Sequence control as a powerful tool for improving the selectivity of antimicrobial polymers

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Experimental

Materials. Acryloyl chloride, chloroform ($CHCl_3$), dichloromethane (DCM), 1, 4 dioxane, ethylacetate (EtOAc), ethylenediamine, triethylamine (NEt_3) and trifluoro acetic acid (TFA) were purchased from Sigma-Aldrich and used without further purification. Sodium chloride (NaCl, Fischer-Scientific, ≥99 %), Boc-anhydride (Fluka, 98 %) and 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044, Wako) were also used without further purification. Mili-Q water was directly used as a solvent for polymerisations. *N*-isopropylacrylamide (NIPAM, Sigma-Aldrich, 97 %) was used after purification by recrystallization in *n*-hexane. All polymerisations were carried out under a nitrogen atmosphere. Lipids 1,2 dioleoyl-*sn*-glycero-3-phosphotehanolamine (PE), 1,2 dioleoyl-*sn*-glycero-3-*rac*(1-glycerol) sodium salt (PG), Cardiolipin sodium salt from bovine heart (CL), Nutrient Agar, Dulbecco's Modified Eagle's Medium (DMEM), Müller-Hinton Broth (MHB), Roswell Park Memorial Institute medium (RPMI-1640) , 3-(*N*-morpholino)propanesulfonic acid (MOPS), Phosphate Buffered Saline (PBS) tablets, Concanavalin A (Con A) and Triton X were purchased from Sigma-Aldrich. The extruder (Avanti Polar Lipids Mini-Extruder, Whatman 400 nm-membranes) was obtained from Avanti Polar lipids, Inc. and used as received. 96-well plates were sourced from Thermo-Fischer. Milli-Q filtered water was used to prepare solutions, according to their recommended concentration and the solutions were autoclaved prior to their usage in order to ensure sterility. The utilised bacteria strains were *P. aeruginosa* ATCC® 27853™, *E. coli* ATCC® 25922™, *S. epidermidis* ATCC® 35984™, *S. aureus* ATCC® 29213™ and *S. aureus* USA 300 (for the development resistance assay). Human red blood cells were obtained from the Australian Redcross.

Methods. *Nuclear Magnetic Resonance (NMR) Spectroscopy.* ^{1}H NMR spectra were recorded on a Bruker Advance 300 spectrometer (300 MHz) at 27 °C in DMSO, $CDCl_3$ or D_2O. For ^{1}H NMR, the delay time (dl) was 2 s. Chemical shift values (δ) are reported in ppm. The residual proton signal of the solvent was used as internal standard.

Molar mass distributions were measured using size exclusion chromatography (SEC) with an Agilent 390-LC MDS instrument equipped with differential refractive index (DRI), viscometry (VS), dual angle light scatter (LS) and dual wavelength UV detectors. The system was equipped with 2 x PLgel Mixed D columns (300 x 7.5 mm) and a PLgel 5 µm guard column. The eluent was DMF with 5 mmol NH_4BF_4 additive. Samples were run at 1 mL min^{-1} at 50°C. Poly(methyl methacrylate) standards (Agilent EasyVials) were used for calibration. 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 using Agilent GPC/SEC software.

Fluorescence spectrometer. The fluorescent intensity was monitored using Agilent Technologies Cary Eclipse Fluorescence Spectrophotometer. The solutions of vesicles were introduced in a polystyrene cuvette for the measurements.

High performance liquid chromatography (HPLC). HPLC was performed using an Agilent 1260 infinity series stack equipped with an Agilent 1260 binary pump and degasser. The flow rate was set to 1.0 mL min^{-1} and samples were injected using Agilent 1260 autosampler with a 100 μL injection volume. The temperature of the column was set at 37 °C. The HPLC was fitted with a phenomenex Lunar C18 column (150 x 4.6 mm) with 5 micron packing (100Å). Detection was achieved using an Agilent 1260 variable wavelength detector. UV detection was monitored at λ = 309 nm. Methods were edited and run using Agilent OpenLAB online software and data was analysed using Agilent OpenLAB offline software. Mobile phase solvents used were HPLC grade (ACN was 'far UV') and consisted of mobile phase A: 100 % ACN, 0.04 % TFA; mobile phase B: 100 % water, 0.04 % TFA with a gradient of 1 to 95 % ACN over 50 minutes.

A retention ratio was calculated from Equation SI-1, relative to the difference in elution times of $pAEAM_{100}$ and $pNIPAm_{100}$.

$$Elution\ ratio\ = \frac{\text{Elution time (sample)} - \text{Elution time (pAEAM}_{100})}{\text{Elution time (pNIPAM}_{100}) - \text{Elution time (pAEAM}_{100})}$$

Equation SI-1. Determination of the elution ratio.

Synthesis of Boc-AEAM.

Boc-AEAM was synthesised according to the literature.[1]

Synthesis of N-t-butoxycarbonyl-1,2-diaminoethane. A solution of ethylenediamine (4.41 g, 4.9 mL, 73 mmol) in 40 mL of DCM was added in a 2-necked 100 mL flask fitted with a condenser, a pressure equalising dropping funnel and nitrogen inlet. After the solution was cooled with an ice-bath, a mixture of Boc-anhydride (3.98 g, 18 mmol) in DCM (20 mL) was added dropwise over 2 hours with stirring. The mixture was allowed to warm to RT and stirred overnight. The solvent was removed by rotary evaporation and a precipitate identified as *N,N'*-(bis-*t*-butoxycarbonyl)-1,2-diaminoethane was observed upon addition of water (50 mL). The filtrate was saturated with NaCl and extracted with EtOAc (3 x 60 mL). The combined organic phases were concentrated under vacuum to obtain a pale oil. Residual NaCl was removed by dissolving the oil in $CHCl_3$ and filtering. The solvent was removed under reduced pressure to give a colourless oil identified as *N-t*-butoxycarbonyl-1,2-diaminoethane (1.51 g, 9 mmol, 50 %). ^{1}H NMR ($CDCl_3$): δ = 1.24 (m, 2H, NH_2); 1.42 (s, 9H, CH_3); 2.77 (m, 2H, CH2), 3.14 (m, 2H, CH2), 5.00 (bs, 1H, amide proton) as shown on Fig. SI-1.

Synthesis of N-t-butoxycarbonyl-N'-acryloyl-1,2-diaminoethane. Acryloyl chloride (0.67 g, 0.6 mL, 7.4 mmol) was dissolved in $CHCl_3$ (30 mL). The solution was cooled in an ice bath and a solution of NEt_3 (0.63 g, 0.9 mL, 6.2 mmol) and *N-t*-butoxycarbonyl-1,2-diaminoethane (1 g, 6.2 mmol) in $CHCl_3$ (15 mL) was added dropwise over an hour and a half. After addition, the reaction mixture was allowed to warm to RT and stirred for an hour before the solvent was removed under reduced pressure. The residue

was washed with water (20 mL) and extracted with $CHCl_3$ (3 x 20 mL). The collected organic fractions were combined and the solvent was removed under vacuum to obtain *N*-*t*-butoxycarbonyl-*N'*-acryloyl-1,2-diaminoethane as a white powder. The product was recrystallized in Et_2O to yield white crystals (1.04 g, 4.9 mmol, 80 %). ^{1}H NMR ($CDCl_3$): δ = 1.37 (s, 9H, CH_3); 3.32 (m, 2H, CH2); 3.45 (m, 2H, CH2); 4.98 (bs, 1H, amide proton); 5.64 (m, 1H, vinyl proton); 6.10 (m, 1H, vinyl proton); 6.27 (m, 1H, vinyl proton); 6.47 (bs, 1H, amide proton) as shown on Fig. SI-2.

Synthesis of (propanoic acid)yl butyl trithiocarbonate PABTC.

The RAFT agent was synthesised according to the literature.[2]

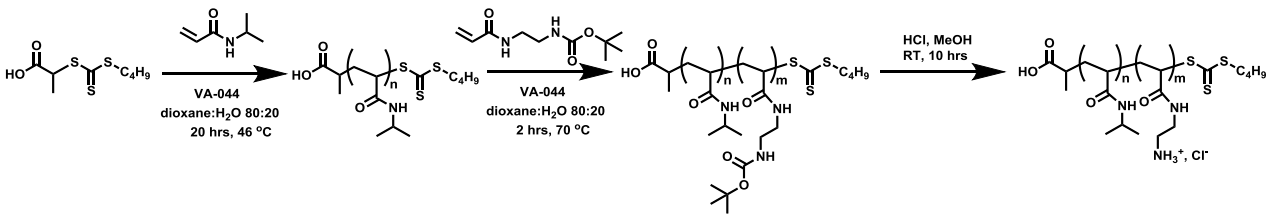
A 50% w/w aqueous sodium hydroxide solution (4.4 g, 2.2 g NaOH, 55 mmol) was added to a stirred mixture of butanethiol (5 g, 5.9 mL, 55 mmol) and water (8.5 mL). Acetone (2.8 mL) was then added, and the resulting clear solution was stirred for 30 min at room temperature. Carbon disulfide (4.75 g, 1.125 eq., 62.4 mmol) was added and the resulting orange solution was stirred for 30 min, then cooled to < 10°C. 2-Bromopropionic acid (8.69 g, 1.025 eq., 56.8 mmol) was slowly added under temperature supervision, followed by the slow addition of a 50% w/w aqueous NaOH solution (4.5 g, 2.25 g NaOH, 57 mmol). When the exotherm stopped, water (8 mL) was added and the reaction was left to stir at RT for 20 hours. A further aliquot of water (15 mL) was added to the reaction mixture, which was subsequently cooled to below 10 °C. A 10 M solution of HCl was slowly added, keeping the temperature below 10 °C and stopping when pH reached 3. The orange solid separated, crystallized and was recovered by filtration under reduced pressure. Yield: 55% (7.2 g, 30.3 mmol). ^{1}H-NMR ($CDCl_3$, 300

MHz, pm): δ = 4.88 (q, 1H, J = 9 Hz, C**H**(CH_3)), 3.39 (t, 2H, J = 9 Hz, S-C**H$_2$**- CH_2-CH_2-CH_3), 1.70 (m, 2H, S-CH_2-C**H$_2$**-CH_2-CH_3), 1.64 (d, 3H, J = 9 Hz, CH(C**H$_3$**)), 1.44 (m, 2H, S-CH_2-CH_2- C**H$_2$**-CH_3), 0.94 (t, 3H, J = 9 Hz, CH_2-C**H$_3$**) .

Typical synthesis of the statistical copolymers.

Monomer(s), initiator, CTA and solvents were introduced in a test tube equipped with a mechanical stirrer and a rubber septum (Table SI-2 for the quantity of reagents needed for the statistical copolymers). The solution was degassed with nitrogen for *ca*. 15 min and the polymerisation was then performed in a thermostated oil bath. After the desired polymerisation time, the test tube was withdrawn from the oil bath.

Multiblock copolymer synthesis by iterative RAFT polymerisation.



Typical synthesis of the initial block. Monomer, initiator, CTA and solvents were introduced in a test tube equipped with a mechanical stirrer and a rubber septum (Tables SI-3, SI-4, SI-5 and SI-6 for the quantity of reagents needed for the diblock and multiblock copolymers). The solution was degassed with nitrogen for *ca*. 20 min and the polymerisation was then performed in a thermostated oil bath at 46 °C. After 20 hours, the test tube was withdrawn from the oil bath and a sample was taken for ^{1}H NMR and SEC analysis.

Typical synthesis of subsequent blocks. The test tube with the reaction mixture was opened and additional monomer, initiator and solvent were introduced. After the mixture was sealed with a septum, the solution was degassed for *ca*. 20 min, then placed in an oil bath set at 70 °C for the polymerisation to occur. The tube was withdrawn from the oil bath after 2 hours and a sample was taken for ^{1}H NMR and SEC analysis.

Determination of monomer conversions. Monomer conversions (p) were calculated from ^{1}H NMR data using equation SI-2.

$$p = 1 - (\int \mathrm{I}_{5.4-6.4\mathrm{ppm}} / \int \mathrm{I}_{\mathrm{a}} / \mathrm{DP}_{\mathrm{targeted}})$$

Equation SI-2. Calculation of monomer conversion p

Where $\int \mathrm{I}_{5.4-6.4\mathrm{ppm}}$ is the integral of the three vinyl protons of the monomer, $\int \mathrm{I}_{\mathrm{a}}$ is the integral of the three methyl protons belonging to the terminal methyl of the Z group of the CTA and $DP_{targeted}$ is the average degree of polymerisation targeted.

Calculation of $M_{n,th}$. The theoretical number-average molecular weight ($M_{n,th}$) is calculated using Equation SI-3.

$$M_{\mathrm{n,th}} = \frac{[\mathrm{M}]_0 \mathrm{p} M_{\mathrm{M}}}{[\mathrm{CTA}]_0 + 2f[\mathrm{I}]_0(1 - \mathrm{e}^{-k_d t})(1 - \frac{f_c}{2})} + M_{\mathrm{CTA}}$$

Equation SI-3. Calculation of $M_{n,th.}$

Where $[M]_0$, $[CTA]_0$, $[I]_0$ are the initial concentrations (in mol.L^{-1}) of the monomer, CTA and the initiator respectively; *p* is the monomer conversion as determined by Equation 2; M_M and M_{CTA} are the

molar masses (in g.mol^{-1}) of the monomer and the CTA, respectively; k_d is the decomposition rate constant (in s^{-1}) of the azo-initiator; and t represents the polymerisation time (in seconds). The factor "2" accounts for the fact that one molecule of initiator yields two primary radicals with the efficiency f (assumed to be equal to 0.5 in this study). The decomposition rate constant for VA-044 at the temperature T ($k_{d,VA\text{-}044(T)}$) was determined from the values obtained from Wako ($k_{d,VA\text{-}044(44\ °C))}$ = 1.92 X 10^{-4} s^{-1} and E_a = 108000 J.mol^{-1}) using the Arrhenius equation ($k_{d,VA\text{-}044(70\ °C))}$ = 4.30 x 10^{-4} s^{-1}). The term 1 – (f_c /2) represents the number of chains produced in a radical-radical termination event with fc representing the coupling factor. An f_c value of 1 means that 100 % of bimolecular terminations occur by combination, whereas a value of 0 indicates that 100 % of bimolecular terminations result in disproportionation. In this study, 100 % terminations y disproportionation are assumed (f_c = 0).

Determination of the livingness (L). The fraction of living chains can be calculated using Equation SI-4, the parameters being $[CTA]_0$ and $[I]_0$ initial CTA and initiator concentration, whereas k_d, f and 1-f_c/2 are related to the thermal decomposition of the initiator.

$$L\ (\%) = \frac{[CTA]_0}{[CTA]_0 + 2f[I]_0(1 - e^{-k_d t})(\frac{1 - f_c}{2})}$$

Equation SI-4. Theoretical determination of the relative amount of living polymer chains using an azo-initiator compound.

Determination of the pKa

20 mg (5 mg.mL^{-1}) of H100 and 1.17 g (0.05 M) of NaCl were dissolved in 40 mL of water. 100 µL of a 6M HCl solution was added to the polymer solution in order to make sure all the amine groups were protonated. The titration was performed manually at room temperature with a syringe pump to control the added volume and a pH meter (HI2211 Hanna Instruments) using a solution of 0.2 M of NaOH as the titrant. For each polymer, the range of pKa was determined using the maximum of the first derivative of the titration curve (Fig. SI-13).

Dynamic Light Scattering measurements

DLS measurements were taken using a Malvern instruments Zetasizer Nano at 37 °C with a 4 mW He-Ne 633 nm laser at a scattering angle of 173° (back scattering). For DLS aggregation studies, 1.024 mg of polymer sample was dissolved in 1 mL of PBS buffer at pH 7.4 and a total of 0.5 mL of the solution was introduced in a 1.5 mL polystyrene cuvette after filtering with a 0.2 µm filter**.**

Dye leakage assays

Formation of vesicles. The synthesis of vesicles was performed according to a protocol detailed by Lienkamp *et al.*.[3] 100 mL of a first buffer (buffer A) was prepared by dissolving 142 mg (1 mmol) of Na_2HPO_4 in 90 mL of H_2O. The pH was then adjusted to 7 with a 1 mol.L^{-1} solution of NaOH. The total volume of the solution was then taken to 100 mL. The calcein solution was obtained by dissolving 249 mg (0.4 mmol) of calcein dye in 8 mL of previously prepared buffer A. The pH of the solution was adjusted to 7.0 with a 1 mol.L^{-1} solution of NaOH in order to dissolve the calcein. The total volume was then taken up to 10 mL in order to yield a buffer of 40 mmol.L^{-1} of calcein.

A second buffer (buffer B) was prepared by dissolving 1.42 g (10 mmol) of Na_2HPO_4 and 5.26 g (90 mmol) of NaCl in 980 mL of H_2O. The pH was adjusted to 7.0 with a 1 mol.L-1 solution of NaOH. The volume of the solution was then taken up to 1000 mL.

For the PE/PG 4:1 vesicles, 6.0 mg (8 mmol) of PE and 1.6 mg (2 mmol) of PG in 0.8 mL of $CHCl_3$, for the CL vesicles, 6 mg (10 mmol) of CL was dissolved in 0.6 mL of $CHCl_3$ in a 25-mL round bottom flask, in order to obtain a solution of roughly 10 mg.mL^{-1}. A film was formed at the bottom of the flask by removing the solvent under reduced pressure, the flask kept as vertical as possible. After the film was dried under vacuum, it was hydrated with 1 mL of buffer A and stirred for an hour with a magnetic stirring bar. After complete dissolution of the lipid, the solution underwent 5 freeze-thaw cycles. The solution was then filtered 15 times by extrusion, using 400 nm membranes. The free dye was filtered through a Sephadex G-50 column using buffer B. The vesicle fraction from the column was diluted for the dye-leakage experiments according to the initial fluorescence of the solution.

Fluorescence monitoring.

Interactions of the polymers with model bacterial membranes composed of lipid bilayers were evaluated using liposomes consisting of a mixture of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) with a ratio of 4 to 1 to model Gram-negative bacteria and Cardiolipin (CL) for Gram-positive bacteria. The fluorescent dye calcein was encapsulated in a self-quenching concentration. When the membrane is compromised by the addition of a sample, the dye leakage would result in an increased fluorescence.

To that end the fluorescence of the vesicle solution was monitored by recording the fluorescence intensity at a wavelength of 537 nm with the excitation wavelength set at 492 nm. The intensity of the vesicle solution was measured, then 20 µL of 1.4 mg mL^{-1} solution of polymer was added 30 seconds after the start of the run, followed by the addition of 20 µL of a 20 % solution of Triton X 9 minutes later. The intensities were normalized by setting the baseline at the intensity before polymer addition and the maximum at the intensity reached after addition of Triton X, corresponding to 100 % leakage.

Antibacterial susceptibility tests

Antibacterial susceptibility was studied using two strains of Gram-negative bacteria: *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*); and two Gram-positive strains: *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*). Minimum inhibitory concentrations (MICs) were determined according to the standard Clinical Laboratory Standards Institute (CLSI) broth microdilution method (M07-A9-2012). A single colony of bacteria was picked up from a fresh (24 hour) culture plate and inoculated in 5 mL of Mueller-Hinton (MH) broth, then incubated at 37 °C overnight. On the next day, the concentration of cells was assessed by measuring the optical density at 600 nm (OD_{600}). Culture suspension was then diluted to an OD_{600} = 0.1 with RPMI with 0.165 mol L^{-1} of MOPS in order to reach a bacterial concentration of ~ 10^8 colony forming unit per mL (CFU mL^{-1}). The solution was diluted further by 100 fold to obtain a concentration of 10^6 CFU mL^{-1}. Polymers were dissolved in distilled water and 100 μL of each test polymer was added to micro-wells followed by the addition of the same volume of bacterial suspension (10^6 CFU mL^{-1}). The micro-wellplates were incubated at 37 °C for 24 hours, and growth was evaluated by measuring the OD_{600} using a plate reader. Triplicates were performed for each concentration and readings were taken twice. The growth in the well was normalised using negative controls, wells without any bacteria introduced, and positive controls, wells only containing bacterial solution.

Hemolysis and hemagglutination assays

Human red blood cells (RBCs) were prepared by washing freshly collected human blood with PBS *via* centrifugation. Polymers were dissolved in PBS. The normalisation was done using positive controls (50 μg mL^{-1} Concanavalin A for hemagglutination and 2 % Triton X-100 in PBS for haemolysis) and negative control (PBS) which were included on each plate. A suspension of 3 % in volume of RBCs was added to each well and the contents were mixed before being incubated at 37°C for 2 hours. The 96-well plates were centrifuged at 600 x *g* for 10 minutes then 100 μL of the supernatant was transferred into a new plate. The absorbance at 540 nm was measured and normalised using the positive and negative control.

Cell Culture

CaCo2 human colorectal adenocarcinoma cells were grown in a 50:50 mixture of Ham's F12 and Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of fetal calf serum, 1% of 2 mM glutamine and 1% penicillin/streptomycin. NIH/3T3 mouse embryonic fibroblasts were grown in DMEM medium supplemented with 10% of bovine calf serum, 1% of 2 mM glutamine and 1% penicillin/streptomycin. Both cell lines were grown as adherent monolayers at 37 °C in a 5% CO_2 humidified atmosphere and passaged at approximately 70-80% confluence.

In vitro growth inhibition assays

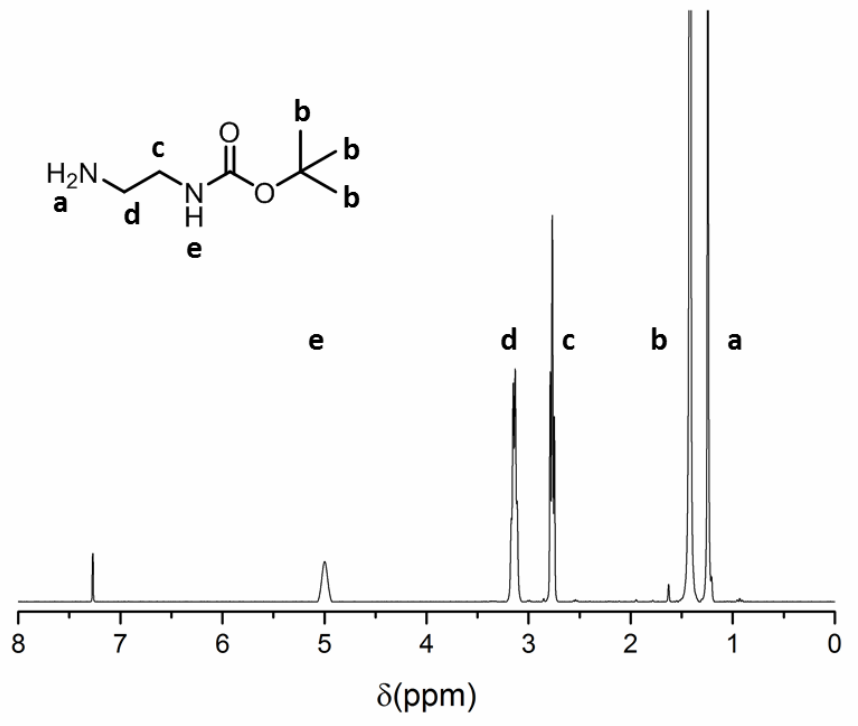
The anti-proliferative activity of the polymers was determined in CaCo2 colorectal cancer cells and NIH/3T3 embryonic fibroblasts. 96-well plates were used to seed 5000 cells per well which were left to pre-incubate with drug-free medium at 37 °C for 24 hours before adding different concentrations of the compounds to be tested (1024 μg mL^{-1} – 32 μg mL^{-1}). A drug exposure period of 72 hours was allowed. The XTT assay was used to determine cell viability. The IC_{50} values (concentrations which caused 50% of cell death), were determined as duplicates of triplicates in two independent sets of experiments and their standard deviations were calculated.

Resistance detection assay.

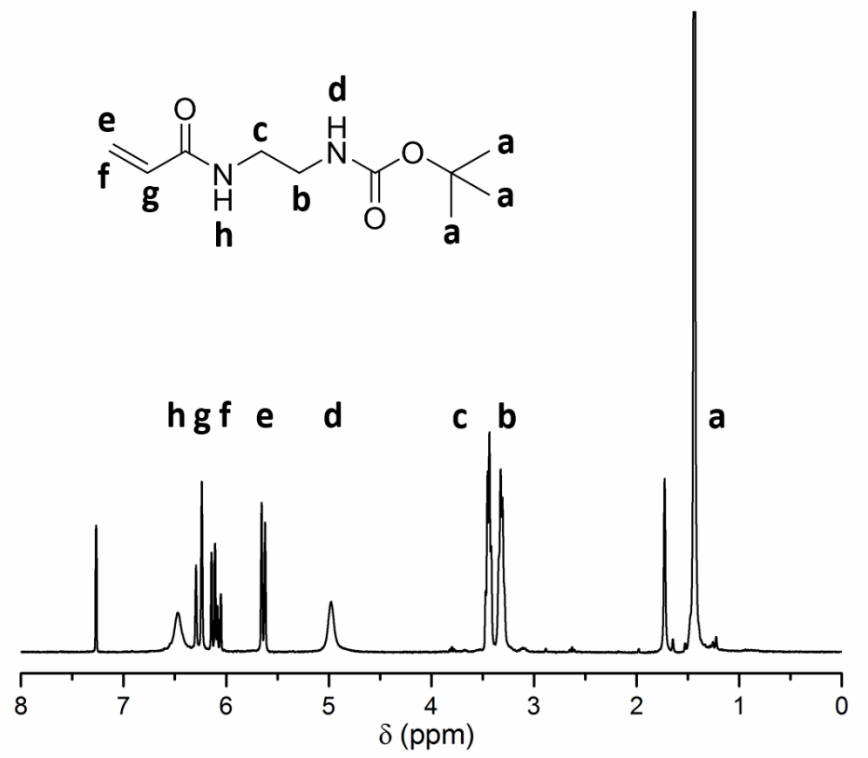
The detection of the development of bacterial resistance was studied using the methodology described by Gullberg *et al.*.[4] Overnight cultures of a methicillin-resistant strain of *S. aureus* (USA 300) in MH broth obtained from agar plates. Cells serially passaged by 400 fold into 1 mL batch cultures every 24 hours for 24 days, in MH broth containing 1/10 MIC value of the antimicrobial agent. After every 100 generations (4 days), an antibacterial susceptibility was performed as described above to observe any variation in the MIC values.

To confirm the absence of any resistant mutants, a further detection method was used. 100 μL of the final bacterial suspension from the resistance generation assay was taken and serially diluted by 10 to 10^7. 100 μL from each dilution was added on an agar plate containing 1 x MIC of the test compound and using a sterile spreader, the solution was spread across the entire agar plate. After incubation at 37 °C for 24 hours, the agar plate with countable single colonies (if present) were used to perform an antibacterial susceptibility test on each colony separately to confirm any increase in resistance from prior MIC values. No resistant mutants were detected.

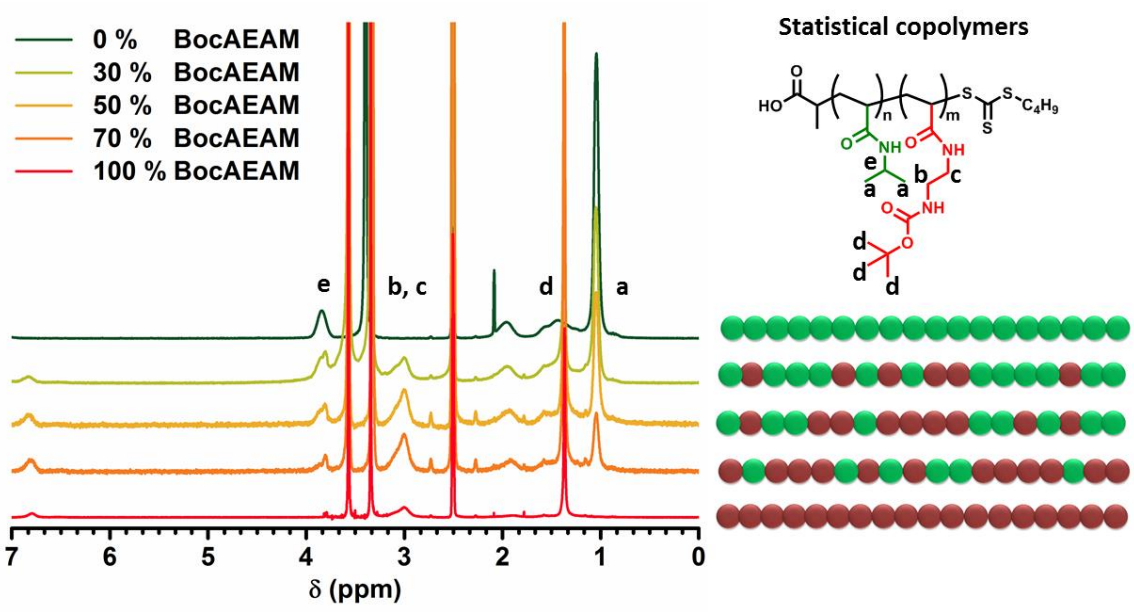
Supporting Figures



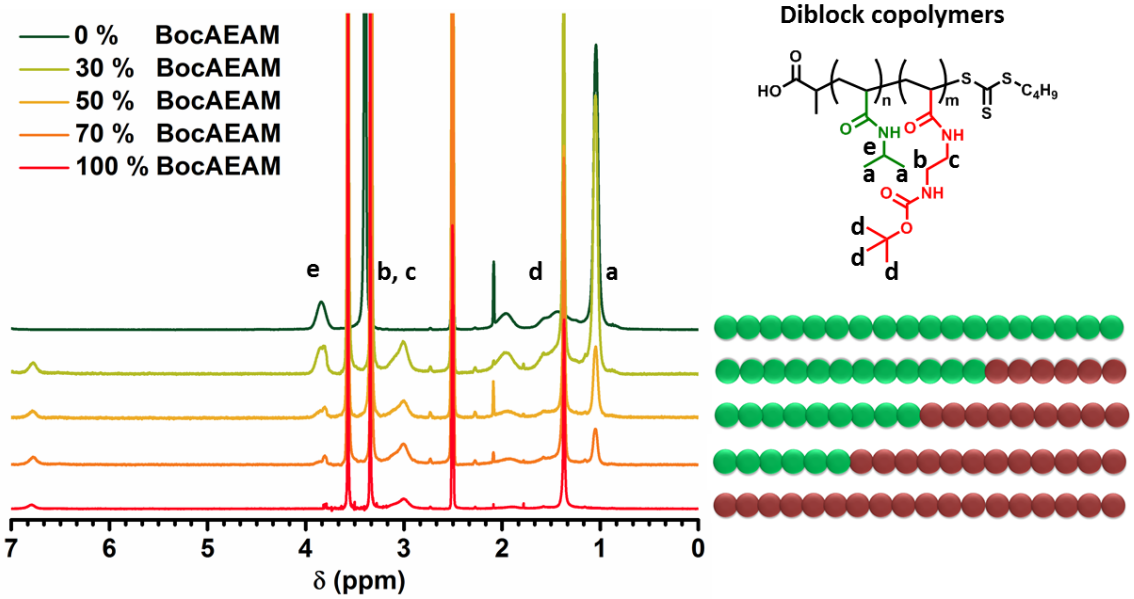
Supporting Figure SI-1. ^{1}H NMR spectrum of the intermediate product N-t-butoxycarbonyl-1,2-diaminoethane.



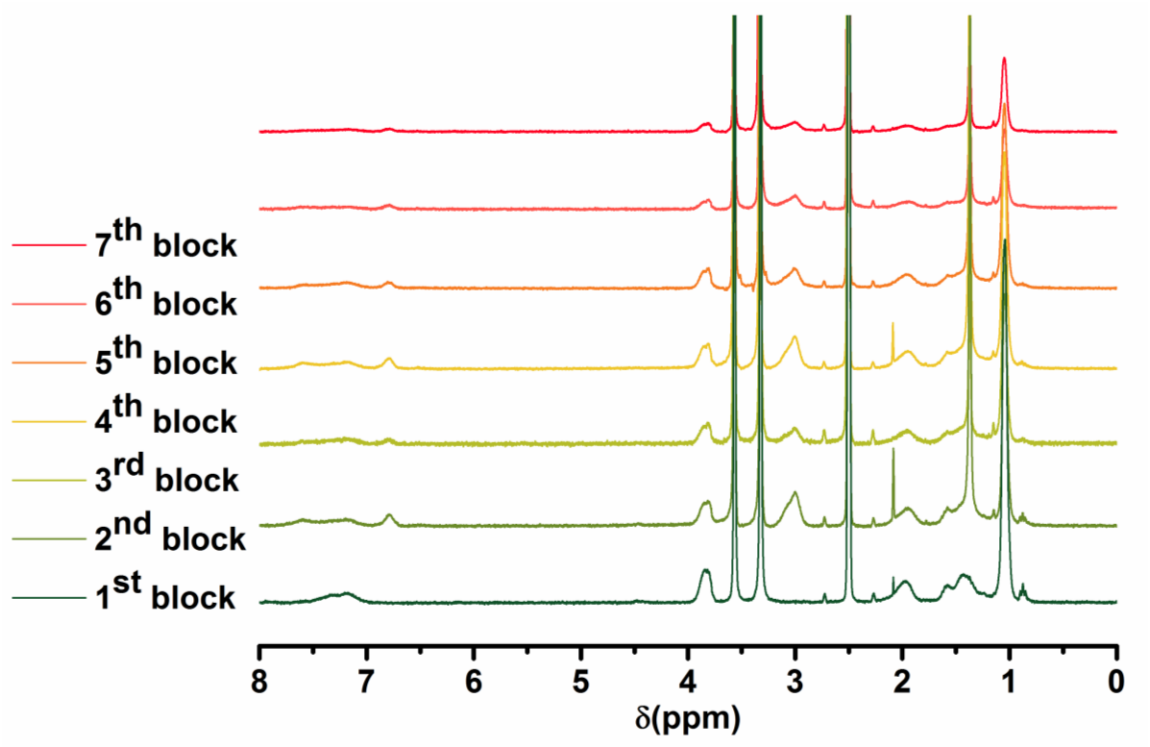
Supporting Figure SI-2. ^{1}H NMR spectrum of Boc-AEAM.



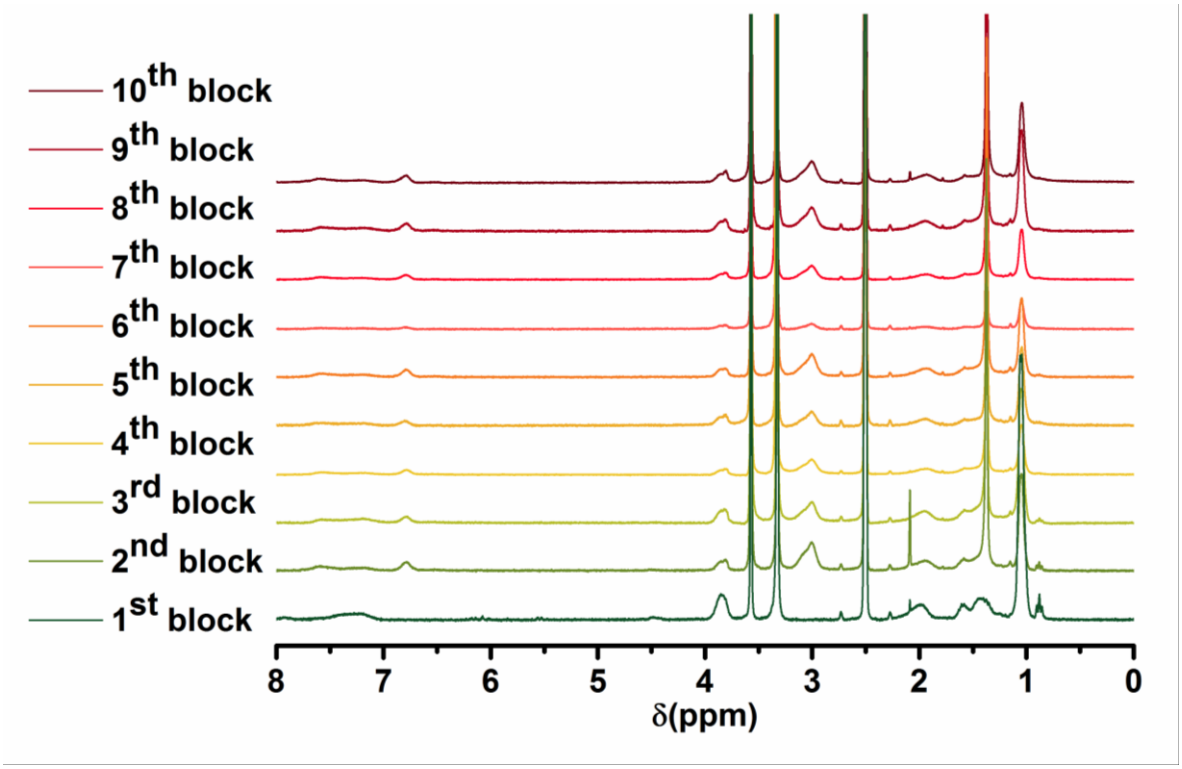
Supporting Figure SI-3. ^{1}H NMR spectra in $DMSO_d$ of Boc-protected statistical copolymers for each composition.



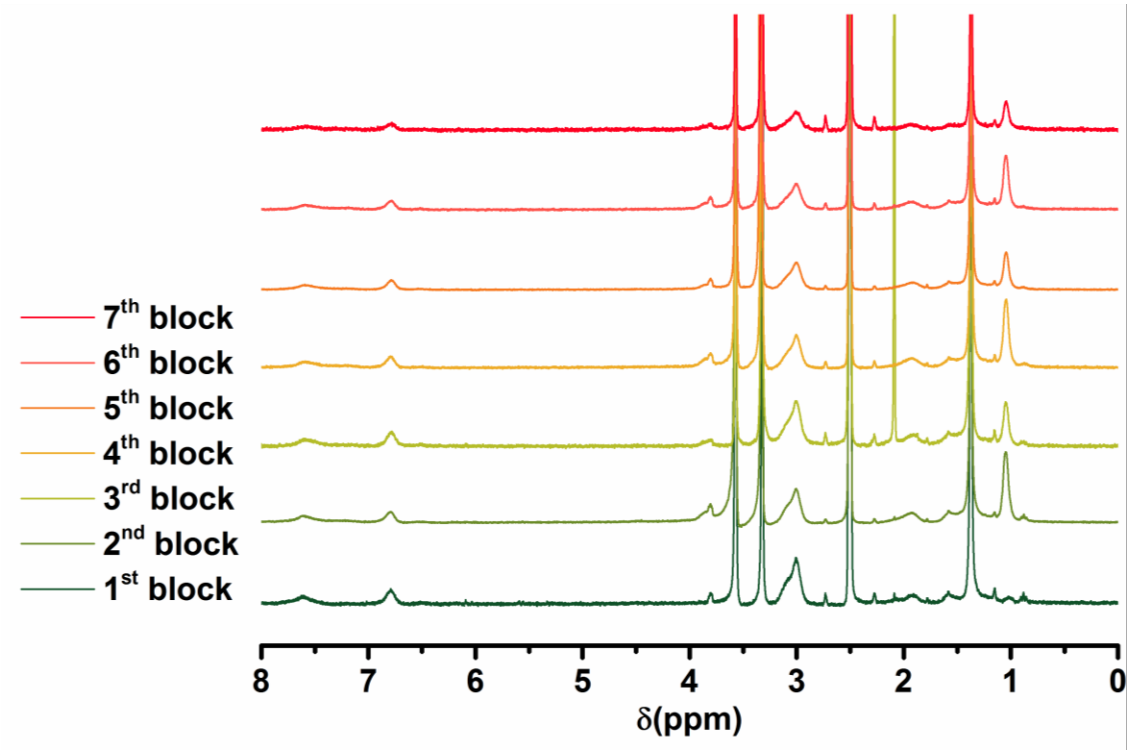
Supporting Figure SI-4. ^{1}H NMR spectra in $DMSO_d$ of Boc-protected diblock copolymers for each composition.



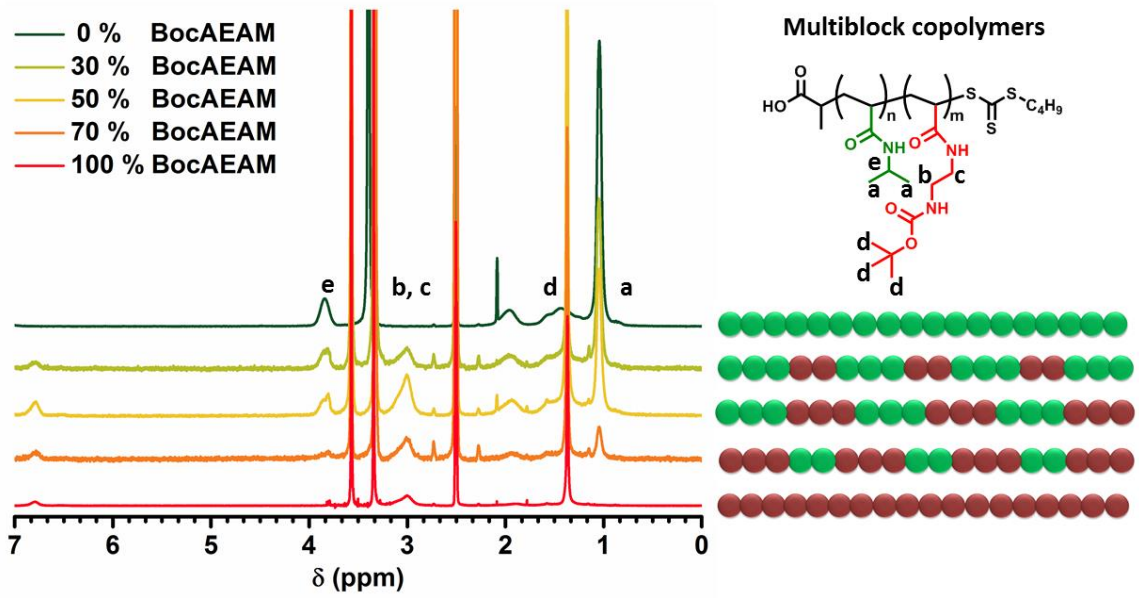
Supporting Figure SI-5. ^{1}H NMR spectra in $DMSO_d$ of $M30^{Bo}$ for each chain extension.



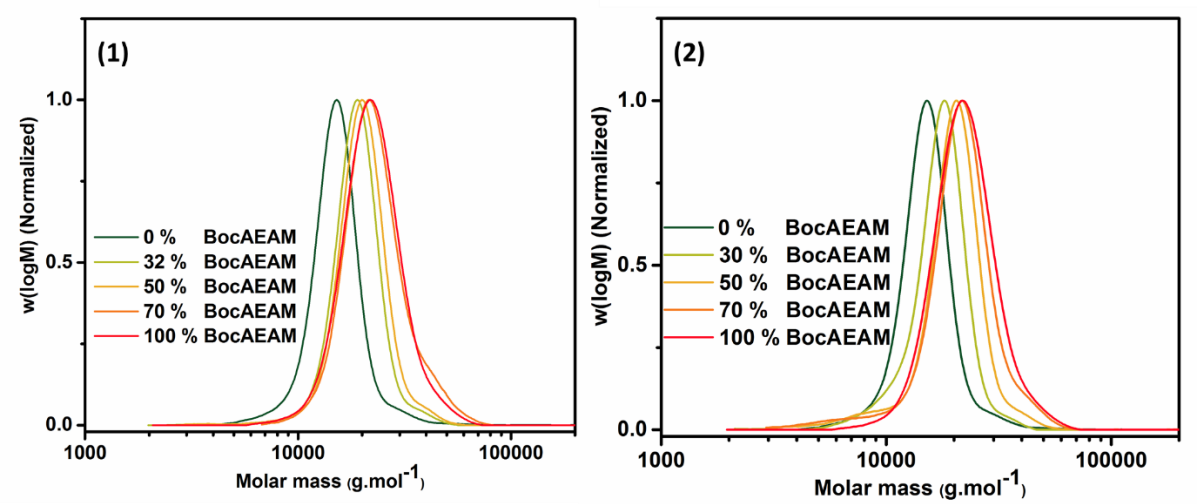
Supporting Figure SI-6. ^{1}H NMR spectra in $DMSO_d$ of $M50^{Boc}$ for each chain extension.



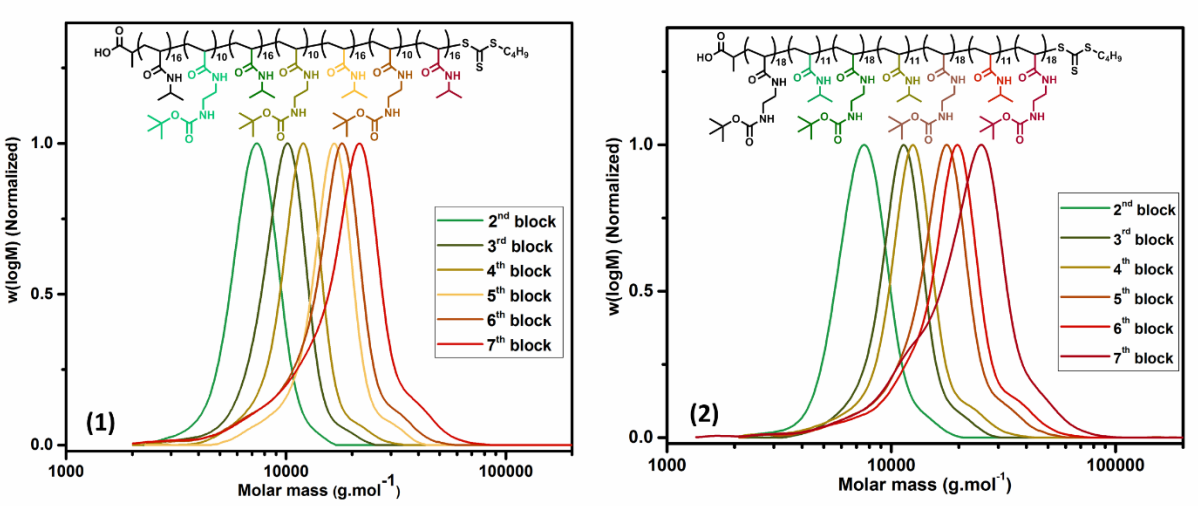
Supporting Figure SI-7. ^{1}H NMR spectra in $DMSO_d$ of $M70^{Boc}$ for each chain extension.



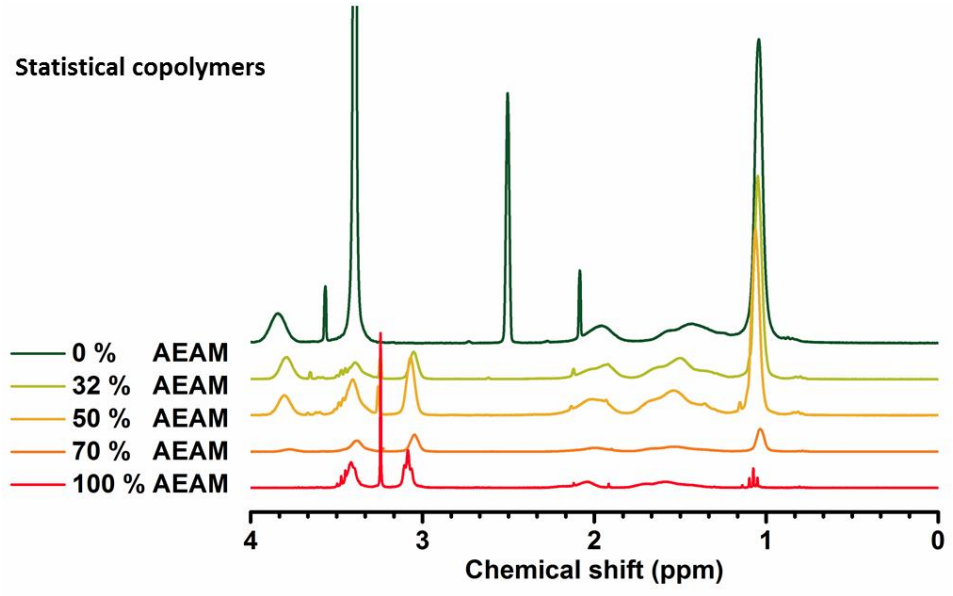
Supporting Figure SI-8. ^{1}H NMR spectra in $DMSO_d$ of Boc-protected multiblock copolymers for each composition.



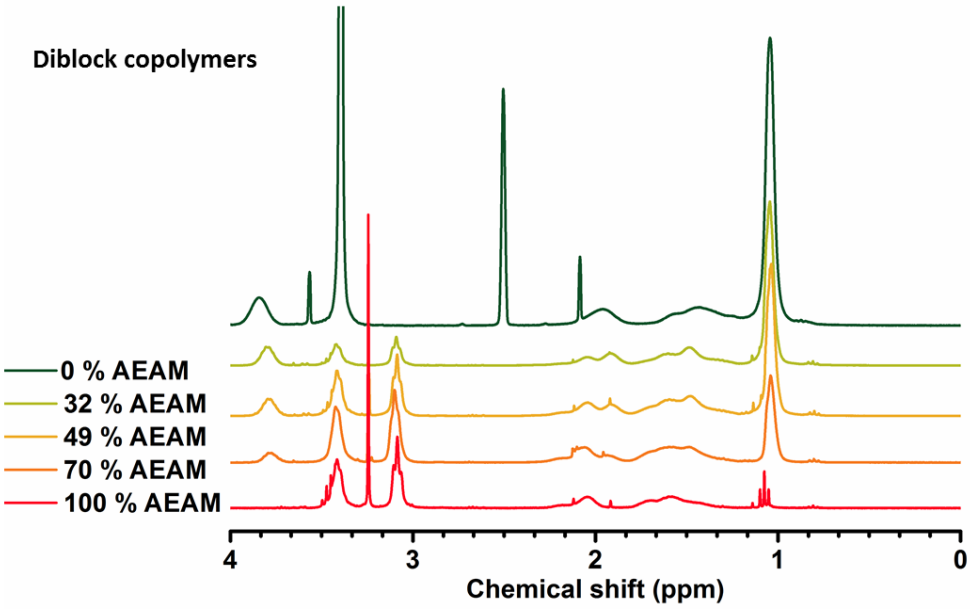
Supporting Figure SI-9. DMF-SEC chromatograms for statistical (1) and diblock (2) copolymers of each composition.



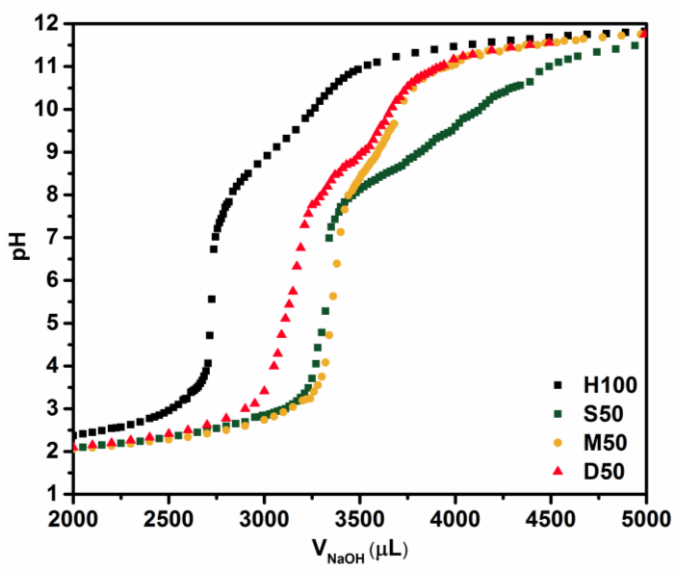
Supporting Figure SI-10. DMF-SEC chromatograms for successive chain extensions of $M30^{Boc}$ (1) and $M70^{Boc}$ (2).



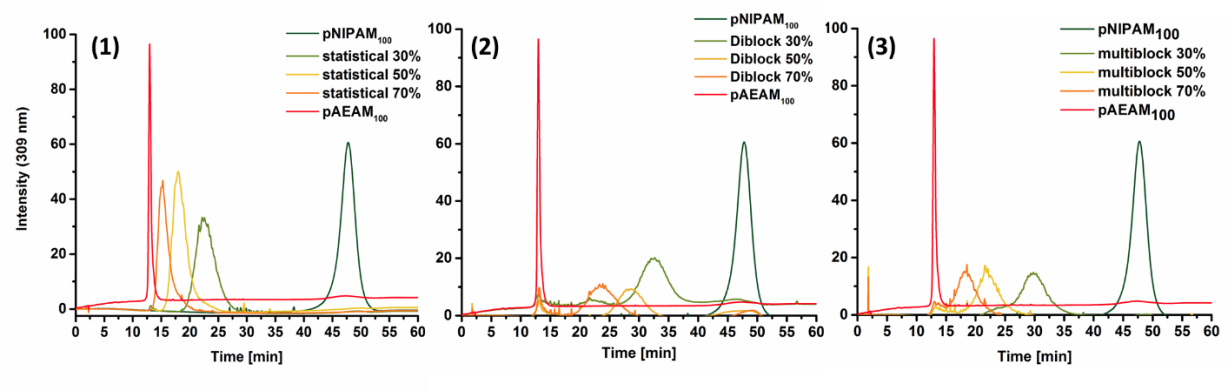
Supporting Figure SI-11. ^{1}H NMR spectra in D_2O of the deprotected statistical copolymers of each composition and in $DMSO_d$ for H0.



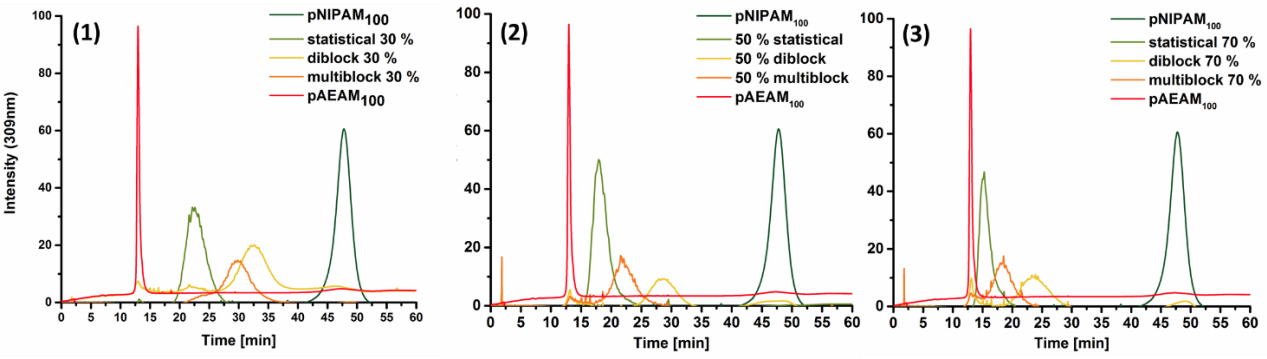
Supporting Figure SI-12. ^{1}H NMR spectra in D_2O the deprotected diblock copolymers of each composition and in $DMSO_d$ for H0.



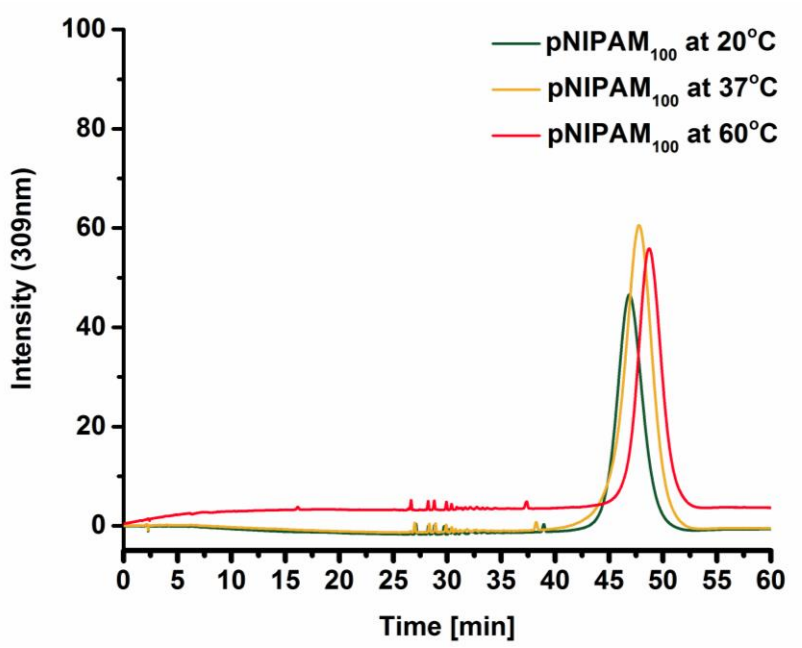
Supporting Figure SI-13. Titration curves of acidified solutions of the cationic polymers H100, S50, D50 and M50 (concentration of around 0.5 mg mL^{-1}) neutralized with sodium hydroxide (0.2 M).



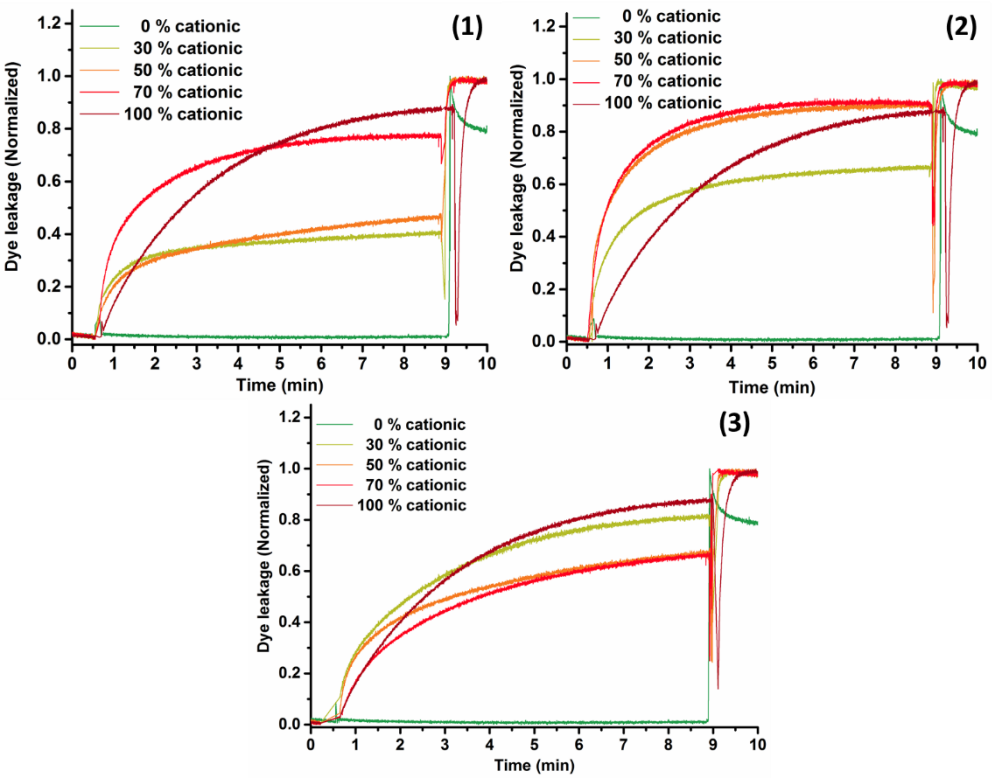
Supporting Figure SI-14. HPLC chromatograms for the various composition of statistical (1), diblock (2) and multiblock (3) copolymers with a gradient of 1 to 95 % ACN over 50 minutes at 37 °C.



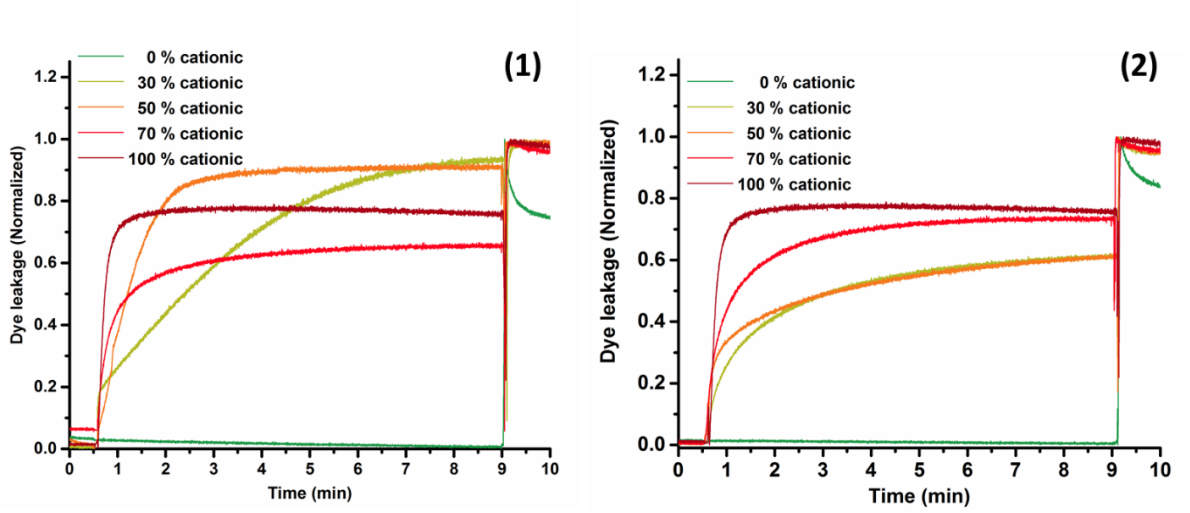
Supporting Figure SI-15. HPLC chromatograms for the various segmentations at 30 % (1), 50 % (2) and 70 % (3) cationic copolymers with a gradient of 1 to 95 % ACN over 50 minutes at 37 °C.



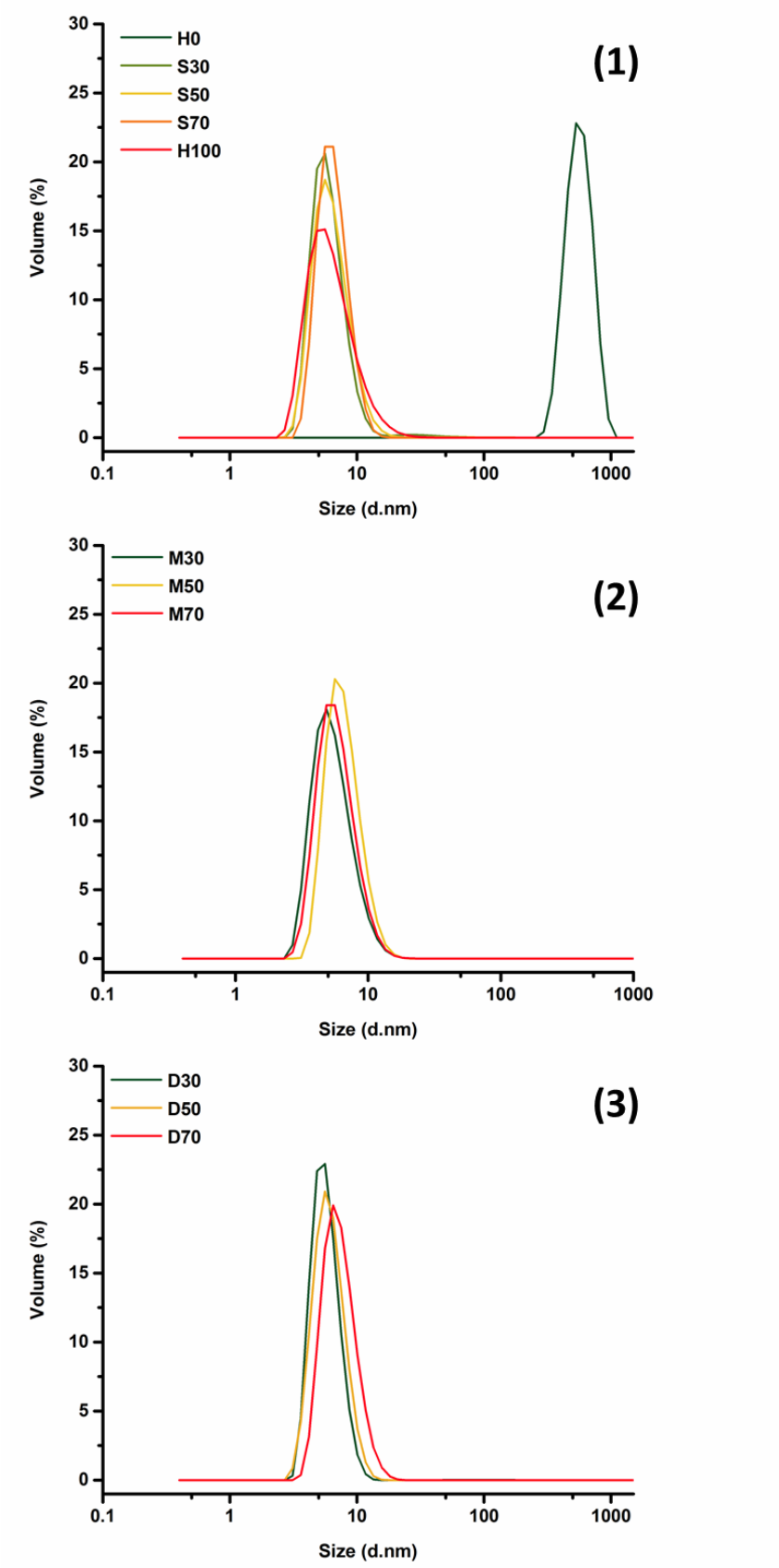
Supporting Figure SI-16. HPLC chromatograms of H0 at 20, 37 and 60 °C with a gradient of 1 to 95 % ACN over 50 minutes.



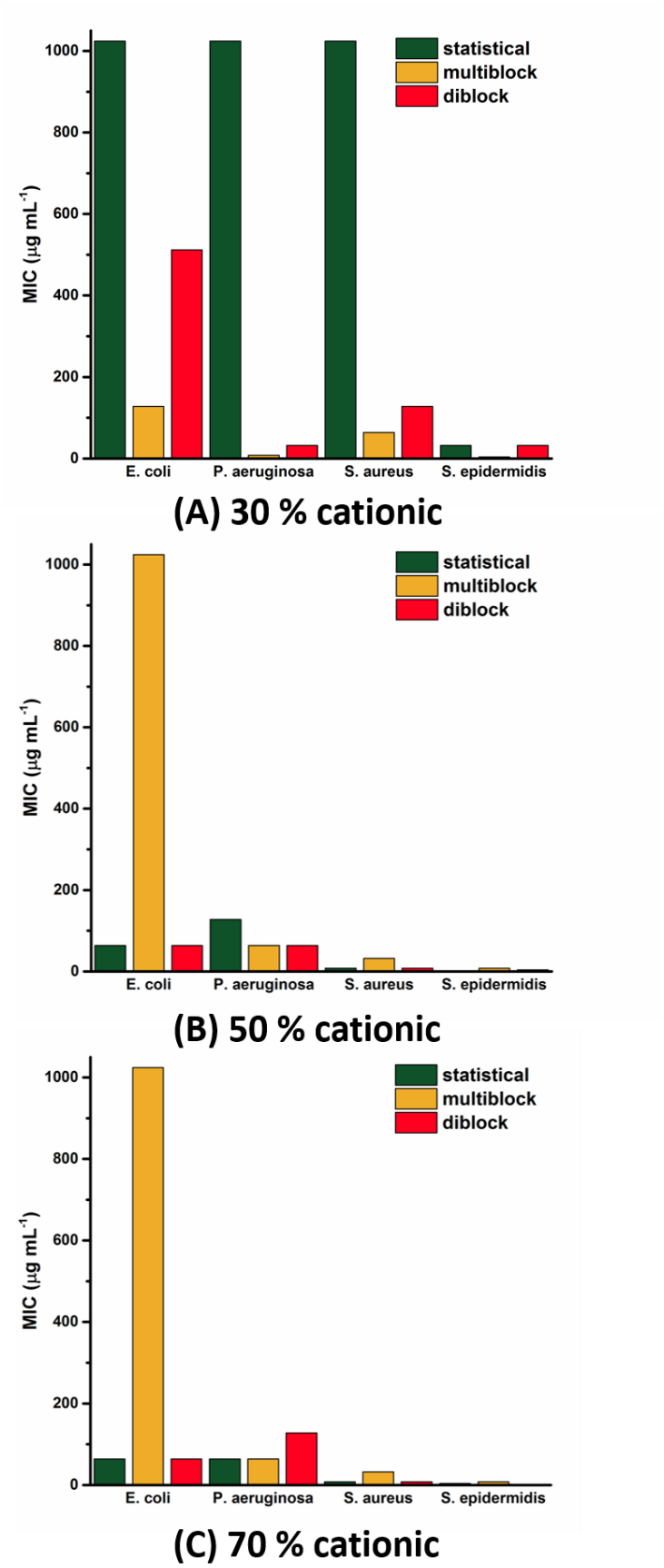
Supporting Figure SI-17. Dye leakage study with statistical (1) diblock (2) and multiblock (3) copolymers on Gram-positive bacteria model. Fluorescence was read at 537 nm (emission) at an excitation wavelength of 492 nm. The Sample was added at 30 s measurement time and vesicles were lysed by addition of Triton X at 9 min.



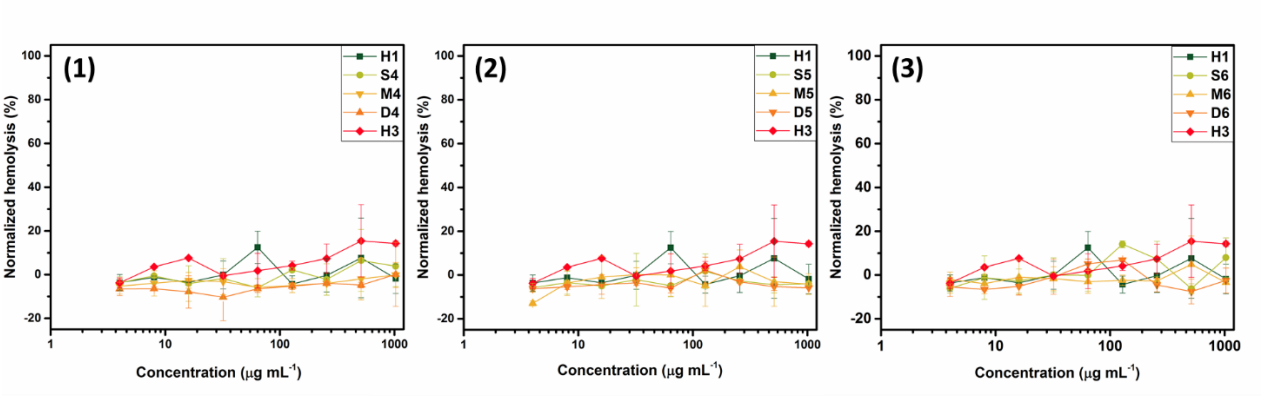
Supporting Figure SI-18. Dye leakage study with statistical (1) diblock (2) copolymers on Gram-negative bacteria model. Fluorescence was read at 537 nm (emission) at an excitation wavelength of 492 nm. The Sample was added at 30 s measurement time and vesicles were lysed by addition of Triton X at 9 min.



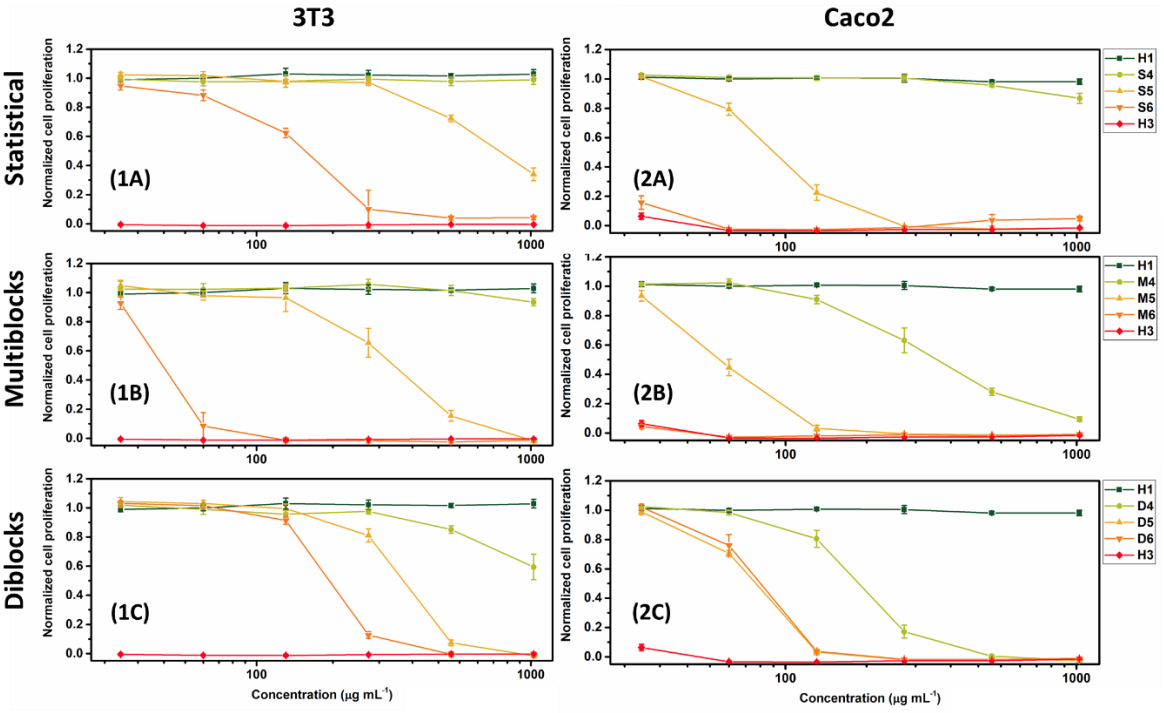
Supporting Figure SI-19. Size distribution by volume of the homopolymers (1), statistical (1), diblock (2) and multiblock (3) copolymers at 1 mg mL^{-1} in PBS.



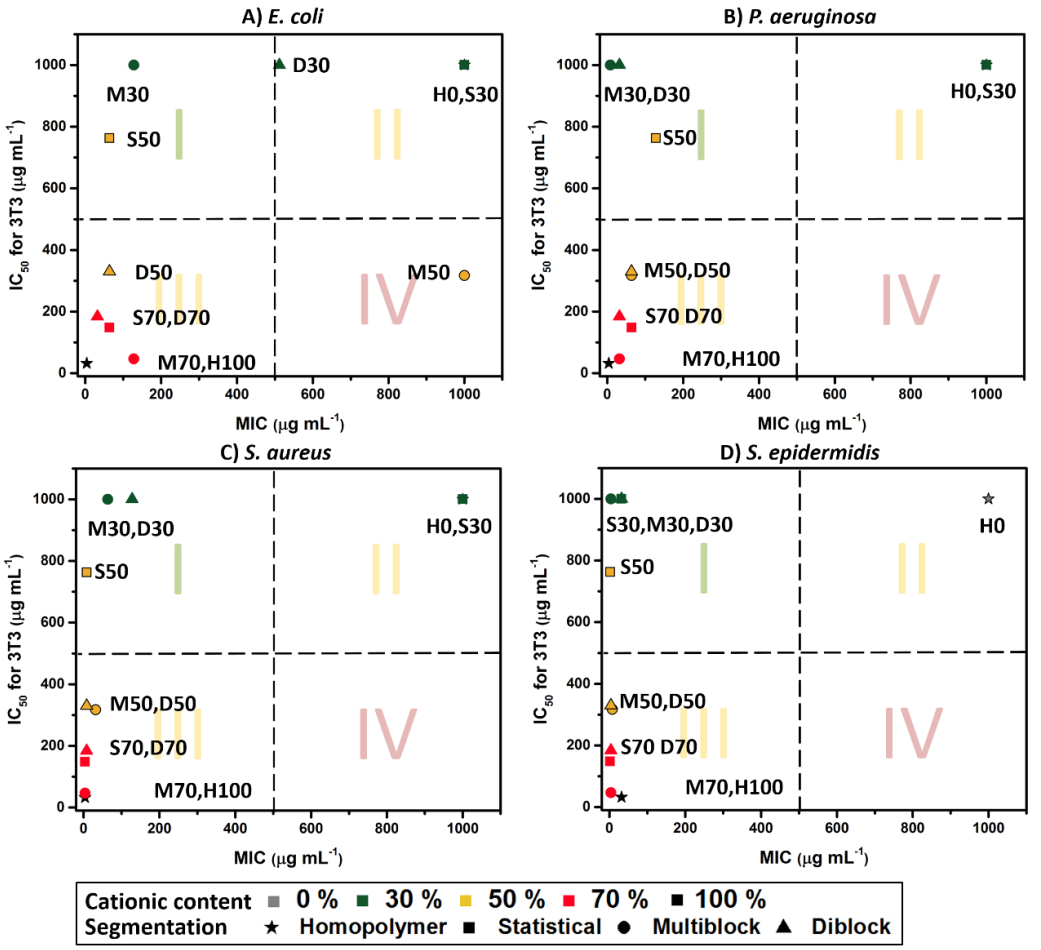
Supporting Figure SI-20. MIC at 30 (A), 50 (B) and 70 % (C) cationic content of various segmentation for each bacteria species.



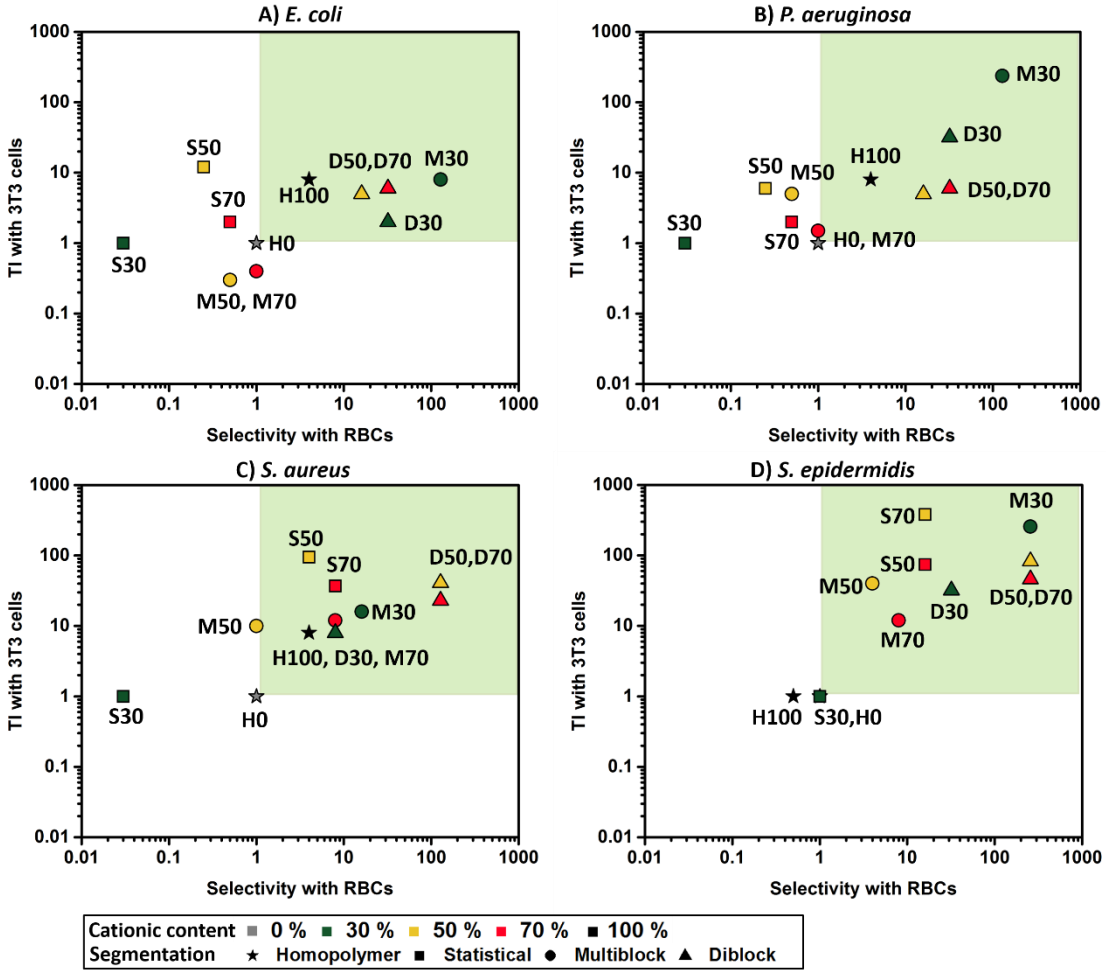
Supporting Figure SI-21. Normalized hemolysis data against 3T3 cells for 30 (1), 50 (2) and 70 % (3) cationic copolymers.



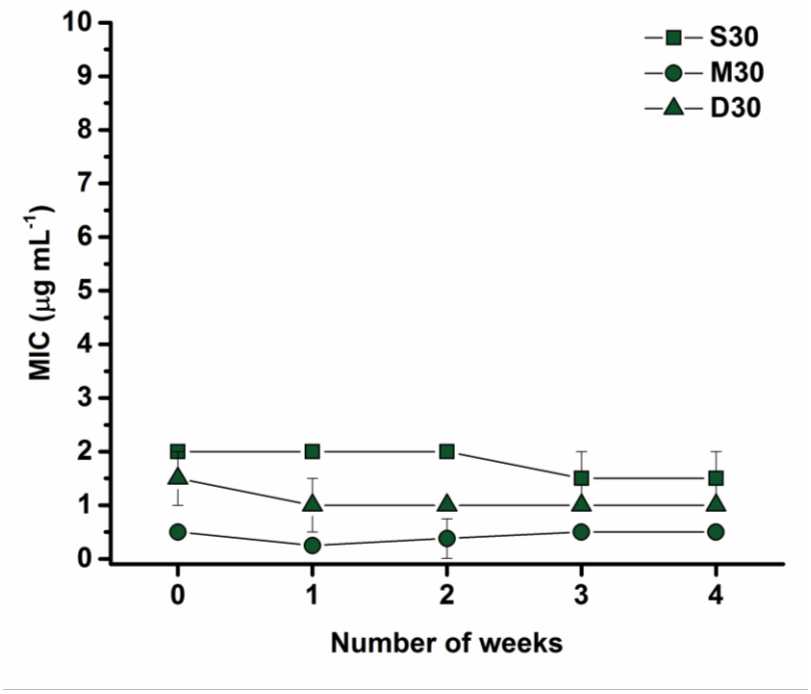
Supporting Figure SI-22. Toxicity data against 3T3 cells for statistical (1A), multiblock (1B) and diblock (1C) copolymers, Caco2 for statistical (2A), multiblock (2B) and diblock (2C) copolymers. A drug exposure period of 72 hours was allowed at 37 °C.



Supporting Figure SI-23. IC_{50} of the SAMPs with NIH 3T3 cells against their MIC for *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C) and *S. epidermidis* (D).



Supporting Figure SI-24. TI of the SAMPs with NIH 3T3 cells against their selectivity with RBCs for *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C) and *S. epidermidis* (D).



Supporting Figure SI-25. Bacterial resistance detection assay towards a MRSA strain USA300 with S30, M30 and D30.

Supporting Tables

Supporting Table SI-1. Synthesized Boc-protected polymers

BocAEAM content (%)	30	50	70
Statistical copolymers	$NIPAM_{70}$-*s*- $BocAEAM_{30}$	$NIPAM_{50}$-*s*- $BocAEAM_{50}$	$NIPAM_{30}$-*s*- $BocAEAM_{70}$
Diblock copolymers	$NIPAM_{70}$-*b*- $BocAEAM_{30}$	$NIPAM_{50}$-*b*- $BocAEAM_{50}$	$NIPAM_{30}$-*b*- $BocAEAM_{70}$
Multiblock copolymers	$NIPAM_{18}$-*b*- $BocAEAM_{10}$-*b*- $NIPAM_{18}$-*b*- $BocAEAM_{10}$-*b*- $NIPAM_{18}$-*b*- $BocAEAM_{10}$-*b*- $NIPAM_{18}$	$NIPAM_{10}$-*b*- $BocAEAM_{10}$-*b*- $NIPAM_{10}$-*b*-$BocAEAM_{10}$-*b*- $NIPAM_{10}$-*b*-$BocAEAM_{10}$-*b*- $NIPAM_{10}$-*b*- $BocAEAM_{10}$-*b*- $NIPAM_{10}$-*b*- $BocAEAM_{10}$	$BocAEAM_{18}$-*b*-$NIPAM_{10}$-*b*- $BocAEAM_{18}$-*b*-$NIPAM_{10}$-*b*- $BocAEAM_{18}$-*b*-$NIPAM_{10}$-*b*- $BocAEAM_{18}$

Supporting Table SI-2. Experimental conditions used for the synthesis of DP 100 homopolymer and statistical copolymers of NIPAM and BocAEAM.

Sample number		H0	$S30^{Boc}$	$S50^{Boc}$	$S70^{Boc}$	$H100^{Boc}$
BocAEAM content (%)		0	32	50	70	100
DP_{total}		104	105	105	105	98
NIPAM	$DP_{targeted}$	104	73	53	32	0
	$m_{monomer\ added}$ (mg)	226	317	226	136	0
BocAEAM	$DP_{targeted}$	0	32	52	73	98
	$m_{monomer\ added}$ (mg)	0	257	429	600	429
$m_{CTA\ added}$ (mg)		4.768	9.535	9.535	9.535	4.768
$V_{dioxane\ added}$ (mL)		0.533	0.98	0.98	0.98	0.533
$V_{water\ added}$ (mL)		0.132	0.266	0.266	0.266	0.132
V_{total} (mL)		1.834	1.246	1.246	1.246	0.665
$m_{VA\text{-}044\ total}$ (mg)		0.647	1.293	1.293	1.293	0.647
$[VA\text{-}044]_0$ (mol L^{-1})		$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$
$[monomer]_0$ (mol L^{-1})		3.00	3	3	3	3
$[CTA]_0/[VA\text{-}044]_0$		10	10	10	10	10
L (%)[a]		92	92	92	92	92

[a] Livingness of the polymers, as defined in Equation SI-4

Supporting Table SI-3. Experimental conditions used for the synthesis DP 100 diblock copolymers of NIPAM (NIP) and BocAEAM (BocA).

Sample number	$D30^{Boc}$		$D50^{Boc}$		$D70^{Boc}$	
BocA content (%)	30		49		70	
DP_{total}	103		90		99	
Cycles	1	2	1	2	1	2
Monomer	NIP	BocA	NIP	BocA	NIP	BocA
$DP_{targeted}$	72	31	46	44	29	70
$m_{monomer\ added}$ (mg)	339	275	226	429	226	1000
$m_{CTA\ added}$ (mg)	10.216	-	9.535	-	15.892	-
$m_{VA\text{-}044\ added}$ (mg)	0.970	0.595	0.862	0.718	1.035	1.839
$V_{dioxane\ added}$ (mL)	0.800	0.125	0.533	0.493	0.640	1.710
$V_{water\ added}$ (mL)	0.200	0.044	0.133	0.173	0.160	0.601
V_{total} (mL)	1.000	1.169	0.666	1.332	0.800	3.111
$m_{VA\text{-}044\ total}$ (mg)	0.970	0.756	0.862	0.862	1.035	2.012
$[VA\text{-}044]_0$ (mol L^{-1})	$3.00\ 10^{-3}$	$2.00\ 10^{-3}$	$4.00\ 10^{-3}$	$2.00\ 10^{-3}$	$4.00\ 10^{-3}$	$2.00\ 10^{-3}$
$[monomer]_0$ (mol L^{-1})	3.00	1.10	3.00	1.50	2.50	1.50
$[CTA]_0/[VA\text{-}044]_0$	14	18	15	15	21	11
L (%)[a]	94	95	95	94	96	92
Cumulative *L* (%)	94	90	95	89	96	88

[a] Livingness of the polymers, as defined in Equation SI-4

Supporting Table SI-4. Experimental conditions used for the synthesis of $M30^{Boc}$, the DP 100 heptablock copolymer of NIPAM (NIP) and BocAEAM (BocA) containing 30 % BocAEAM.

Cycles	1	2	3	4	5	6	7
Monomer	NIP	BocA	NIP	BocA	NIP	BocA	NIP
$DP_{targeted}$	18	10	18	10	18	10	18
$m_{monomer\ added}$ (mg)	226	238	226	238	226	238	226
$m_{CTA\ added}$ (mg)	26.49	-	-	-	-	-	-
$m_{VA\text{-}044\ added}$ (mg)	1.078	0.799	1.280	1.681	1.998	2.360	2.670
$V_{dioxane\ added}$ (mL)	0.533	0.261	0.460	0.405	0.351	0.350	0.281
$V_{water\ added}$ (mL)	0.132	0.082	0.129	0.173	0.136	0.157	0.180
V_{total} (mL)	0.665	1.008	1.597	2.175	2.662	3.169	3.630
$m_{VA\text{-}044\ total}$ (mg)	1.078	0.98	1.552	2.113	2.587	3.079	3.527
$[VA\text{-}044]_0$ (mol L^{-1})	$5.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$
$[monomer]_0$ (mol L^{-1})	3.00	1.10	1.25	0.51	0.75	0.35	0.55
$[CTA]_0/[VA\text{-}044]_0$	33	37	23	17	14	12	10
L (%)[a]	98	98	96	95	94	92	91
Cumulative *L* (%)	98	95	91	86	81	75	68

[a] Livingness of the polymers, as defined in Equation SI-4

Supporting Table SI-5. Experimental conditions used for the synthesis of $M50^{Boc}$, the DP 100 decablock copolymer of NIPAM (NIP) and BocAEAM (BocA) containing 50 % BocAEAM.

Cycles	1	2	3	4	5	6	7	8	9	10
Monomer	NIP	BocA	NIP	BocA	NIP	BocA	NIP	BocA	NIP	BocA
$DP_{targeted}$	10	10	10	10	10	10	10	10	10	10
$m_{monomer\ added}$ (mg)	113	214	113	214	113	214	113	214	113	214
$m_{CTA\ added}$ (mg)	23.84	-	-	-	-	-	-	-	-	-
$m_{VA\text{-}044\ added}$ (mg)	0.862	0.601	0.747	0.815	1.002	1.147	1.235	1.394	1.594	2.069
$V_{dioxane\ added}$ (mL)	0.247	0.349	0.267	0.222	0.169	0.180	0.121	0.160	0.212	0.492
$V_{water\ added}$ (mL)	0.086	0.087	0.075	0.096	0.069	0.077	0.084	0.094	0.110	0.139
V_{total} (mL)	0.333	0.769	1.111	1.429	1.667	1.924	2.129	2.383	2.705	3.336
$m_{VA\text{-}044\ total}$ (mg)	0.862	0.746	1.078	1.293	1.617	1.865	2.064	2.31	2.622	3.233
$[VA\text{-}044]_0$ (mol L^{-1})	$8.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$
$[monomer]_0$ (mol L^{-1})	3.00	1.30	0.90	0.70	0.60	0.52	0.47	0.42	0.37	0.30
$[CTA]_0/[VA\text{-}044]_0$	38	43	30	23	20	17	16	14	12	10
L (%)[a]	98	98	97	96	95	95	94	94	93	91
Cumulative *L* (%)	98	96	93	89	85	81	76	71	66	60

[a] Livingness of the polymers, as defined in Equation SI-4

Supporting Table SI-6. Experimental conditions used for the synthesis of $M70^{Boc}$, the DP 100 heptablock copolymer of NIPAM (NIP) and BocAEAM (BocA) containing 70 % BocAEAM.

Cycles	1	2	3	4	5	6	7
Monomer	BocA	NIP	BocA	NIP	BocA	NIP	BocA
$DP_{targeted}$	18	11	18	11	18	11	18
$m_{monomer\ added}$ (mg)	429	126	429	126	429	126	429
$m_{CTA\ added}$ (mg)	26.487	-	-	-	-	-	-
$m_{VA\text{-}044\ added}$ (mg)	1.078	1.132	1.552	1.804	2.160	2.486	2.823
$V_{dioxane\ added}$ (mL)	0.533	0.261	0.460	0.405	0.351	0.350	0.281
$V_{water\ added}$ (mL)	0.132	0.082	0.129	0.173	0.136	0.157	0.180
V_{total} (mL)	0.665	1.008	1.597	2.175	2.662	3.169	3.630
$m_{VA\text{-}044\ total}$ (mg)	1.078	1.4	1.94	2.343	2.812	3.266	3.731
$[VA\text{-}044]_0$ (mol L^{-1})	$5.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$	$3.00\ 10^{-3}$
$[monomer]_0$ (mol L^{-1})	2.00	0.77	1.00	0.46	0.69	0.33	0.52
$[CTA]_0/[VA\text{-}044]_0$	22	26	19	15	13	11	10
L (%)[a]	96	96	95	94	93	92	91
Cumulative *L* (%)	96	93	88	83	77	71	65

[a] Livingness of the polymers, as defined in Equation SI-4

Supporting Table SI-7. Characterisation data of Boc-protected polymers.

	Sample	BocAEAM content (%)	$M_{n,th}$ [a] (g mol^{-1})	$M_{n,SEC}$ [b] (g mol^{-1})	$Đ$ [b]
Homopolymer	H0	0	12000	14400	1.10
	H100^{Boc}	100	21200	21000	1.11
Statistical	S30^{Boc}	32	15400	17900	1.09
	S50^{Boc}	50	17400	18800	1.09
	S70^{Boc}	70	19600	21600	1.12
Multiblock	M30^{Boc}	30	13900	15800	1.29
	M50^{Boc}	50	16000	17000	1.38
	M70^{Boc}	70	19400	17800	1.31
Diblock	D30^{Boc}	30	15000	16000	1.10
	D50^{Boc}	49	14900	18000	1.17
	D70^{Boc}	71	18500	19000	1.20

[a] Theoretical molecular weight of the protected polymers calculated from Equation 2 (SI); [b] determined for the protected polymers by SEC/RI in DMF using PMMA as molecular weight standards.

Supporting Table SI-8. Characterisation data of deprotected polymers.

	Sample	Cationic content (%)	$M_{n,th}$[a] (g mol^{-1})	Retention ratio[b] (%)	Z-average[c] (nm)	PDI[c]
Homopolymer	H0	0	12000	100	580	0.016
	H100	100	15000	0	7	0.223
Statistical	S30	32	13300	40	6	0.536
	S50	50	14070	20	7	0.695
	S70	70	14850	10	7	0.654
Multiblock	M30	30	12900	73	6	0.326
	M50	50	13400	37	7	0.615
	M70	70	14800	23	6	0.393
Diblock	D30	30	13000	87	6	0.808
	D50	49	12100	67	5	0.311
	D70	71	14000	50	8	0.313

[a] Theoretical molecular weight of the protected polymers calculated from Equation 2 (SI); [b] From HPLC data measured in water/ACN in a C18 column (gradient 1 to 95 % ACN in 50 minutes). Retention ratio was calculated according to Equation 3 (shown in the Supplementary Information using the retention time); [c] Measured by DLS in Phosphate Buffer Saline (PBS) at 37˚C and 1 mg mL^{-1}

Supporting Table SI-9. Hemagglutination observation

sample /concentration (μg/mL)	1024	512	256	128	64	32	16	8	4	2
H0	-	-	-	-	-	-	-	-	-	-
H100	+++	+++	+++	+++	+++	++	+	-	-	-
S30	++	++	++	++	+	+	-	-	-	-
S50	+++	+++	+++	++	+	+	-	-	-	-
S70	+++	+++	+++	++	++	+	-	-	-	-
D30	-	-	-	-	-	-	-	-	-	-
D50	-	-	-	-	-	-	-	-	-	-
D70	-	-	-	-	-	-	-	-	-	-
M30	-	-	-	-	-	-	-	-	-	-
M50	+++	+++	++	++	++	+	-	-	-	-
M70	+++	+++	+++	+++	++	+	-	-	-	-

Hemagglutination strength: +++ strong; ++ moderate; + weak; - none.

Supporting Table SI-10. Hemocompatibility concentration across the SAMPs of various composition and structure determined by hemagglutination assay. All polymers showed no haemolytic activity within the tested concentration range (except H100, for which HC_{10} was still higher than c_H).

Sample	Hemocompatibility concentration (μg mL^{-1})
H0	> 1024
H100	16
S30	32
S50	32
S70	32
M30	> 1024
M50	32
M70	32
D30	> 1024
D50	> 1024
D70	> 1024

Supporting Table SI-11. Toxicity values of the SAMPs against 3T3 and Caco2 cells obtained using XTT assays.

Sample name	IC_{50} [a] (μg mL^{-1})		Therapeutic Index (TI) [b]							
			3T3				Caco2			
	3T3	Caco2	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*
H0	> 1024	> 1024	> 1	> 1	> 1	> 1	> 1	> 1	> 1	> 1
H100	< 32	< 32	< 8	< 8	< 8	< 1	< 8	< 8	< 8	< 1
S30	> 1024	> 1024	> 1	> 1	> 1	> 1	> 1	> 1	> 1	> 1
S50	763	90	12	6	95	381	1.4	0.7	11	45
S70	148	< 32	2	2	37	74	< 0.5	< 0.5	< 8	< 16
M30	> 1024	307	> 8	> 128	> 16	> 256	2	38	5	77
M50	317	61	0.3	5	10	40	0.1	1	2	7.7
M70	47	< 32	0.4	1.5	12	12	< 0.3	< 1	< 8	< 8
D30	> 1024	179	> 2	> 32	8	> 32	1.4	22	3	45
D50	330	76	5	5	41	83	1.2	1.2	10	19
D70	184	77	6	6	23	46	2	2	10	19

[a] IC_{50} was determined as the concentration at which 50 % inhibition occurred; [b] Therapeutic index (TI) was calculated as the ratio of the lowest IC_{50} (between 3T3 and Caco2) of the SAMP to the MIC of the bacteria species.

Supporting References

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