Cationic Acrylate Oligomers Comprising Amino Acid Mimic Moieties Demonstrate Improved Antibacterial Killing Efficiency

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MATERIALS AND METHODS

Materials. *Tris*(2-(dimethylamino)ethyl) amine (Me_6TREN) was synthesized according to literature procedures with modifications.[1] **(**2-Boc-amino)ethyl acrylate (2-BocAEA) was synthesized via published methods[2-3] and kindly supplied by Dr. Jeroen Goos of ARC CoE CBNS, Monash University. Dichloromethane (DCM, Merck Millipore), copper (II) bromide ($CuBr_2$, Sigma-Aldrich, 98%), ethyl α-bromoisobutyrate (EBiB, Sigma-Aldrich, 98%), dodecyl 2-bromoisobutyrate (DBiB, Sigma Aldrich), trifluoroacetic acid (TFA, Sigma Aldrich), 1H-pyrazole-1-carboxamidine hydrochloride (Sigma Aldrich), anhydrous ethanol (EtOH), N,N-diisopropylethylamine (DIEA, Sigma Aldrich) were used as received. 2-(Dimethylamino)ethyl acrylate (DMAEA, Sigma-Aldrich) was de-inhibited by percolating over a column of basic alumina. Copper wire was activated by washing in sulfuric acid for 10 min, followed by the removal of the acid with water and drying of the copper wire.

The bacteria strains used in the primary panel were *Escherichia coli* ATCC® 25922™, MDR *Klebsiella pneumoniae* ATCC® 700603™, *Acinetobacter baumannii* ATCC® 19606™, *Pseudomonas aeruginosa* ATCC® 27853™, and methicillin resistant *Staphylococcus aureus* (MRSA) ATCC® 43300™. The bacteria strains used in the extended panel were: *P. aeruginosa* FADDI-PA070 (polymyxin resistant), *K. pneumoniae* ATCC® BAA-2146™ (NDM-1 positive), *S. aureus* NRS 17 (GISA), *S. aureus* NRS 1 (GISA, MRSA), *Streptococcus pneumoniae* ATCC® 700677™ (MDR), and *S. aureus* NARSA-VRS1 (VRSA). Colistin (Sulfate) (Sigma C4661), Polymyxin B (sulfate) (Sigma PO972-IMV), Vancomycin (HCl) (Sigma 861987), and Daptomycin (Molekula 64342447) were used as the control compounds for the antibacterial tests.

The cell lines used in the cytotoxicity assay were HEK293 ATCC® CRL-1573™ and HepG2 ATCC® HB-8065™.

Polymer Synthesis. A typical example of a Cu(0)-mediated polymerization of **(**2-Boc-amino)ethyl acrylate is detailed below. 2-BocAEA (0.5 g, 2.32 mmol, 25 eq.), DMSO (1.0 mL), EBiB (0.0136 mL, 0.0929 mmol, 1.00 eq.), Me_6TREN (0.0040 mL, 0.0149 mmol, 0.16 eq.), $CuBr_2$ (1.0 mg, 0.00465 mmol, 0.05 eq.), and a magnetic stir bar were charged to a polymerization flask fitted with a rubber septum and the mixture degassed via nitrogen sparging for 15 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. 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 with $CDCl_3$, while the sample for GPC was first diluted with DMAc then passed over a neutral aluminium oxide column to remove metal salts. The polymers synthesized were purified by diluting with THF and passing over a neutral aluminium oxide column to remove metal salts and remaining monomer, followed by removal of the solvent by evaporation under a stream of air. This was then repeated for the DBiB initiator under the same conditions.

Synthesis of Primary Amine Polymers by Deprotection of Poly((2-Boc-amino)ethyl acrylate). Poly((2-Boc-amino)ethyl acrylate) (200 mg) was dissolved in DCM (2.0 mL) in a 20 mL glass vial, in which TFA (2.0 mL) was added and allowed to react overnight. The resulting deprotected polymer solution was

then evaporated to dryness under a stream of air, 5.0 mL of acetone was added and evaporated to dryness under a stream of air, and this process was repeated three times. Finally, the polymer was dried in the vacuum oven at 25°C for a week to remove residual solvent. Complete deprotection was confirmed by ^{1}H NMR using MeOD.

Synthesis of Guanidine Functionalized Polymers. 1H-pyrazole-1-carboxamidine hydrochloride (0.75 g) was weighed out and dissolved in anhydrous ethanol (23 mL) to give a stock solution of 0.033 mg/mL. A representative procedure is shown below, using a literature method.[4] The reaction scheme is shown in Scheme 1.

1H-pyrazole-1-carboxamidine hydrochloride in anhydrous ethanol (1.97 mL, 0.44 mmol) was added to Polymer 2D (C_2-AEA_{21}) (0.0592 g). Then, *N, N*-diisopropylethylamine base (~0.138 mL, 0.79 mmol) was added to the vial, and the reaction was heated to 55°C overnight under positive nitrogen pressure. The solvent was removed by evaporation under a steam of air, and remaining excess reactants were removed by trituration using acetone and then placed in the vacuum oven to remove residual solvent. Successful guanylation was confirmed by ^{1}H NMR and ATR FTIR.

Polymer Characterization. All NMR spectra were recorded on a Bruker Advance III 400 MHz spectrometer using an external lock and referenced to the residual nondeuterated solvent. Chemical shifts (δ_H) are reported in parts per million (ppm). NMR solvents (CD_3OD and $CDCl_3$) were purchased from Sigma-Aldrich and used as received.

Gel Permeation Chromatography (GPC). GPC 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 *N,N*-dimethylacetamide (CHROMASOLV Plus for HPLC) and the flow rate was kept at 1.0 mL min^{-1} using an 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^{-1}. Polymer solutions at approx. 2 mg mL^{-1} were prepared and filtered through 0.45 µm PTFE filters before injection.

ATR-FTIR spectra were obtained using a Shimadzu IRTracer-100 Fourier Transform Infrared Spectrophotometer with an MCT detector using a resolution of 16 cm^{-1}.

Single Point Bacterial Inhibition Assay. Bacteria were cultured in Mueller Hinton broth (MHB) at 37 °C overnight. A sample of each culture was then diluted 40-fold in fresh MHB and incubated at 37°C for 1.5-3 h. The compounds were plated at a single concentration of 64 µg/mL in a 96-well plate (Corning 3370, polystyrene plates). The resultant mid-log phase cultures were diluted to the final concentration of 1x10^6 CFU/mL, then 50 µL was added to each well of the compound containing plates, giving a final compound concentration of 32 µg/mL, and a final cell density of 5x10^5 CFU/mL. All the plates were covered and incubated at 37 °C for 24 h.

Inhibition of bacterial growth was determined visually**,** classifying wells into; 1) full growth, 2) partial growth or 3) without any growth.

Following the single point bacterial inhibition assay, compounds with partial or complete growth inhibition were further tested for minimum inhibitory concentration (MIC) against the strains where the activity was seen.

MIC (Minimum Inhibitory Concentration) Assay. Bacteria were cultured in Mueller Hinton broth (MHB) at 37°C overnight. A sample of each culture was then diluted 40-fold in fresh MHB and incubated at 37°C for 1.5-3 h. The compounds were serially diluted two-fold across the wells of 96-well plates (Corning 3370, polystyrene plates), with compound concentrations ranging from 0.03 μg/mL to 64 μg/mL, plated in duplicate. The resultant mid-log phase cultures were diluted to the final concentration of $1x10^6$ CFU/mL, then 50 μL was added to each well of the compound-containing 96-well plates, giving a final compound concentration range of 0.015 μg/mL to 32 μg/mL, and a final cell density of $5x10^5$ CFU/mL. All plates were covered and incubated at 37°C for 24 h.

Inhibition of bacterial growth was determined visually after 24 h**,** where the MIC is recorded as the lowest compound concentration with no visible growth. The MIC assays were carried out in duplicates on 2 different occasions (total of n=4).

Following MIC determination of the primary panel, compounds with MIC ≤16 μg/mL against any of the Gram-negative strains were further tested against 2 other Gram-negative strains from an extended panel. Likewise, those with MIC ≤16 μg/mL against *S. aureus* ATCC 43300 (MRSA) were further tested against 5 other Gram-positive strains from an extended panel.

Haemolysis Assay. 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 (excluding the PBS and Triton-X wells) 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. Each well except for the PBS only well was diluted 2-fold by the addition of 100 μL of 8% red blood cells. The plates were then incubated for 1 hour at 37°C, followed by centrifugation at 1000 g for 5 minutes. 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 Assay (Resazurin Assay). Cytotoxicity to HepG2 cells was determined using a resazurin assay.[5-6] In brief, HepG2 cells were seeded as 5000 cells per well in black wall clear bottom 384 well plates and incubated for 24 hours at 37°C, 5% CO_2. Then, a series concentrations of compound was added into each well. After 24 hours incubation, 5 μM resazurin were added per well and incubated at 37°C for 2 hours. The fluorescence intensity was read using Polarstar Omega plate reader with excitation/emission 560/590. This method was then repeated for HEK293 cells.

Membrane Perturbation Assays. 3,3'-Dipropylthiadicarbocyanine iodide (diSC3-5) assay (Anderson *et al.*[7]) and 1-*N*-phenylnaphthylamine (NPN) uptake assay (Helander *et al.*[8]) were performed as previously described with 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.

RESULTS

Table SI 1 Polymer Characterization using ^{1}H NMR and GPC

Polymer №	Monomer	Initiator chain length	^{1}H NMR		GPC	
			DP	Mn	Mn	PDI
1	DMAEA	12	5	1100	900	1.05
2	(2-Boc-amino)ethyl acrylate	2	21	4700	5200	1.17
3	(2-Boc-amino)ethyl acrylate	2	12	2800	4700	1.16
4	(2-Boc-amino)ethyl acrylate	2	11	2600	4400	1.10
5	(2-Boc-amino)ethyl acrylate	2	5	1300	2900	1.08
6	(2-Boc-amino)ethyl acrylate	12	27	6100	10200	1.19
7	(2-Boc-amino)ethyl acrylate	12	18	4200	6100	1.10
8	(2-Boc-amino)ethyl acrylate	12	13	3100	4100	1.14
9	(2-Boc-amino)ethyl acrylate	12	7	1800	3200	1.07

Table SI 2 Standard Panel of Bacteria

Bacteria	**Description**
Escherichia coli	ATCC® 25922™ FDA Control Strain
Klebsiella pneumoniae	ATCC® 700603™ MDR
Acinetobacter baumannii	ATCC® 19606™ Type Strain
Pseudomonas aeruginosa	ATCC® 27853™ Type Strain
Staphylococcus aureus	ATCC® 43300™ MRSA

Table SI 3 Extended Panel of Bacteria

Bacteria	**Description**
Pseudomonas aeruginosa	FADDI-PA070 (polymyxin resistant)
Klebsiella pneumoniae	ATCC® BAA-2146™ (NDM-1 positive)
Staphylococcus aureus	NRS 17 (GISA)
Staphylococcus aureus	NRS 1 (GISA, MRSA)
Streptococcus pneumoniae	ATCC® 700677™ (MDR)
Staphylococcus aureus	NARSA-VRS1 (VRSA)

Table SI 4 Single point bacterial inhibition at 32 µg/mL of the polymers against the primary panel of bacteria

Polymer №	Monomer	Initiator chain length	DP	***E. coli***	***K. pneumoniae MDR***	***A. baumannii***	***P. aeruginosa***	***S. aureus (MRSA)***

				Growth Inhibition at 32 µg/mL				
1	DMAEA	12	5	Active	Inactive	Active	Inactive	Inactive
2D	2-aminoethyl acrylate	2	21	Inactive	Inactive	Inactive	Partial	Active
3D	2-aminoethyl acrylate	2	12	Inactive	Inactive	Inactive	Inactive	Inactive
4D	2-aminoethyl acrylate	2	11	Inactive	Inactive	Inactive	Inactive	Inactive
5D	2-aminoethyl acrylate	2	5	Inactive	Inactive	Inactive	Inactive	Inactive
6D	2-aminoethyl acrylate	12	27	Inactive	Inactive	Inactive	Inactive	Inactive
7D	2-aminoethyl acrylate	12	18	Inactive	Inactive	Inactive	Inactive	Partial
8D	2-aminoethyl acrylate	12	13	Inactive	Inactive	Inactive	Inactive	Inactive
9D	2-aminoethyl acrylate	12	7	Inactive	Inactive	Inactive	Inactive	Inactive
2G	2-guanidinoethyl acrylate	2	21	Inactive	Inactive	Inactive	Inactive	Inactive
3G	2-guanidinoethyl acrylate	2	12	Inactive	Inactive	Inactive	Inactive	Inactive
4G	2-guanidinoethyl acrylate	2	11	Inactive	Inactive	Inactive	Inactive	Inactive
5G	2-guanidinoethyl acrylate	2	5	Inactive	Inactive	Inactive	Inactive	Inactive
6G	2-guanidinoethyl acrylate	12	27	Active	Inactive	Active	Active	Active
7G	2-guanidinoethyl acrylate	12	18	Active	Inactive	Active	Active	Active
8G	2-guanidinoethyl acrylate	12	13	Active	Active	Active	Active	Active
9G	2-guanidinoethyl acrylate	12	7	Active	Active	Active	Active	Active

Table SI 5 MIC Assay of the polymers against the primary panel of bacteria

Polymer №	Compound/Monomer	Initiator chain length	DP	*E. coli*	*K. pneumoniae MDR*	*A. baumannii*	*P. aeruginosa*	*S. aureus (MRSA)*
				MIC (µg/mL)				
-	Colistin	-	-	0.125-0.25	0.125-0.25	0.125-0.5	0.25-05	-
-	Polymyxin B	-	-	0.25	0.125-0.5	0.06-0.125	0.25-0.5	-
-	Vancomycin	-	-	-	-	-	-	1
-	Daptomycin	-	-	-	-	-	-	1-2
1	DMAEA	12	5	>32	-	>32	-	-
2D	2-aminoethyl acrylate	2	21	-	-	-	-	>32
7D	2-aminoethyl acrylate	12	18	-	-	-	-	>32
6G	2-guanidinoethyl acrylate	12	27	32	>32	16	>32	32
7G	2-guanidinoethyl acrylate	12	18	16-32	>32	8-16	>32	8-16
8G	2-guanidinoethyl acrylate	12	13	32	32	16	>32	4-8
9G	2-guanidinoethyl acrylate	12	7	8-16	>32	32	>32	8

Table SI 6 Antibacterial testing of lead compounds against the extended panel of bacteria

Polymer №	Compound/ Monomer	Initiator chain length	DP	*P. aeruginosa (Polymyxin resistant)*	*K. pneumoniae (NDM-1 positive)*	*S. aureus (GISA)*	*S. aureus (GISA, MRSA)*	*S. pneumoniae (MDR)*	*S. aureus (VRSA)*
				MIC [µg/mL]					
-	Colistin	-	-	>32	0.125-0.5	-	-	-	-
-	Polymyxin B	-	-	32	0.125-0.5	-	-	-	-
-	Vancomycin	-	-	-	-	4	4-8	1-2	>32
-	Daptomycin	-	-	-	-	8	8-16	2	2
7G	2-guanidino ethyl acrylate	12	18	>32	>32	16	16	16	16
8G	2-guanidino ethyl acrylate	12	13	>32	>32	8	16	8-16	8
9G	2-guanidino ethyl acrylate	12	7	>32	>32	4-8	8	8	4-8

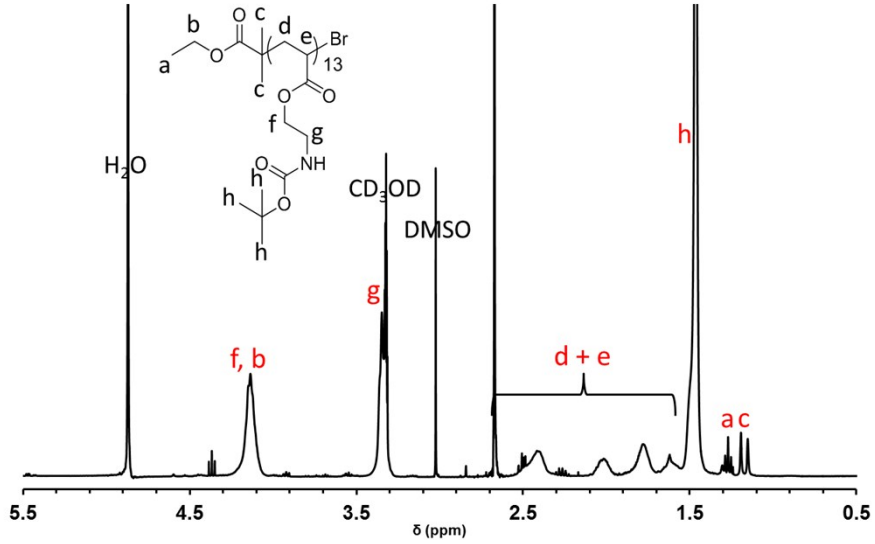

Figure SI 1 ^{1}H NMR spectra of Poly((2-Boc-amino)ethyl acrylate) in CD_3OD

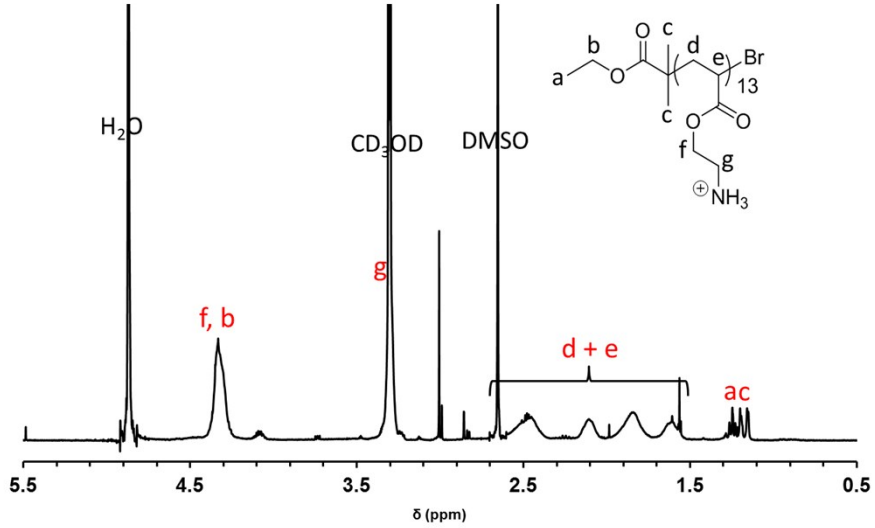

Figure SI 2 ^{1}H NMR spectra of the deprotected Poly((2-Boc-amino)ethyl acrylate) in CD_3OD

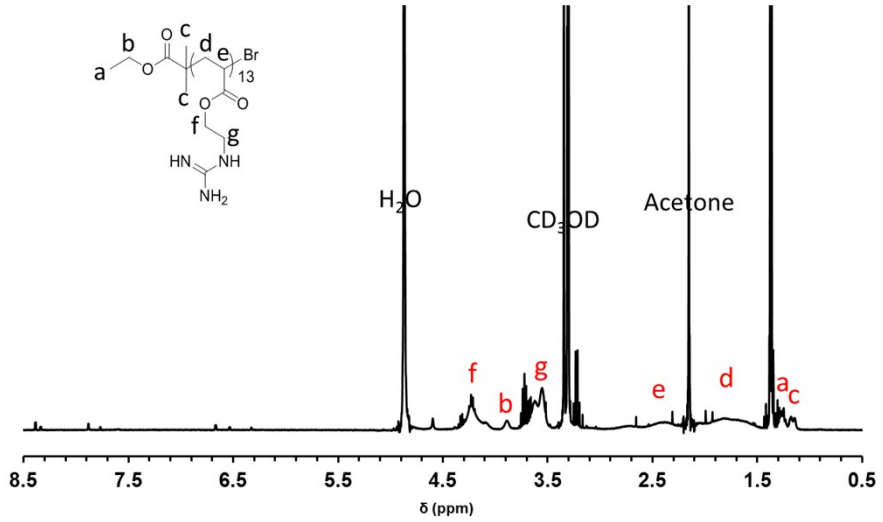

Figure SI 3 ^{1}H NMR spectra of the guanylated polymer in CD_3OD

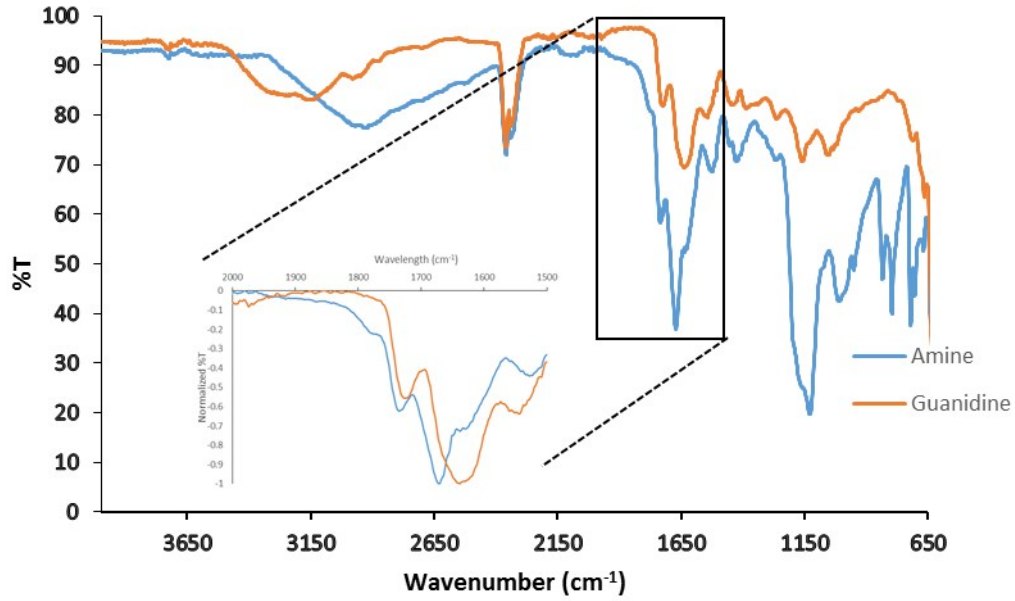

Figure SI 4 ATR FT-IR spectra for primary amine polymer (blue) and for guanidine polymer (orange)

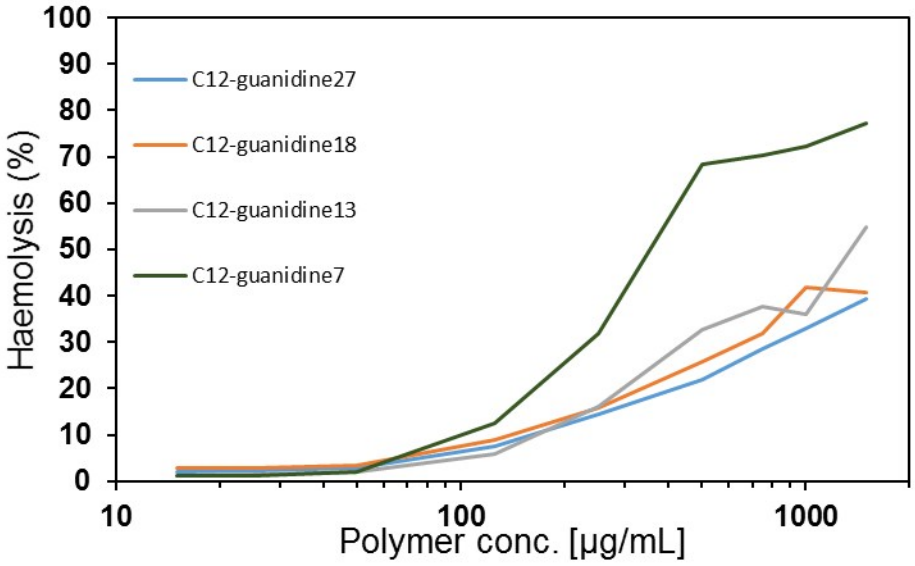

Figure SI 5 Haemolytic effect of dodecyl tailed guanidine polymers after 1 hour exposure to human red blood cells

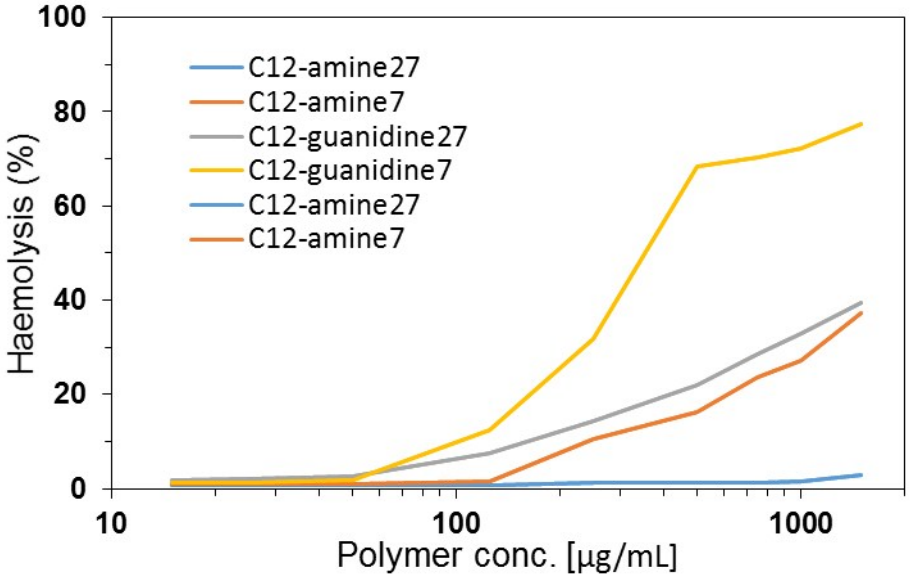

Figure SI 6 Haemolytic effect of high and low DP dodecyl tailed amine polymers after 1 hour exposure to human red blood cells compared to dodecyl tailed guanidine polymers with the same DP

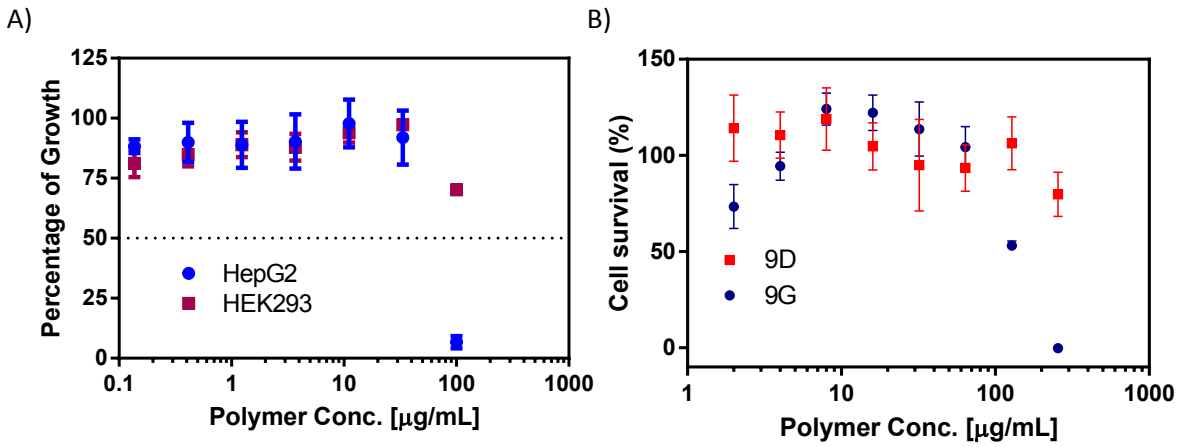

Figure SI 7 A) Comparison of cell viability for HepG2 and HEK293 cells against Polymer 9G. B) Comparison of cell viability for HEK293 cells against C_{12} tail low DP amine and guanidine polymers. Data are presented as Mean ± Standard Deviation

Table SI 7 Selectivity index values[a] for the C_{12} guanidine polymers. MIC values are located in Table SI 3.

Polymer Nº	HC50 (µg/mL)[b]	*E. coli*	*K. pneumoniae MDR*	*A. baumannii*	*P. aeruginosa*	*S. aureus (MRSA)*
6G	>1500	>47	NS[c]	>94	NS[c]	>47
7G	>1500	>94	NS[c]	94-188	NS[c]	94-188
8G	1374	43	43	86	<43	172-343
9G	374	23-47	<12	12	<12	47

[a]The selectivity index is obtained by dividing the HC_{50} by the MIC value for a given bacterium; and these values are shown in Table SI 5. The selectivity index is used routinely[9-10] to describe the selectivity of the compound towards a particular bacterium. [b]HC_{50} values were calculated by interpolation of results in Figure SI 5. [c]NS (No-selectivity) was given to polymers that had both a HC_{50} value and an MIC value above the concentration range tested.

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