Supporting information for:

Photochemical 'In-Air' Combinatorial Discovery of Antimicrobial Polymers

Sarah-Jane Richards,[(a)] Adam Jones[(a)] Ruben Tomás[(a)] and Matthew I. Gibson*[(a),(b)]

[a)] Department of Chemistry, University of Warwick, Coventry, CV4 7AL, United Kingdom

[b)] Warwick Medical School, University of Warwick, Coventry, CV4 7AL, United Kingdom

CORRESPONDING AUTHOR DETAILS

E-mail: m.i.gibson@warwick.ac.uk

Experimental Section

Materials

2-(Dimethylamino)ethyl methacrylate (DMAEMA) (98 %), methyl methacrylate (MMA) (99 %), ethyl methacrylate (EMA) (99 %), isopropyl 2-methacrylate (iPMA), cyclohexyl methacrylate (cHMA) (≥97 %), 2-hydroxyethyl methacrylate (HEMA) (97 %), di(ethylene glycol) methyl ether methacrylate (DEGMA) (95 %), poly(ethylene glycol) methyl ether methacrylate (PEGMA) (average M_n ~300), poly(propylene glycol (PPGMA) (average M_n ~375), triethanolamine (98 %), 2-cyano-2-propyl dodecyl trithiocarbonate (97 %), mesitylene (98 %), gold (III) chloride trihydrate (>99.9%) and deuterated chloroform $CDCl_3$ (99.8 atom % D) were all purchased from Sigma-Aldrich as used as received. Dimethyl sulfoxide (DMSO) (analytical grade) was purchased from Fischer Chemical. The blue LED source was a commercial strip lighting (300 LEDs, 12V, 4.8 mW cm^{-2}).

Physical and Analytical Methods

SEC was performed using a Agilent 390-LC MDS instrument equipped with differential refractive index (DRI), viscometry (VS), dual angle light scatter (LS) and UV detectors. The system was equipped with 2 × PLgel Mixed D columns (300 × 7.5 mm) and a PLgel 5 μm guard column. The eluent is DMF with 5 mmol NH_4BF_4 additive. Samples were run at 1mL.min^{-1} at 50°C. Poly(methyl methacrylate) standards (Agilent EasyVials) were used for calibration between 955,000 – 550 g.mol^{-1} . Analyte samples were filtered through a nylon membrane with 0.22 μm pore size before injection. Respectively, experimental molar mass (M_n, SEC) and dispersity (Đ) values of synthesized polymers were determined by conventional calibration and universal calibration using Agilent GPC/SEC software.NMR spectroscopy was conducted on a

Bruker DPX-300 using deuterated chloroform as solvent. Fluorescence microscopy was performed using a Zeiss LSM 710 microscope. SYTO 9 dye was imaged by excitation at 488 nm and emission at 530 nm for green fluorescence. Propidium iodide was imaged by excitation at 561 nm and emission at 646 nm for red fluorescence. Liquid handing was achieved using a Gilson PipetteMax.

Synthetic section

General procedure for the photo-CRP of DMAEMA

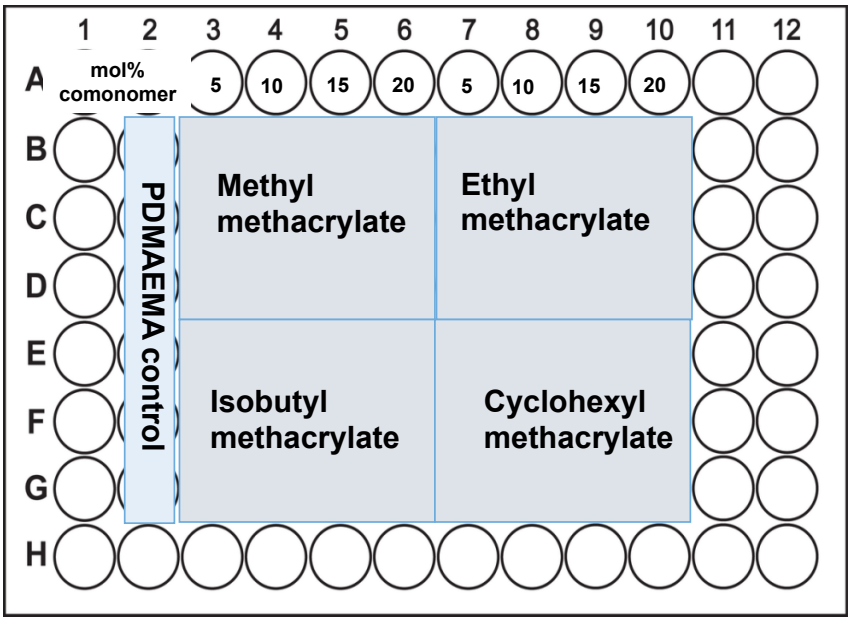
A deep well, polypropylene 96-well plate was charged with monomer (DMAEMA: 19 mg, 0.12 mmol in 180 μL DMSO), 2-cyano-2-propyl dodecyl trithiocarbonate (0.6 mg, 1.6 μmol in 200 μL DMSO), TEOA (45 mg, 0.3 mmol in 150 μL in DMSO), 20 μL mesitlyene and DMSO (180 μL). An aliquot was taken for ^{1}H NMR in $CDCl_3$, then the plate was covered with a sealing film. The blue LED light source was switched on and left to polymerise at room temperature for 24 hours. Immediately an aliquot was taken for ^{1}H NMR in $CDCl_3$ and conversion was determined against mesitylene standard. An aliquot was taken for SEC in DMF.

General procedure for the photo-CRP of copolymers

A deep well, polypropylene 96-well plate was charged using a Gilson Pipetmax 268 liquid handling robot with monomer (DMAEMA: 19 mg, 0.12 mmol in 180 μL DMSO), comonomer (MMA, EMA, iPMA, cHMA, HEMA, DEGMA, PEGMA, PPGMA) (5-20 mol % in 20 – 80 μL DMSO), 2-cyano-2-propyl dodecyl trithiocarbonate (0.6 mg, 1.6 μmol in 200 μL DMSO), TEOA (45 mg, 0.3 mmol in 150 μL in DMSO), 20 μL mesitlyene and DMSO (180-100 μL). An aliquot was taken for

^{1}H NMR in $CDCl_3$, then the plate was covered with a sealing film. The blue LED light source was switched on and left to polymerise at room temperature for 24 hours. Polymers were diluted in to PBS for biological testing.

The polymers were made in triplicate. The plates were laid out as follows. Outside well were not used, but filled (as these are not used in antimicrobial screening hence to reduce plate-replication errors were not used in synthesis either):



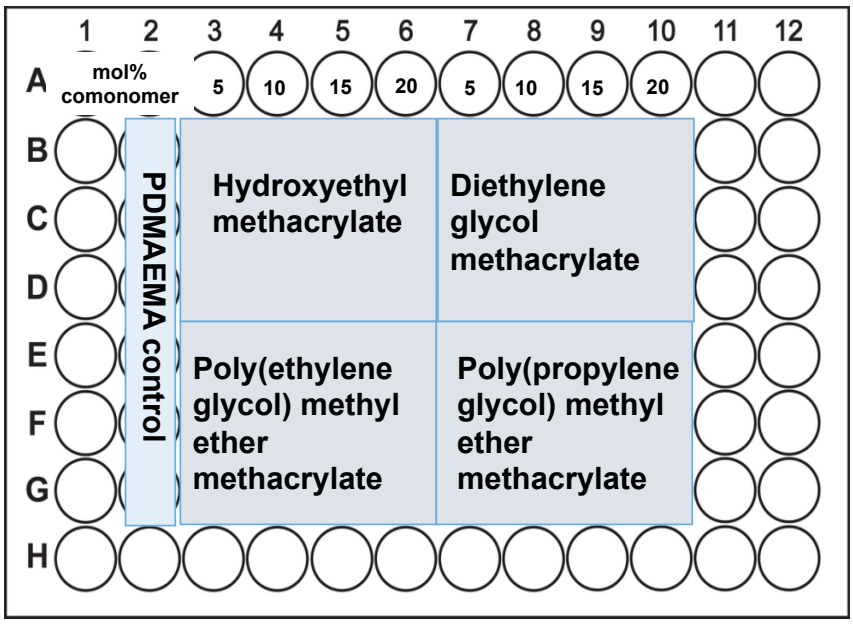


Figure S1: Plate layout for photo-polymerizations of copolymers.

Microbiology Section

Bacterial Strains and Growth Conditions

Mycobacterium smegmatis MC^2155 was grown in Middlebrook 7H9 media supplemented with 0.2 % glycerol and 0,05 % Tween 80. *Escherichia coli* Top10 were grown in Luria-Bertani (LB) media. For MBCs and CFUs bacteria were grown on LB agar containing no antibiotics. AHD solution for haemolysis determination was made up as follows, 2.5 g Triton X-100 and 0.4 g NaOH in 100 mL water

Heamolysis and Heamagglutiniation

Ovine red blood cells were pelleted by centrifugation a 6000 g for 5 min and the supernatant was removed and resuspended in PBS buffer. 100 μL copolymers at 2 $mg.mL^{-1}$ were added to 100 μL of ovine red blood cells in a 96 well plate and incubated at room temperature for 1 hour. For haemagglutination determination 25 μL of this mixture was added to 75 μL of PBS in a U-bottom 96-well plate and incubated for a further hour at room temperature. Polyethyleneimine was used as a positive control for haemagglutination and cells in PBS alone as a negative control. Haemagglutination was determined visually by creation of a pellet (haemagglutination negative) or formation of a blood film (haemagglutination positive)

For haemolysis testing the rest of the particle-blood mixture was centrifuged at 2000 g for 5 min. 20 μL of the supernatant was added to 750 μL of AHD solution. The absorbance of 200 μL of the AHD solution was measured at 580 nm and compared

against deionized water as a positive control for lysis and PBS as a negative control to determine % haemolysis.

Determination of Antibacterial Activities of copolymers

PDMAEMA copolymers were tested for the increased minimum inhibitory concentration (MIC_{99}) compared to PDMAEMA homopolymer against *M. smegmatis* and *E. coli* by single concentration testing at 0.5 × MIC_{99} of homopolymer. The bacteria were cultured to mid-log phase and the inoculum was standardized to 1 x 10^5 $CFU.mL^{-1}$ before addition to a 96-well microtiter plate of the polymers at 250 μL for *E. coli* and 31.3 μL. Control wells contained homopolymers of PDMAEMA and culture controls. The plates were incubated in a static incubator at 37 °C for 16 h for *E. coli* and 24 h *M. smegmatis*. Following this incubation period, 25 μL of resazurin (one tablet (VWR) in 30 mL of sterile PBS) was added and left for a further incubation of 2 h for *E. coli* and 24 h for *M. smegmatis*. The polymer was determined as a hit if it the color of the resazurin did not change from blue to pink (*ie* no bacterial growth).

Determination of MIC_{99} of hit copolymers

Minimum inhibitory concentration (MIC_{99}) of the hit copolymers was determined against *E. coli* by single concentration testing at 0.5 × MIC_{99} of homopolymer. The bacteria were cultured to mid-log phase and the inoculum was standardized to 1 x 10^5 $CFU.mL^{-1}$ before addition to a 96-well microtiter plate in which the copolymers were serially diluted two-fold across the plate. Control wells contained culture controls. The plates were incubated in a static incubator at 37 °C for 16 h for *E. coli*. Following this incubation period, 25 μL of resazurin (one tablet (VWR) in 30 mL of sterile PBS) was added and left for a further incubation of 2 h. The MIC_{99} values were determined as the

lowest concentration of polymer at which the resazurin did not change from blue to pink (*ie* no bacterial growth). The MIC_{99} values were determined in triplicate.

Minimum Bactericidal Concentration Determination

Minimum bactericidal concentration (MBC) of the PDMAEMA and P(DMAEMA-co-PPGMA) were determined against *E. coli.* The bacteria were cultured to mid-log phase and the inoculum was standardized to 1×10^5 CFU.mL^{-1} before addition to a 96-well microtiter plate in which the polymers were serially diluted two-fold across the plate. The plate were incubated in a static incubator for 16 h. Following the incubation period, 100 μL of each culture was plated onto LB agar and incubated at 37 °C for 24 h. The MBC values were determined as the lowest concentration of polymer that resulted in the observation of no bacterial growth. The MBC values were determined in triplicate.

Fluorescence Microscopy

LIVE/DEAD viability testing was carried out using a protocol from Molecular probes. *E. coli* was grown to late-log phase (0.7) before being harvested by centrifugation at 3300 g for 15 min at room temperature. The cell pellet was resuspended in 0.85 % NaCl, then aliquoted and incubated with 0.5 × MIC_{99} (125 mg.mL^{-1} for PDMAEMA and 7.8 mg.mL^{-1} for P(DMAEMA-co-PPGMA)) and 2 × MIC_{99} (500 mg.mL^{-1} for PDMAEMA and 31.3 mg.mL^{-1} for P(DMAEMA-co-PPGMA)) for 15 min at room temperature. An aliquot was used as a live cell control and incubated with no polymer and a further aliquot was used as a heat-killed cells control, incubated at 80 °C for 30 min. After incubation all samples were washed with 0.85 % NaCl twice and resuspended in 0.85 % NaCl, LIVE/DEAD bacterial viability dyes were used to determine membrane viability. In brief, SYTO 9 (3.34 mM in DMSO) and propidium iodide (20 mM in DMSO) were used in a 1:1 ratio, and 3 μL of the dye mixture was

added for 1 mL of the bacterial suspension in 0.85 % NaCl before incubating for 15 min in the dark. Samples were then analysed using a Zeiss LSM 710 confocal microscope to check for green (Ex 488 nm, Em 520) and red (Ex 561 nm, Em 646 nm) fluorescence.

Cell Culture

Cell culture. Human Caucasian lung carcinoma cells (A549) were obtained from European Collection of Authenticated Cell Cultures (Public Health England, UK) and grown in 175 cm^2 Nunc cell culture flasks (ThermoFisher, Rugby, UK). Standard cell culture medium was composed of Ham's F-12K (Kaighn's) Medium (F-12K) (Gibco, Paisley, UK) supplemented with 10% USA-origin fetal bovine serum (FBS) purchased from Sigma Aldrich (Dorset, UK), 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (PSA) (HyClone, Cramlington, UK). A549 cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C and the culture medium was renewed every 3–4 days. The cells were subcultured every 7 days or before reaching 90% confluency. To subculture, cells were dissociated using 0.25% trypsin plus 1 mM EDTA in balanced salt solution (Gibco) and reseeded at $1.87X10^5$ cells per 175 cm^2 cell culture flasks.

Solution preparation. Solutions for cell incubation experiments were prepared by dissolving the individual compounds in F-12K supplemented with 10% FBS and 1X PSA followed by filter sterilisation to remove biological contaminants. Compound A (DP75 PDMAEMA) and compound B (D75 15% PPGMA) were diluted to a final concentration range of 5 mg/mL to 2.44 μg/mL.

Cell toxicity screening. Cells were seeded at 6 x 10^4 cells per well in 200 μL of cell culture medium in 96-well plates (ThermoFisher). Plates had an approximate growth

area of 0.32 cm^2 and plates were used with the accompanying lid. Cells were allowed to attach for 2 h to the entire free surface, forming a confluent layer with maximum one cell thickness, in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cell culture medium of experimental cells was subsequently exchanged for medium containing the varying concentrations of compounds A and B. Control cells received no additional solutes. All cells were subsequently incubated for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Following the incubation period, resazurin sodium salt (Sigma Aldrich) was dissolved in phosphate buffered saline (pH 7.4; Sigma Aldrich) and added to wells in an amount of $1/10^{th}$ initial well volume. Readings were taken using the Synergy HTX Multi-Mode Reader (BioTek, Swindon, UK) at 570/600 nm absorbance every 30 minutes until control cells reached ~70% reduction.

Statistical analyses. Data were analysed with a one-way analysis of variance (ANOVA) on ranks followed by comparison of experimental groups with the appropriate control group (Tukey's post hoc test). R (R Foundation for Statistical Computing, Vienna, Austria) was used for the analyses.

Additional Data.

SEC analysis using UV detection at 309 nm showed that the RAFT end groups are intact upon polymerisation.

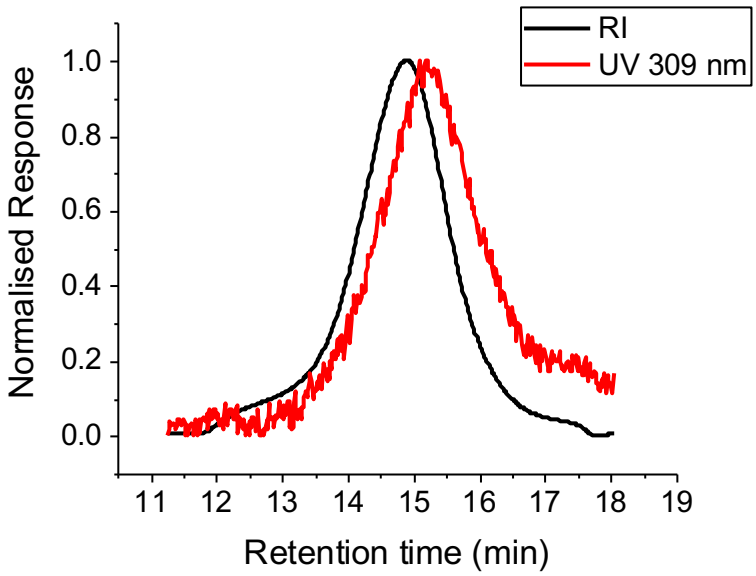


Figure S2: RI (black) and UV at 309 nm (red) SEC traces of PDMAEMA produced by photo-RAFT polymerisation.

Figures S3 and S4 show photos of the AHD assay plates where green is positive for lysis and clear is no lysis.

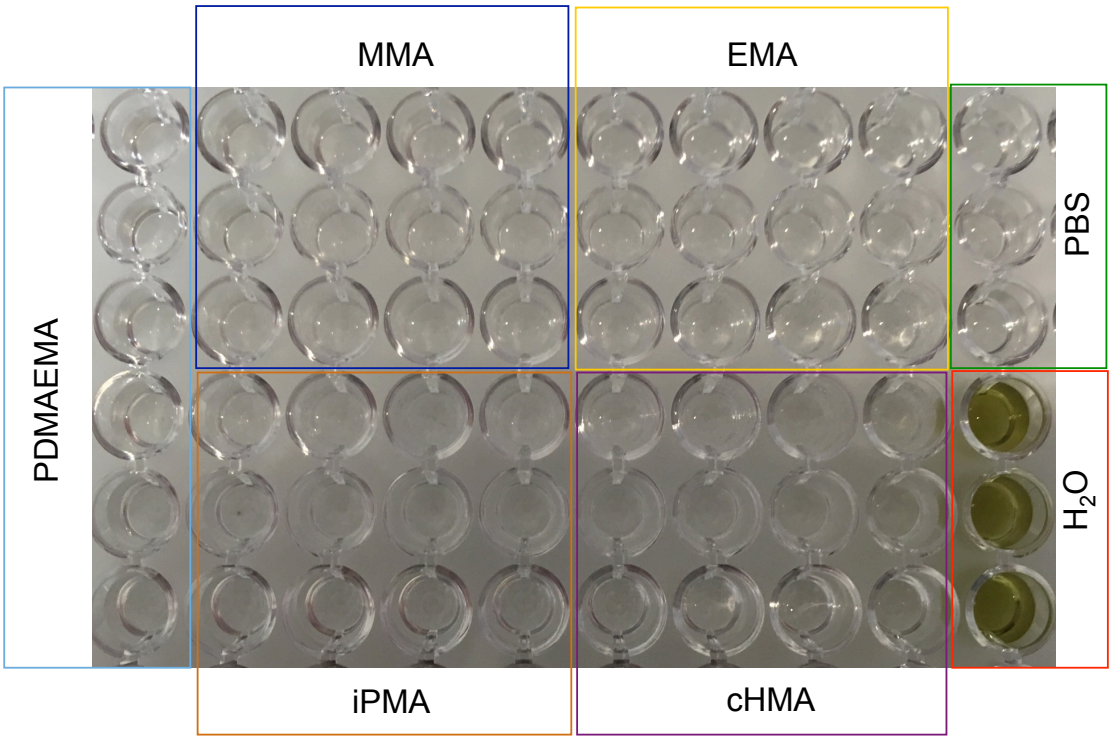


Figure S3: Typical AHD assay plate to determine % haemolysis for polymer plate 1

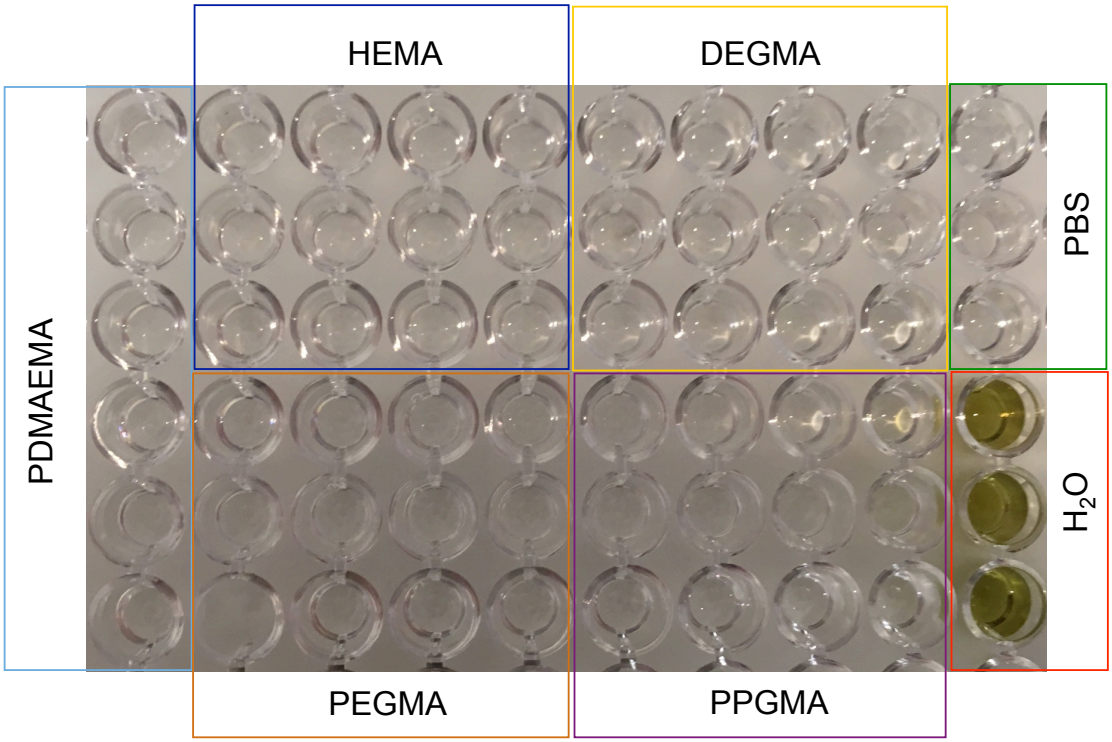


Figure S4: Typical AHD assay plate to determine % haemolysis for polymer plate.

Table 1: %Heamolysis compared to PBS 0 % haemolysis and H_2O 100 % haemolysis.

		Repeat			
Co-monomer	**mol% co-monomer**	**1**	**2**	**3**	**Average**
		0.5	0.6	0.6	0.6
MMA	5	0.1	0.0	0.3	0.2
	10	0.1	0.1	0.6	0.3
	15	0.1	0.2	0.0	0.1
	20	0.0	0.0	0.0	0.0

EMA	5	0.9	0.0	0.0	0.3
	10	0.2	0.6	0.0	0.3
	15	0.1	0.6	0.7	0.5
	20	0.0	0.0	0.2	0.1
iPMA	5	0.4	0.1	0.0	0.2
	10	0.0	0.0	0.6	0.2
	15	0.2	0.2	0.0	0.1
	20	0.0	0.2	0.0	0.1
cHMA	5	0.2	0.0	0.0	0.1
	10	0.1	1.0	0.1	0.4
	15	0.6	0.5	0.4	0.5
	20	0.3	0.1	0.4	0.3
HEMA	5	0.8	0.3	0.5	0.6
	10	0.0	0.1	0.0	0.0
	15	0.0	0.1	0.5	0.2
	20	0.0	0.1	0.02	0.1
DEGMA	5	0.1	0.0	0.1	0.1
	10	0.1	0.2	0.3	0.2

	15	0.3	0.2	0.5	0.3
	20	0.3	0.1	0.1	0.2
PEGMA	5	0.7	0.0	0.6	0.4
	10	1.4	0.2	0.0	0.5
	15	0.8	0.1	0.1	0.3
	20	1.6	0.2	1.6	1.1
PPGMA	5	0.1	0.5	0.0	0.2
	10	0.4	0.3	0.4	0.3
	15	0.1	0.4	0.4	0.3
	20	0.2	0.7	0.4	0.4

All polymers showed less that 2 % haemolysis.

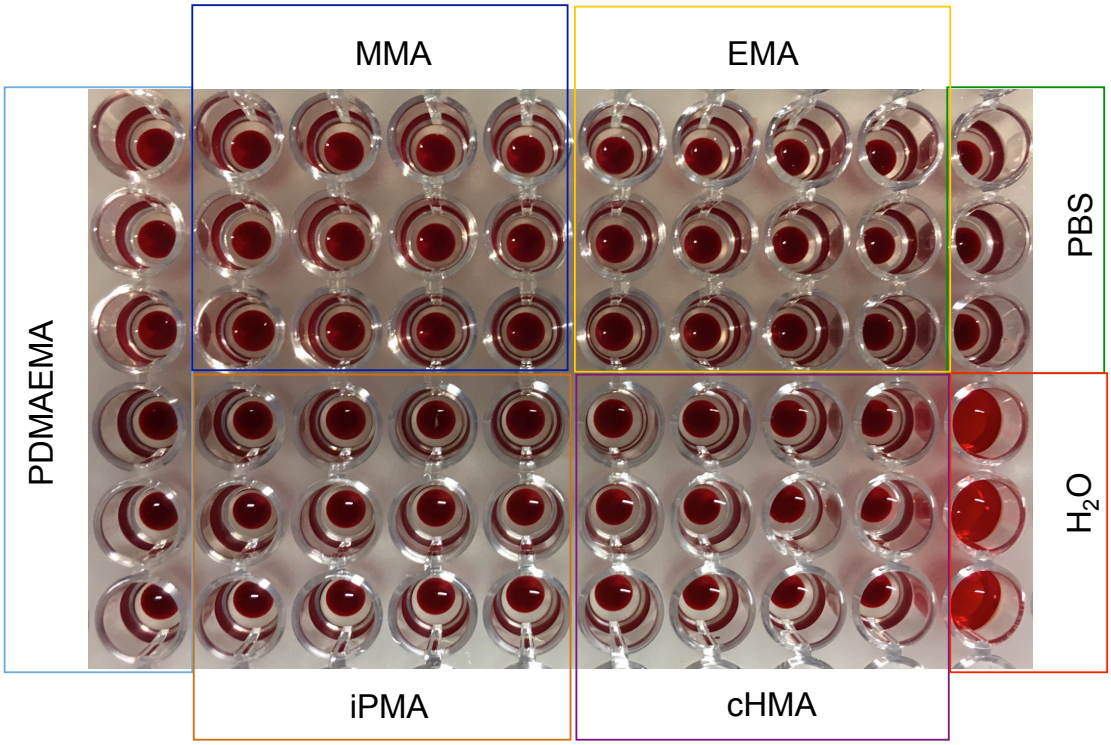


Figure S5: Typical haemagglutination assay plate for polymer plate 1.

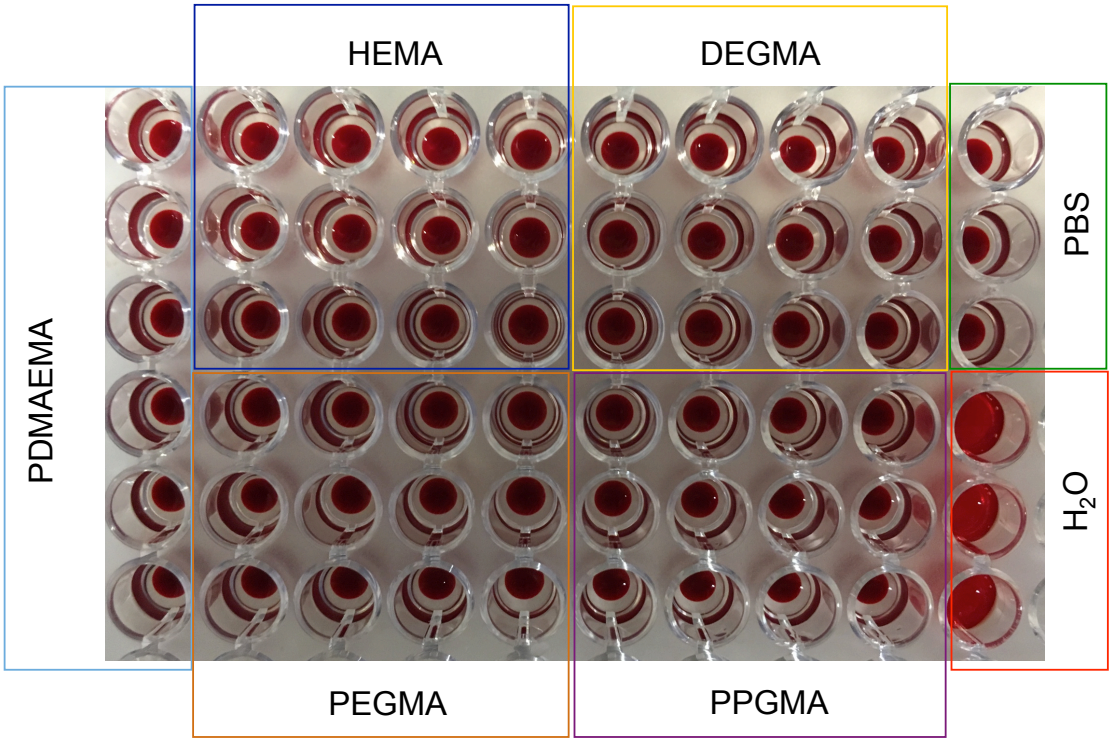


Figure S6: Typical haemagglutination assay plate for polymer plate 2.

Haemagglutination is determined visually Figures S5 and S6. The formation of a button at the bottom of the plate suggests no haemagglutination.

Impact of Polymerization Additives on Screening Protocol

The polymerization mixture was directly sampled in the screening procedure and diluted into buffer for testing. This results in residual DMSO/TEOA. The impact of this additive on antimicrobial activity was therefore tested to ensure the screening protocol was valid. Tertiary amines are used as sacrificial electron donors in the reaction. DMAEMA in this case will also act as an electron donor. Polymerizations were carried out in varying ratios of TEOA:PDMAEMA from 0.3:1 to 3.6:1. The MIC_{99} of PDMAMEA against *M. smegmatis* has previously been determined as 31.3 $\mu g.mL^{-1}$.[2] Below 1.2:1 TEOA:PDMAEMA the MIC_{99} for PDMAEMA is lower than 31.3 $\mu g.mL^{-1}$ suggesting that the DMAEMA is becoming oxidised (Figure S7).

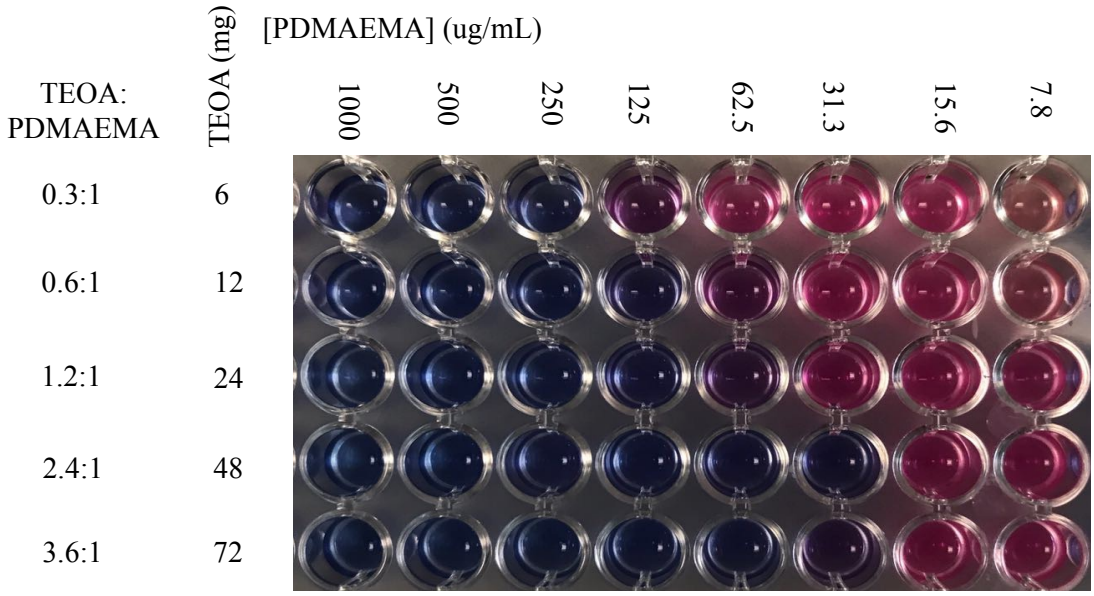


Figure S7: MIC_{99} determination of polymers made with varying TEOA added against *M. smegmatis*.

This was also carried out after filtering the polymers using 3 kDa centrifugal filters to remove the TEOA, and any excess monomer and RAFT agent and the results were the same (Figure S8).

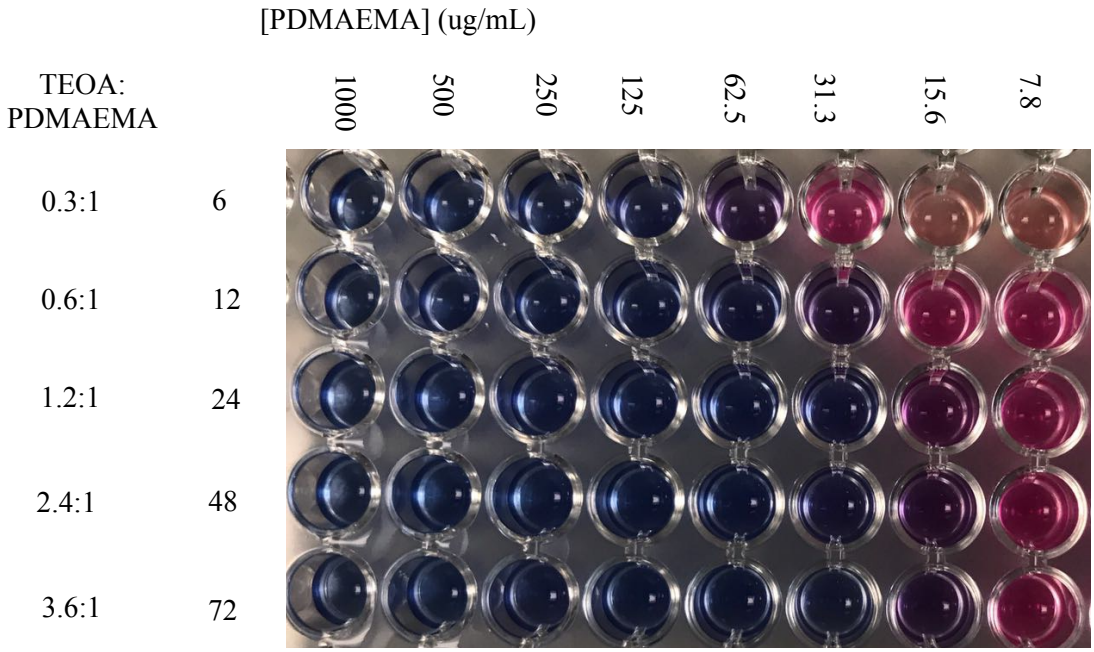


Figure S8: MIC_{99} determination of polymers made with varying TEOA added against *M. smegmatis* post filtering using centrifugal dialysis filters.

The components were tested as if they were used directly from the reaction mixture with 100 % DMSO and 20 $mg.mL^{-1}$ DMAEMA and 48 mg TEOA (Figure S9) Therefore not toxic at the 5 % DMSO used once the reaction was diluted. The top TEOA concentration would be 2.4 $mg.mL^{-1}$ and the DMAEMA would be much less than 0.1 $mg.mL^{-1}$.

Figure S9: Determination of the toxicity of the reaction components.

LogP Calculations

LogP of the comonomers were estimated to make comparisons between hydrophilicity and antimicrobial activity (Figure S10).

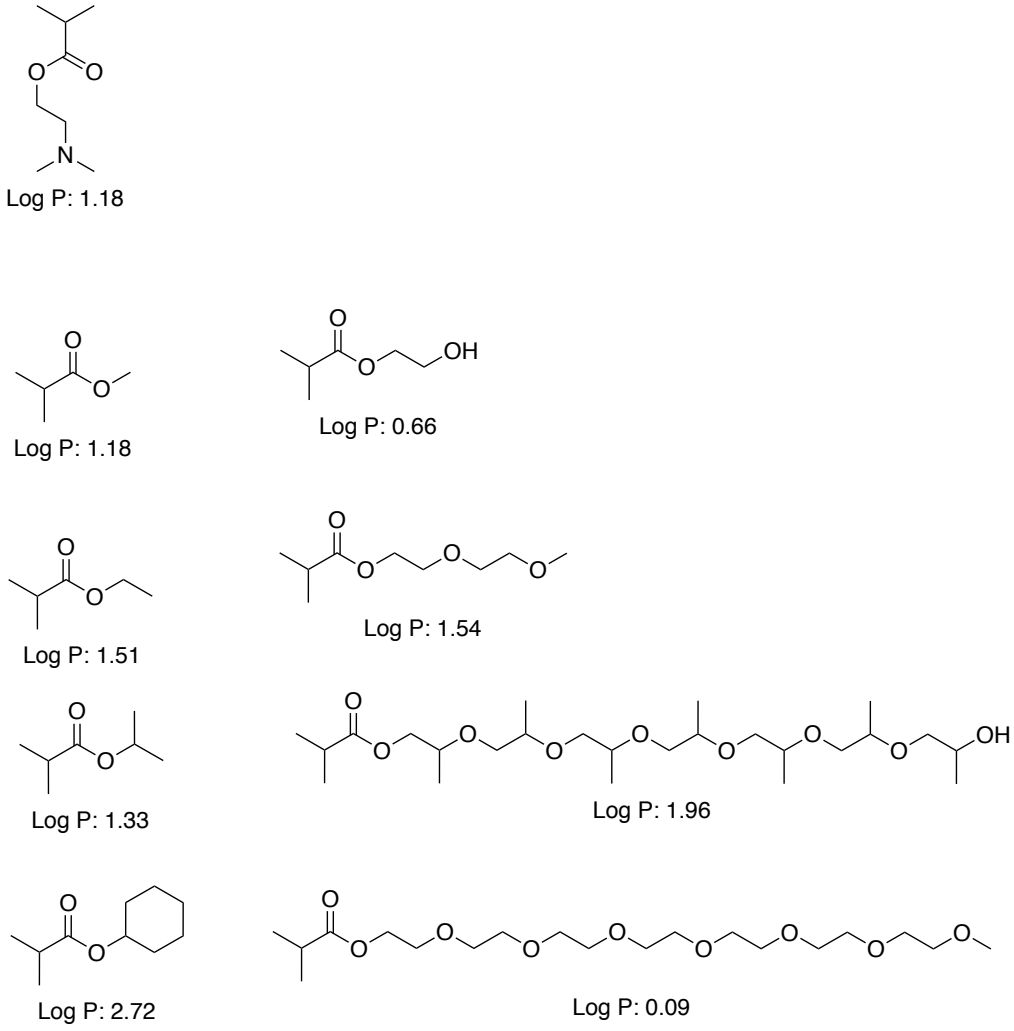


Figure S10: Estimated LogP of PDMAEMA and comonomers.

Copolymers were tested at 0.5 × MIC_{99} of the PDMAEMA homopolymer (125 μg.mL^{-1}) and hits were determined when bacterial growth was inhibited if resazurin (blue) was not reduced to resorufin (pink) for all three repeats (Figures S11 and S12).

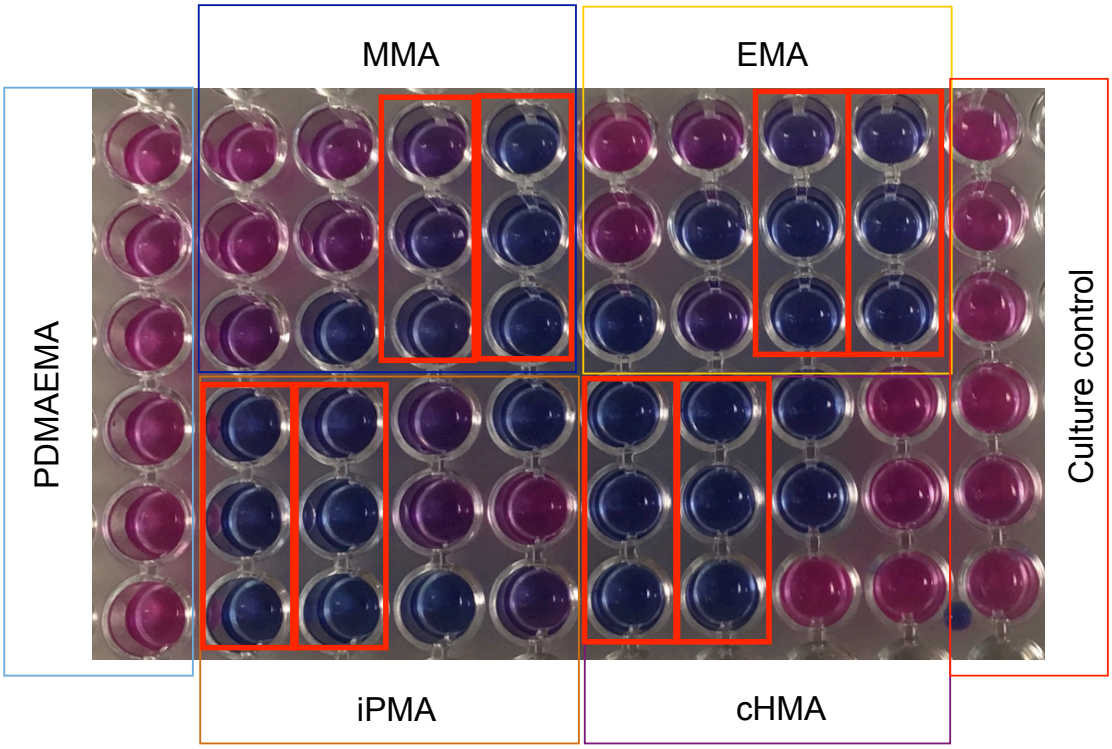


Figure S11: Determination of hits against *E. coli* for plate 1. Hits are highlighted in red.

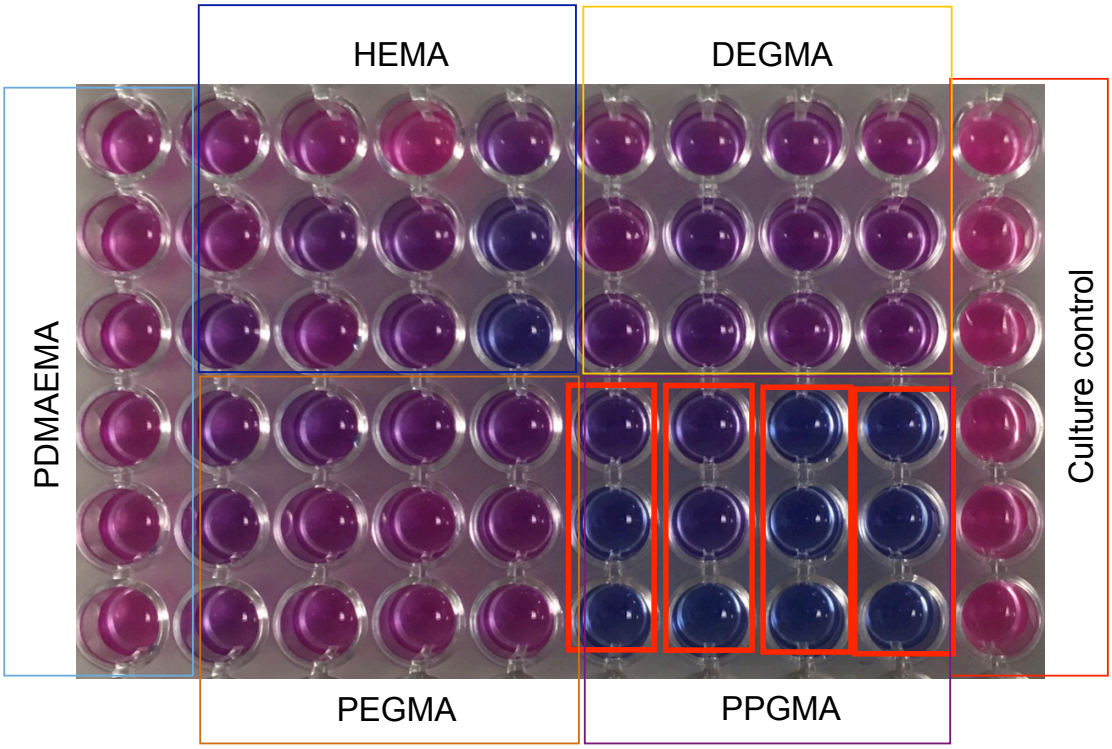


Figure S12: Determination of hits against *E. coli* for plate 1. Hits are highlighted in red.

The hits from Figures S11 and S12 were tested for their MIC_{99} against *E. coli.* The MIC_{99} are shown numerically in the main text (Figure 2) and graphically in Figure S13.

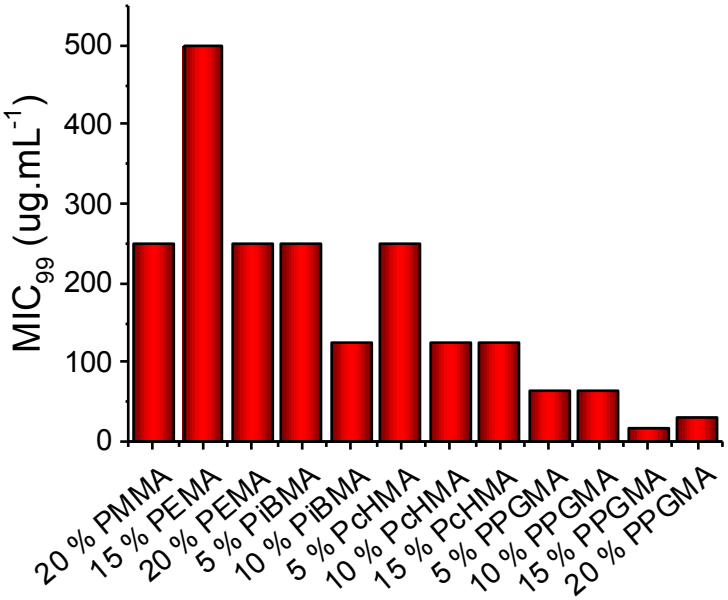


Figure S13: MIC_{99} of hit polymers against *E.coli*.

Cytotoxicity against a mammalian cell line (A459)

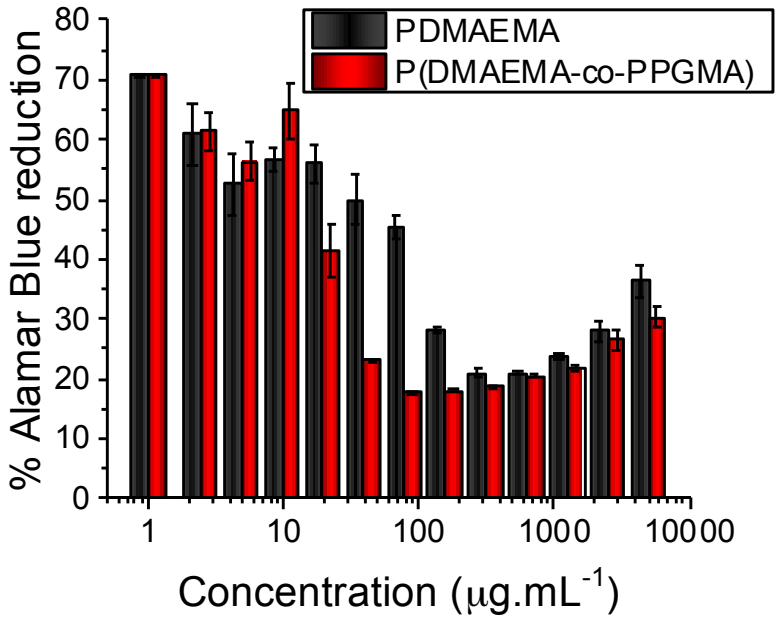


Figure S4: Cytotoxicity of PDMAEMA and P(DMAEMA(85 %)-co-PPGMA(15%)) against a mammalian cell line (A459) after 24 hours of incubation.