Supporting Information

Three-dimensionally printable shear-thinning triblock copolypeptide hydrogels with antimicrobial potency

Robert Murphy, Shadi Kordbacheh, Dimitrios Skoulas, Simon Ng, Kasinan Suthiwanich, Andrea M. Kasko, Sally-Ann Cryan, Deirdre Fitzgerald-Hughes, Ali Khademhosseini, Amir Sheikhi* and Andreas Heise*

Materials

All chemicals were obtained from Sigma Aldrich unless otherwise noted. Benzyl-L-glutamate was purchased from Iris Biotech. S-benzyl-L-cysteine, ε-carbobenzyloxy-L-lysine and O-benzyl-L-tyrosine were purchased from Bachem. Water was removed from tetrahydrofuran (THF) and ethyl acetate using molecular sieves. All other solvents used were anhydrous. Reference bacterial strains were obtained from the American Tissue Type Collection (ATCC), *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *E. coli* ATCC 700926. Brilliance™ UTI Agar (Oxoid), SA-Select Agar (BioRad) and Mueller-Hinton II (MH) Agar (Fannin) were used for hydrogel contact assays.

Methods

^{1}H NMR spectra of the monomers and synthesized polymers were recorded on a Bruker Avance™ 400 (400 MHz) spectrometer at room temperature with DMSO-d^6 and a 5:1 mixture of $CDCl_3$/TFA-*d* as solvents, with tetramethylsilane (TMS) used as the internal reference. Attenuated total reflection (ATR) FTIR was recorded using a Thermo Scientific™ iS10 spectrometer in the region of 4000-400 cm^{-1}. A background measurement was initially performed before analyzing the sample. Sixteen scans were completed using a resolution of 2 cm^{-1}. Size exclusion chromatography (SEC) was used to determine the dispersities ($Đ_M$) and molecular weights of polymers. SEC was conducted in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFiP) using an PSS SECurity™ GPC system equipped with a PFG 7 µm 8 × 50 mm pre-column, a PSS 100 Å, 7 µm 8 × 300 mm and a PSS 1000 Å, 7 µm 8 × 300 mm column in series and a differential refractive index (RI) detector at a flow rate of 1.0 mL min^{-1}. The systems were calibrated against Agilent Easi-Vial linear poly(methyl methacrylate) (PMMA) standards and analyzed by the software package PSS winGPC UniChrom. Rheological measurements were completed on a MCR 301 digital rheometer (Anton Paar™). All experiments were conducted at room temperature using a conical plate (CP50-1, Anton Paar™, Physica, Australia) consisting of a 50 mm diameter geometry and a gap length of 0.097 mm and a protective hood was used to prevent evaporation.

3D extrusion printing

The polymers were loaded into the printing cartridge (Cellink™) using a spatula. The cartridge was then connected to the air system and placed in the printhead. The 3D printing was conducted at room temperatures, using a Cellink™ INKREDIBLE+ 3D bioprinter operated at printing pressure ~ 200 kPa and printing speed ~ 60 mm/min. The nozzle diameter was 23G. The hydrogel inks were printed in different architectures, ranging from meshed constructs to a complex inverse pyramid without further modification.

Zeta-potential (ζ)

Zeta-potential measurements were conducted on a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, UK). The samples for P1and P2 were made in deionized water, at concentrations relevant to the MIC and cell viability studies, namely 0.43 μM, 1.74 μM, 6.94 μM and 27.77 μM. The sample volume inside the Zetasizer cell was 1 ml.

Contact antimicrobial assay (hydrogel phase)

E. coli ATCC 25922 and *S. aureus,* ATCC 29213 were grown overnight at 37ºC on MH agar. Pure colonies were suspended in 0.95% NaCl to the density of a 0.5 McFarland standard (1.5×10^8 CFU/mL approximately) using a Densichek meter (Biomérieux, Ireland). Aliquots (50μl) of Hydrogels P1 (active) and P2 (control) at 1.5 wt % were added to the wells of a 96-well round bottom plate. Plates were centrifuged at 4000 g before overlaying the wells with 200μl of bacterial suspension. Plate controls were included in which bacterial suspension was placed directly into wells. Plates were incubated for 40 min at ambient temperature (14ºC approximately). Aliquots from the bacterial suspensions were removed from wells and serial 10-fold dilution were prepared in 0.9% NaCl. Dilutions (100μl) were spread onto MH agar and incubated at 37°C for 18 h. Resulting colonies forming units (CFU) were counted and the $\log_{10}$ reduction in CFU/mL was calculated for each polymer with respect to plate controls.

Visualization of antimicrobial activity on chromogenic agar (hydrogel phase)

To further demonstrate the ability of the P1 hydrogel to prevent bacterial growth, high density inocula (approx. 1.5 x 10^8 CFU/ml) of *S. aureus* and *E. coli* cultures were lawned onto chromogenic agar plates (SASelect and Brilliance UTI respectively) using a cotton swab. P1 and P2 hydrogels (1.5 %) samples were extruded in short lines onto each half of the inoculated plates before overnight incubation. *S. aureus* grow as pink colonies on SASelect and *E. coli* grow as pink colonies on Brilliance UTI.

Minimum inhibitory concentration (MIC) assay (solution phase)

MICs of the soluble polypeptide (P1) was determined in a standard microbroth dilution assay as described elsewhere.[1] Briefly, an overnight culture (37ºC, 18 h, 150 rpm) of *E. coli* ATCC700926 was

prepared from pure colonies on LB agar and diluted with Luria-Bertani (LB) broth (Fisher BioReagents) and grown to mid-log phase (approximately 3 h, OD600 ~ 0.5-0.6, measured using UV-visible spectrophotometer, BIOMATE 3S, Thermo Scientific). Cultures were diluted 1/50 in LB broth and 100 µl added to the wells of a 96-well tissue culture plate (approximately 10^4 CFU/well) containing doubling serial dilutions of P1. Plates were incubated at 37 °C for 18 h in a static incubator (Sterilemax) and the OD600 was measured using Synergy H1 hybrid reader (BioTek). The MIC value was determined as the lowest concentration that inhibited bacterial growth relative to growth controls (no polypeptide).

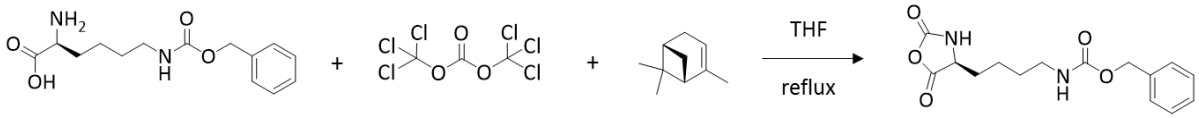
Bacteria killing assays (solution phase)

Overnight cultures (37ºC, 18 h, 150 rpm) of *E. coli* ATCC 700926 from pure colonies on LB agar were grown in LB. Further 1/100 dilutions were prepared in LB and incubated at 37 °C to mid-log phase (O.D. 600 =0.4-0.5). The mid-log phase culture was diluted in Difco™ M9 salt solution (40x) to 1 x 10^7 CFU/mL and20 µL was added to the wells of 96-well plates containing varying concentrations of P1 in a total volume of 200 µL of M9 salt (1 x 10^6 CFU/well). The plates were incubated at 37 °C in a microplate shaker for 1 h. Serial 10-fold dilutions were prepared in Difco™ M9 minimal salt, BD and 100 µL aliquots were spotted onto LB agar plates and incubated overnight at 37 °C to yield visible colonies. *E. coli* killing assays were conducted in duplicate.

Live/dead viability assay (solution phase)

The cytotoxicity of the P1 towards NIH/3T3 fibroblast cells (ATCC) was investigated using the live/dead viability/cytotoxicity kit (Molecular Probes). The assay detects intracellular esterase activity and plasma membrane integrity as markers of cell viability. Live cells appear green due to esterase-mediated fluorescent of calcein and dead cells appear red due to interaction of ethidium homodimer with damaged cell membranes. Approximately, 10,000 cells/well were cultured in a 96-well plate using DMEM complete (Gibco, 10% FBS) media containing 0.43 µM (MIC) of P1 for 24, 48, and 72 h at 37 °C. Subsequently, the cells were stained with 0.43 µL of the live/dead assay reagents (2 µM of calcein and 4 µM of ethidium homodimer) and incubated at 37 °C for 20 min. The stained cells were imaged using a Zeiss Axiovert Observer Z1 inverted fluorescent microscope.

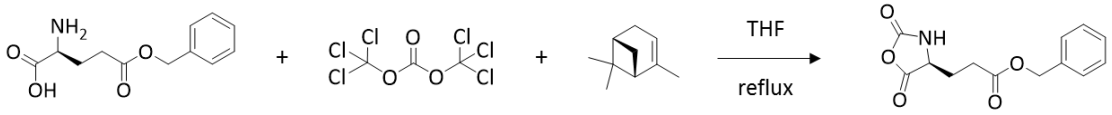
Synthesis of ε-carbobenzyloxy-L-lysine (ZLL) NCA



ε-Carbobenzyloxy-L-lysine (15 g, 53.51 mmol) and α-pinene (18.22 g, 133.78 mmol) were suspended in 180 mL of dry THF and heated under reflux. A solution of triphosgene (7.15 g, 24.08 mmol) in 30 mL dry THF was added dropwise to the suspension. The suspension was refluxed until all solids disappeared and the solution became clear (5 h). The solution was then cooled, filtered and 2/3 of the volume was removed under vacuum. It was then precipitated by addition of 250 mL hexane and stored

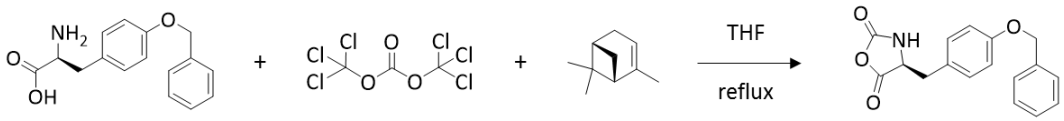
overnight at -18 °C. The NCA solid was dried, re-dissolved in dry ethyl acetate and filtered. The NCA solution was then recrystallized thrice in ethyl acetate/hexane (1:1.5) and subsequently washed with hexane to remove any trace impurities. It was vacuum dried to afford a colourless fluffy solid (yield 83%). For the ^{1}H-NMR spectrum, see Figure S1.

Synthesis of γ-benzyl-L-glutamate (BLG) NCA



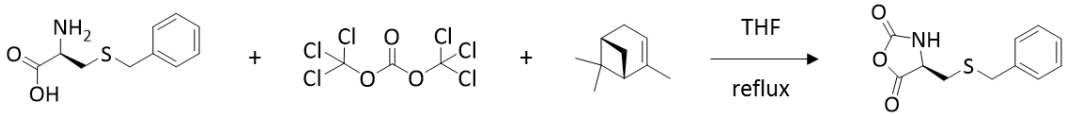
γ-benzyl-L-glutamate (10 g, 42.15 mmol) and α-pinene (14.73 g, 92.73 mmol) were suspended in 120 mL of dry THF and heated under reflux. A solution of triphosgene (5.25 g, 17.70 mmol) in 20 mL dry THF was added drop-wise to the suspension. The suspension was refluxed until all solids disappeared and the solution became clear (3 h). The solution was then cooled, filtered and reduced to 1/3 of its original volume under vacuum. It was then precipitated by addition of 180 mL hexane and stored overnight at -18 °C. The NCA solid was dried, re-dissolved in dry ethyl acetate and filtered. The NCA solution was then precipitated into excess hexane, dried, and then recrystallized twice in ethyl acetate/hexane (1:2). It was vacuum dried to afford a colourless solid (yield 85%). For the ^{1}H-NMR spectrum, see Figure S2.

Synthesis of O-benzyl-L-tyrosine (BLT) NCA



O-benzyl-L-tyrosine (8 g, 29.49 mmol) and α-pinene (8.83 g, 64.87 mmol) were suspended in 110 mL of dry THF and heated under reflux. A solution of triphosgene (4.37 g, 14.74 mmol) in 20 mL dry THF was added dropwise to the suspension. The suspension was refluxed until all solids disappeared and the solution became clear (3 h). The solution was then cooled, filtered and reduced to 1/3 of its original volume under vacuum. It was then precipitated by addition of 150 mL hexane and stored overnight at -18 °C. The NCA solid was dried, re-dissolved in dry ethyl acetate and re-precipitated in excess hexane. The NCA was then recrystallized twice in a mixture of ethyl acetate/hexane followed by vacuum drying to afford off-white colorless crystals (yield 79%). For the ^{1}H-NMR spectrum, see Figure S3.

Synthesis of S-benzyl-L-cysteine (BLC) NCA



S-benzyl-L-cysteine (11 g, 52.06 mmol) and α-pinene (15.60 g, 114.54 mmol) were suspended in 130 mL of dry THF and heated under reflux. A solution of triphosgene (6.64 g, 22.39 mmol) in 25 mL dry

THF was added drop-wise to the suspension. The suspension was refluxed until all solids disappeared and the solution became clear (3.5 h). The solution was then cooled, filtered and reduced to 1/3 of its original volume under vacuum. It was then precipitated by addition of 180 mL hexane and stored overnight at -18 °C. The NCA solid was dried, re-dissolved in dry ethyl acetate and re-precipitated in excess hexane and allowed crystallize at -18 °C. The NCA was then recrystallized twice in a mixture of ethyl acetate/hexane followed by vacuum drying to afford shiny long crystals (yield 88%). For the ^{1}H-NMR spectrum, see Figure S4.

Synthesis of block copolymers

The NCA of benzyl-L-cysteine (449 mg, 1.89 mmol) was dissolved in 15 mL of anhydrous DMF (0.1 M LiBr) under a N_2 atmosphere in a schlenk flask and placed in a thermostatically controlled bath (0 °C). Allylamine (4.70 μL, 6.33×10^{-2} mmol) in 1 mL of anhydrous DMF (0.1 M LiBr) was then quickly charged to the Schlenk flask. The flask was continuously evacuated under vacuum to remove CO_2 and was allowed to stir for 18 hours at 0 °C. The solution was allowed to stir until full monomer consumption was confirmed by Fourier-transform infrared (FTIR) spectroscopy. An aliquot was then taken directly via syringe to monitor the molecular mass using size exclusion chromatography (SEC). The NCA of ε-carbobenzyloxy-L-lysine (2.90 g, 9.49 mmol) was then dissolved in 35 mL of anhydrous DMF and charged to the flask, and stirring was continued until complete consumption was confirmed by FTIR. A second aliquot was taken to monitor the molecular mass of the diblock using SEC. Finally, the NCA of benzyl-L-tyrosine (847 mg, 2.85 mmol) was dissolved in 15 mL of anhydrous DMF and charged to the flask, which was stirred until conversion was observed by FTIR. The triblock copolymer was then diluted with $CHCl_3$, precipitated into excess diethyl ether, and dried under vacuum in a desiccator to give the triblock copolymer (yield: 3.1 g, 80 %). The triblock copolymer (2.5 g) was solubilized in 35 mL of trifluoroacetic acid and 9 mL of HBr (33 wt% in acetic acid) was then added dropwise to the copolymer solution in a six-fold excess amount with respect to the carbobenzyloxy and benzyl protecting groups and allowed to stir for 18 hours. The polymer was precipitated into excess diethyl ether, and the supernatant decanted and washed 3 more times with diethyl ether. It was then dried under vacuum and subsequently dissolved in deionized water and neutralized with NaOH to pH 7.5. Dialysis was performed against deionized water for 5 days using a 10,000 MWCO membrane (Thermo Scientific™), with frequent water replacement. Lyophilization then afforded the P1 triblock copolymer (yield: 1.15 g, 86%). *for the P2 triblock copolymer, γ-benzyl-L-glutamate NCA replaced ε-carbobenzyloxy-L-lysine NCA as the second monomer to afford the anionic triblock copolymer (yield: 2.6 g, 82 %).

Table S1: Characteristics of triblock copolypeptides.

Entry	Polymer block	M_n^a	M_n^b	M_n^c	$Đ_M^c$
P1	P(BLC_{30})	5800	5400	12,100	1.06
	P(BLC_{30}-*b*-ZLL_{150})	45,100	34,300	36,600	1.15
	P(BLC_{30}-*b*-ZLL_{150}-*b*-BLT_{45})	56,500	42,900	39,400	1.21
P2	P(BLC_{30})	5800	4,800	12,400	1.06
	P(BLC_{30}-*b*-BLG_{150})	38,700	35,100	38,100	1.14
	P(BLC_{30}-*b*-BLG_{150}-*b*-BLT_{45})	50,100	42,900	40,300	1.25

[a] Molar mass theoretical expectation based on stoichiometric feed ratio. [b]Molecular mass as determined by ^{1}H-NMR monomer ratio. [c]Molecular mass and dispersity ($Đ_M$) determined by SEC (PMMA standards).

Table S2: Determination of MIC value of P1 against *E. coli.* OD600 values of *E. coli* culture incubated with increasing concentrations of P1.

Polypeptide concentration (µM)	OD600	Cell number (CFU/mL)
27.77	0.05	5×10^7
13.89	0.05	5×10^7
6.94	0.07	7×10^7
3.47	0.08	8×10^7
1.74	0.06	6×10^7
0.88	0.04	4×10^7
0.43	0.10	1×10^8
0	0.29	2.9×10^8

Table S3: Zeta-potential measurements for diluted P1 and P2 hydrogels

Concentration (µM)	P1 ζ (mV)	P2 ζ (mV)
27.77	45.1 ± 0.65	-39.8 ± 1.34
6.94	35.2 ± 0.40	-11.5 ± 0.41
1.74	0.2 ± 0.28	-6.62 ± 0.27
0.43	0.07 ± 0.15	-1.97 ± 3.75

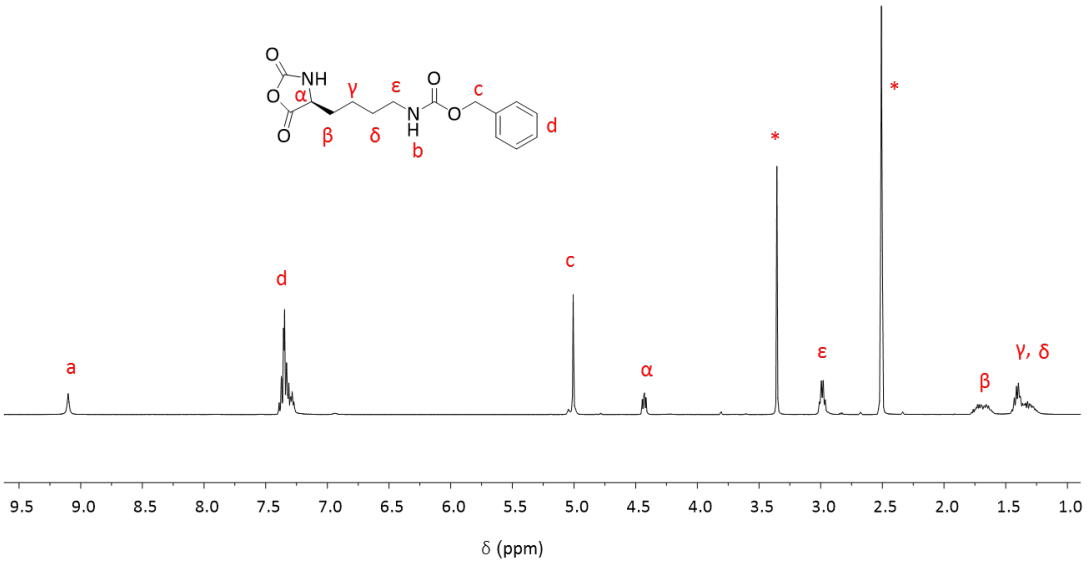


Figure S1. [1]H-NMR of ε-carbobenzyloxy-L-lysine *N*-carboxyanhydride in DMSO-d^6.

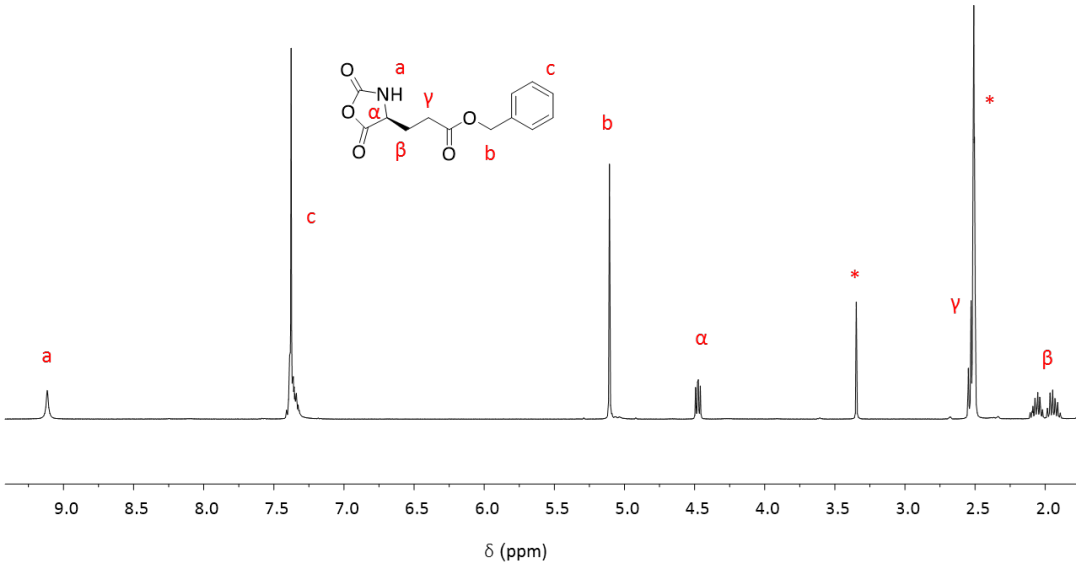


Figure S2. [1]H-NMR of γ-benzyl-L-glutamate *N*-carboxyanhydride in DMSO-d^6.

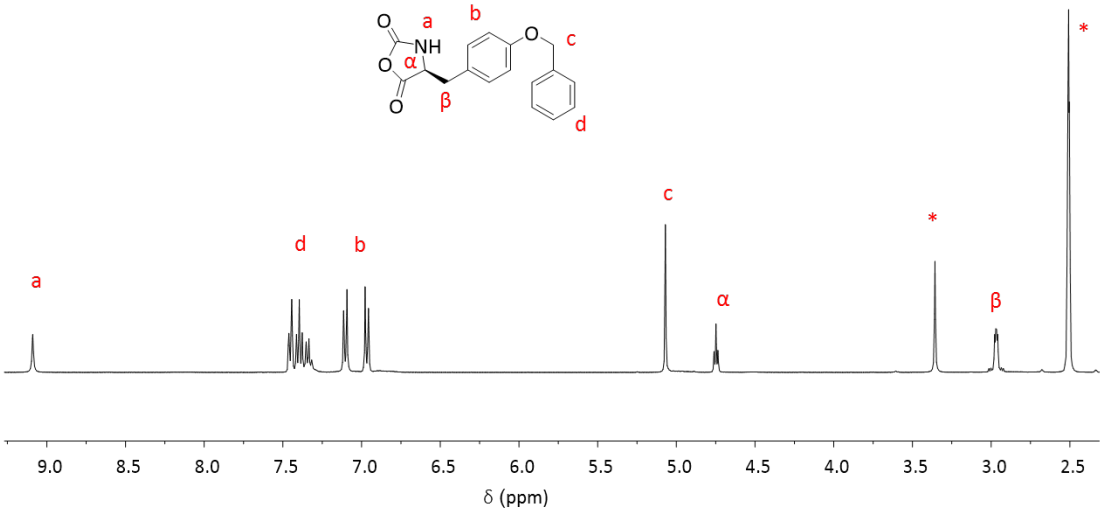


Figure S3. [1]H-NMR of O-benzyl-L-tyrosine *N*-carboxyanhydride in DMSO-d^6.

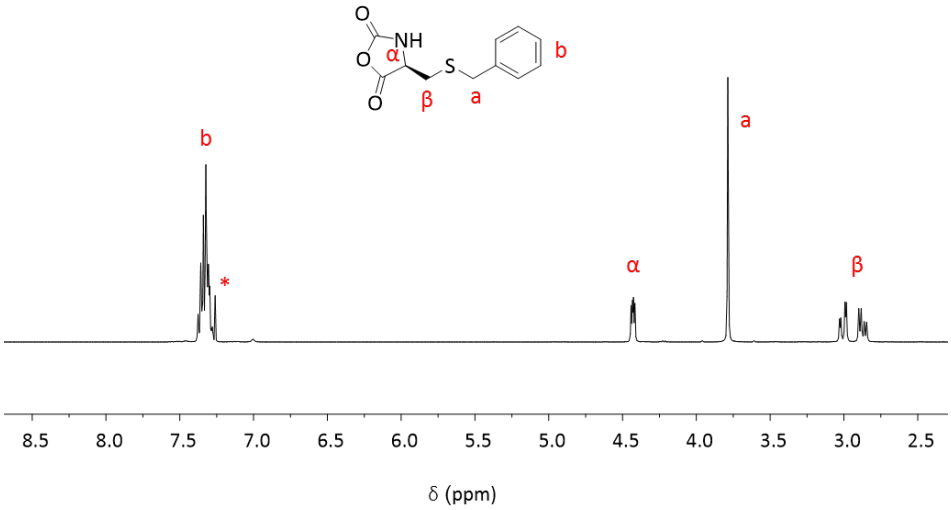


Figure S4. [1]H-NMR of S-benzyl-L-cysteine *N*-carboxyanhydride in $CDCl_3$/TFA-*d* (5:1).

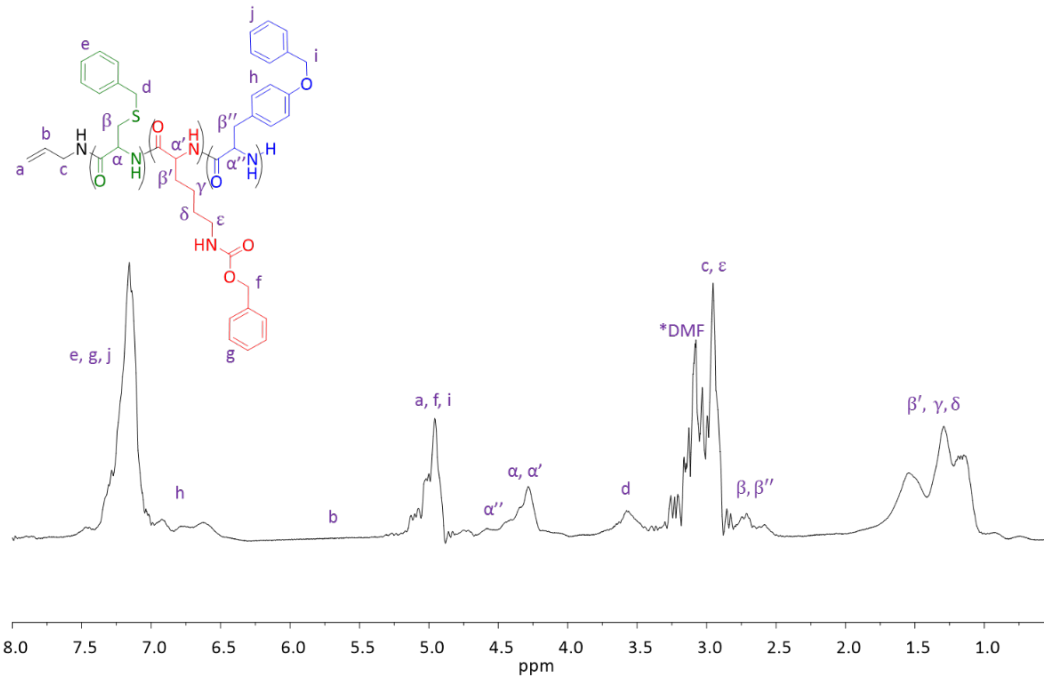


Figure S5. ^{1}H-NMR of protected P1 copolymer in $CDCl_3$/TFA-*d* (5:1).

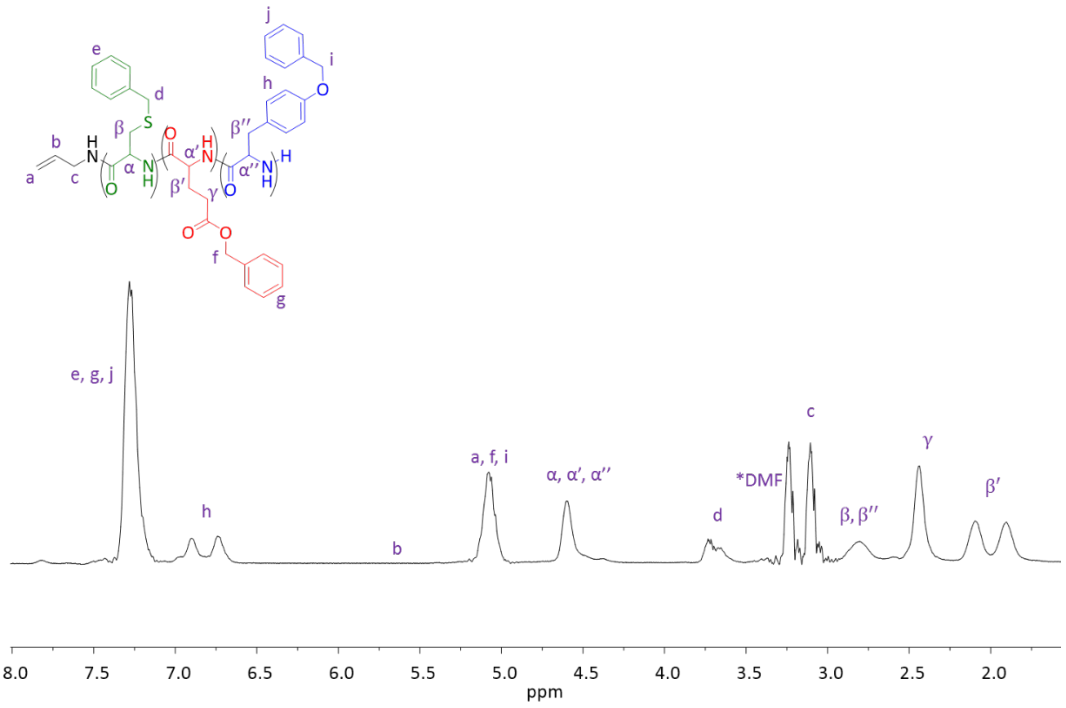


Figure S6. ^{1}H-NMR of protected P2 copolymer in $CDCl_3$/TFA-*d* (5:1).

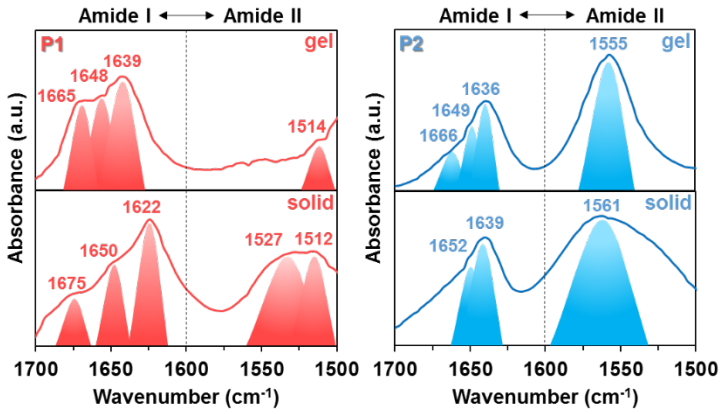


Figure S7. FTIR spectra of P1 and P2 pre- (solid) and post-self-assembly (gel), showing the conformational switches in the amide I and II regions between solid and gel states. The highlighted regions correspond to β-motif and other secondary structurally changes with numbers illustrating the relevant conformations.

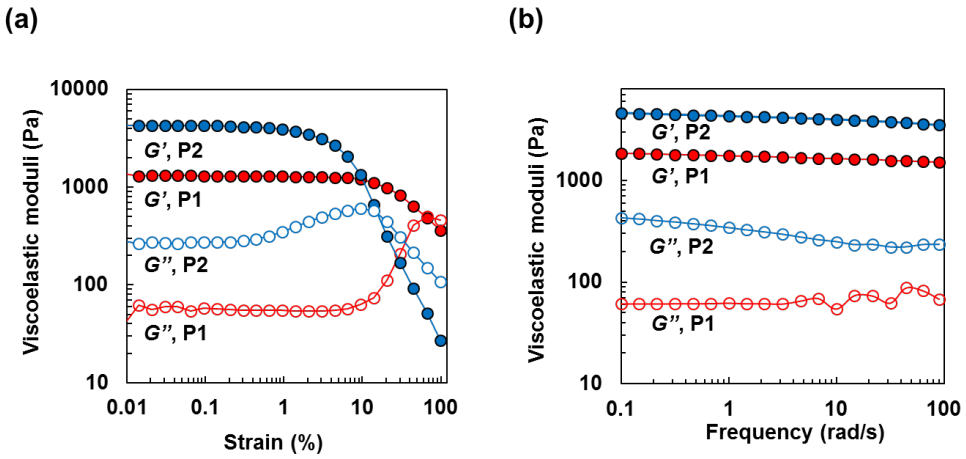


Figure S8. Rheological amplitude sweeps (a) at 10 rad/s showing the viscoelastic moduli versus oscillatory shear strain. Rheological frequency sweeps (b) at 0.1% strain showing hydrogel viscoelastic moduli over broad frequency range.

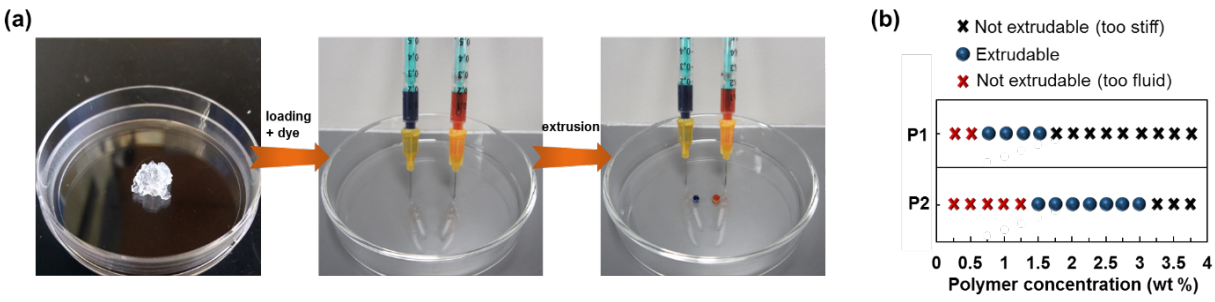


Figure S9. (a) Image of transparent P1 hydrogel at its relative critical gel forming concentration (CGC) and (b) demonstration of loading and extrusion of P1 (blue) and P2 (red) hydrogels from a 23G needle and syringe.

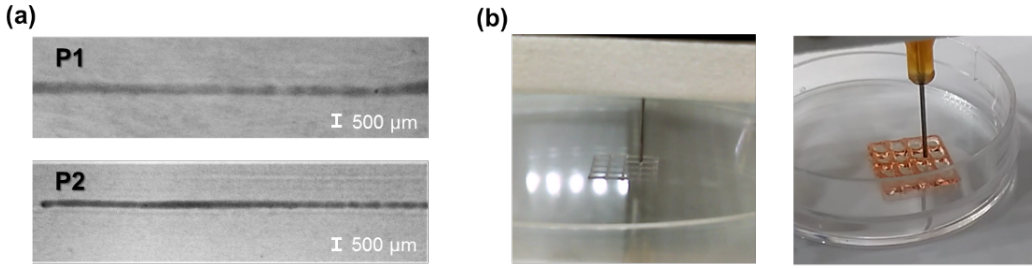


Figure S10. (a) Hydrogel filament width of both P1 (0.75 wt%, 2700 µM) and P2 (2 wt%, 8000 µM) hydrogels. (b) Representative images during 3D printing of lattice structures.

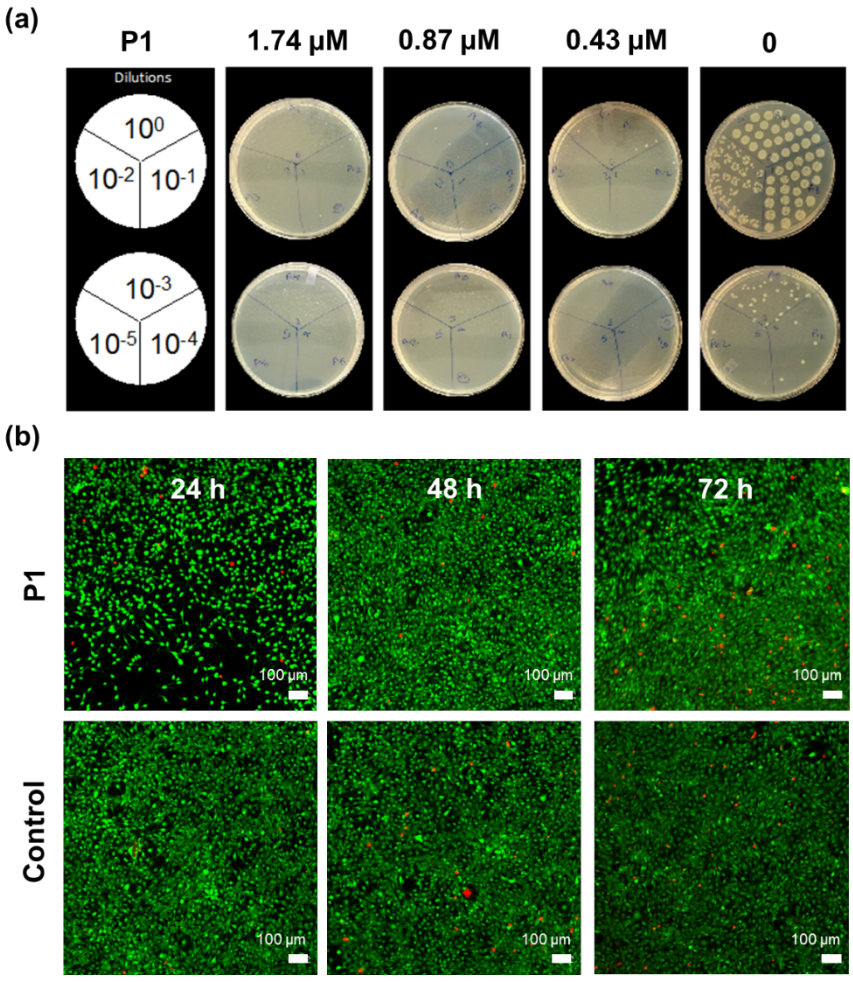


Figure S11. (a) Determination of minimum bactericidal concentration (MBC) of P1 against *E. coli.* Assay was performed in duplicate and a representative assay is shown. (b) *In vitro* live/dead fluorescent images of NIH/3T3 fibroblast cell monolayer cultured in the presence of 0.43 µM of P1 after 24, 48, and 72 h. The controls show the same cells cultured in polymer-free media.

References

(1) S. Deshayes, Wujing Xian, N. W. Schmidt, S. Kordbacheh, J. Lieng, J. Wang, S. Zarmer, S. St. Germain, L. Voyen, J. Thulin, G. C. L. Wong, A. M. Kasko, *Bioconjugate Chem.* **2017**, *283*, 793.