Supporting Information

Antimicrobial and Degradable Triazolinedione (TAD) Crosslinked Polypeptide Hydrogels

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Materials

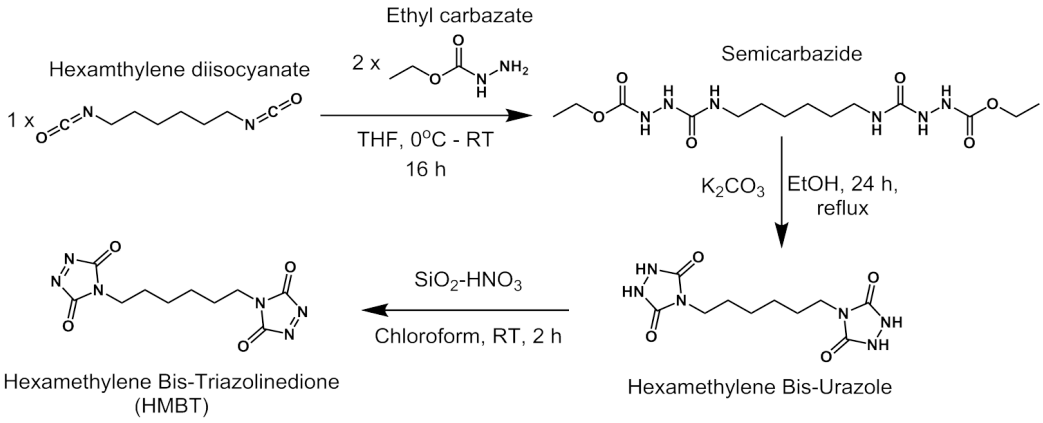
All reagents and chemicals were purchased from Sigma-Aldrich, unless otherwise stated. D-Tryptophan, L-Lysine, L-Tryptophan, Benzyl-L-Glutamate and Triphosgene were purchased from Fluorochem Ltd. Hexane, dichloromethane, ethanol, and anhydrous potassium carbonate were purchased from Fisher Scientific Ireland Ltd. N, N-Dimethylformamide (99.8%, extra dry over molecular sieves, AcroSeal™) was purchased from Acros Organics. All chemicals were used as received, unless stated otherwise. Silica nitric acid was prepared as previously described in the literature (described briefly below).[1,2] Hexamethylene bis-triazolinedione (HMBT) was synthesized following previously published procedures.[1] Protected amino acids were converted into NCAs using triphosgene following methods described previously.[3,4] Z-D-Lysine and D-Tryptophan NCAs were prepared in the same way as for their L enantiomeric counterparts.

Methods

^{1}H, ^{13}C and COSY NMR spectra were obtained on a Bruker DPX-400 spectrometer (400 MHz) at 293 K. All chemical shifts are reported as δ in parts per million (ppm) and were referenced to the residual solvent signal $(CD_3)_2SO$: ^{1}H, δ 2.50 ppm and ^{13}C, δ 39.52 ppm. Gel permeation chromatography (GPC) was carried out in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) using a PSS SECurity GPC system equipped with a PFG 7 µm 8 × 50 mm pre-column, a PSS 100 Å, 7µm 8 × 300 mm and a PSS 1000 Å, 7µm 8 × 300 mm column in series and a differential refractive index (RI) detector at a flow rate of 1.0 mL min^{-1}. The GPC system was calibrated against Agilent Easi-Vial linear poly(methyl methacrylate) (PMMA) standards and analysed by PSS winGPC UniChrom software package. The morphology of copolypeptide hydrogel materials was investigated using a Zeiss ULTRA Plus scanning electron microscope (SEM) operating at 5kV with a 30 µm aperture. Sections of hydrogel were sliced while hydrated and then freeze dried. They were then placed onto stubs and sputter coated using a Cressington 208HR sputter coater for 15 seconds with 80/20 Au/Pd to give a coating of approximately 5 nm prior to imaging. Attenuated total reflection (ATR) FT-IR spectra were recorded on a Thermo Scientific iS10 spectrometer in the region of 4000 - 500 cm^{-1}. A background was performed prior to placing a sample onto the ATR for analysis. 16 scans were completed with a resolution of 2 cm^{-1}. Rheological measurements were completed on an MCR 301 digital rheometer (AntonPaar) at room temperature using a conical plate (CP50-1, Anton Paar) consisting of a 50 mm diameter geometry with a gap length of 0.1 mm. A protective hood was used to prevent evaporation. For the gelation study, two separate solutions, 100 mg/mL copolypeptide in 1:1 v/v acetonitrile:chloroform and 33 mg/mL HMBT in 2:1 v/v acetonitrile:chloroform respectively, were mixed with molar ratio of Trp:HMBT of 2:1 for all three polymers tested (LZK_{35}-LW_{15}, LZK_{70}-LW_{30}, and LZK_{80}-LW_{20}) (total volume 400 µL on the plate) on the rheometer plate and analysed immediately using the "fast moving profile".

Synthesis and characterization of HMBT[1,2,5]

Scheme S1: Synthesis of HMBT



Step 1 - Synthesis of hexamethylene bis-semicarbazide

Ethyl carbazate (10.04 g, 96.4 mmol) was added to a 250 mL round bottom flask and dissolved with 60 mL anhydrous THF under a nitrogen atmosphere. Then a solution of hexamethylene diisocyanate (8.11 g, 48.2 mmol) in THF (60 mL) was added dropwise to the stirred reaction solution at 0°C for 10 minutes (after 2-3 minutes a precipitate was observed). This suspension was then stirred for a further 2 hours, filtered and then the product was washed with THF. The semicarbazide was then dried under vacuum to give a white product (17.6 g, 46.8 mmol, 97% yield).

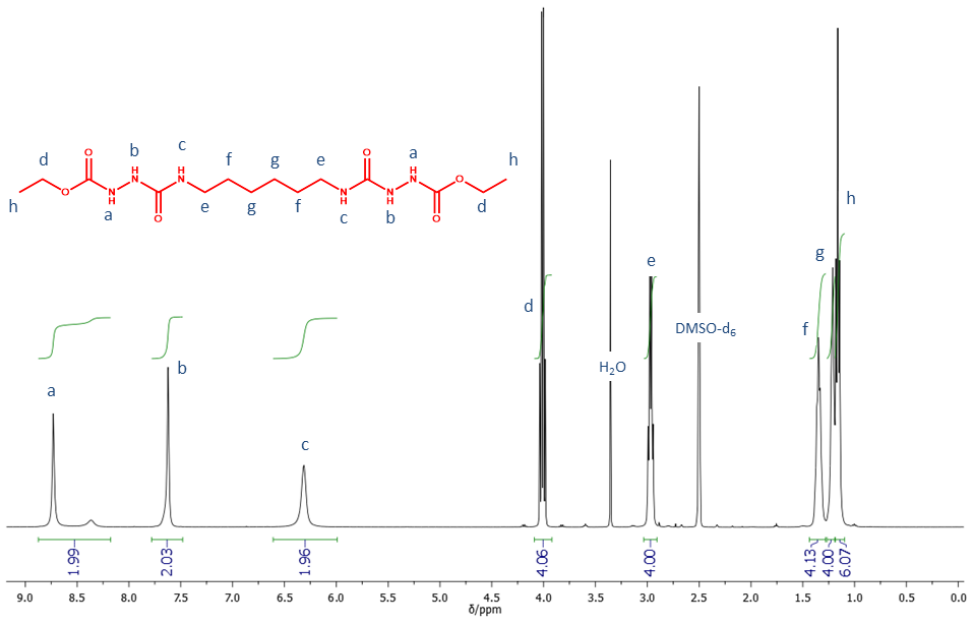


Figure S1: ^{1}H NMR semicarbazide in DMSO-d_6.

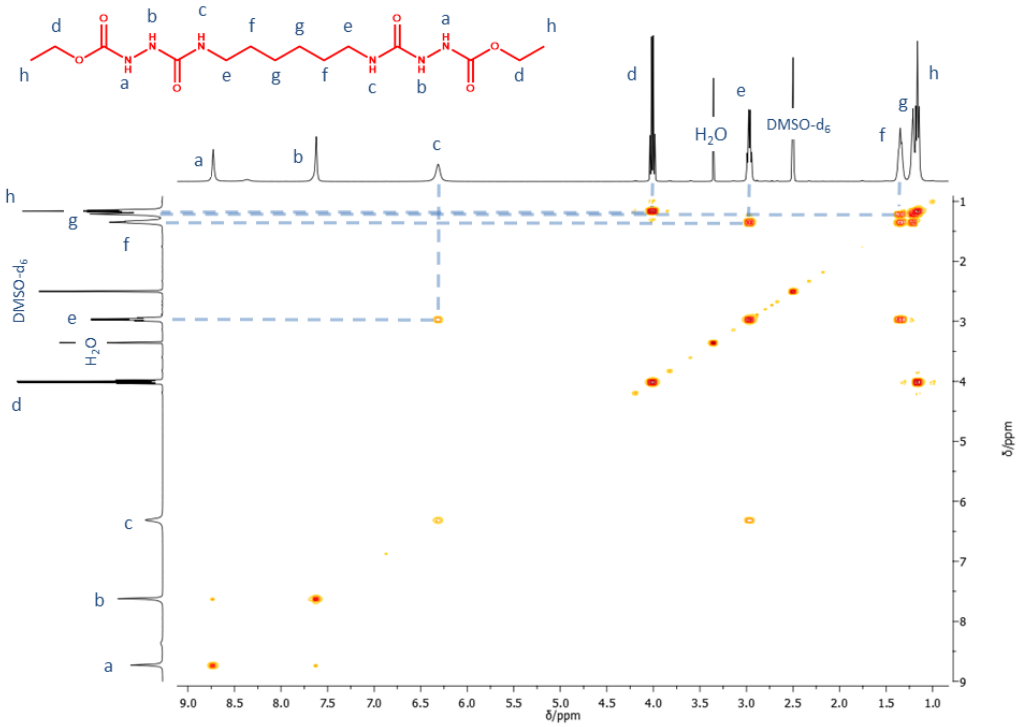


Figure S2: COSY ^{1}H NMR semicarbazide in DMSO-d_6.

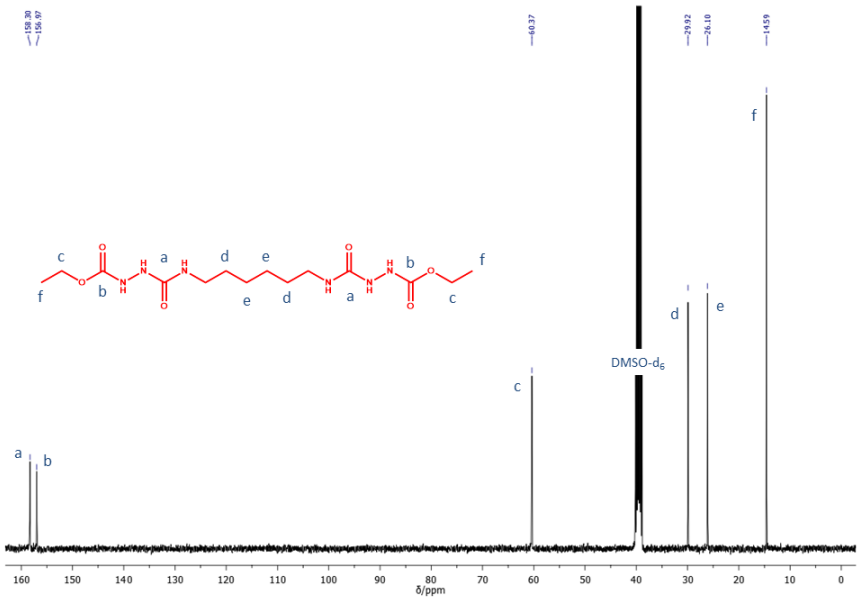


Figure S3: ^{13}C NMR semicarbazide in DMSO-d_6.

Step 2 - Synthesis of Hexamethylene Bis-Urazole

10 g (26.6 mmol) of semicarbazide was suspended in 300 mL of absolute ethanol. 12.4 g (90 mmol) of anhydrous potassium carbonate was added to the solution and heated to reflux and mixed for one day. Then the ethanol was evaporated and the crude material was dissolved in ice/water. Then concentrated HCl was added dropwise at 0ºC until the pH of the solution was between 1-2. The precipitate is filtered and the product washed with excess deionized water. Finally, the product was dried under vacuum (4.1 g, 14.4 mmol, 54% yield).

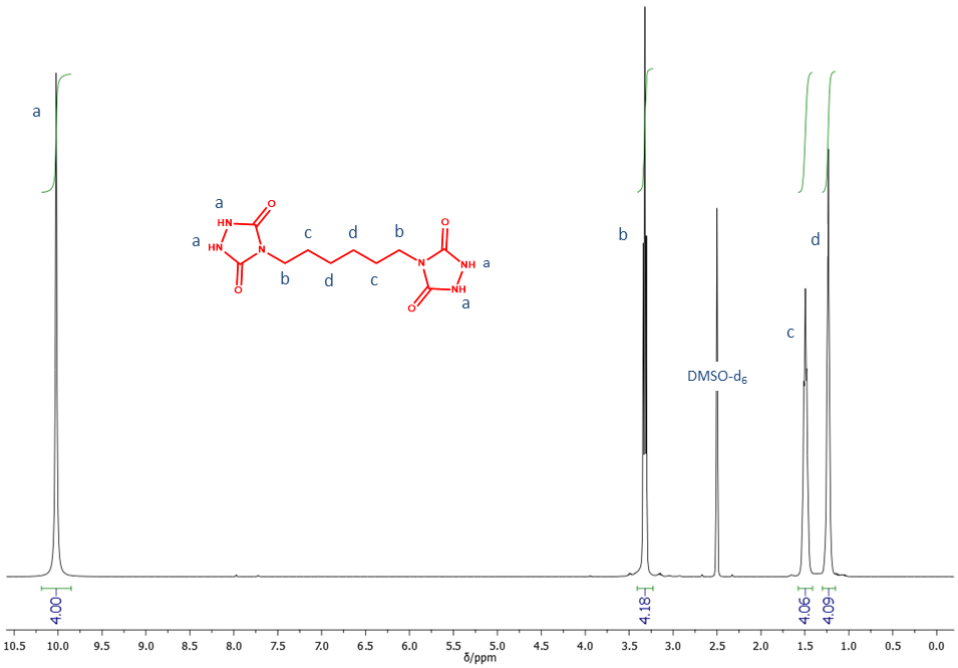


Figure S4: ^{1}H NMR hexamethylene bis-urazole in DMSO-d_6.

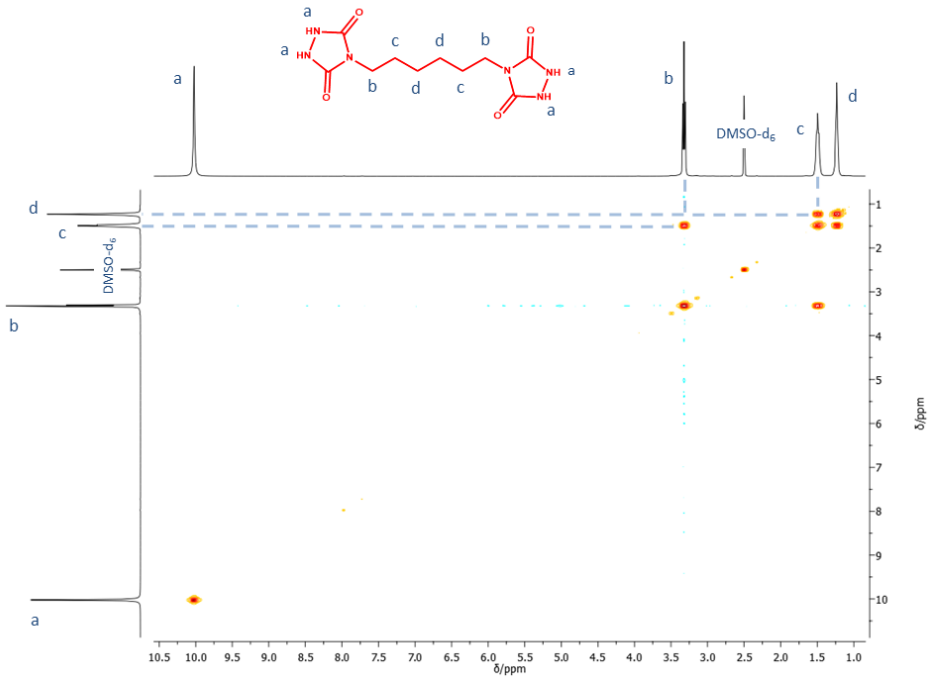


Figure S5: COSY ^{1}H NMR hexamethylene bis-urazole in DMSO-d_6.

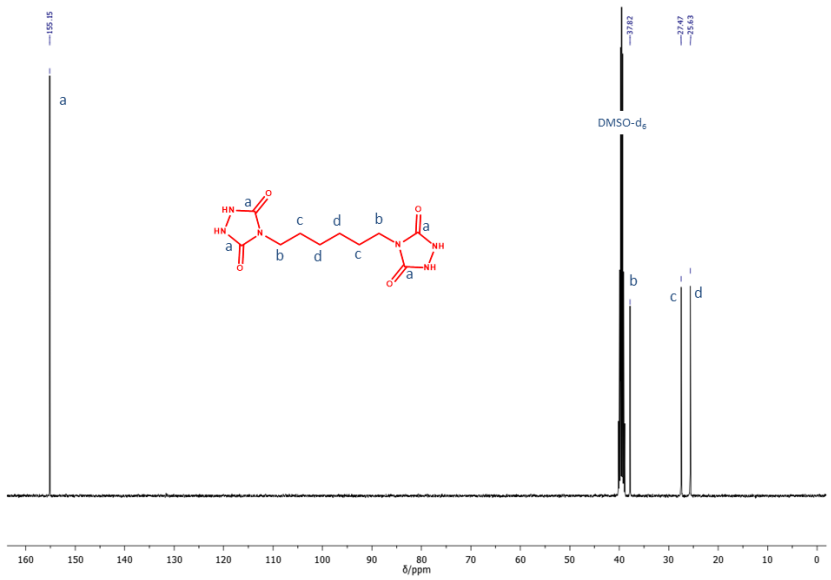


Figure S6: ^{13}C NMR hexamethylene bis-urazole in DMSO-d_6.

Step 3 - Synthesis of HMBT

Initially silica nitric acid was prepared following published procedures,[2] in brief, 3.2 mL concentrated nitric acid (65%, 4.5g) was added dropwise to 3.2 g of silica gel in a 40 mL glass vial and mixed with a glass rod over 15 minutes. The silica nitric acid (SiO_2-HNO_3) was then washed with dichloromethane and filtered under vacuum, 1.2 g of SiO_2-HNO_3 was then added

to a mixed suspension of 500 mg of hexamethylene bis-urazole in dichloromethane over 10 minutes and then mixed at room temperature for 2 hours. A bright pink solution is formed with silica nitric acid suspended within it. The solution is then filtered and $MgSO_4$ is added to the filtered solution and mixed for a minimum of 10 minutes. This is then filtered and $MgSO_4$ is washed three times with DCM. The solution is then evaporated using a rotary evaporator to leave a red powder (note it is important that the bath temperature is no higher than 30ºC otherwise this can result in the degradation of the final product). Finally, the product was then stored at -18ºC under nitrogen to prevent degradation (370 mg, 1.32 mmol, 75 % yield).

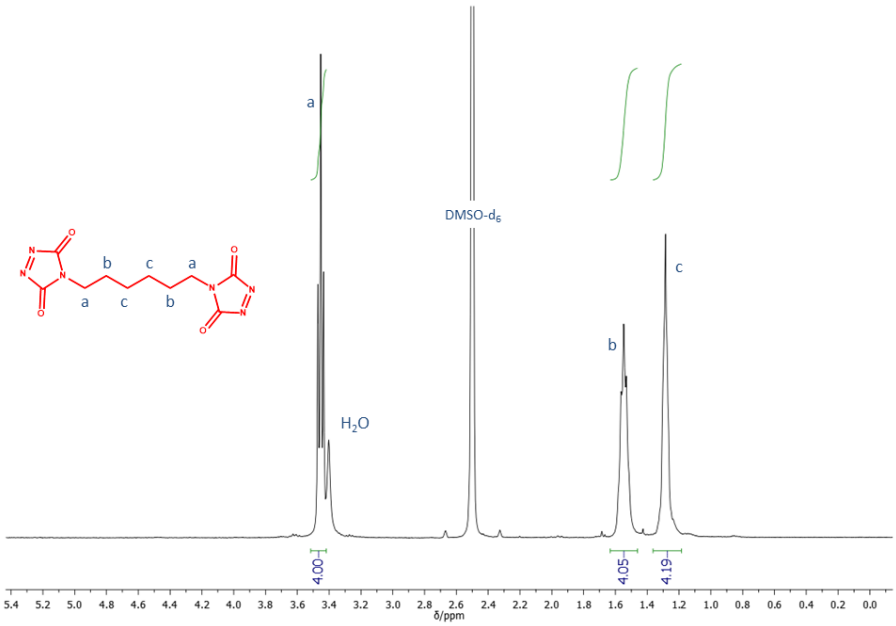


Figure S7: ^{1}H NMR hexamethylene bis-triazolinedione in DMSO-d_6.

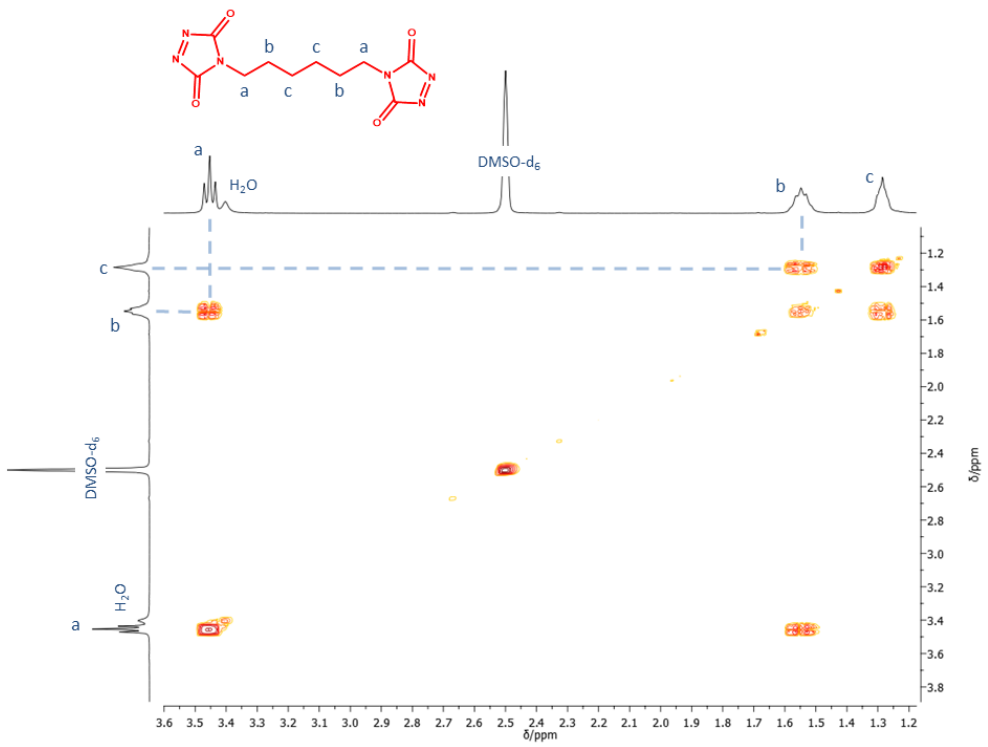


Figure S8: COSY ^{1}H NMR hexamethylene bis-triazolinedione in DMSO-d_6.

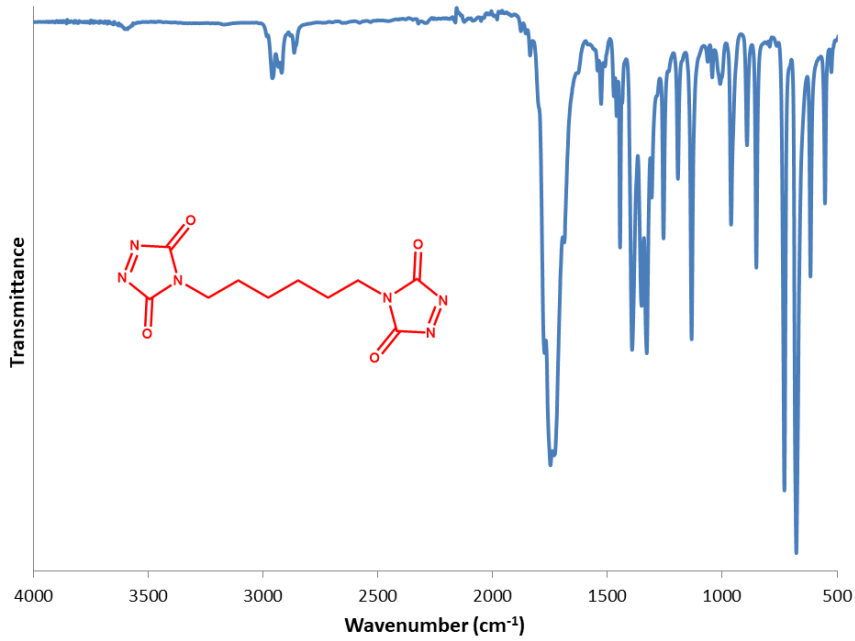


Figure S9: FTIR spectrum hexamethylene bis-triazolinedione.

Synthesis and characterization of NCAs

The synthesis of NCAs followed previous literature.[3,4] The synthesis of N-ε-carbobenzyloxy-L-lysine N-carboxyanhydride ZLLys NCA is given as a representative example. In brief,

ZLLysine (20 g, 43.8 mmol) and α-pinene (20.24 g, 148.6 mmol) was suspended in 250 mL of anhydrous THF in a 500 mL three-necked round bottom flask and heated to reflux. Then triphosgene (9.2 g, 31 mmol) was dissolved in 80 mL anhydrous THF and added dropwise over 90 minutes to the round bottom flask. After a total of 4 hours the clear solution was cooled, filtered and then reduced under vacuum (approximately 2/3 volume) and then precipitated into hexane (10 x volume of THF mixture) and then stored overnight at -18ºC. Then the NCA was dried under vacuo and then re-dissolved in anhydrous THF and filtered. The NCA solution was then recrystalised three times using hexane and washed with hexane to remove any trace impurities and then dried in vacuo to produce a white fluffy solid (yield 72%). Other NCAs including D enantiomers were prepared using a similar method. ^{1}H-NMR, COSY ^{1}H-NMR and FT-IR of Z-Lys NCA and other NCAs prepared are shown below (Figs. S10-S27).

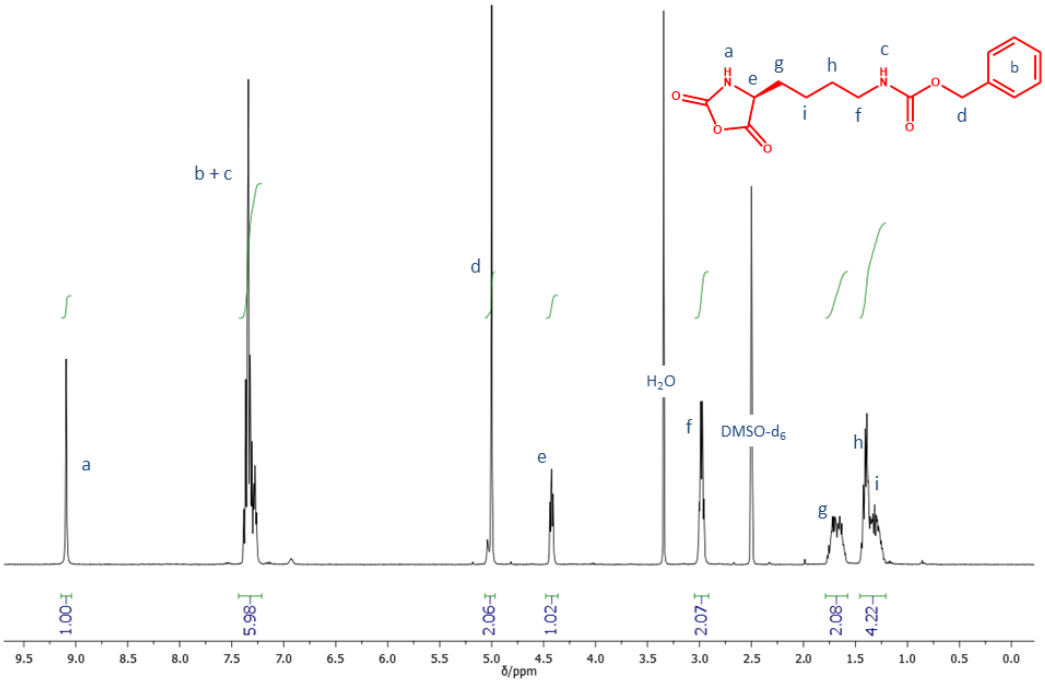


Figure S10: ^{1}H NMR of LZ-Lys NCA in DMSO-d_6.

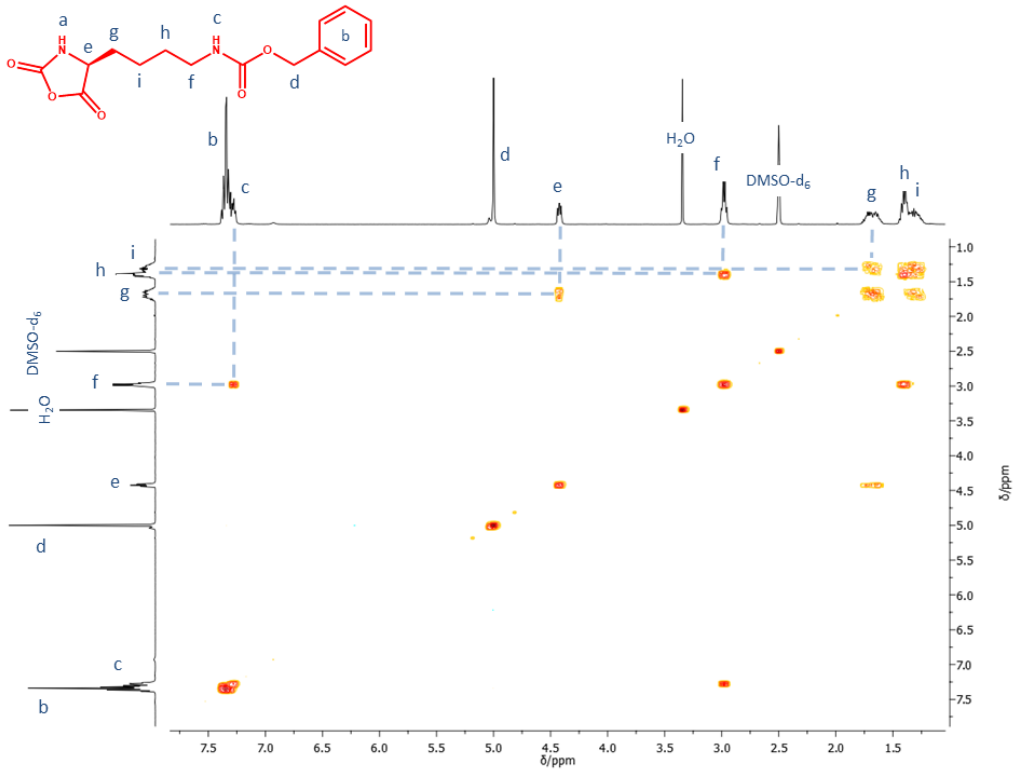


Figure S11: COSY ^{1}H NMR of LZ-Lys NCA in DMSO-d_6.

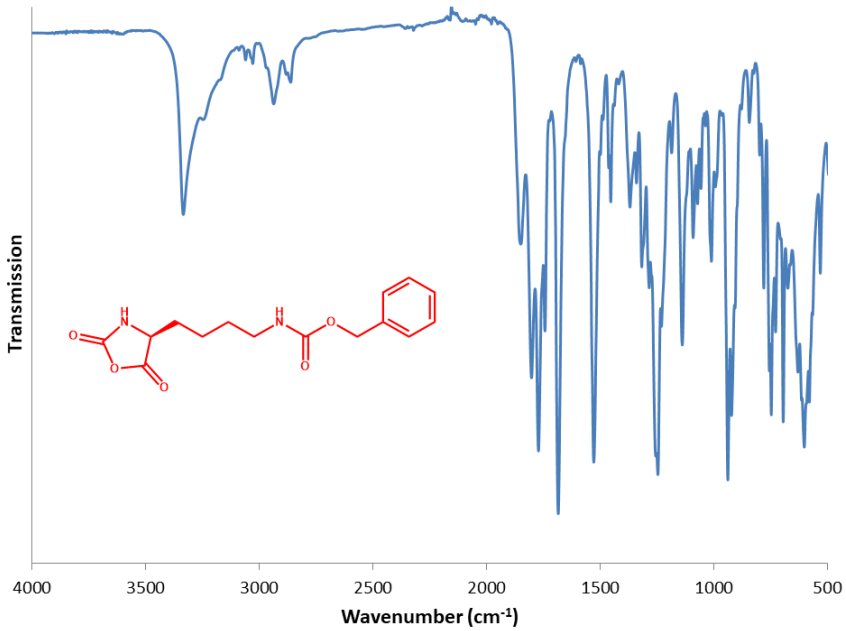


Figure S12: FTIR LZ-Lys NCA.

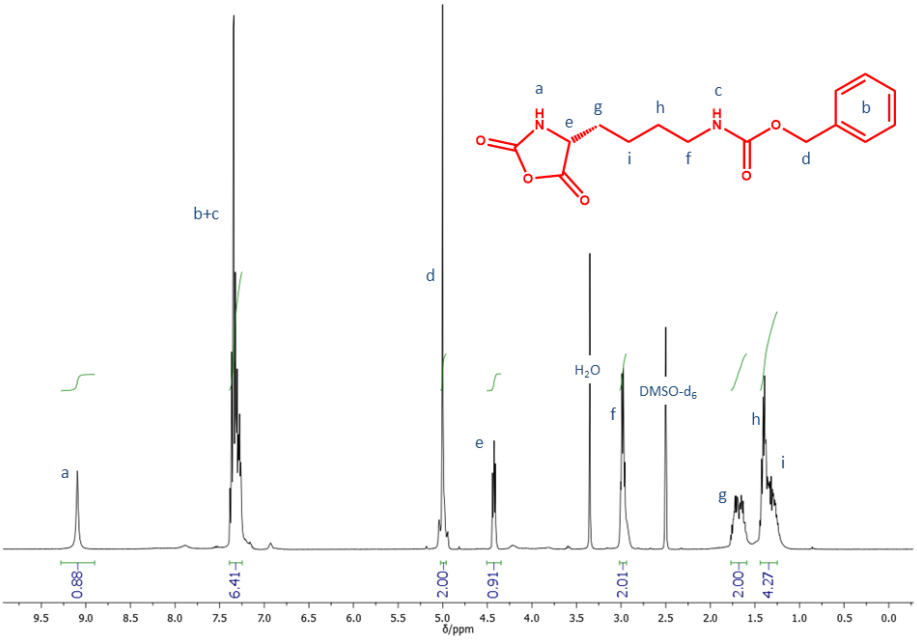


Figure S13: ^{1}H NMR DZ-Lys NCA in DMSO-d_6.

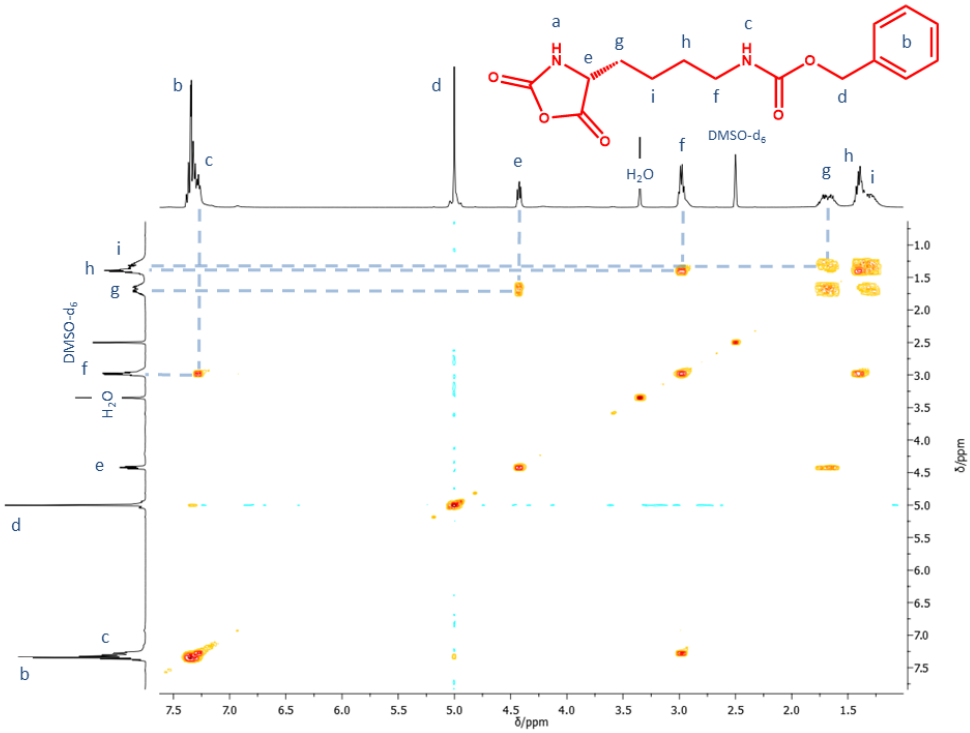


Figure S14: COSY ^{1}H NMR DZ-Lys NCA in DMSO-d_6.

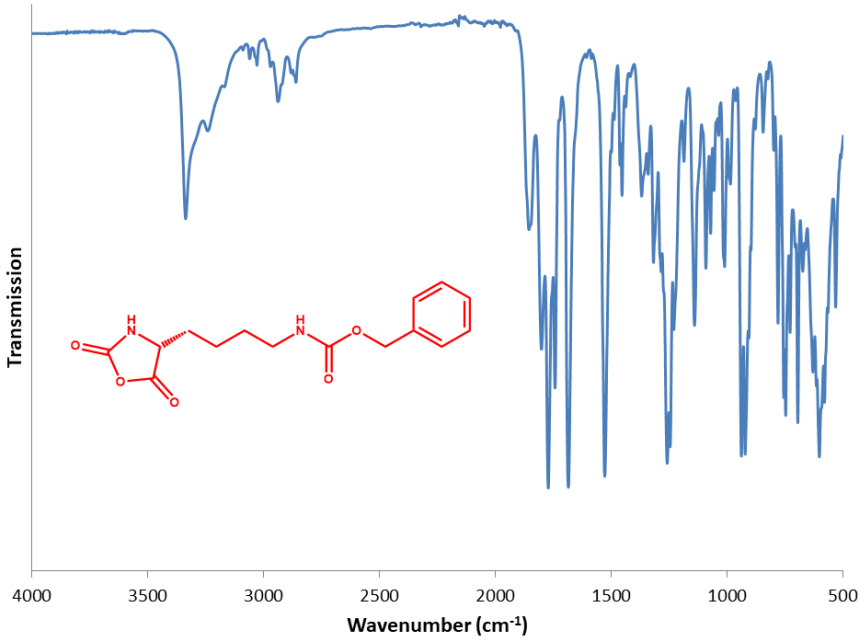


Figure S15: FTIR DZ-Lys NCA.

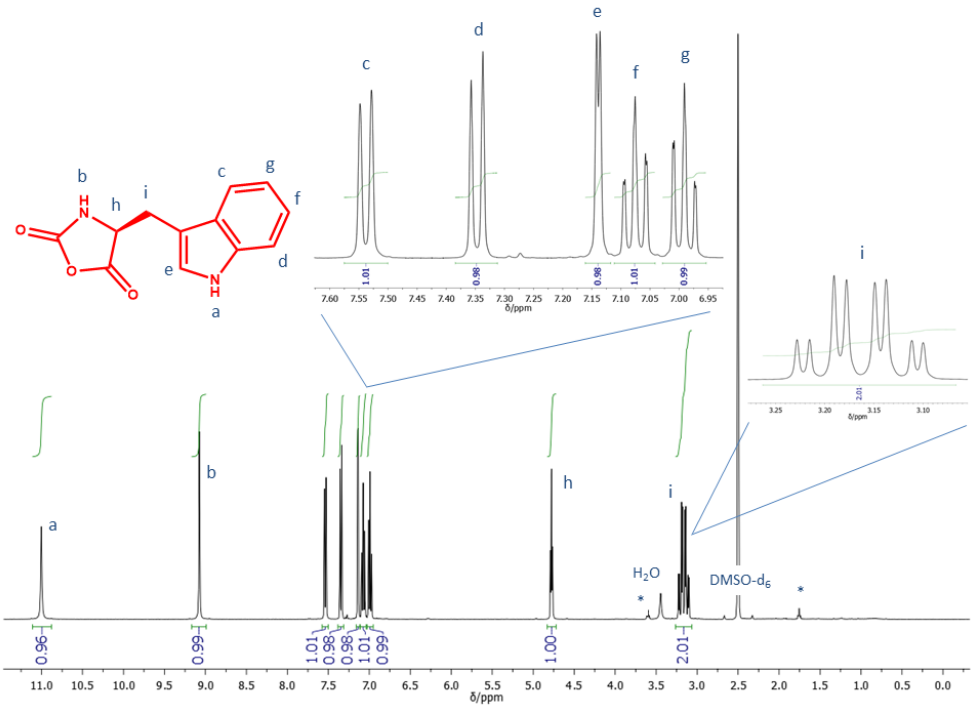


Figure S16: ^{1}H NMR L-Trp NCA in DMSO-d_6.

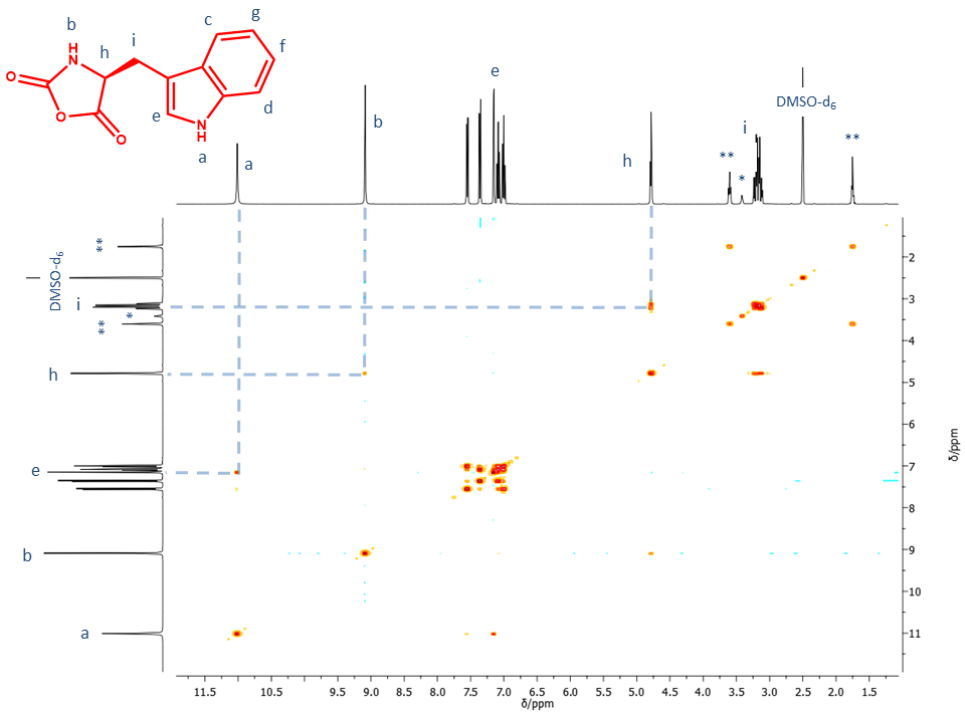


Figure S17: COSY ^{1}H NMR L-Trp NCA in DMSO-d_6.

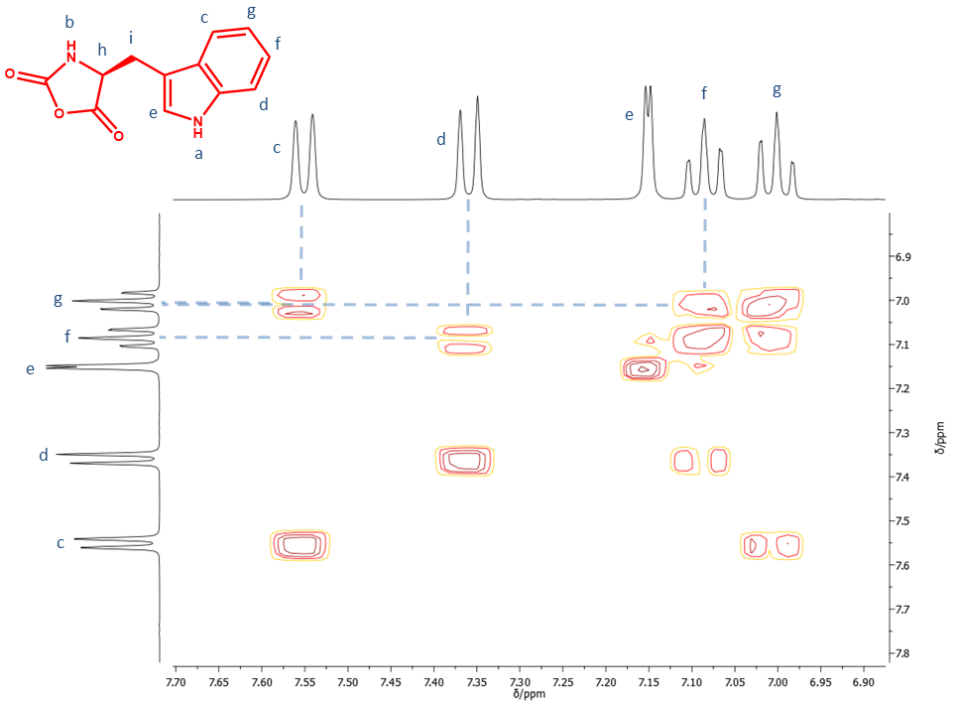


Figure S18: COSY ^{1}H NMR L-Trp NCA in DMSO-d_6.

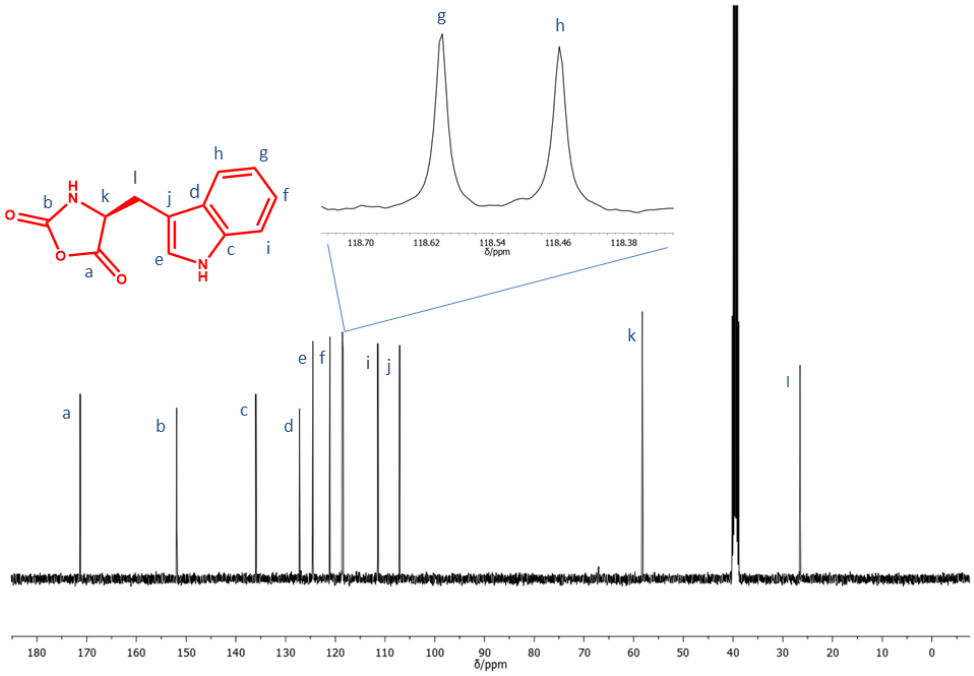


Figure S19: ^{13}C NMR L-Trp NCA in DMSO-d_6.

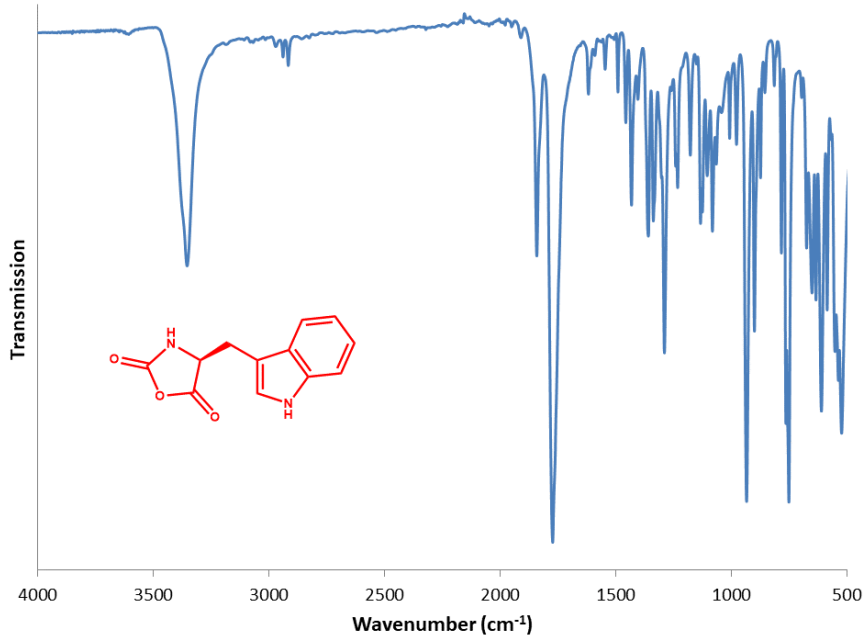


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Figure S20: FTIR L-Trp NCA.

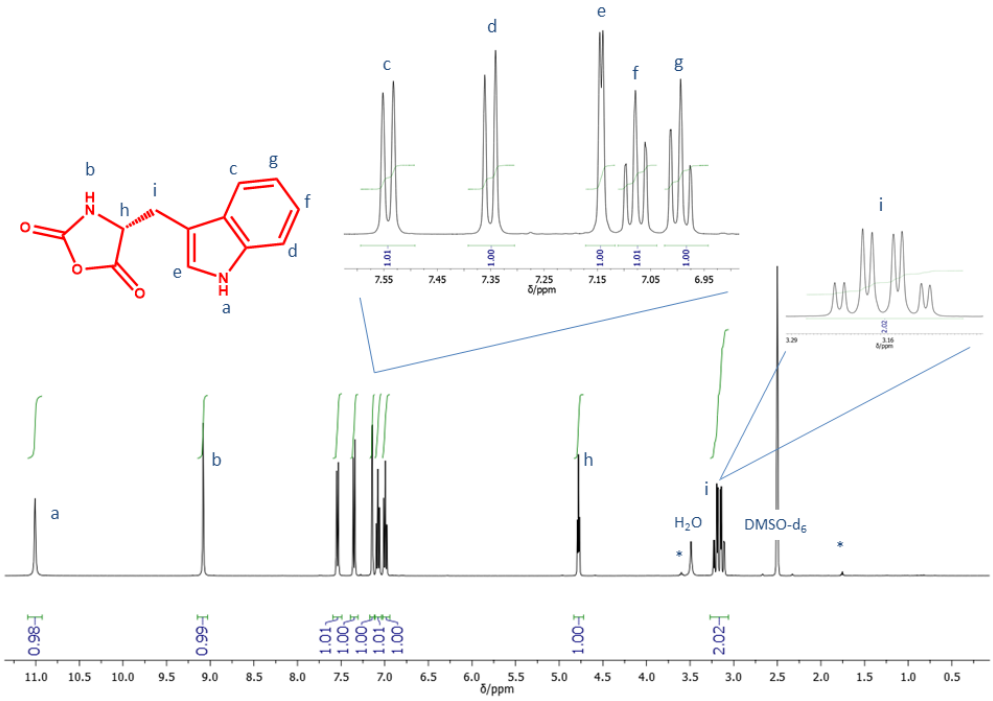

Figure S21: ^{1}H NMR D-Trp NCA in DMSO-d_6.

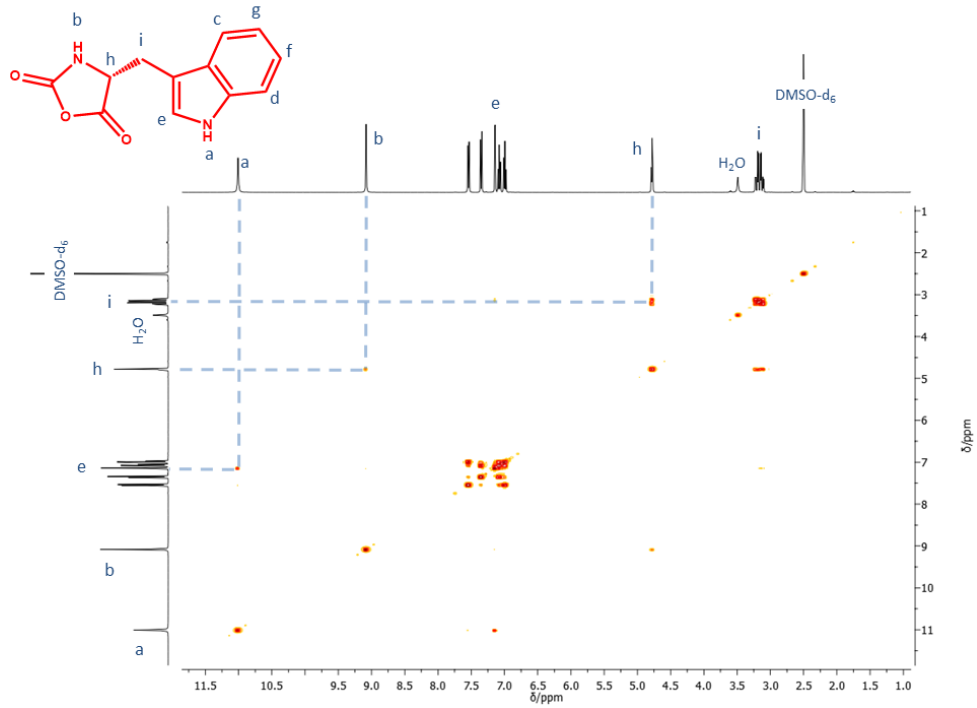

Figure S22: COSY ^{1}H NMR D-Trp NCA in DMSO-d_6.

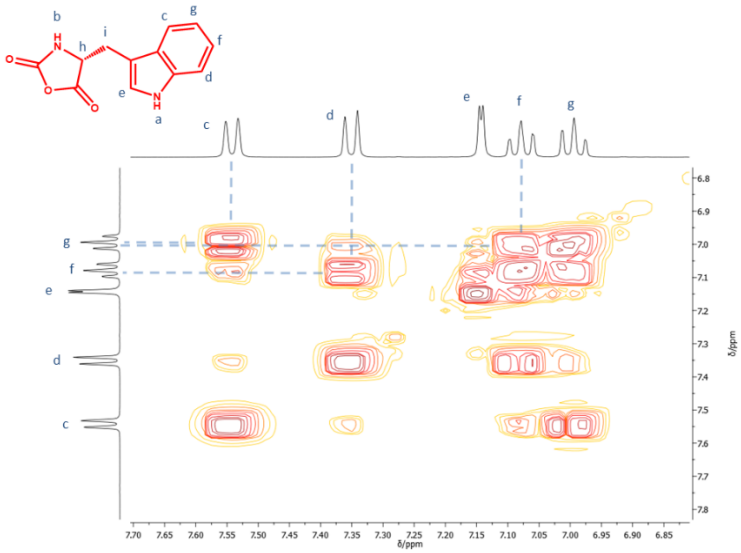

Figure S23: COSY ^{1}H NMR D-Trp NCA in DMSO-d_6.

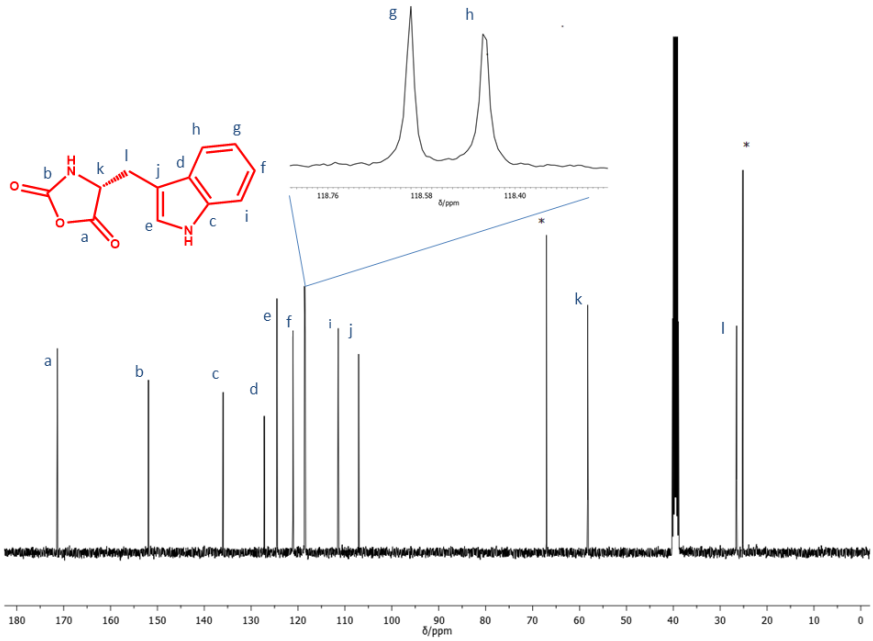

Figure S24: ^{13}C NMR D-Trp NCA in DMSO-d_6.

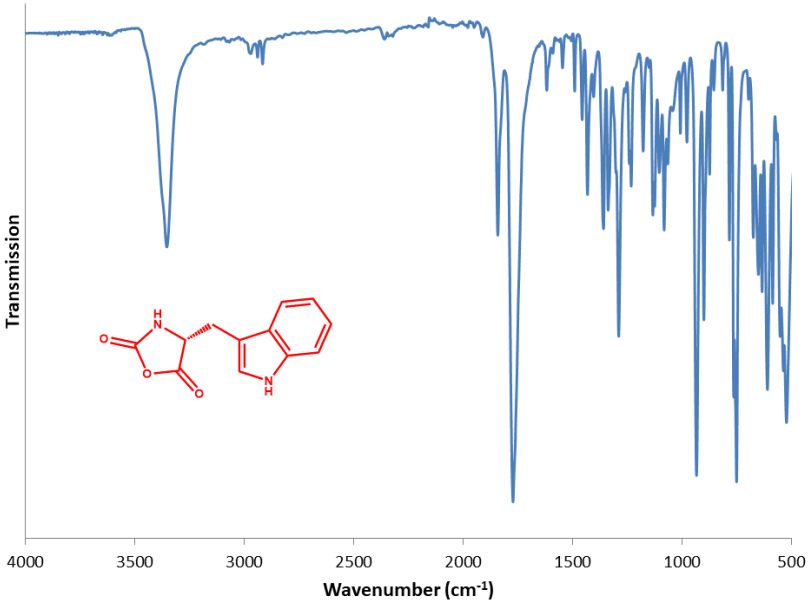

Figure S25: FTIR D-Trp NCA.

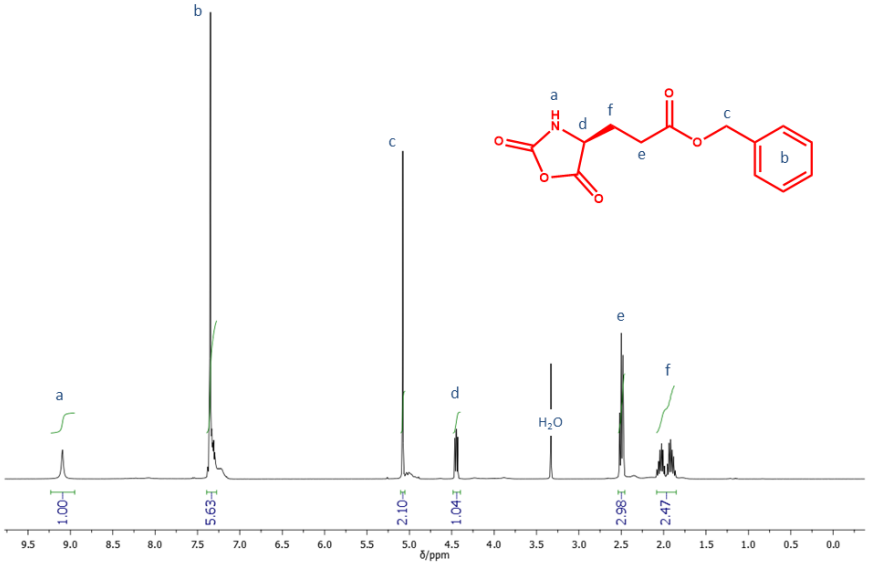

Figure S26: ^{1}H NMR BLG NCA in DMSO-d_6.

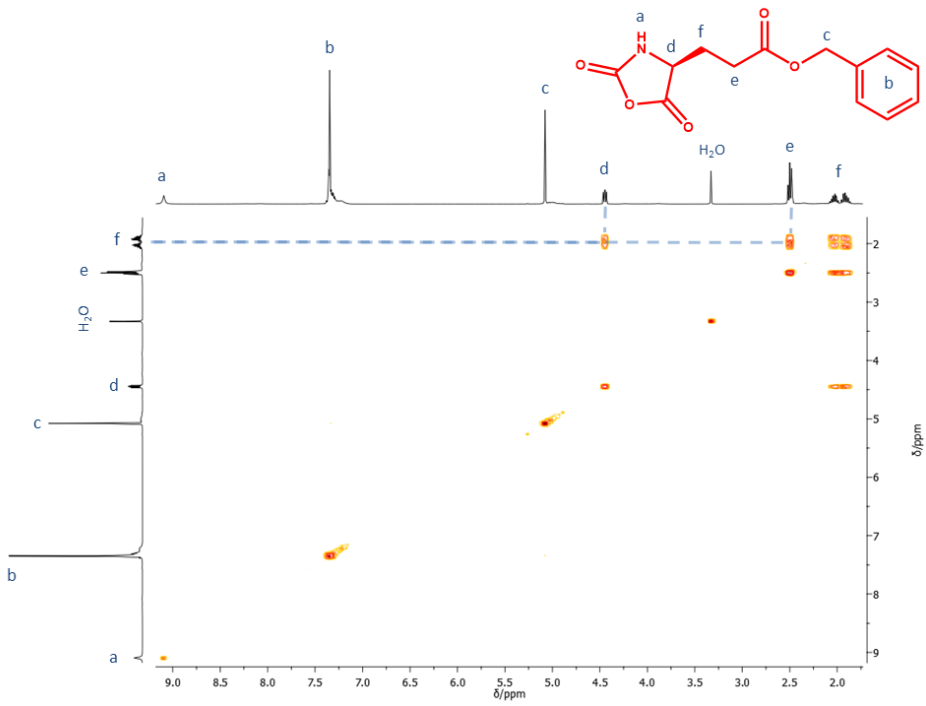

Figure S27: COSY ^{1}H NMR BLG NCA in DMSO-d_6.

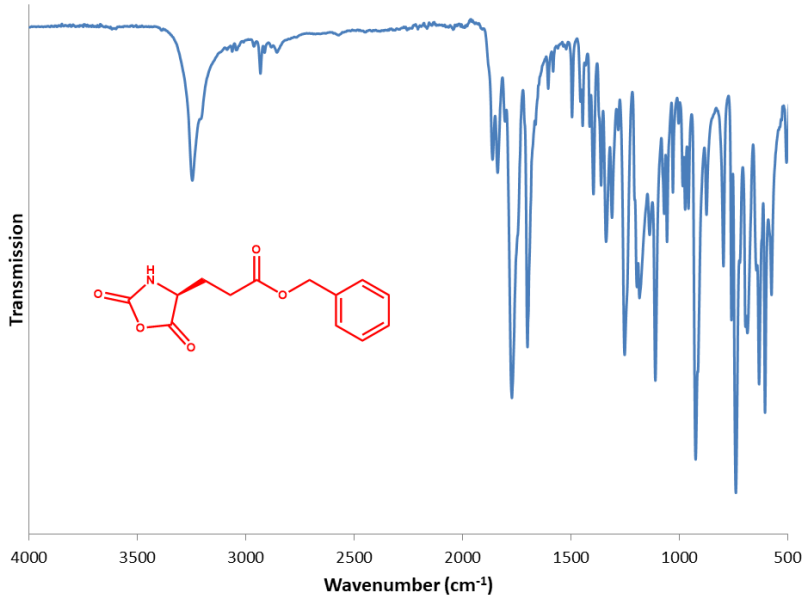

Figure S28: FTIR BLG NCA.

Copolypeptide characterisation

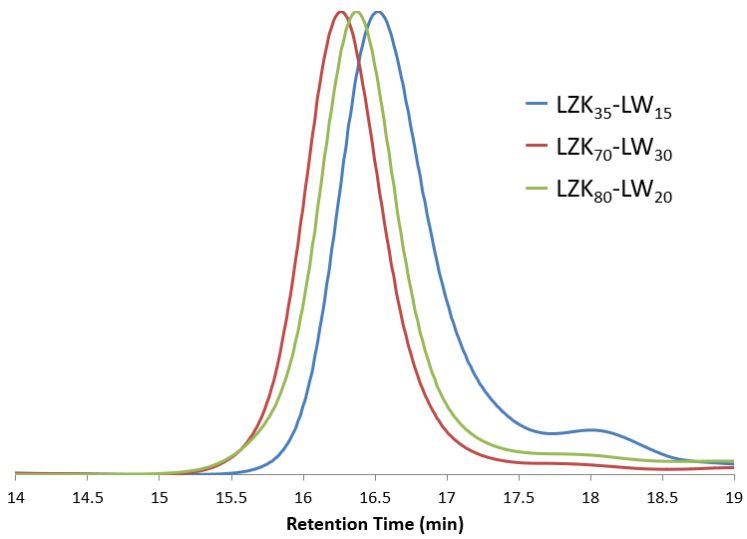

Figure S29: GPC of polymers LZK_{35}-LW_{15}, LZK_{70}-LW_{30} and LZK_{80}-LW_{20}.

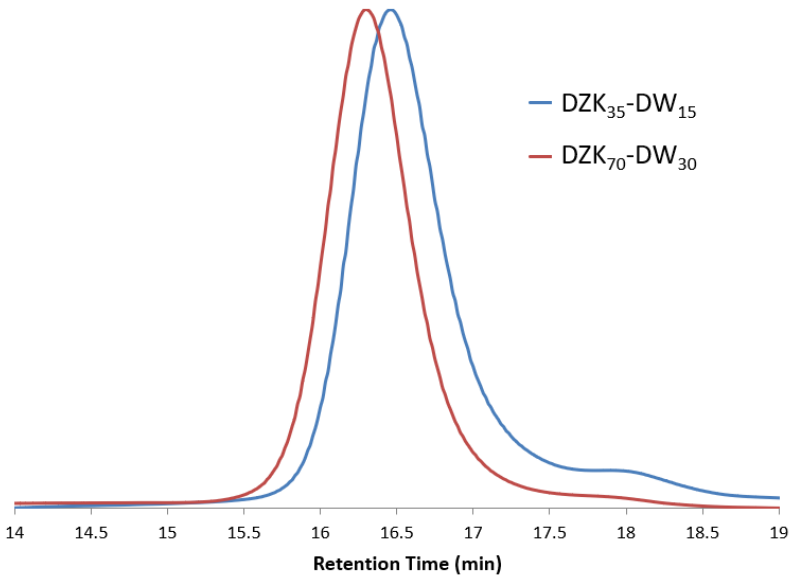

Figure S30 GPC of polymers DZK_{35}-DW_{15} and DZK_{70}-DW_{30}.

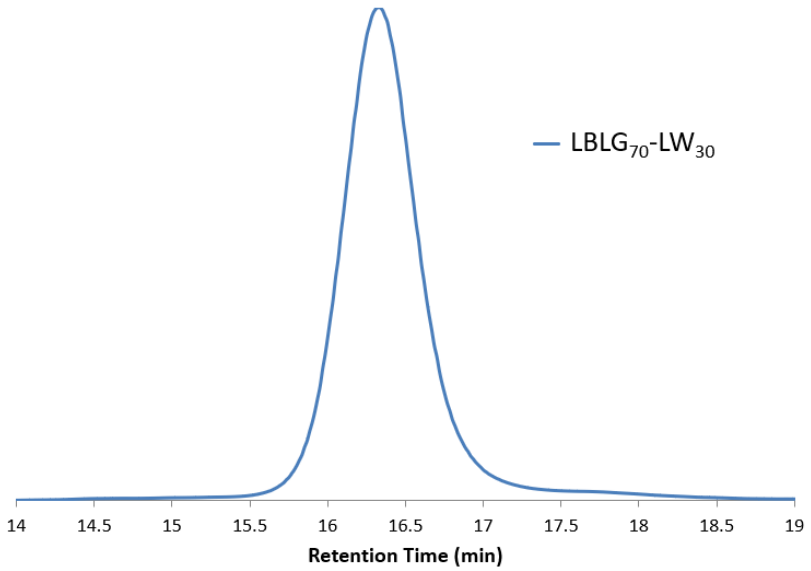

Figure S31: GPC of polymer $LBLG_{70}$-LW_{30}

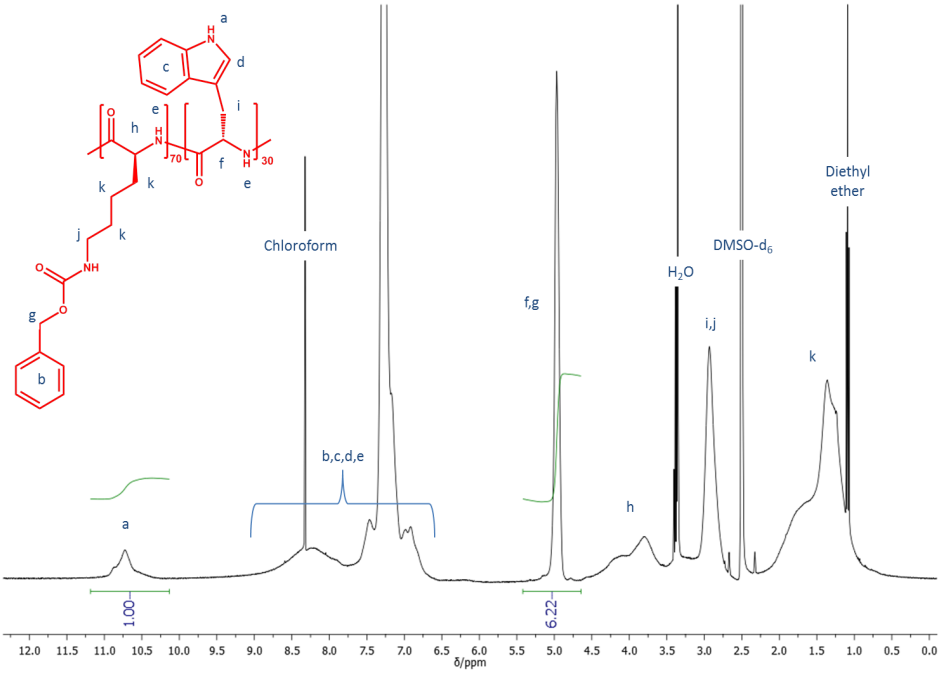

Figure S32: ^{1}H NMR polymer LZK_{70}-LW_{30} in DMSO-d_6.

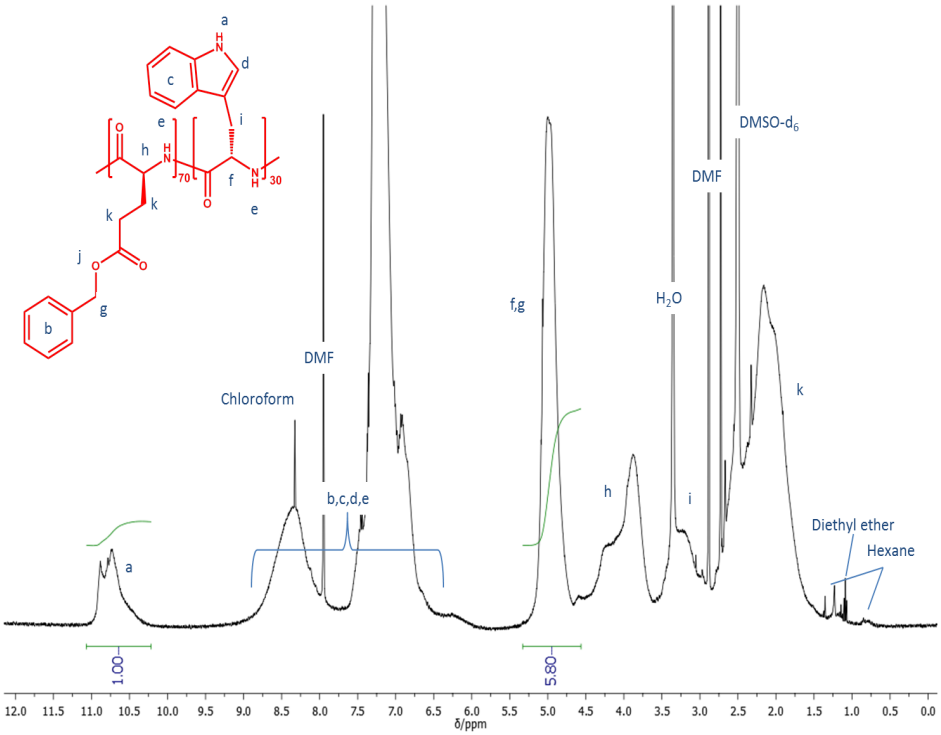

Figure S33: ^{1}H NMR Polymer LBLG$_{70}$-LW$_{30}$ in DMSO-d$_6$.

Example of calculation of polymer composition ^{1}H NMR integration for LZK$_{70}$-LW$_{30}$ (Figure S32): Copolymer composition (Lys$_x$Trp$_y$) was calculated by setting a=1 and y=1. Then, f+g= 6.22, it is assumed that f is also equal to 1 as a=1, then g=5.22 and x=2.61 as this is an integral of two protons. The normalized ratio is x+y=1 and therefore x=0.723 and y=0.277 and if total degree of polymerisation is assumed to be 100, then x=72 and y=28.

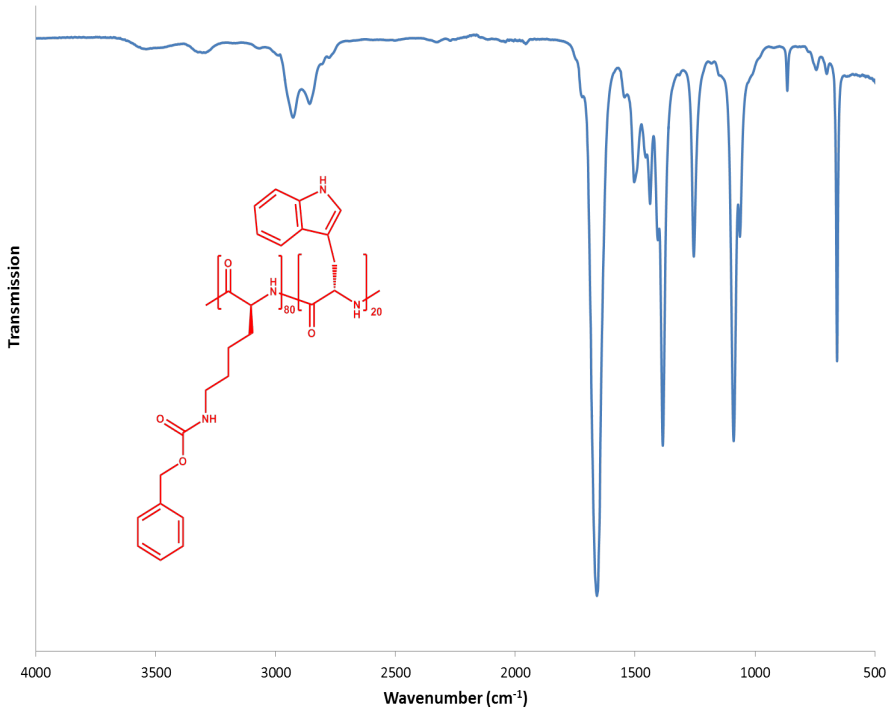

Figure S34: FTIR Polymer LZK_{80}-LW_{20}.

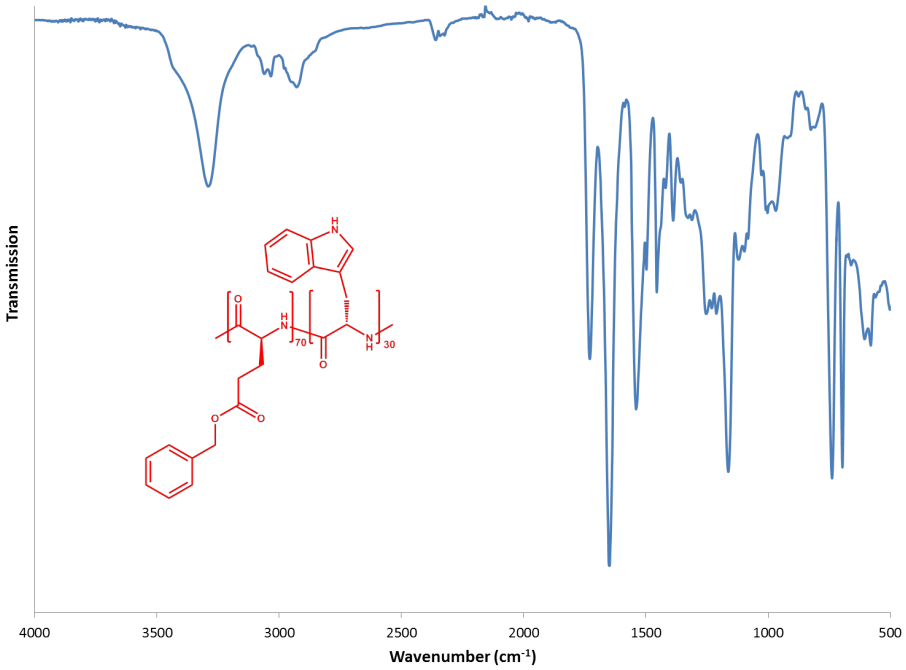

Figure S35: ^{1}H NMR Polymer $LBLG_{70}$-LW_{30}.

Swelling Ratio Testing

Polypeptide hydrogels in a disc-like shape and already swollen in deionized water (coded below with respective polymer composition and molar ratio of HMBT to Trp groups) were then subsequently freeze dried in triplicate for each entry. Typical weight of hydrogels after freeze drying was around 3 mg. These hydrogels were then swollen in a water solution overnight before being removed and carefully swabbed with tissue paper to remove free water and then re-weighed to obtain the swollen weight.[6,7] The degree of swelling (Q) (or swelling ratio) in water was calculated from the equation ($Q = (W_S - W_D)/W_D$, where W_D is the mass of the hydrogel in the dry state (after freeze drying) and W_S is the hydrogel mass in the swollen state (n=3).

Table S1: Water Uptake Ratio of polypeptide hydrogels. *50:50 polymer blend of LK_{70}-st-LW_{30} and DK_{70}-st-DW_{30}

Entry	Molar Ratio HMBT:Trp	Water Uptake Ratio
LK_{35}-LW_{15}	1:2	69 ± 9
LK_{70}-LW_{30}	1:2	41 ± 4
LK_{70}-LW_{30}	1:3	39 ± 3
LK_{70}-LW_{30}	1:4	62 ± 6
LK_{80}-LW_{20}	1:2	121 ± 10
L50-D50*	1:2	43 ± 5

Degradation testing

Three hydrogels for each sample for both hydrolytic and enzymatic degradation were tested. Initial wet weight of each polypeptide hydrogel and vial was determined (in PBS buffer). For the hydrolytic degradation, 2 mL of PBS (pH 7.4) was added to each vial and the samples were placed into a shaker bath set to 37°C. Then at predetermined time points the buffer was carefully removed from the vial and weighed, the buffer replaced and then the sample was returned to the shaker bath at 37°C. The same procedure was used for the enzymatic study using PBS that contained trypsin (28.5 U/mL).

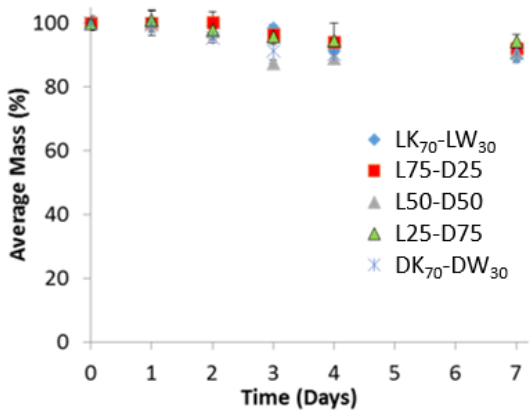

Figure S36: Hydrolytic degradation of pure and blended hydrogels at 37°C (n=3).

Antimicrobial Testing - Preliminary Qualitative Test

A preliminary qualitative test (n=1) was performed to assess the antibacterial activity of the hydrogels based on the JIS Z 2801 (2010)/ ISO 22196 (2011) test.[8,9] Briefly, the prepared hydrogels were sectioned into discs measuring approximately 10 mm ⌀ and 5 mm in height using a fine blade. Discs of parafilm (Sigma Aldrich, Ireland) were cut using an 8 mm ⌀ biopsy punch (Kai Medical, Japan) (total surface area = 201 mm). These were washed in 100% ethanol for 2 hours followed by 2 x washes in Phosphate Buffered Saline (PBS) and dried under laminar airflow. 25 µL of bacterial suspension (*Escherichia coli* CFT 073) was added to the surface of each hydrogel disc and a parafilm disc was placed on top (1 x 10^4 CFU/cm^2). The inoculated hydrogels were incubated at 37°C in a humid environment for 24 hours. After the incubation period, the hydrogels along with the parafilm discs were each added to 5 mL PBS, vortexed briefly, and sonicated for 5 mins. This solution was then serially diluted, plated, and colonies were counted as per the Miles and Misra method.

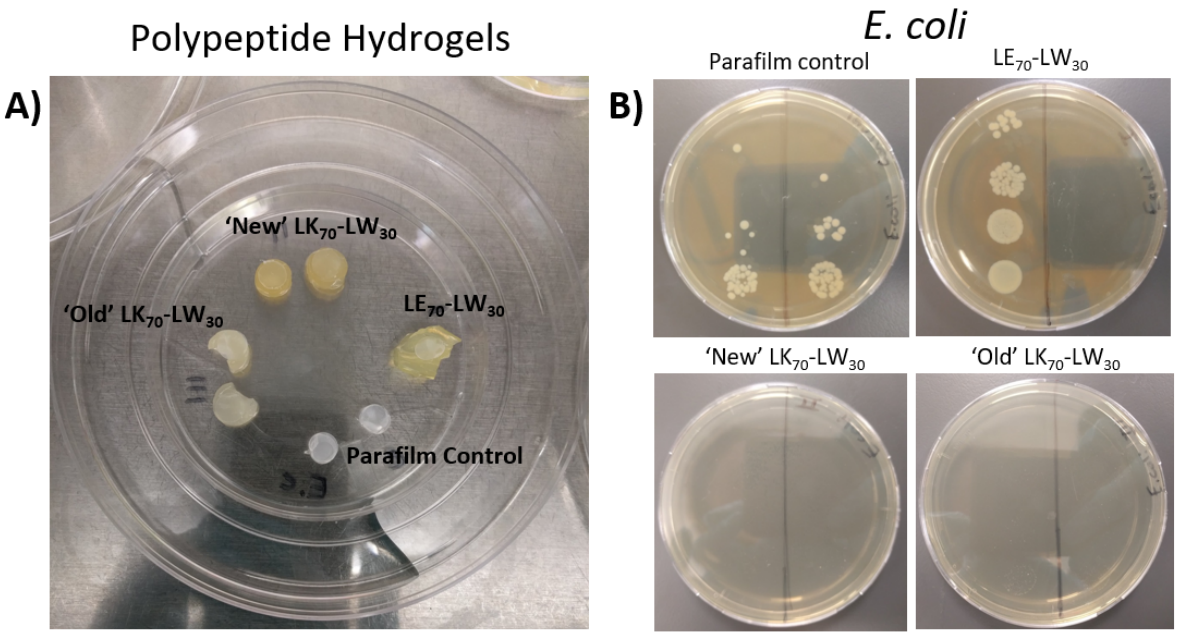

Figure S37: A) Image of four polypeptide hydrogels tested in the preliminary antimicrobial study, including 'New' LK_{70}-LW_{30} that was prepared and tested on the day, 'Old' LK_{70}-LW_{30} was left in water for over 3 months before testing, LE_{70}-LW_{30} (prepared and tested on the day) and a parafilm control. B) Shows the *E.Coli* growth with the hydrogels in comparison to a parafilm control. Please note that only with the parafilm control sample, both sides of the petri dish were tested, with all other samples only the left-hand side (of the line) was tested.

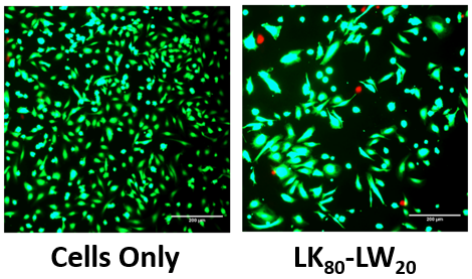

Figure S38: Live/Dead fluorescence imaging of HUVEC cells after 72 hours. Scale bar 200 μm.

References

1 S. B. Hanay, B. Ritzen, D. Brougham, A. A. Dias and A. Heise, *Macromol. Biosci.*, 2017, **17**, 1700016.

2 A. Ghorbani-Choghamarani, Z. Chenani and S. Mallakpour, *Synth. Commun.*, 2009, **39**, 4264–4270.

3 S. B. Hanay, J. O'Dwyer, S. D. Kimmins, F. C. S. de Oliveira, M. G. Haugh, F. J. O'Brien, S.-A. Cryan and A. Heise, *ACS Macro Lett.*, 2018, **7**, 944–949.

4 R. P. Brannigan, S. D. Kimmins, E. Bobbi, S. Caulfield and A. Heise, *Macromol. Chem. Phys.*, 2019, **220**, 1900067.
5 T. Little, J. Meara, F. Ruan, M. Nguyen and M. Qabar, *Synth. Commun.*, 2002, **32**, 1741–1749.
6 J. Wang, C. Liu, X. Lu and M. Yin, *Biomaterials*, 2007, **28**, 3456–3468.
7 A. M. Oelker, S. M. Morey, L. G. Griffith and P. T. Hammond, *Soft Matter*, 2012, **8**, 10887–10895.
8 *JIS Z 2801 (2010) - Antibacterial products - Test for antibacterial activity and efficacy*.
9 *ISO 22196:2011 - Measurement of antibacterial activity on plastics and other non-porous surfaces*.