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Biocompatible polypeptide-based interpenetrating network (IPN) hydrogels with enhanced mechanical properties

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Materials and Methods

All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise noted. Protected amino acid, L-glutamic acid γ-benzyl ester (BLG), was purchased from Carbosynth. Triphosgene was purchased from Fluorochem Ltd. Chloroform-*d*,dimethyl sulfoxide-d_6 and deuterium oxide were all purchased from Apollo Scientific Ltd. Anhydrous tetrahydrofuran (THF), dimethylformamide (DMF) and, ethyl acetate were used as received under inert conditions. NMR spectra were recorded using Bruker Advance 400 spectrometer at 298.19 K (400 MHz). All chemical shifts are reported as δ in parts per million (ppm) and referenced to the residual solvent signal ($CDCl_3$: ^{1}H, δ= 7.26 ppm; ^{13}C, δ= 77.16 ppm, $(CD_3)SO$: ^{1}H, δ= 2.50 ppm; ^{13}C, δ= 39.52, D_2O: ^{1}H, δ = 4.79 ppm).A Perkin-Elmer Spectrum 100 was used for collecting attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra in the spectral region of 650–4000 cm^{-1}. Gel permeation chromatography (GPC) was used to determine the dispersities ($Đ_M$) and molecular weights of polymers. GPC 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, 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 $mL{\cdot}min^{-1}$. The system was calibrated against Agilent Easi-Vial linear poly(methyl methacrylate) (PMMA) standards and analyzed by PSS winGPC UniChrom. All GPC samples were prepared using a concentration of 2 $mg{\cdot}mL^{-1}$, and were filtered through a 0.2 µm millipore filter prior to injection. Compression testing was performed to evaluate the mechanical properties of a single polypeptide network (SN) and double network (IPN) gels. Hydrogels were prepared into cylinders with a diameter of 6 mm and height of 2 mm. A preload force of 0.1 N was set and each test was carried out at a compression velocity of 5 $mm{\cdot}min^{-1}$. Each gel was subject to a point-break test in order to determine the ultimate compressive stress and strain. All compression tests were repeated 3 times and an average of data was taken to find the ultimate compressive stress and strain. These tests were carried out using an M100-1CT Testometric single column universal materials testing machine with a load cell of 100kN. Data was analyzed using Wintest analysis software. Cell studies were carried out to assess cell biocompatibility properties of IPN gels. AlamarBlue® assay was performed to evaluate the effect of the IPN gel on cell metabolic activity and LIVE/DEAD assay was utilized to visualize the distribution of living and dead cells in the IPN gel.

Synthetic procedures

Synthesis of γ-propargyl-L-glutamate

γ-propargyl-L-glutamate was synthesized using a modified method previously reported by Hammond and co-workers.[1] L-Glutamic acid (10 g, 68 mmol) was suspended in propargyl alcohol (300 mL, 5.2 mol) under an inert atmosphere and chlorotrimethylsilane (17 mL, 134 mmol) was added dropwise to the suspension. The suspension was left stirring for 48 hours and over which time the L-glutamic acid gradually dissolved. The crude modified amino acid was recovered by precipitation into cold diethyl ether. To remove any free propargyl alcohol, the product was extensively washed with diethyl ether before being recrystallized from ethanol and diethyl ether, and dried under vacuum to yield the final product γ-propargyl- L-glutamate as a white solid (8.806 g, 47.6 mmol, 70% yield). ^{1}H NMR (400 MHz, D_2O, 293 K): δ = 4.74 (d, $^3J_{H\text{-}H}$ = 2.5 Hz, OCH_2), 4.06 (t, $^3J_{H\text{-}H}$ = 6.7 Hz, CHCH$_2$CH$_2$), 2.94 (t, $^3J_{H\text{-}H}$= 2.6 Hz, alkyne-CH), 2.65 (m, CHCH_2CH$_2$), 2.22 (m, CHCH$_2$CH_2) ppm.^{13}C NMR (400 MHz, D_2O, 293 K): δ = 173.4 (*C*(O)OH), 171.3 (*C*(O)OCH$_2$), 77.6 (CH$_2$*C*H), 75.8 (C*C*H), 52.3 (O*C*H$_2$), 51.8 (*C*H), 29.2 (*C*H$_2$CH$_2$), 24.3 (*C*H$_2$CH) ppm.

γ-Propargyl-L-glutamate NCA (2)

γ-Propargyl-L-glutamate (1.35 g, 7.29 mmol) was dissolved in anhydrous ethyl acetate (50 mL) and the solution heated to reflux under nitrogen. Once the reaction has been refluxing for 30 min, a solution of triphosgene (0.6 g, 2.04 mmol) in anhydrous ethyl acetate(20 mL) was added dropwise to the solution and the reaction was left to reflux for 5 hours. The reaction solution was left to cool to room temperature and was filtered to remove any unreacted γ-propargyl-L-glutamate before being cooled to 4 °C and washed with 50 mL of H_2O, 50 mL of a saturated $NaHCO_3$ solution and 50 mL of brine at 4 °C. The organic phase was then dried over anhydrous $MgSO_4$, filtered, and evaporated to give the final product γ-propargyl-L-glutamate NCA as a viscous oil (0.65 g, 3.08 mmol, 59% yield) ^{1}H NMR (400 MHz, $CDCl_3$, 293 K): δ = 6.98 (s, NH), 4.68 (m, OCH_2), 4.45 (m, CHCH$_2$CH$_2$), 2.60 (m, alkyne CH), 2.51 (m, CHCH_2CH$_2$), 2.22 (m, CHCH$_2$CH_2) ppm.^{13}C NMR (400 MHz, $CDCl_3$, 293 K): δ = 171.5 (*C*(O)CH), 169.3 (*C*(O)OCH$_2$), 152.2 (NH*C*(O)), 77.4 (CH$_2$*C*H), 75.4 (C*C*H), 56.8 (O*C*H$_2$), 52.6 (*C*H), 29.2 (*C*H$_2$CH$_2$), 24.3 ppm (*C*H$_2$CH).

γ-Benzyl-L-glutamate NCA

γ-Benzyl-L-glutamate (3.0 g, 12.6 mmol) was suspended in anhydrous THF (100 mL), and α-pinene (3.34 g, 25.2 mmol) was added. The suspension was heated to reflux and stirred under N_2. After 30 min., a solution of triphosgene (1.86 g, 6.3 mmol) in anhydrous THF (10 mL) was added dropwise. The reaction was stirred for 4 hours under until the solution became clear and all solids disappeared. The reaction was allowed to cool down and filtered before being reduced to 1/3 its volume*in vacuo*. 50 mL ofhexane was added to precipitate the crude NCA before being recrystallized × 3 from hexane and THF and dried under vacuum to yield γ-benzyl-L-glutamate NCA as a pure white solid (2.78 g, 10.6 mmol, 84% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 293 K): δ = 7.36 (m, ArH), 6.40 (s, NH), 5.14 (s, OCH_2), 4.37 (m, CHCH$_2$CH$_2$), 2.30 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, CHCH_2CH_2), 2.21 (m, CHCH_2CH_2) ppm. ^{13}C NMR (400 MHz, $CDCl_3$, 293 K): δ = 173 (*C*(O)CH), 169.2 (*C*(O)OCH$_2$), 151.8 (NH*C*(O)), 135.5 (C$_5$H$_4$*C*H$_2$), 128.8, 128.5, 128.4 (*C*$_5$H$_4$), 67.1 (OCH$_2$), 56.9 (*C*H), 30.03 (*C*H$_2$CH$_2$), 26.4 (*C*H$_2$CH) ppm.

Ring-opening copolymerization of PLG NCA and BLG NCA

PLG NCA (1.85 g, 11.1 mmol) and BLG NCA (2.43 g, 11.1 mmol) were dissolved in dry DMF (40 mL); the solution was degassed and kept under vacuum. The polymerization was initiated by the addition of a single aliquot of hexylamine (0.22 mmol, 29.17 µL) with stirring vigorously. The reaction was left to stir until the NCA monomer had been completely consumed as monitored by ATR FT-IR spectroscopy. Afterwards the polypeptide was precipitated into an excess of cold diethyl ether before being filtered and dried under vacuum to yield Poly (BLG$_{39}$-*co*-PLG$_{31}$) (3) as a white solid (3.6476 g, 0.188 mmol, 85% yield). ^{1}H NMR (400 MHz, d_6- DMSO, 293 K): δ = 7.31 (m, ArH), 5.06 (m, CH_2), 4.64 (m, CH_2), 4.17 (m, CH), 3.92 (m, CH), 3.56 – 3.45 (m, CH), 1.39 (m, CH_2), 1.23 (m, 3CH_2), 0.84 (m, CH_3) ppm

Synthesis of azide terminated multi-arm PEG

Glycerol ethoxylate (*tris*-PEG-OH) M_n = 1,000 g·mol^{-1} (3 g, [OH] 9 mmol) was dissolved in dry DCM (30mL) and cooled on an ice-bath before the addition triethylamine (24 mL, 172.2 mmol). Methanesulfonyl chloride (13.8 mL, 0.18 mol) was added dropwise to the cooled solution and the reaction was left at 0 °C for 3 hours before being allowed to warm to room temperature for 16 hours.

After the reaction was complete, a white precipitate was formed. DCM (135 mL) was added and the precipitate was removed by filtration over a bed of celite. The filtrate was washed with 1MHCl (3 × 100 mL), brine (3 × 100 mL), 1M NaOH (3 × 100 mL) and brine. The organic phase was then dried over anhydrous $MgSO_4$, filtered and evaporated to yield a light brown oil was obtained (1.76 g, 1.37 mmol, 46% yield).
The isolated intermediate, *tris*-PEG-mesylate, (1 g, [OSO_2CH_3] 2.34 mmol) was dissolved in DMF (16 mL) before the addition of NaN_3 (1.82 g, 28.2 mmol). The reaction was brought to 80 °C and allowed to react for 20 hours before the DMF was removed under vacuum. The impure product was redissolved in 100 mL of DCM and washed with saturated NaCl, the organic phase was then dried over anhydrous $MgSO_4$, filtered, and its volume reduced under vacuum and precipitated into cold diethyl ether to yield *tris*-PEG-N_3) as a an oil (128.6 mg, 1.19 mmol, 16% yield). *Tetrakis*-PEG-N_3 M_n = 2,000 g·mol^{-1} was synthesized using a similar procedure. ^{1}H NMR (400 MHz, d_6-DMSO, 293 K): δ = 3.39 (m, 2H, C$H_2$$N_3$), 3.51 (m, 16H, -OC$H_2CH_2$O-), 3.6 (s, 2H, OC$H_2CH_2$ N_3) ppm.

<u>*Tetrakis*-PEG-N_3 M_n = 2,000 g·mol^{-1}</u>: (56 mg, 26.7 µmol, 13% yield) ^{1}H NMR (400 MHz, d_6-DMSO, 293 K): δ = 3.39 (m, 2H, C$H_2$$N_3$), 3.50 (m, 32H, -OC$H_2CH_2$O-), 3.6 (s, 2H, OC$H_2CH_2$$N_3$) ppm.

Hydrogel formation

Polypeptide network

Hydrogels were prepared *via* 'click'-like copper catalyzed alkyne-azide cycloaddition (CuAAC) by dissolving polypeptide – Poly(BLG$_{39}$-*co*-PLG$_{31}$) (3)– (50 mg, 5% w/v) and *bis*-PEG-N_3 (M_w = 1,000 g·mol^{-1}, 143 mg, 174 µMol) in DMF in a glass vial, bath sonication as used to ensure complete dissolution. Copper (I) bromide (2.08 mg, 14.5µMol) catalyst and ascorbic acid (2.4 mg, 13.6 µMol) were added to the degassed solution and then *N,N,N',N'',N''*-pentamethyldiethylenetriamine PMDETA (2.09µLs, 14.5 µMol); after which the vial was shaken to make certain thorough mixing of the components. Finally, the gels were dialyzed against demonized water for 5 days to remove the copper catalyst, PMDETA, and DMF, with frequent water replacement.

<u>Fabrication of interpenetrating double network hydrogels</u>

IPN hydrogels were prepared in a two-stage method. Firstly, the polypeptide CuAAC single network was prepared as previously described and freeze-dried. The lyophilized material was then swollen in a solution of PEG dithiol (M_w = 2,000 g·mol^{-1}, 5%w/v), pentaaerythritoltetraacrylate - (at a target feed ratio thiol: acrylate 1:0.5, 1:1, 1:2) in DMF and photoinitiator benzophenone (1% w/v), in a glass vial. Once the gels were swollen to maximum capacity they were exposed to UV radiation for 60 min. Photoreactions were performed using a handheld UV lamp UVP-100AP, 254/365 nm, 6 watt.

Compression Analyses

Hydrogels were prepared into cylinders with a diameter of 6 mm and height of 2 mm. A preload force of 0.1 N was set and each test was carried out at a compression velocity of 5 mm.min^{-1}. Each gel was subject to a point-break test in order to determine the ultimate compressive stress, Young's modulus and fracture strain and hysteresis to determine the ultimate tensile strength at 2% strain. All compression tests were repeated 5 times and an average of data was taken. These tests were carried out using an M100-1CT Testometric single column universal materials testing machine with a load cell of 100 kN. Data was analyzed using Wintest analysis software

Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out on a Zeiss Ultra Plus Scanning Electron Microscope. All samples were freeze dried prior to sputter coating. The coating was performed on a 208HR Cressington Sputter Coater using a Gold/Palladium target for approximate 30 - 40 seconds as the sample is insulating and this reduces charging effects. Pore sizes were determined using ImageJ software and a fixed scale bar – carried out in triplicate

Swelling studies

Hydrogels were fabricated as stated in the above procedure. To determine the degree of swelling (DS) the hydrogels were lyophilized and their weights (W_d) recorded. Then placed in PBS solution and at preset time intervals, the hydrogel was removed, gently blotted dry to remove excess surface water and their weights (W_s) recorded. All measurements were repeated in triplicate. The degree of swelling was expressed as:

$$DS(\%) = \frac{W_s - W_d}{W_s} \times 100$$

Small-angled X-ray scattering

Synchrotron-basedsmall-angle X-ray scattering (SAXS) measurements were performed on BM26B (DUBBLE) at the European Synchrotron Radiation Source, Grenoble, France. The sample to SAXS detector distance was 3 m using a wavelength λ = 0.9994 Å. A Dectris-Pilatus 1 M detector with a resolution of 981 × 1043 pixels and a pixel size of 172 × 172 µm was employed to record the 2D SAXS scattering patterns. Standard corrections for sample absorption and background subtraction have been performed.The data were normalized to the intensity of the incident beam (in order to correct for primary beam intensity fluctuations) and were corrected for absorption, background scattering. The scattering pattern from AgBe was used for the calibration of the wavenumber ($q = 4\pi sin\theta/\lambda$) scale of the scattering curve.All the sample wereswollen inside water for 24 hours before the measurement, and placed in a capillary (*d* =2 mm) and kept at 20 °C using a Linkam stage SAXS data frames were acquired each 5 min during the process.

SAXS profile fitting:

The SAXS curves of the single network were modeled with the following function:

$$I(q) = \frac{8\pi\langle\eta^2\rangle/\xi}{a^2 - 2bq^2 + q^4} + \frac{I_{OZ}}{1 + (\xi_{Oz}q)^2} + I_{bkg}$$

Where $a^2 = (k^2 + 1/\xi^2)$, $b = k^2 - 1/\xi^2$,$d = 2\pi/k$ and represents the average distance between crosslinking junctions, η^2 squared scattering length density contrast, ξ correlation length in Teubner-Strey model, I_{OZ} is the asymptotic value of Ornstein-Zernike intensity at $q \longrightarrow 0$, ξ_{Oz} is the correlation length of OZ and indicates the size of dangling chain.

^{1}H NMR diffusometry

All NMR experiments were performed on 11.4T Bruker Avance III NMR spectrometer equipped with a four channel (^{1}H, ^{13}C, ^{15}N, ^{2}H) 4 mm CMP MAS probe, fitted with actively shielded gradient. All samples were swollen in D2O, and kept locked by deuterium throughout the whole experiment. The spinning rate of the rotor was kept at 10 kHz. The ^{1}H DOSY (Diffusion Ordered Spectroscopy) experiment was performed using the pulsed-field gradient (PFG) stimulated-echo (STE) sequence with phase cycling of radio frequency pulses and a spoil gradient to suppress artifacts. A total of 32 increments were collected for the DOSY dimension with 32 transients collected for each increment. The gradient was ramped in a linear fashion from 2 to 98% of full the gradient strength 30G/cm/A. Two sine shaped gradient pulses of 1.1 ms duration was used for both coding and decoding. Typical acquisition parameters are as following: 1) 16k acquisition points 2) 2.0s recycle delay and 200ms diffusion time 3) 10kHz spectral width. All the NMR measurements were carried out at 20 °C

Cell metabolic activity assay

Human Mesenchymal Stem Cells (hMSCs) were expanded under normal conditions 37 °C 5 % CO2. To evaluate the effect of IPN gels on cell metabolic activity, pre-formed hydrogels (with a diameter of 6 mm and height of 2 mm) were placed in 24-well Corning Costar® cell culture plates. hMSCs were seeded directly on to the hydrogel cylinders (n=3) at a seeding density of 3 x 106hMSCs per hydrogel portion. All IPN hydrogels were washed in a solution of PBS for 24 hrs prior to any testing and cell analysis, to ensure excess material was removed and the networks environment resembles an *in vivo*

setting (i.e. aqueous conditions). 1 ml of normal hMSC medium (Dulbecco's Modified Eagle's Medium D6046, supplemented with 10 % Fetal Bovine Serum and 1 % penicillin/streptomycin) was added to the wells and the plates were placed in the incubator at 37 °C, 5 % CO2. On days 2, 5, 7 and 10 the medium was removed from each well and the gels were washed with Phosphate Buffered Saline. 100µL of alamarBlue Cell Viability solution (ThermoFisher) was added to 900µL of fresh medium in each well. The plate was placed back in the incubator for two hours. Following this, the alamarBlue/medium solution from each well was placed in separate wells of a black 96 well plate. Fluorescence was measured on a Varioskan plate reader with excitation at 560 nm and emission at 590 nm.

Live/dead viability assay

Live/dead staining was utilized to visualize the distribution of living and dead cells in the IPN gel. Following ten days of culture, medium was removed from wells containing the cell-seeded hydrogels. The gels were washed three times with PBS. 1 ml of Live/Dead solution (2µMcalcein AM, 4µM ethidium homodimer) was added to each well and incubated for 15 minutes at room temperature, protected from light. Fluorescence images were taken using a Leica microscope and merged using ImageJ software.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 26 statistical analysis software package. T-tests were conducted between SN1 and IPN 1-3. A value of $P < 0.05$ was considered statistically significant.

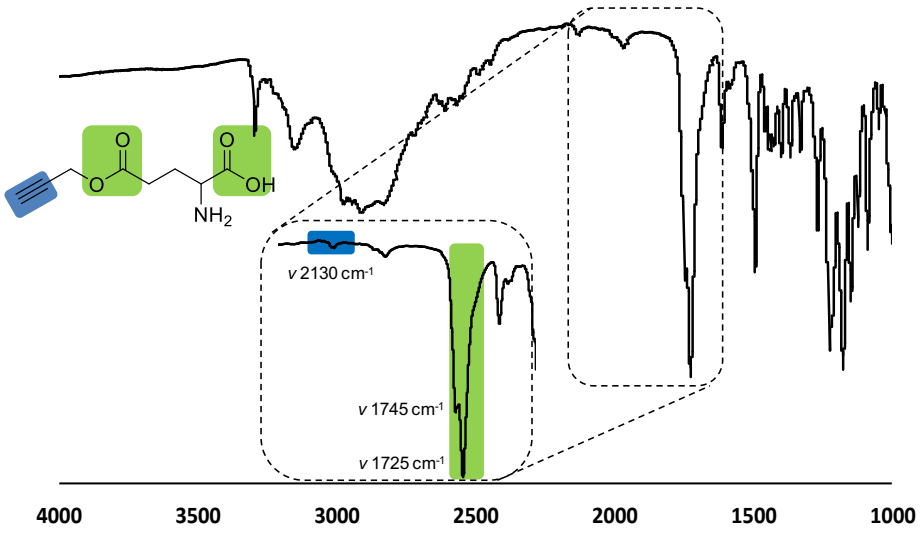

Figure S1. FT-IR spectrum of γ-propargyl-l-glutamate (PLG).

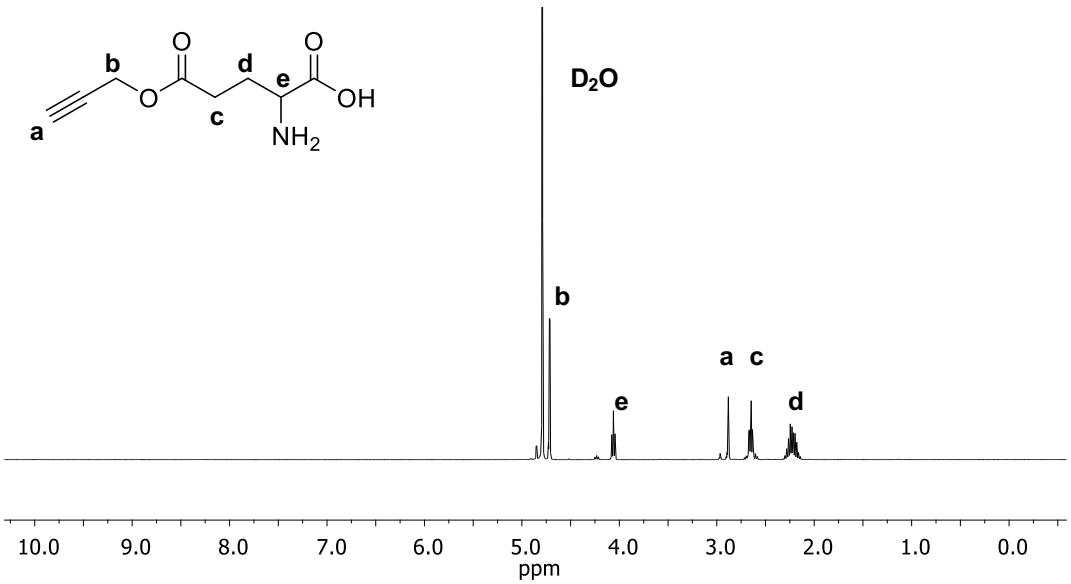

Figure S2. ^{1}H NMR spectrum of PLG (400 MHz, 298 K, D_2O)

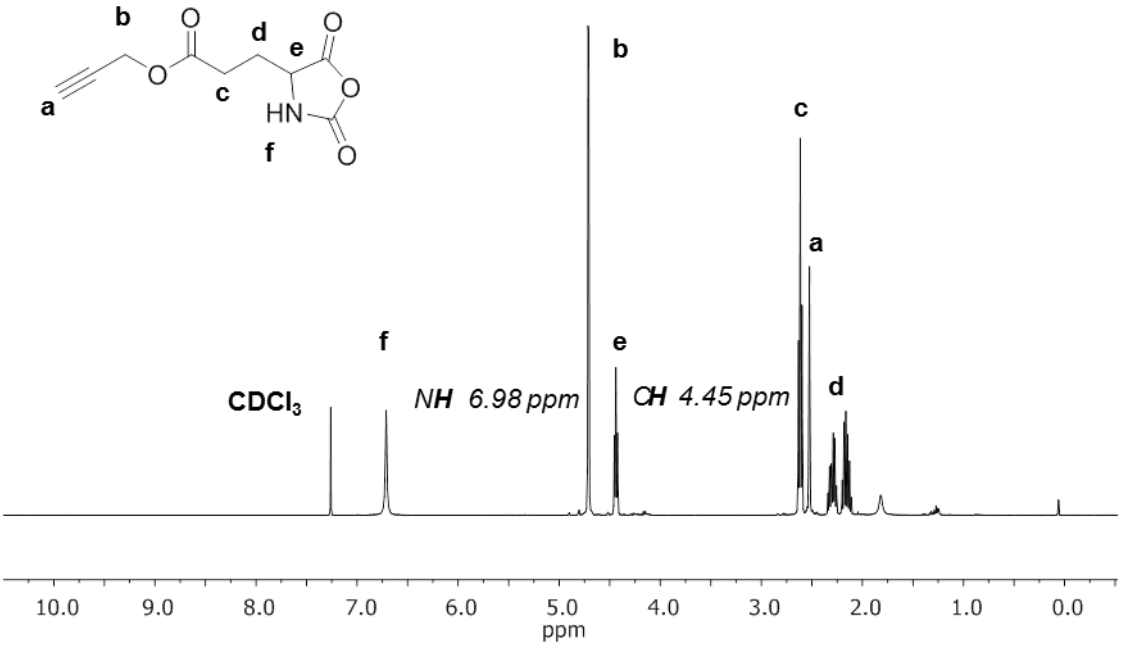

Figure S3. ^{1}H NMR spectrum of γ-propargyl-l-glutamate N-carboxyanhydride (PLG NCA) (400 MHz, 298 K, $CDCl_3$).

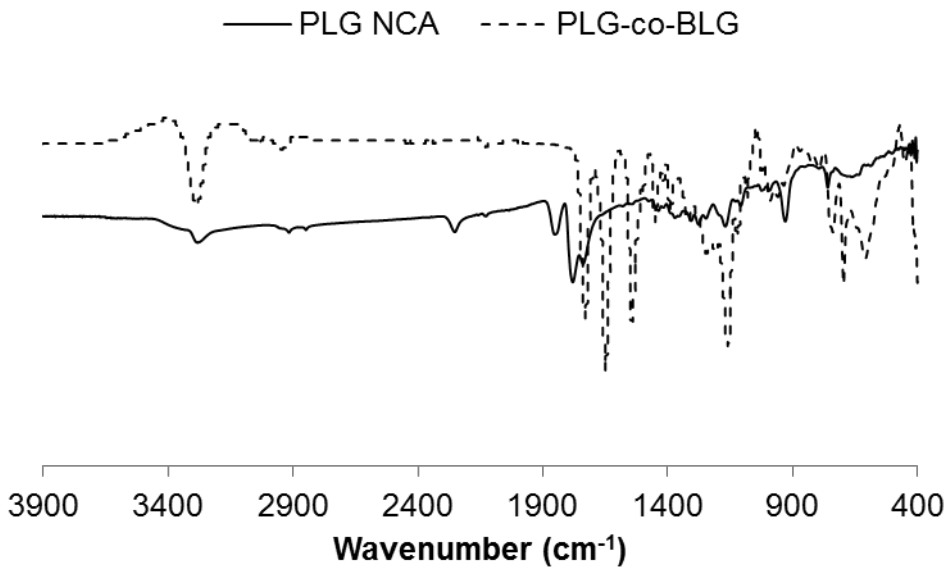

Figure S4. FT-IR spectrum of PLG NCA monomer and resultant poly(γ-propargyl-l-glutamate-co-γ-benzyl-l-glutamate) (P(PLG-co-BLG), (3)).

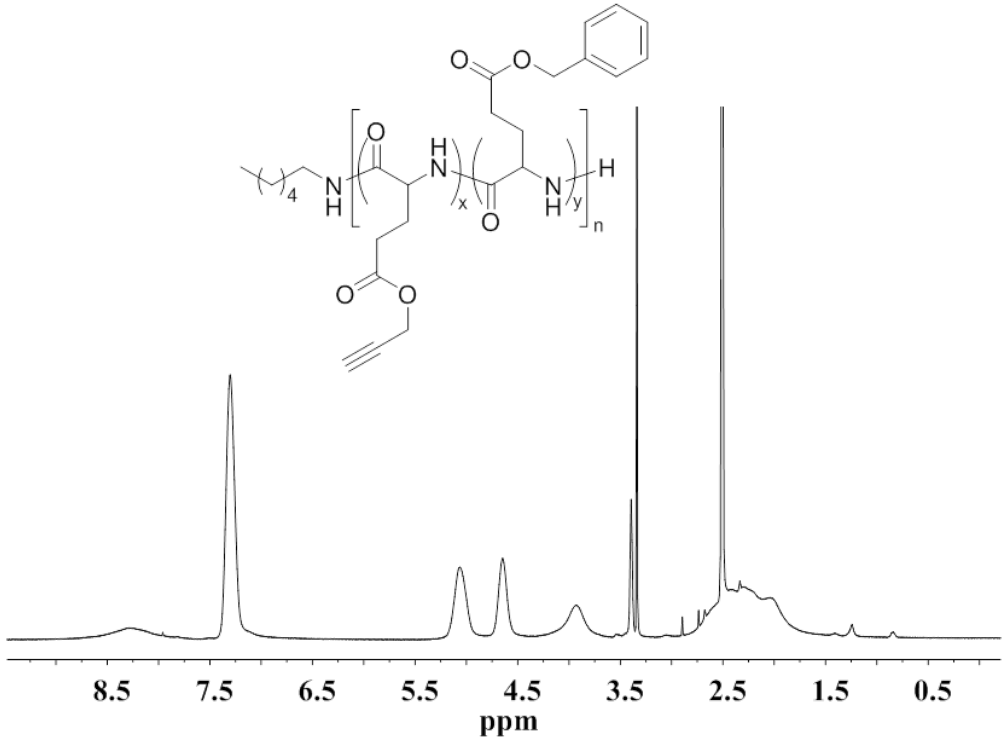

Figure S5. 1H NMR spectrum of P(PLG-co-BLG) (3) (400 MHz, 298 K, d6-DMSO)

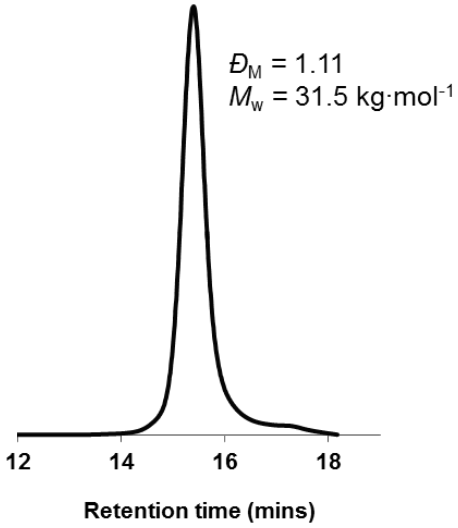

Figure S6. GPC trace of P(PLG-coBLG) (conducted in HFiP at a flow rate of 1.0 mL·min^{-1} against linear poly(methyl methacrylate) (PMMA) standards).

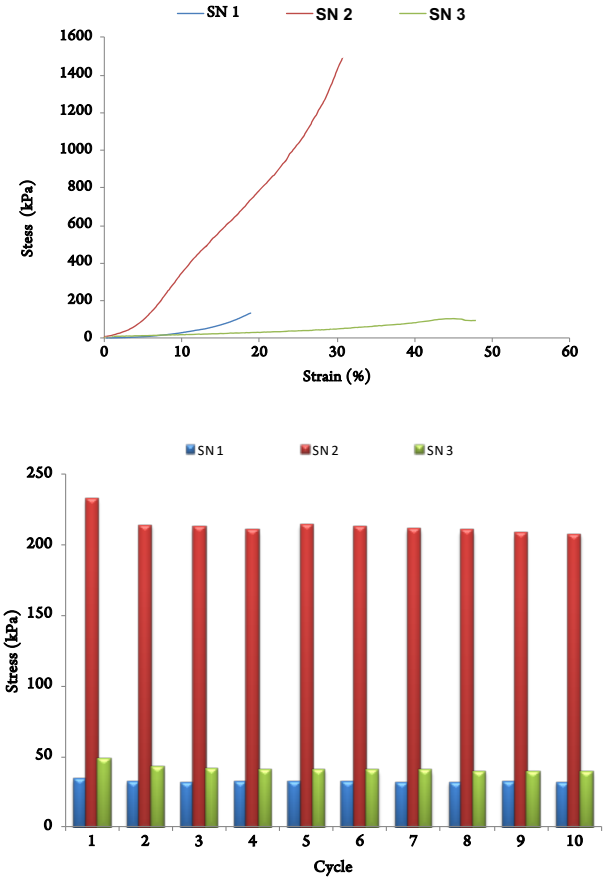

Figure S7. (top) Stress-strain curves and (bottom) hysteresis profile of single network peptide-based hydrogel synthesized from bis-PEG-N3 (M_w = 1 kDa; SN1), tris-PEG-N3 (Mw = 1 kDa; SN2) and tetrakis-PEG-N3 (M_w = 2 kDa; SN3).

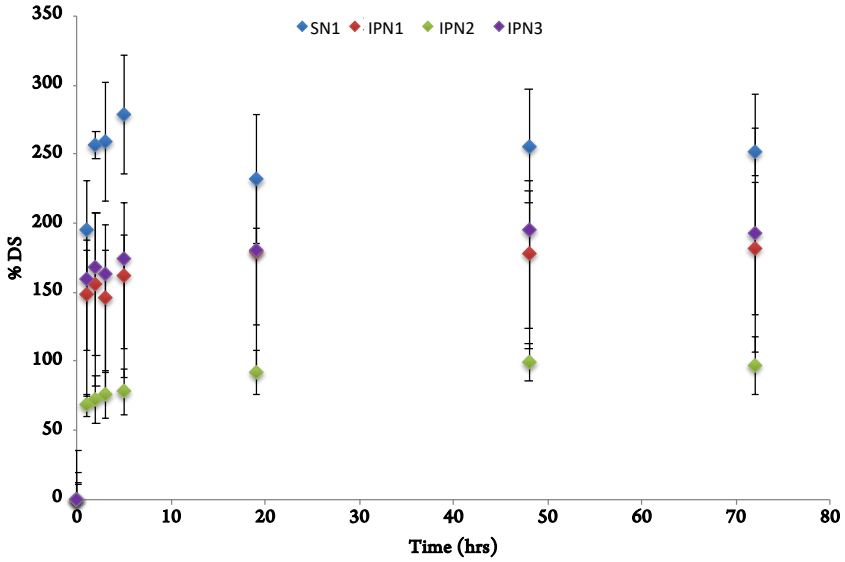

Figure S8. Degree of swelling of single network SN1 and double networks IPN1-3.

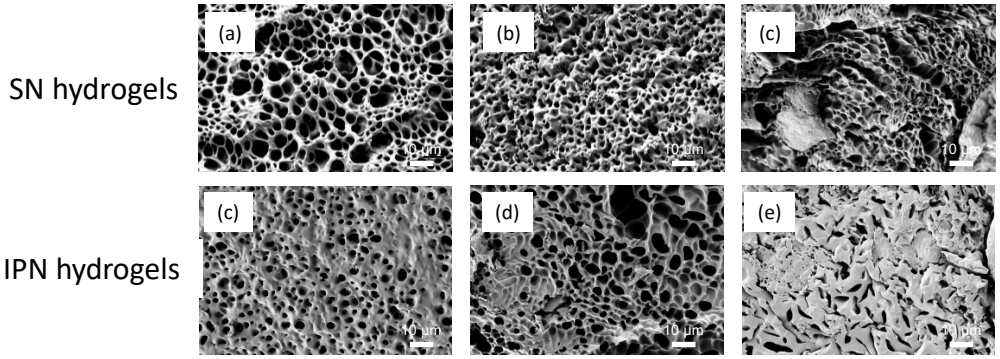

Figure S9. Scanning electron microscopy (SEM) cross-sectional images single network polypeptide-based hydrogels synthesized from (a) bis-PEG-N_3, (b) tris-PEG-N_3, (c) tetrakis-PEG-N_3 and IPN polypeptide-based hydrogels (d) IPN1, (e) IPN2 and (f) IPN3.

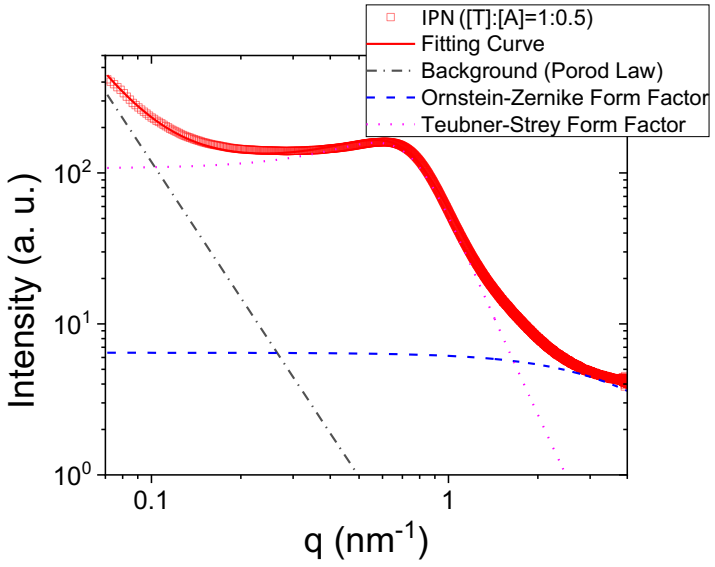

Figure S10. Contributions to the model function for sample IPN1. Square symbols: experimental data; Lines: full fitting function and contributions

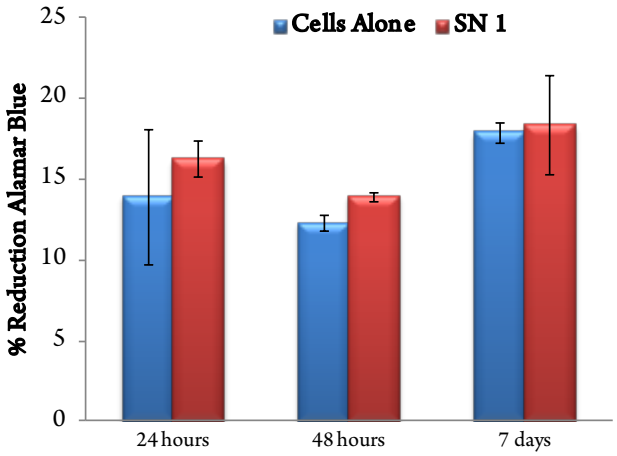

Figure S11: Metabolic activity of human Mesenchymal Stem Cells (MSCs) seeded onto SN1 hydrogels, compared to metabolic activity of cells not exposed to the gels (n=3; error bars represent standard deviation).

References

1 1. A. C. Engler, A. Shukla, S. Puranam, H. G. Buss, N. Jreige and P. T. Hammond, *Biomacromolecules*, 2011, **12**, 1666-1674.