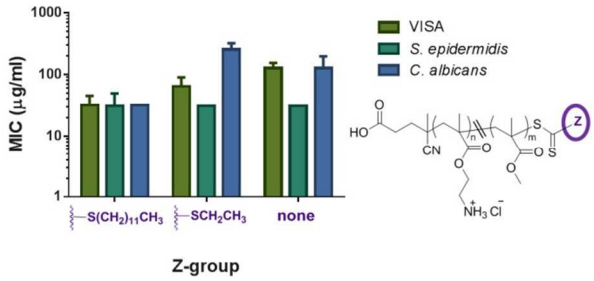


Figure 5B: Antimicrobial activity correlated to structural changes in the Z end-group of the guanidine polymers

In both cases, the polymers containing the long alkyl Z-group had the highest activity, particularly for VISA and *C. albicans*. The role that these groups play in the underlying mechanism may arise from the fact that the lipophilic component of polymers is thought to be responsible for processes leading to membrane insertion and permeabilisation. The added lipophilic bulk of the dodecylsulfanyl group might assist in this process, perhaps even promoting the anchoring of the polymers in the membrane. This would facilitate the subsequent interaction between the polymer chain and the phospholipid layer, accounting for the increased activity observed with PA1 and PG1. This hypothesis is supported by the fact that the complete removal of the RAFT end-group led to a decrease in activity in all cases but one (PA4 for *S. epidermidis*) for both the amines and guanidines. Interestingly adding or removing hydrophobic

bulk at the Z-terminus had little to no effect on the haemolytic activity of the polymers as can be seen in Figures 6A and 6B.

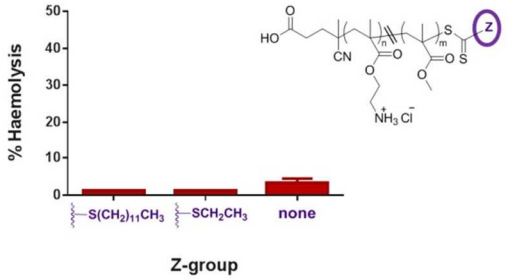


Figure 6A: Haemolytic activity correlated to structural changes in the Z end-group of the amine polymers

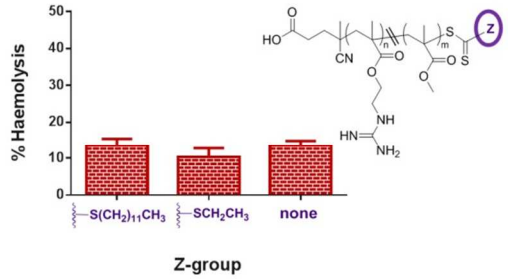


Figure 6B: Haemolytic activity correlated to structural changes in the Z end-group of the guanidine polymers

This finding is in contradiction to findings by Gellman and co-workers who showed for antimicrobial β-lactam polymers that an increase of hydrophobic bulk in the end-group led to higher haemolysis[11], although this finding relates to longer alkyl chains (dodecyl up to octadecyl) than studied here. Presumably below this threshold, the introduction of smaller lipophilic groups does not have a significant effect on the haemolytic profile of polymers. Our findings therefore suggest that while the R-group appears to have the dominant influence on the haemolytic profile of the polymethacrylates studied here, the Z-group may have the more important role in determining the antimicrobial potency. Thus, the polymers tested in this study, which were synthesized using RAFT agents, that contained a dodecylsulfanyl Z-group appeared to be the most active against the bacterial and fungal species tested.

End-Group Effect on Solution Behaviour of Polymers

Dynamic light scattering (DLS) experiments under physiological salt conditions were also conducted as a means to identify the potential for RAFT end-groups to mediate polymer association in solution (see supporting information for relevant results). Irrespective of the choice of R- and Z-groups, all polymers tested were found to agglomerate in solution, however, different trends were observed across the series. Almost identical DLS spectra were obtained for the R-group series (PA1 versus PA3 and PG1 versus PG3) indicating that the presence of a terminal carboxylic acid did not appear to effect polymer agglomeration. This suggests that the role of the R-group in haemotoxicity is not based on the meditation of polymer association in solution. In contrast, the results obtained with DLS experiments were observed to vary dramatically across the Z-group series. In the absence of lipophilic Z-groups (PA4 and PG4), the polymers were found to strongly agglomerate at all concentrations tested. The presence of either long or short lipophilic groups seemed to mediate agglomeration behaviour in a concentration dependant fashion. These findings suggest that the Z-group trends for antimicrobial activity observed may be the result of a complex interplay of structure-based membrane binding affinity as well as end-group mediated solution behaviour of polymers. The question of which is the dominant factor behind the mechanism of these polymers is the subject of current work.

Conclusions

Numerous studies have shown that the synthesis of highly active and specific antimicrobial polymers requires control over a range of structural aspects. Whereas a significant amount of research has been carried out towards understanding the influence of chemical changes to the polymer chain on activity [16, 49, 50], relatively little attention had been paid to the role that end-groups play in activity profiles.[22]

Owing to the wide range of RAFT CTAs available and chemically accessible end-group modification methods, we were able to synthesize a series of well-defined, well-matched polymers where the R and Z end-groups were systematically varied. This allowed the elucidation of the relative roles of the anionic cyanovaleric acid R-group and the lipophilic dodecylsulfanyl Z-group in the activity of recently described polymers.[23-25]

The biological testing of the synthesized polymers against the clinically relevant pathogens vancomycin and methicillin resistant *S. aureus* (VISA), a vigorously biofilm forming strain of *S. epidermidis* and the opportunistic fungus *C. albicans* as well as haemotoxicity assays, revealed a number of relevant trends. While the activity of the majority of candidates was potent against all three species examined, a number of end-group mediated effects were observed.

On one hand, the RAFT R-group appeared to play the dominant role in relation to haemolytic activity. A significantly lower level of haemolysis was observed for polymers that contained a cyanovaleric acid R-group than those containing an isobutyronitrile R-group. While the mechanism underlying this observation is the subject of further work, analysis of the results presented here suggests that this may relate to a carboxylic acid mediated interaction with red blood cells rather than through alteration of agglomeration behaviour of the polymers in solution. Overall it appeared that the presence of a cyanovaleric acid in the R-group was beneficial in both increasing the potency of polymers against the fungus *C. albicans* and in lowering the haemolytic activity.

On the other hand, the Z-group appeared to mainly affect the antimicrobial activity of the polymers. The presence of a longer lipophilic chain seemed to be associated with greater antimicrobial activity, especially against VISA and *C. albicans*. As the lipophilic components of antimicrobial polymers are thought to be involved in mechanisms underlying bacterial membrane insertion and permeabilisation, it is thought that the added lipophilic bulk of the dodecylsulfanyl Z-group may assist with the process. This may, in part, account for the increased activity observed with these polymers. Furthermore, DLS measurements demonstrated that a longer alkyl chain in the Z-group appeared to mediate agglomeration of the polymers molecules in solution, potentially also explaining the Z-group mediated effects observed. The exact mechanisms and implications thereof are the focus of future research. In summary, our results suggest that for highly potent and selective antimicrobial RAFT polymers, a cyanovaleric acid in the R-group and a longer alkyl chain in the Z-group appear optimal for the polymers examined in this study.

In essence, this study establishes an important aspect in the SAR governing antimicrobial polymers; i.e. even relatively small alterations to the chemistry of polymer end-groups can result in changes in the activity profiles observed and thus contributing another aspect that scientists need to consider when designing new antimicrobial polymers. By utilizing RAFT polymerization we were able to systematically probe these influences in order to identify the end-group properties which resulted in a high activity and selectivity of the resulting antimicrobial polymer. By combining these aspects with our previous studies, we have been able to investigate both the optimal polymer chain and end-group compositions necessary to obtain highly potent antimicrobial polymers that also show low haemotoxicity.

Notes and references

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† Electronic Supplementary Information (ESI) available: NMR, UV-Vis and DLS spectra and additional biological assay results. See DOI: 10.1039/b000000x/

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