Enzyme-responsive polyion complex (PIC) nanoparticles for the targeted delivery of antimicrobial polymers

Ignacio Insua,[a] Evangelos Liamas,[b] Zhenyu Zhang,[b] Anna F. A. Peacock,[a] Anne Marie Krachler[c,d] and Francisco Fernandez-Trillo[a,d]*

[a] *School of Chemistry,* [b] *School of Chemical Engineering,* [c] *School of Biosciences and* [d] *Institute of Microbiology and Infection, University of Birmingham, Edgbaston, B15 2TT Birmingham, United Kingdom. E-mail: f.fernandez-trillo@bham.ac.uk*

1. Materials

Fmoc-protected-L-amino acids were purchased from Merck Millipore. Dimethylformamide (DMF), piperidine 20% v/v in DMF, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and linear poly(ethylene imine) 2.5 KDa average molecular weight (L-$PEI_{2.5}$) were purchased from Sigma-Aldrich®. H_2N-L-Glu(O^tBu)-2-chlorotrityl resin (0.58 mmol/g) was bought from AGTC Bio Products Ltd. 2-chlorotrityl chloride resin (1.49 mmol/g) was obtained from Iris Biotech Gmbh. Linear poly(ethylene imine) 25 KDa average molecular weight (L-PEI_{25}), branched poly(ethylene imine) 1.2 KDa average molecular weight (B-$PEI_{1.2}$), *N,N*-diisopropylethylamine (DIPEA), triisopropylsilane (TIPS), 1,2-ethanedithiol (EDT), trifluoroacetic acid (TFA), casein from bovine milk (technical grade) and succinic anhydride were bought from Alfa Aesar®. *N*,*N*,*N*',*N*'-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate

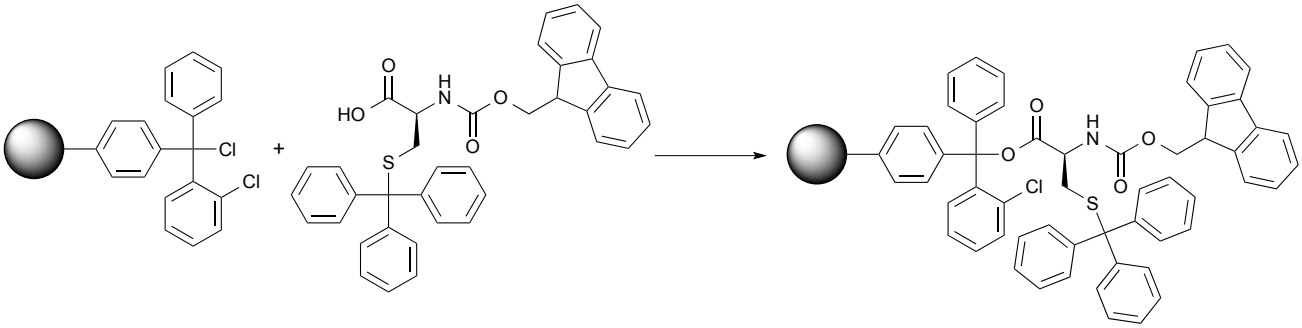
(HBTU) was purchased from Carbosynth Ltd. Spectra/Por® 6 dialysis membranes (molecular weight cut off 1 KDa) were purchased from Spectrum® Laboratories. Penta(ethylene imine) (L-PEI_{5n}) was purchased from Acros Organics™. All other chemicals were purchased from Fisher Scientific UK Ltd. and were used without further purification.

2. Instrumentation

NMR data was acquired on a Bruker Avance III operating at 400 MHz and fitted with a 5 mm DUL probe ($^{1}H/^{13}C$). MS spectra were obtained on a Xevo® G2-XS ToF (Waters) from electrospray ionisation (ESI) and time-of-flight (TOF) measurement in positive ion mode. High-resolution MS data was calculated by comparison with leucine-enkephalin as internal standard. Reverse phase (RP) HPLC analysis was run through a Kinetex® C18-EVO column (Phenomenex®): 5 µm, 100 Å, 250 x 4.60 mm. A gradient from 3 to 20% of (CH_3CN + 0.05% TFA) in (H_2O + 0.05% TFA) was used at 1 mL/min. The column was maintained at 35°C and UV-VIS detection was set at 210 nm. UV-VIS spectra were acquired on a Cary 5000 NIR (Agilent) in polystyrene cuvettes of 1 mm light paths. Dynamic light scattering (DLS) and ζ-potential measurements were carried out in a Zetasizer Nano ZS (Malvern Instruments Ltd) stabilised at 37°C. DLS was read at 173° (backscattering) for 60 seconds in triplicate and ζ-potentials were recorded 30 times at 140 V.

3. Synthesis and characterisation of peptides

3.1. Loading of Fmoc-L-Cys(Trt)-OH on 2-chlorotrityl chloride resin



A solution of Fmoc-L-Cys(Trt)-OH (1.10 g, 1.9 mmol) in 10 mL of DCM:anhydrous DMF (9:1) was purged with argon for 10 min. Then, the solution was added under inert conditions into a sealed flask with commercial preswollen 2-chlorotrityl chloride resin (1.00 g, 1.5 mmol), and DIPEA (1.3 mL, 7.5 mmol) was added to the mixture. The reaction was left under stirring at room temperature for 2 hours. After this time, the resin was filtered and washed with 10 mL of DCM:MeOH:DIPEA (17:2:1) 3 times. Finally, the resin was washed with 10 mL of DCM (x3) and 10 mL of Et_2O (x3) and dried under vacuum. Amino acid loading was calculated following the protocol described by Kay et al.,[1] giving a final value of 0.44 mmol/g of resin.

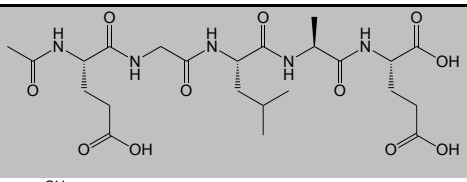
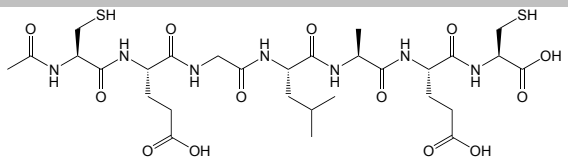
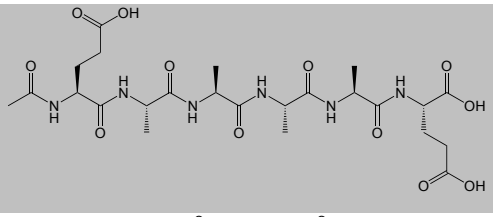
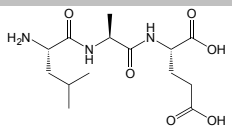
3.2. Solid-phase synthesis of peptides

For peptides without cysteine (**P1**, **P2** and **P3**), commercial H_2N-L-Glu(O^tBu)-2-chlorotrityl resin (250-400 mg, 0.14-0.23 mmol) was swollen in 4 mL of DMF for 30 minutes. Then, solutions of Fmoc-L-amino acid (3 eq), *N*,*N*,*N*',*N*'-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) (2.8 eq) and DIPEA (2.8 eq) in DMF were added to a final volume of 4 mL. The reaction mixture was shaken for 1 hour at room temperature, after which a negative chloranil test[2] indicated the reaction had gone to completion. *N*-terminal Fmoc protecting group was removed with 4 mL of piperidine in DMF 20% v/v during 10 minutes. A positive chloranil test confirmed the removal of the Fmoc group, and the previous steps were repeated to couple all amino acids in the sequence. Following coupling of the last amino acid, the Fmoc group was removed and, if necessary, the terminal amine in the peptide was capped with 4 mL of Ac_2O:DIPEA:DMF (1:1:3) for 1 hour at room temperature. Then, the resin was thoroughly

washed with Et_2O and the peptide was cleaved from the resin with 4 mL of aqueous TFA (95%) for 1 hour. The solution obtained was concentrated under argon and precipitated in chilled Et_2O. Finally, this suspension was centrifuged and the pellets were freeze-dried from acidic water.

For peptide **$P1_{SH}$** (containing cysteine), Fmoc-L-Cys(Trt)-OH loaded 2-chlorotrityl chloride resin (1.00 g, 0.44 mmol), prepared as indicated in 3.1., was deprotected twice with 10 mL of piperidine in DMF 20% v/v before coupling subsequent amino acids. The elongation and capping of the peptide was performed as indicated above for **P1**-**P3**, and the peptide was cleaved from the resin with 10 mL of TFA (80%), TIPS (8%) and EDT (12%) for 2 hours to protect thiols from side reactions. This solution was concentrated under argon and precipitated in chilled Et_2O:hexane (1:4). Finally, this suspension was centrifuged and the pellets were freeze-dried from acidic water. All peptides were isolated as white solids, regardless of the synthetic method. Purity was determined by HPLC.

Table S1 Sequences and structures of the peptides prepared in this work.

Peptide	Sequence	Structure
P1	Ac-EGLAE-OH	
$P1_{SH}$	Ac-CEGLAEC-OH	
P2	Ac-EAAAAE-OH	
P3	H_2N-LAE-OH	

3.3. Characterisation of peptides

P1, Ac-E-GLA-E-OH (83.8 mg, 96% yield) **^{1}H-NMR** (400 MHz, DMSO-d_6): δ 0.84(dd, *J*=15.1, 6.5 Hz, 6H, H^{δ}-Leu); 1.21(d, *J*=7.0 Hz, 3H, H^{β}-Ala); 1.36-1.49(m, 2H, H^{β}-Leu); 1.51-1.61(m, 1H, H^{γ}-Leu); 1.67-2.00(m, 4H, H^{β}-Glu); 1.85(s, 3H, Ac); 2.20-2.33(m, 4H, H^{γ}-Glu); 3.68(d, *J*=5.8 Hz, 2H, H^{α}-Gly); 4.15-4.34(m, 4H, H^{α}); 7.83(d, *J*=8.2 Hz, 1H, NHCO); 7.96(d, *J*=7.8 Hz, 1H, NHCO); 8.04(d, *J*=7.4 Hz, 1H, NHCO); 8.11(d, *J*=7.4 Hz, 1H, NHCO); 8.21(t, *J*=5.8 Hz, 1H, NHCO) ppm. **^{13}C-NMR** (400 MHz, DMSO-d_6): δ 17.9(C^{β}-Ala); 21.6(C^{δ}-Leu); 22.5(Ac); 23.2(C^{δ}-Leu); 24.1(C^{γ}-Leu); 26.4(C^{β}-Glu); 27.1(C^{β}-Glu); 30.0(C^{γ}-Glu); 30.2(C^{γ}-Glu); 40.8(C^{β}-Leu); 42.0(C^{α}-Gly); 48.0(C^{α}); 50.8(C^{α}); 51.1(C^{α}); 52.3(C^{α}); 168.6(NHCO); 169.8(Ac); 171.6(NHCO); 171.8(NHCO); 172.2(NHCO); 173.1(*C*-term); 173.8(C^{δ}-Glu); 174.0(C^{δ}-Glu) ppm. **MS** (ESI-TOF, +eV): m/z 614.2 $[M+Na+CH_3OH]^+$; 604.2 $[M-H+2Na]^+$; 582.2 $[M+Na]^+$. **HR-MS** (ESI-TOF, +eV): m/z 582.2387 (calculated for $[M+Na]^+$) 582.2385 (found). Purity by **HPLC** = 88%.

$P1_{SH}$, Ac-C-E-GLA-E-C-OH (214.3 mg, 64% yield). **^{1}H-NMR** (400 MHz, DMSO-d_6): δ 0.84(dd, *J*=14.8, 6.5 Hz, 6H, H^{δ}-Leu); 1.20(d, *J*=7.1 Hz, 3H, H^{β}-Ala); 1.43(t, *J*=7.0 Hz, 2H, H^{β}-Leu); 1.50-1.63(m, 1H, H^{γ}-Leu); 1.67-1.83(m, 2H, H^{β}-Glu); 1.84-1.98(m, 2H, H^{β}-Glu); 1.87(s, 3H, Ac); 2.18-2.29(m, 4H, H^{γ}-Glu); 2.34(t, *J*=8.5 Hz, 1H, SH); 2.44(t, *J*=8.5 Hz, 1H, SH); 2.62-2.90(m, 4H, H^{β}-Cys); 3.70(dd, *J*=11.2, 5.7 Hz, 2H, H^{α}-Gly); 4.19-4.41(m, 6H, H^{α}); 7.87-7.92(m, 2H, NHCO); 8.07-8.19(m, 5H, NHCO) ppm. **^{13}C-NMR** (400 MHz, DMSO-d_6): δ 17.7(C^{β}-Ala); 21.6(H^{δ}-Leu); 22.6(Ac); 23.1(H^{δ}-Leu);

24.1(H^{γ}-Leu); 25.4(C^{β}-Cys); 26.0(C^{β}-Cys); 27.1(C^{β}-Glu); 27.5(C^{β}-Glu); 29.9(C^{γ}-Glu); 30.0(C^{γ}-Glu); 41.0(C^{β}-Leu); 41.9(C^{α}-Gly); 48.2(C^{α}); 50.8(C^{α}); 51.6(C^{α}); 52.2(C^{α}); 54.3(C^{α}); 55.1(C^{α}); 168.4(NHCO); 169.7(Ac); 170.2(NHCO); 171.1(NHCO); 171.3(NHCO); 171.4(NHCO); 171.8(NHCO); 172.0(*C*-term); 174.0(C^{δ}-Glu) ppm. **MS** (ESI-TOF, +eV): m/z 810.2 $[M-H+2Na]^+$, 788.3 $[M+Na]^+$. **HR-MS** (ESI-TOF, +eV): m/z 788.2571 (calculated for $[M+Na]^+$) 788.2568 (found). Purity by **HPLC** = 92%.

P2, Ac-E-AAAA-E-OH (118.8 mg, 97% yield) **^{1}H-NMR** (400 MHz, DMSO-d_6): δ 1.18-1.21(m, 12H, H^{β}-Ala); 1.64-2.00(m, 4H, H^{β}-Glu); 1.84(s, 3H, Ac); 2.17-2.33(m, 4H, H^{γ}-Glu); 4.16-4.30(m, 6H, Hα); 7.91(d, J=7.4 Hz, 2H, NHCO); 7.99(d, *J*=7.3 Hz, 1H, NHCO); 8.03(d, *J*=8.1 Hz, 1H, NHCO); 8.05(d, *J*=7.7 Hz, 2H, NHCO) ppm. **^{13}C-NMR** (400 MHz, DMSO-d_6): δ 17.9(C^{β}-Ala); 18.1(C^{β}-Ala); 18.1(C^{β}-Ala); 22.5(Ac); 26.4(C^{β}-Glu); 27.4(C^{β}-Glu); 30.0(C^{γ}-Glu); 30.2(C^{γ}-Glu); 47.8(C^{α}); 48.0(C^{α}); 48.1(C^{α}); 48.1(C^{α}); 51.1(C^{α}); 51.8(C^{α}); 169.4(Ac); 171.1(NHCO); 171.6(NHCO); 171.8(NHCO); 171.9(NHCO); 172.2(*C*-term); 173.1(NHCO); 173.7(C^{δ}-Glu); 174.0(C^{δ}-Glu) ppm. **MS** (ESI-TOF, +eV): m/z 647.2 $[M-H+2Na]^+$; 625.3 $[M+Na]^+$. **HR-MS** (ESI-TOF, +eV): m/z 625.2445 (calculated for $[M+Na]^+$) 625.2444 (found). Purity by **HPLC** = 98%.

P3, H_2N-LA-E-OH (50.4 mg, 66% yield) **^{1}H-NMR** (400 MHz, DMSO-d_6): δ 0.88(dd, *J*=8.8, 6.4 Hz, 6H, H^{δ}-Leu); 1.24(d, *J*=7.0 Hz, 3H, H^{β}-Ala); 1.45-1.59(m, 2H, H^{β}-Leu); 1.61-1.69(m, 1H, H^{γ}-Leu); 1.71-1.81(m, 1H, H^{β}-Glu); 1.91-2.00(m, 1H, H^{β}-Glu); 2.19-2.32(m, 2H, H^{γ}-Glu); 3.77(t, *J*=6.6 Hz, 1H, H^{α}-Leu); 4.16-4.21(m, 1H, H^{α}-Glu); 4.38(p, *J*=7.1 Hz, 1H, H^{α}-Ala); 8.21(d, *J*=7.8 Hz, 1H, NHCO); 8.69(d, *J*=7.4 Hz, 1H, NHCO) ppm. **^{13}C-NMR** (400 MHz, DMSO-d_6): δ 18.1(C^{β}-Ala); 22.0(H^{δ}-Leu); 22.7(H^{δ}-Leu); 23.6(C^{γ}-Leu); 26.5(C^{β}-Glu); 30.1(C^{γ}-Glu); 40.3(C^{β}-Leu); 48.2(C^{α}-Ala); 50.8(C^{α}-Leu); 51.3(C^{α}-Glu); 168.3(NHCO); 171.7(NHCO); 173.1(*C*-term); 173.9(C^{δ}-Glu) ppm. **MS** (ESI-TOF, +eV): m/z 332.2 $[M+H]^+$. **HR-MS** (ESI-TOF, +eV): m/z 332.1822 (calculated for $[M+H]^+$) 332.1824 (found). Purity by **HPLC** = 99%.

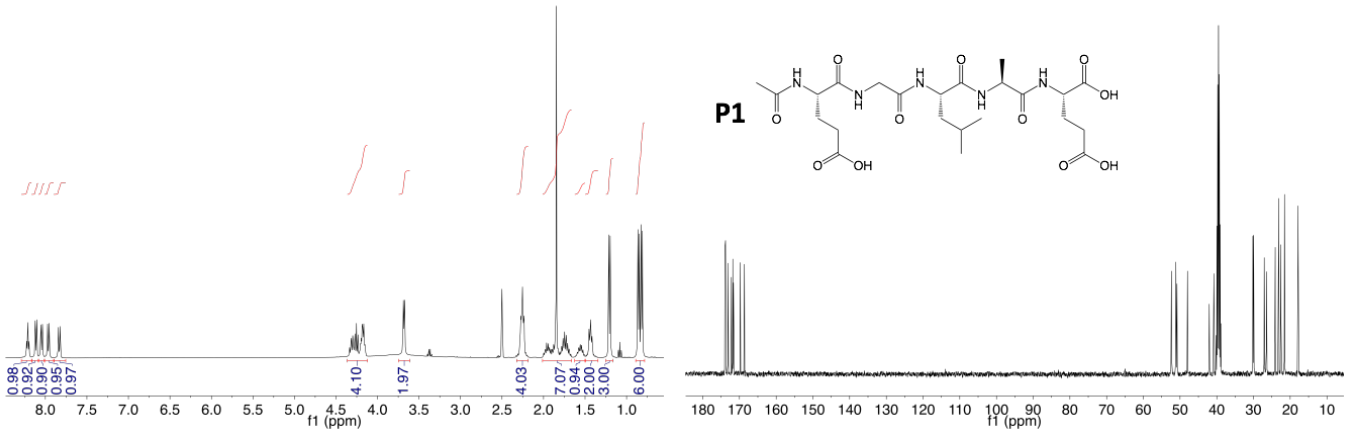


Fig. S1 ^{1}H (left) and ^{13}C (right) NMR (400 MHz, DMSO-*d6*) spectra of **P1** (Ac-E-GLA-E-OH).

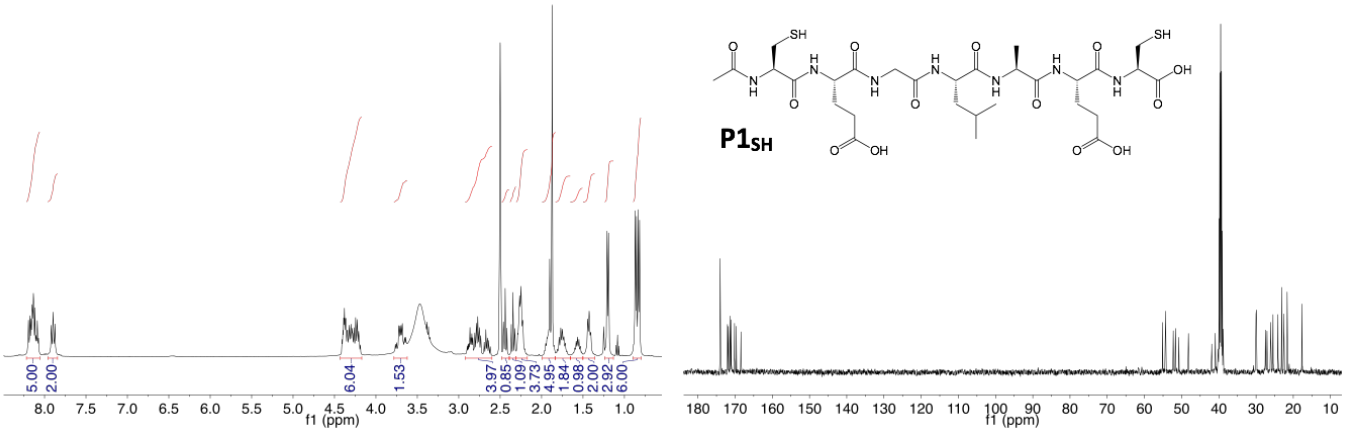


Fig. S2 ^{1}H (left) and ^{13}C (right) NMR (400 MHz, DMSO-*d6*) spectra of **P1$_{SH}$** (Ac-C-E-GLA-E-C-OH).

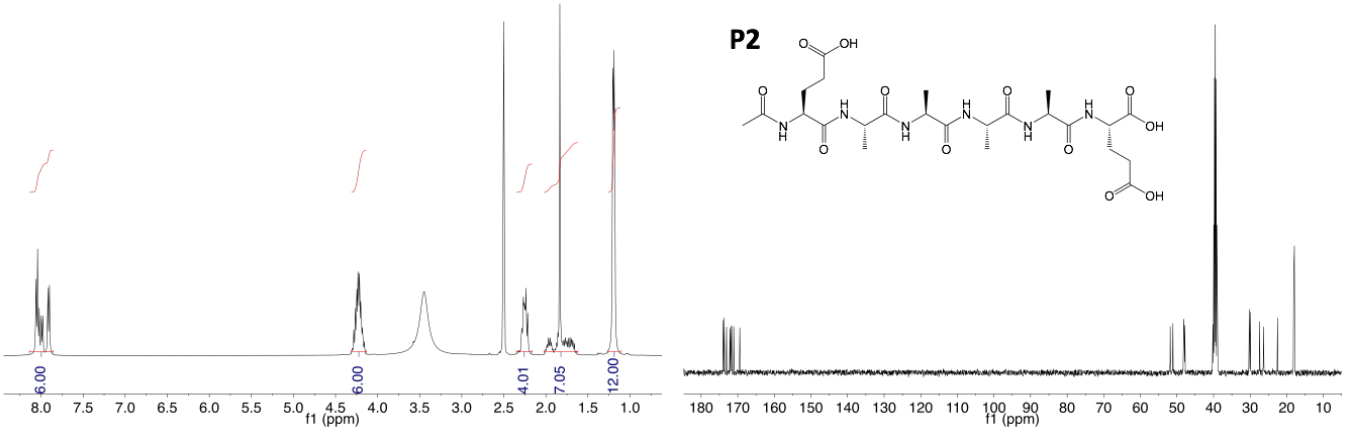


Fig. S3 ^{1}H (left) and ^{13}C (right) NMR (400 MHz, DMSO-*d6*) spectra of **P2** (Ac-E-AAAA-E-OH).

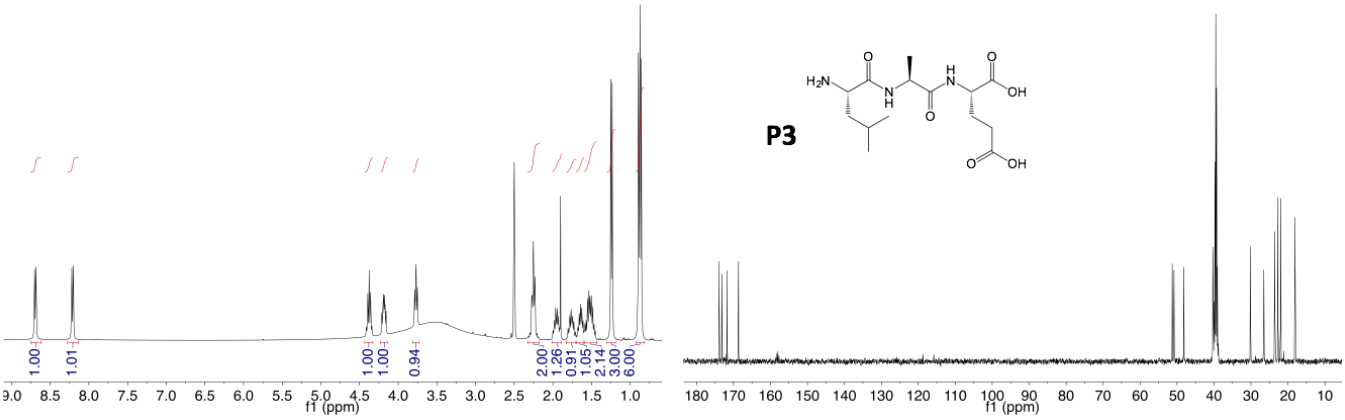


Fig. S4 ^{1}H (left) and ^{13}C (right) NMR (400 MHz, DMSO-*d6*) spectra of **P3** (H_2N-LA-E-OH).

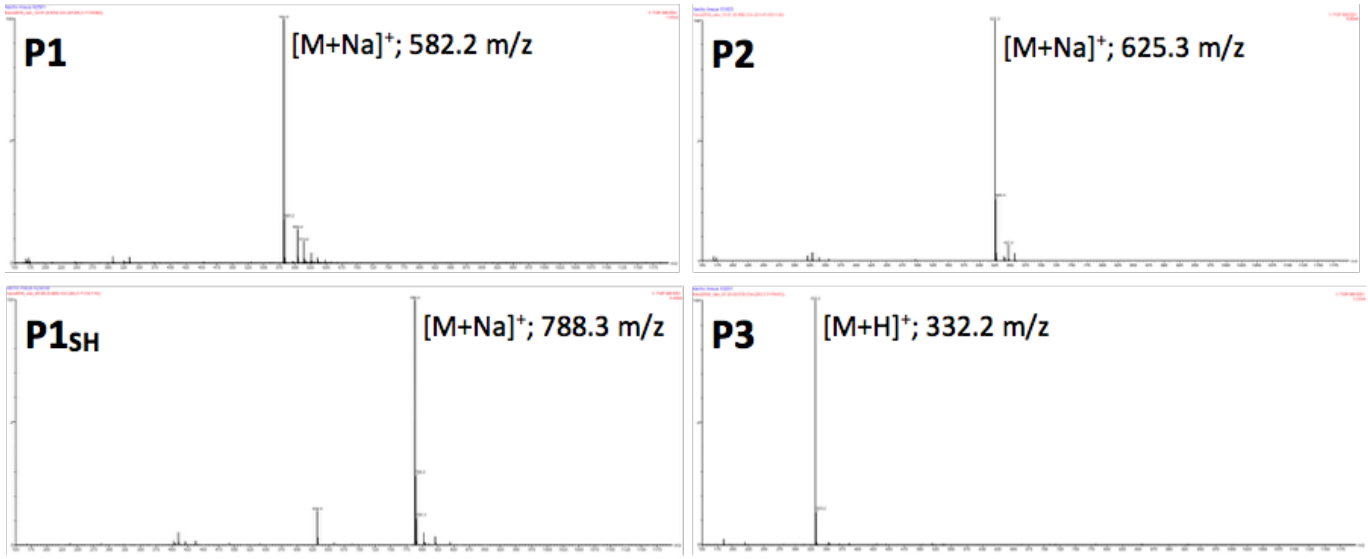


Fig. S5 ESI-TOF mass spectra of **P1**(Ac-E-GLA-E-OH), **P1$_{SH}$**(Ac-C-E-GLA-E-C-OH), **P2**(Ac-E-AAAA-E-OH) and **P3**(H_2N-LA-E-OH).

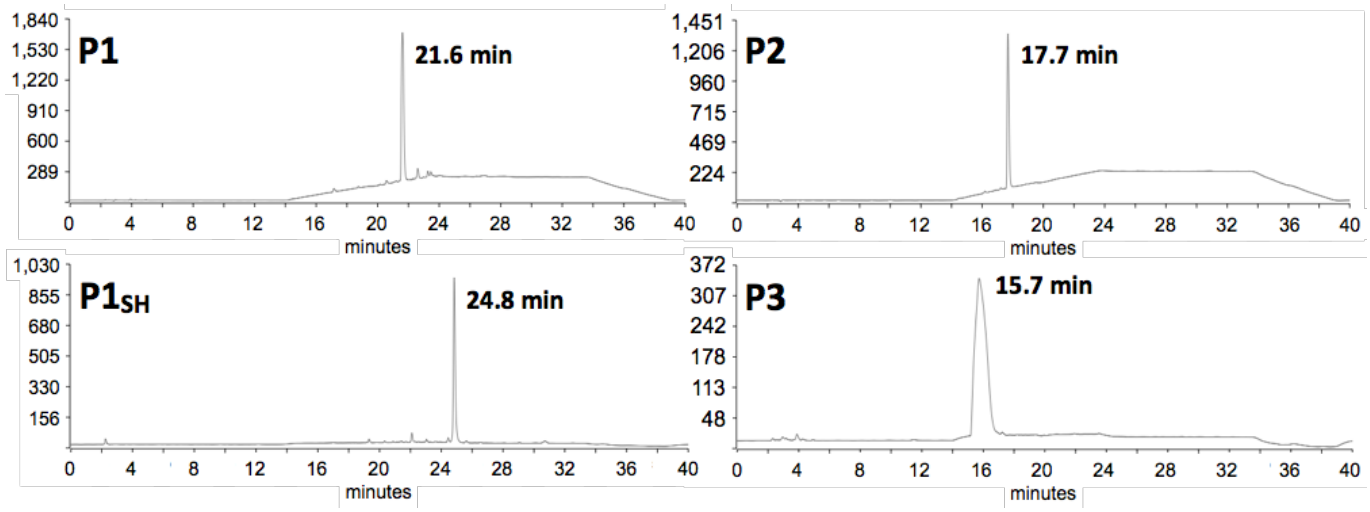


Fig. S6 RP-HPLC chromatograms of **P1**(Ac-E-GLA-E-OH), **P1$_{SH}$**(Ac-C-E-GLA-E-C-OH), **P2**(Ac-E-AAAA-E-OH) and **P3**(H_2N-LA-E-OH).

4. Enzymatic degradation of peptides

4.1. Synthesis of succinyl casein (SC)

A modified procedure from the literature was used for the preparation of SC:[3] commercial casein (500 mg, 0.02 mmol) was heated at 60°C with succinic anhydride (400 mg, 4.00 mmol) in 100 mL of $NaHCO_3$ buffer at pH 8.0. The pH of the reaction was maintained at 8.0 by addition of NaOH 1M. After 1 hour, the reaction mixture was left to reach room temperature and dialysed against deionised water for 2 days. Then, the suspension was freeze-dried to give a white solid (481.8 mg).

SC was used as a control to monitor enzymatic activity because SC is degraded by both proteases. For each experiment, degradation of SC was monitored to ensure that enzymatic activity for LasB and HLE was not lost upon storage. In general, we observed a higher degradation of SC in the presence of HLE (**Fig. S7**). This increase in enzymatic degradation by HLE can be due to a higher content of HLE cleavable sites in SC. Thus, we prepared a model degradation peptide (H_2N-LA-E-OH, **P3**) that corresponds to the product obtained upon enzymatic hydrolysis of **P1**.[4] This peptide has a terminal amine and is not cleaved by any of the elastases. Thus, fluorescence intensity can be normalised to that observed in the presence of this peptide, which corresponds to 100% degradation.

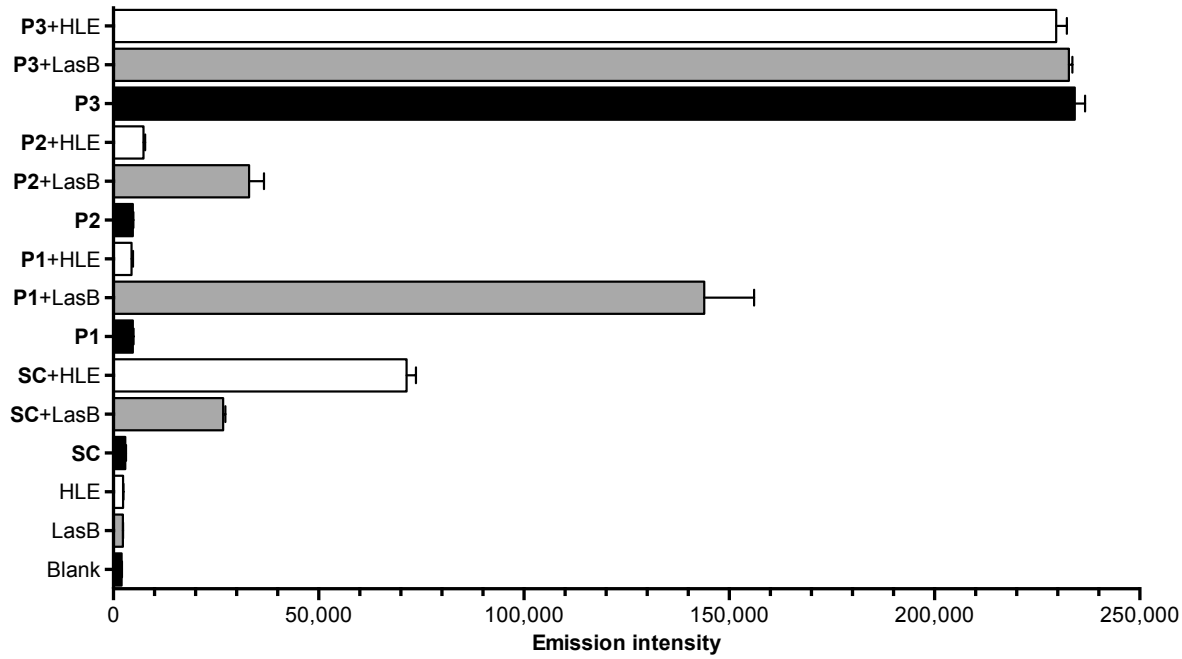


Fig. S7 Emission intensity (λ_{exc} 355 nm, λ_{em} 460 nm) of fluorescamine conjugates for $Na_2B_4O_7$ buffer (blank), succinyl casein (SC) as a control for enzymatic activity, LasB responsive anionic peptides (Ac-E-GLA-E-OH, **P1**; Ac-E-AAAA-E-OH, **P2**) and degradation peptide (H_2N-LA-E-OH, **P3**) as a control to normalise fluorescence intensity. All substrates were evaluated in the absence and presence of LasB or HLE. Incubation time: 4 hours. n=3.

5. Antimicrobial activity of commercial PEIs

1 mL aliquots of a *P. aeruginosa* PAO1V culture in LB broth (OD_{600} = 1.0) were centrifuged and resuspended in the same volume of a PEI solution prepared in DMEM. 5 different PEIs were tested: 3 linear (25 KDa, 2.5 KDa and pentamer) and 2 branched (25 and 1.2 KDa) at 3 different concentrations. Bacteria were also resuspended in pure DMEM and 70% v/v aqueous 2-propanol as live and dead controls, respectively. All samples were incubated at room temperature for twenty hours. After this time, 300 µL of each sample were reacted in the dark with 1 µL of a 1:1 mixture of BacLight™ probes for 10 minutes. Then, samples were analysed by FACS setting gates in green *versus* red emission dot plots from the live and dead controls. For some of the higher concentrations, aggregation, biofilms or bacterial debris were observed in the culture well before FACS analysis.

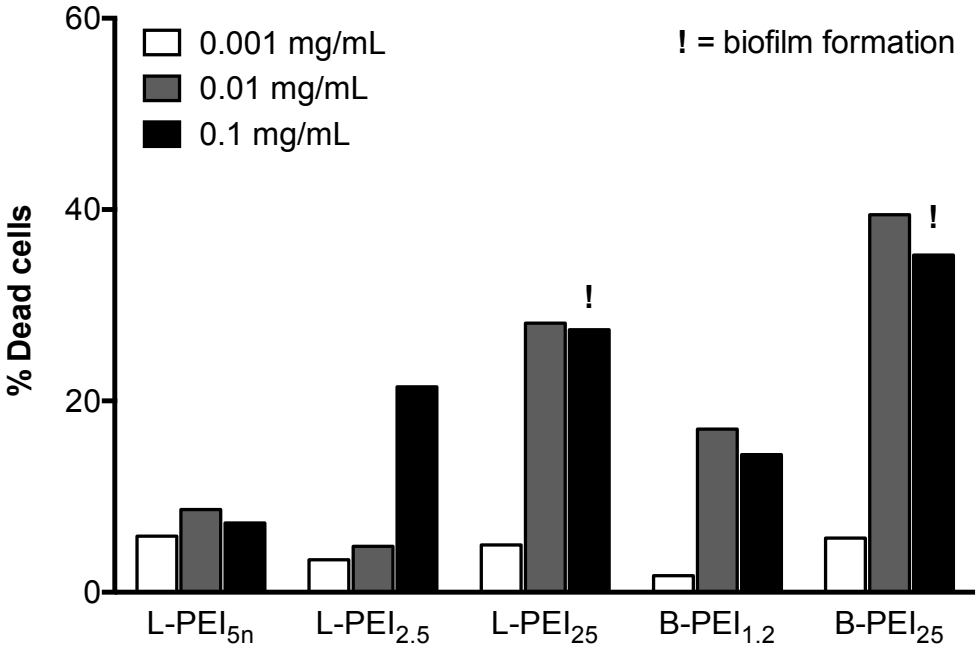


Fig. S8 Normalised population of *P. aeruginosa* PAO1V presented as the percentage of red (dead) cells. n=1.

6. Characterisation of PIC nanoparticles

Table S2 Hydrodynamic diameter (D_H) and ζ-potential of PIC nanoparticles prepared at different N:COOH ratios. SD indicates the dispersion in D_H or ζ of the only size or charge population fitted by the software.

N:COOH RATIO	D_H ± SD (nm)	ζ ± SD (mV)	Notes
1:2.0	-	-	No PIC particles formed
1:1.5	-	-	No PIC particles formed
1:1.0	-	-	No PIC particles formed
1:0.8	582 ± 79	6.7 ± 4.7	-
1:0.7	487 ± 67	9.8 ± 3.2	-
1:0.6	431 ± 59	11.3 ± 4.5	-
1:0.5	379 ± 68	13.4 ± 4.7	-
1:0.4	230 ± 56	17.2 ± 7.1	-
1:0.3	111 ± 35	18.9 ± 6.4	-
1:0.2	-	-	No PIC particles formed

6.1. Cross-linking kinetics of PIC nanoparticles

Ellman's assay was used to quantify by UV-VIS spectroscopy the amount of accessible sulfhydryl groups present in the sample. PIC nanoparticles were prepared at a 1:0.3 N:COOH ratio as described in the main text. 300 µL of sample were taken at different time points and mixed with 300 µL of 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) solution in degassed 100 mM phosphate buffer at pH 7.27 containing with 1 mM EDTA. Absorbance was measured at 412 nm. Thiol concentration in a solution of $\mathbf{P1_{SH}}$ at the same concentration was used as a control.

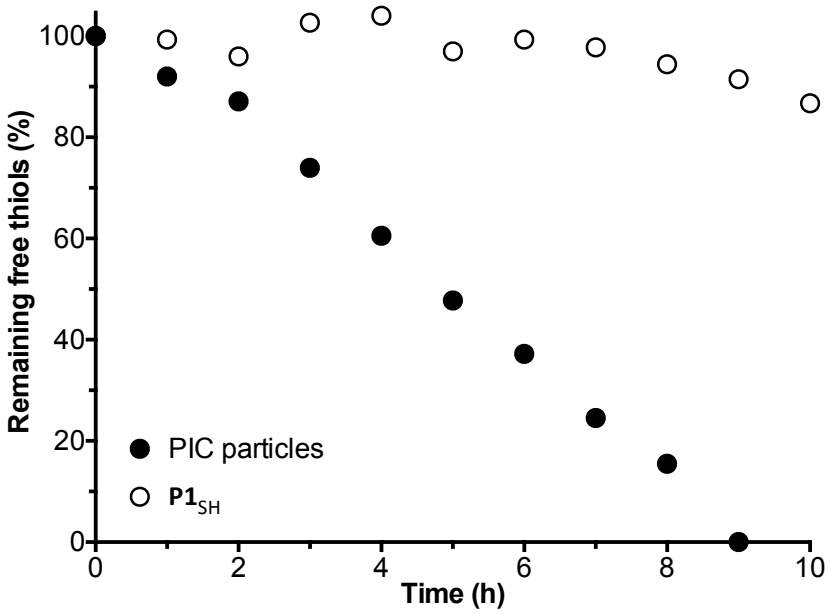


Fig. S9 Amount of accessible thiols, as monitored using Ellman's assay, following incubation of $\mathbf{P1_{SH}}$ (○) or PIC nanoparticles (●) prepared at a 1:0.3 N:COOH ratio in phosphate/EDTA buffer (pH 7.27) under stirring and open to air. n=1.

7. Physiological stability of PIC nanoparticles

1 mL of a PIC nanoparticles (**Table S2**), prepared as described in the main text, was diluted with 182 µL of a 1 M NaCl solution in water to give a final NaCl concentration of 154 mM (physiological osmolarity).[5] All samples were incubated at 37°C and analysed by DLS over time for up to 4 hours. Autocorrelation function (ACF), intensity distributions and detection counts for these samples were compared to that of a control in the absence of NaCl (**Fig. S10**-**Fig. S12**).

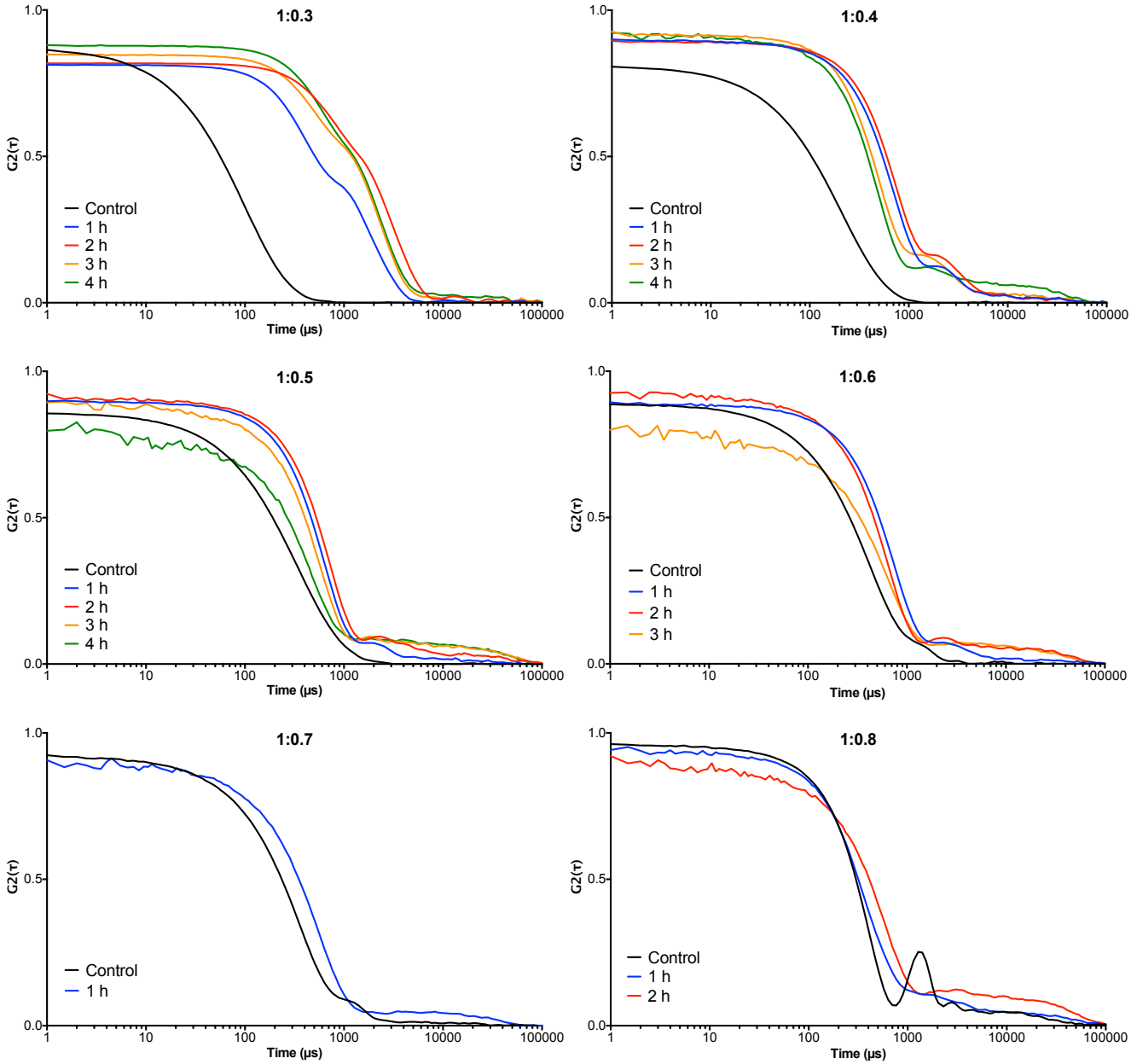


Fig. S10 Representative autocorrelation function[†] (ACF) curves for **P1$_{SH}$** PIC nanoparticles prepared using six different N:COOH ratios (**Table S2**) in the absence (control) and presence of 154 mM NaCl at 37°C over time (1-4 hours).

[†] Because of the dispersion in DLS measurements observed for unstable particles (e.g. 1:0.6 N:COOH ratio at 3 hours) only representative plots are shown. 3 technical replicates were recorded for each sample.

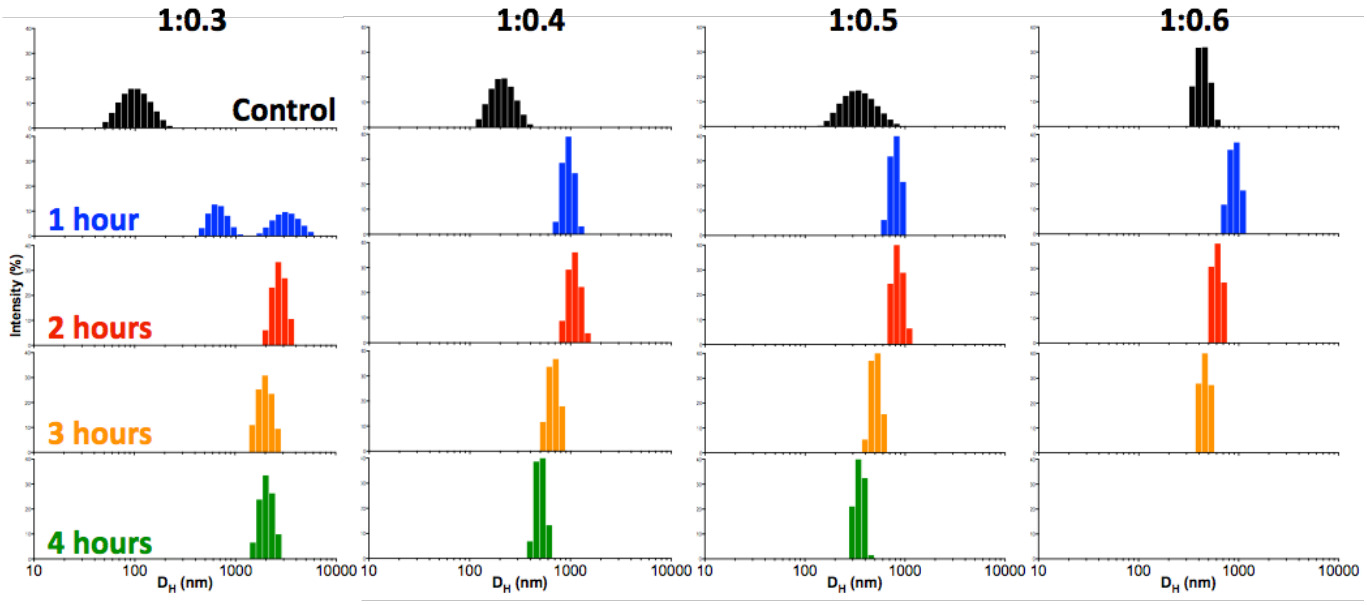


Fig. S11 Representative intensity distributions[†] of sizes for the four most stable **P1$_{SH}$** PIC nanoparticles analysed (**Table S2** and **Fig. S10**) in the absence (control) and presence of 154 mM NaCl at 37°C over time (1-4 hours).

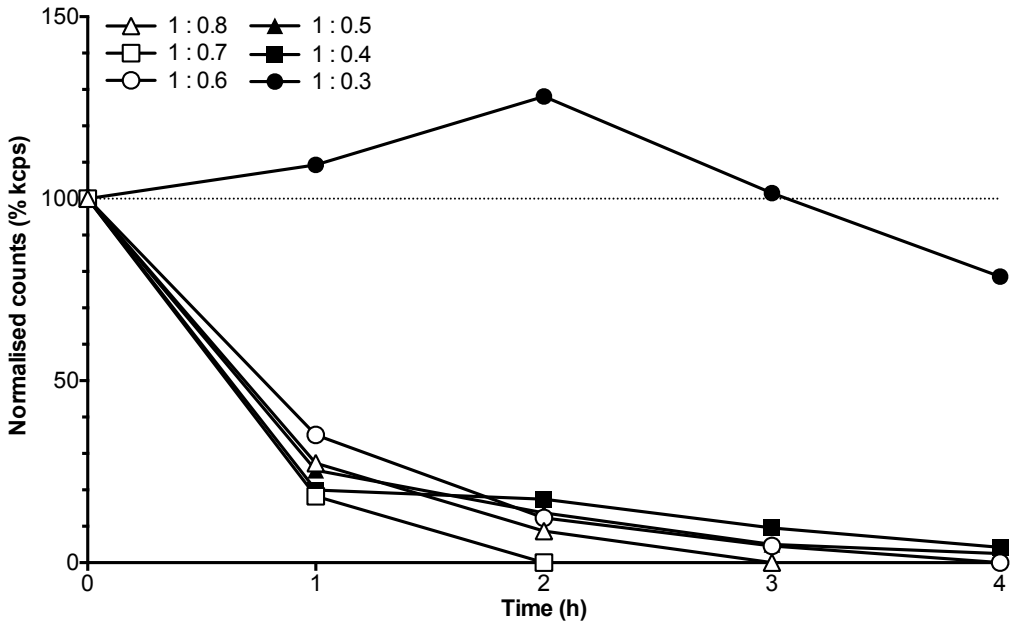


Fig. S12 Normalised detection counts for **P1$_{SH}$** PIC nanoparticles prepared using six different N:COOH ratios (**Table S2**) in the absence (0 h) and presence of 154 mM NaCl at 37°C over time (1-4 hours). Data was normalised to the size of the particles in the absence of NaCl (100%). Values plotted are the average of 3 technical replicates.

8. Stability in the presence of divalent cations – Treatment with CaCl2

To 500 µL of PIC particles (1:0.6 in N:COOH), prepared as described in the main text, a 1 M solution of $CaCl_2$ in water was added to give a final concentration of 10, 50 and 100 mM in Ca^{2+}. The samples were analysed by DLS immediately after addition of $CaCl_2$.

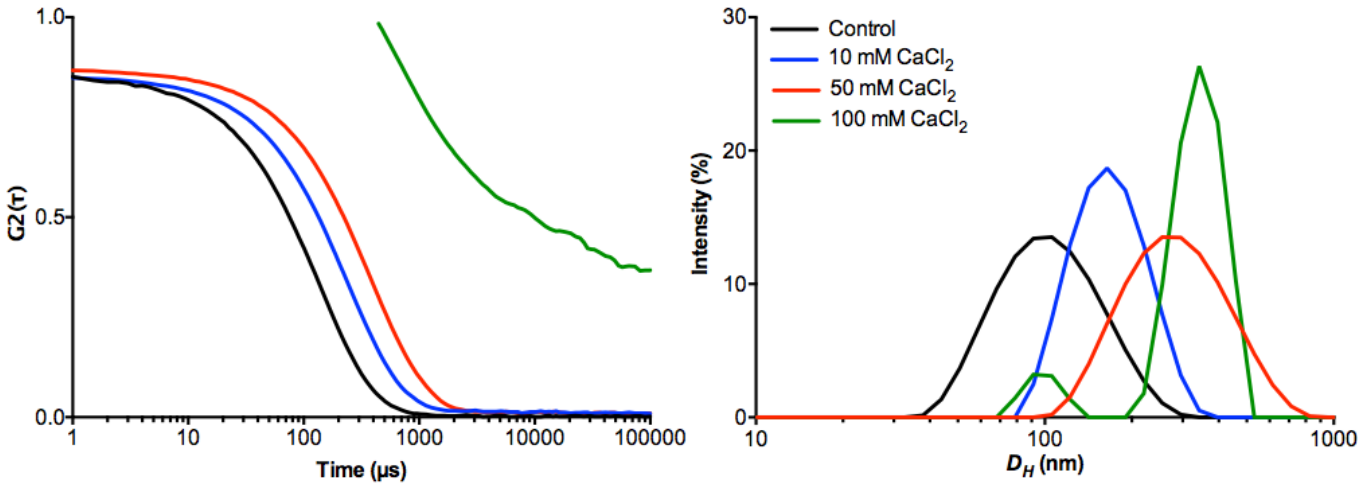


Fig. S13 Representative DLS autocorrelation function (ACF) curves (left) and intensity distributions of sizes (right) of PIC particles (1:0.6 in N:COOH) in the absence (control) and presence of increasing concentrations of $CaCl_2$.

9. Enzymatic degradation of PIC nanoparticles

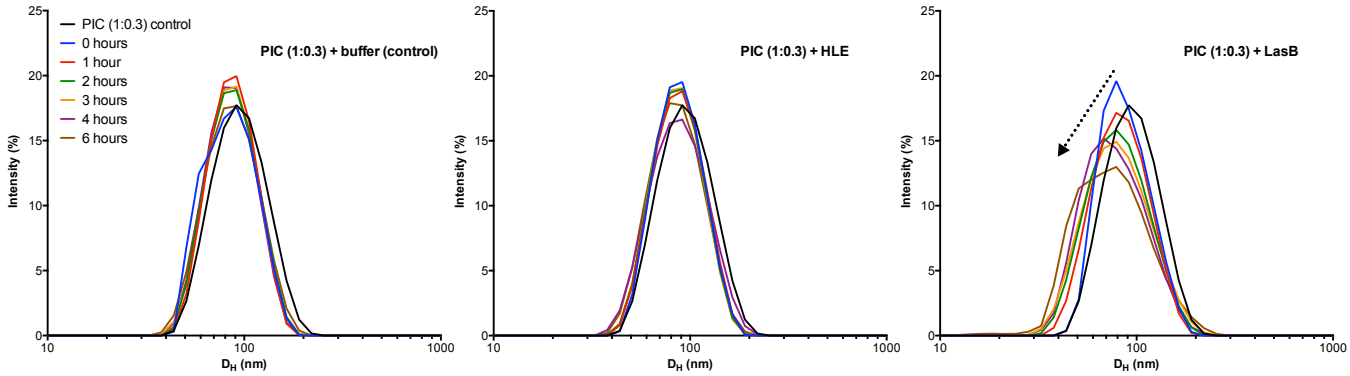


Fig. S14 Representative intensity distributions of sizes for $\mathbf{P1_{SH}}$ PIC nanoparticles prepared at a 1:0.3 N:COOH ratio incubated with $Na_2B_4O_7$ buffer (left), HLE (middle) and LasB (right) over time. Distribution for LasB broadens upon increasing incubation time (arrow). Values plotted are the average of 3 sample replicates, each measured 3 times. n=9.

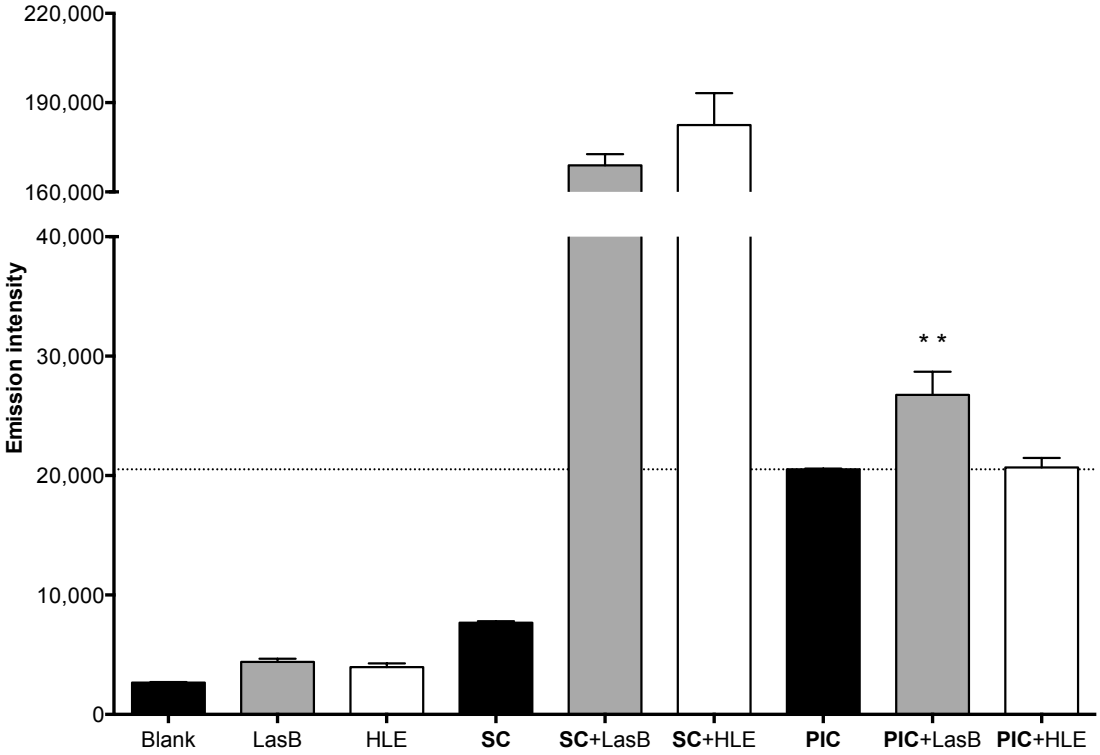


Fig. S15 Emission intensity (λ_{exc} 355 nm, λ_{em} 460 nm) of fluorescamine conjugates for enzymes, succinyl casein (SC) as a control for enzymatic activity and **P1$_{SH}$** PIC nanoparticles prepared at a 1:0.3 N:COOH ratio. All substrates were evaluated in the absence and presence of LasB or HLE and incubated for 4 hours. ** $p < 0.01$ between PIC and PIC + LasB. n=3.

10. Antimicrobial activity of PIC nanoparticles

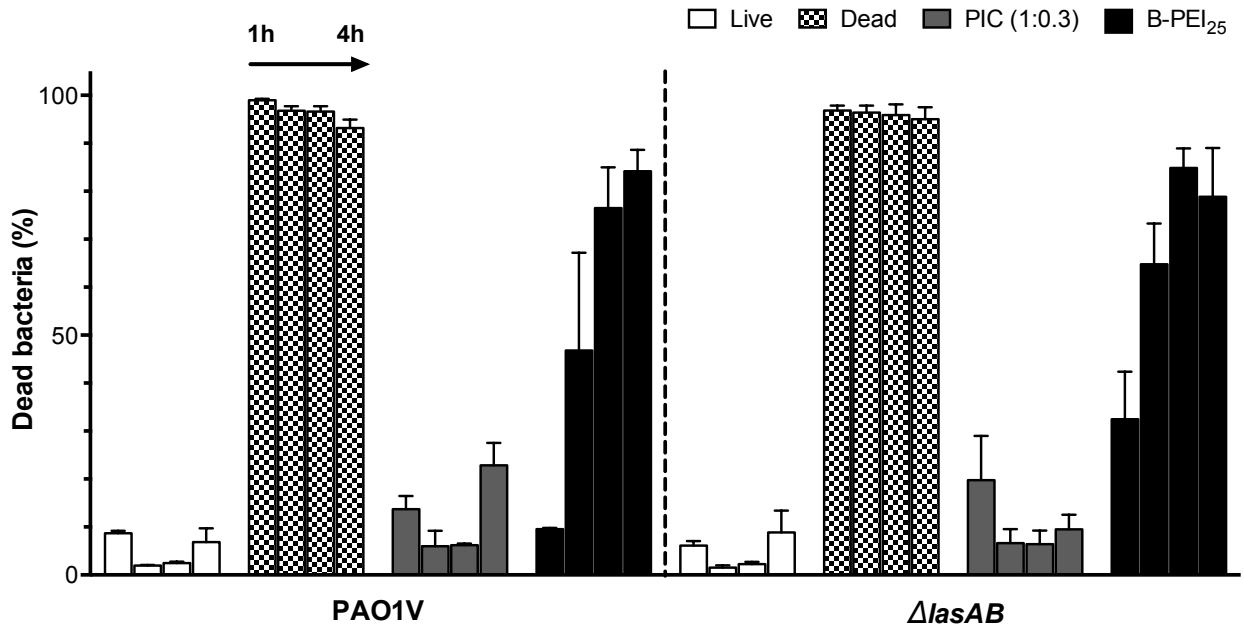


Fig. S16 Raw FACS data for *P. aeruginosa* wild type (PAO1V) and *ΔlasAB* mutant in the absence (live control, white bars) and presence of **P1$_{SH}$** PIC nanoparticles (1:0.3 N:COOH ratio, grey bars), B-PEI$_{25}$ (black bars) and 70% v/v aqueous 2-propanol (dead control, dotted bars). Population presented as the percentage of red (dead) cells. In each group of four, each bar represents a time point (1, 2, 3 and 4 hours from left to right). Toxicity of B-PEI$_{25}$ after one hour was not statistically different from the live control and thus that time point has not been included in **Fig. 5**. n=3.

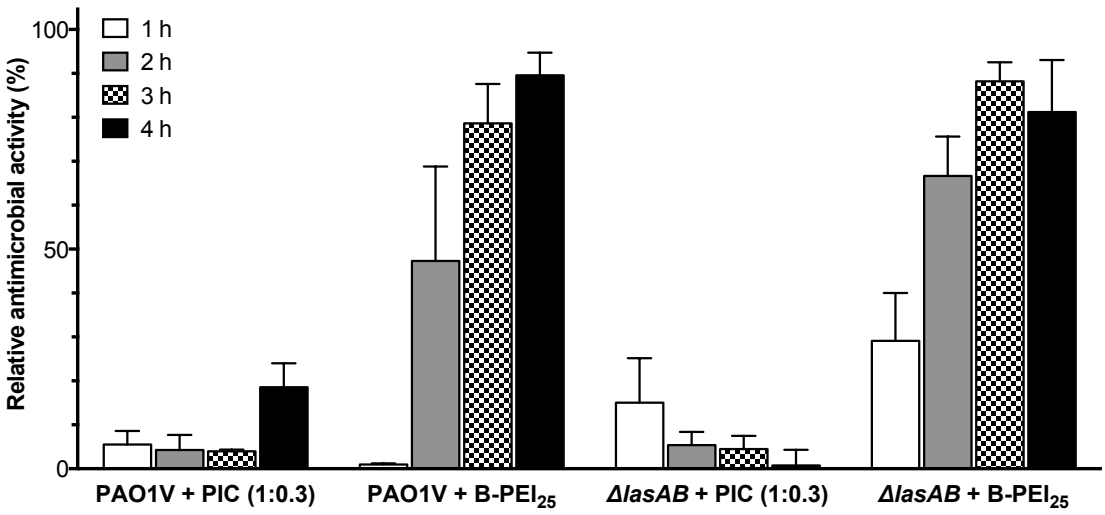


Fig. S17 Relative antimicrobial activity for *P. aeruginosa* wild type (PAO1V) and *ΔlasAB* in the presence of $\mathbf{P1_{SH}}$ PIC nanoparticles (1:0.3 N:COOH ratio, grey bars) and B-PEI_{25} (black bars). Relative antimicrobial activity was calculated normalising data from **Fig. S16** to live control (0% of antimicrobial activity) and dead control (100% of antimicrobial activity) at each time point. n=3.

11. Supplementary references

[1] C. Kay, O. E. Lorthioir, N. J. Parr, M. Congreve, S. C. McKeown, J. J. Scicinski and S. V. Ley, *Biotechnol. Bioeng.*, 2001, **71**, 110.
[2] T. Vojkovsky, *Pept. Res.*, 1995, **8**, 236.
[3] T. Hatakeyama, H. Kohzaki and N. Yamasaki, *Anal. Biochem.*, 1992, **204**, 181.
[4] K. Morihara and H. Tsuzuki, *Arch. Biochem. Biophys.*, 1971, **146**, 291–296.
[5] E. Kessler and D. E. Ohman, in *Handbook of Proteolytic Enzymes - (Third Edition) - ScienceDirect*, eds. N. D. Rawlings and G. Salvesen, Academic Press, London, 3rd edn. 2013, vol. 1, pp. 582–592.