Supporting Information

Controlling Ligand Density and Viscoelasticity in Synthetic Biomimetic Polyisocyanide Hydrogels for Studying Cell Behaviours: The Key to Truly Biomimetic Hydrogels

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List of abbreviations

σ_c	Critical stress
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
Et_3N	Triethylamine
FTIR	Fourier-transform infrared
G_0	Bulk stiffness
G'	Storage modulus
G''	Loss modulus
GRGDS	Peptide sequence Gly-Arg-Gly-Asp-Ser
K'	Differential modulus
LC–MS	Liquid chromatography-mass spectrometry
LCST	Lower critical solution temperature
M_{co}	Molecular weight of the average repeating copolymer subunit
MeCN	Acetonitrile
M_v	Viscosity average molecular weight
PFA	Paraformaldehyde
PIC	Polyisocyanopeptide
SANS	Small-angle neutron scattering
SDD	Sample-to-detector distance
SPAAC	Strain-promoted azide-alkyne cycloaddition
SSD	Source-to-sample
$T_{1/2}$	Stress relaxation half-life
T_{gel}	Gelation temperature

1 Peptide-linker Synthesis

1.1 Non-aqueous methodology

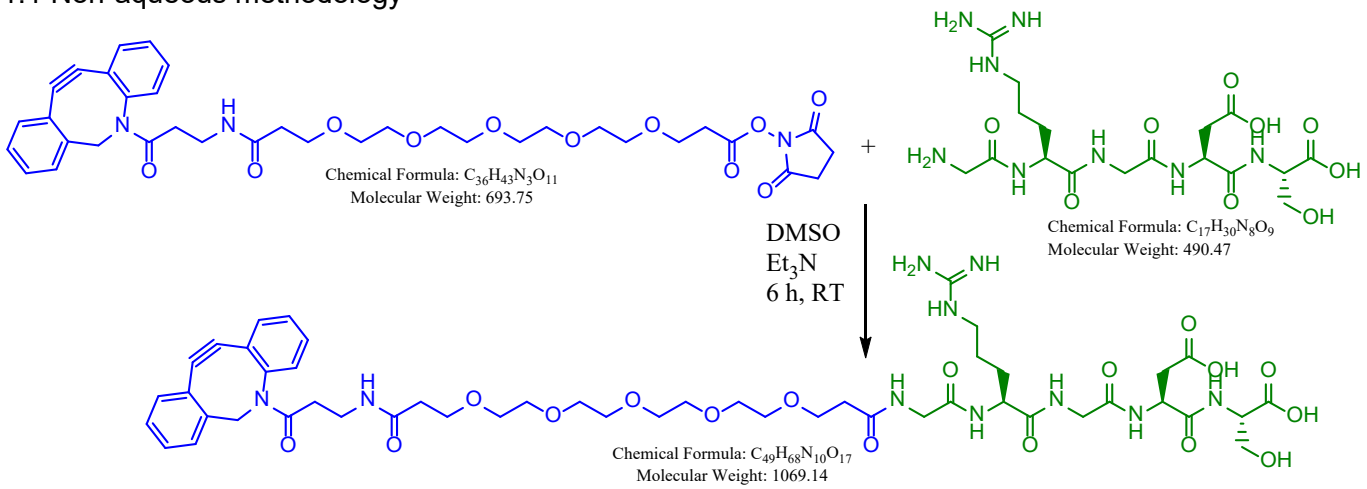

Figure S1: GRGDS-DBCO peptide-linker synthesis in non-aqueous conditions

The GRGDS-DBCO linker was synthesised by combining DBCO-PEG5-NHS (0.055 g, 0.079 mmol) with GRGDS (0.054 g, 0.111 mmol) in anhydrous DMSO (4 mL) and triethylamine (Et_3N, 0.05 mL, 0.396 mmol). The reaction mixture was stirred at room temperature for 4 h protected from light. The efficacy of the peptide-linker synthesis and the ratio of product to starting material was determined by liquid chromatography - mass spectrometry (LC-MS) conducted using a Dionex HPLC module equipped with a 4.6 x 50 mm C18 Kintetex column and UV-Vis detector connected to a ThermoFisher TSQ Quantum Ultra QqQ MS. The crude mixture was separated through the C18 column with an eluent system starting at 95% A: 5% B and moving through a gradient to 100% B (A = 0.2% formic acid in H_2O, B = 0.2% formic acid 80% MeCN in H_2O). m/z: calculated for $C_{49}H_{68}N_{10}O_{17}$ 1068.48, found 1069.69 ($[M + H]^+$).

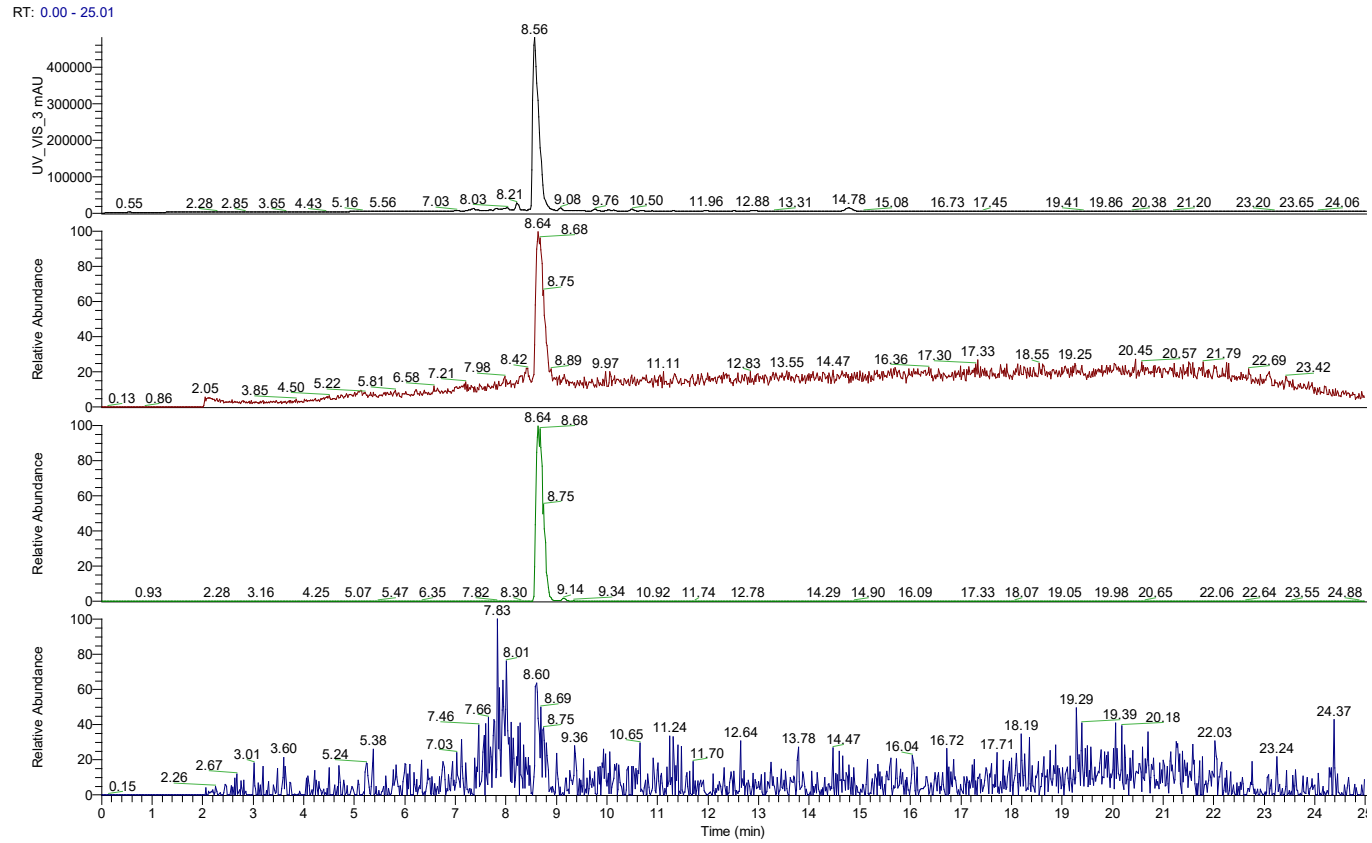

Figure S2: HPLC chromatogram of the crude GRGDS-DBCO peptide-linker reaction mixture synthesised in anhydrous DMSO. UV-Vis detector channel 310 nm, total ion count chromatogram, ion count chromatogram for product (mass range 1068-1070 m/z) and, ion count chromatogram for unreacted reagent (mass range 693-695 m/z), top to bottom respectively. Eluent system; A = 0.2% formic acid in H_2O, B = 0.2% formic acid, 80% MeCN in H_2O. Gradient eluent starting at 95% A 5% B to 100% B over 25 minutes.

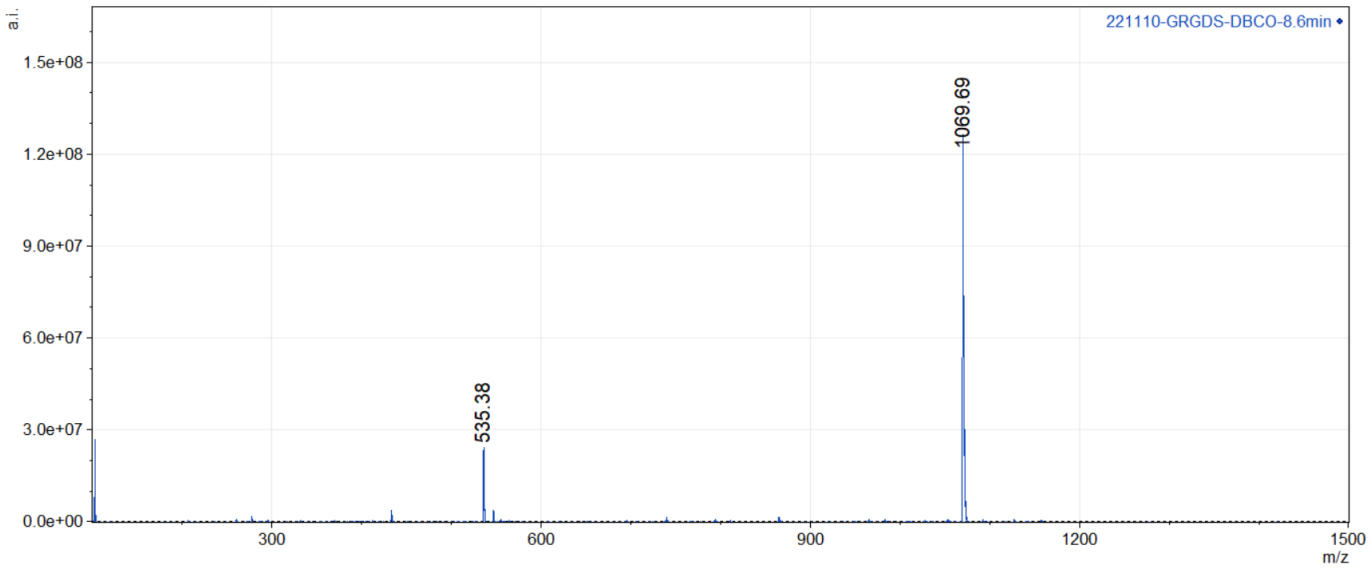

Figure S3: Mass spectra snapshot of HPLC chromatogram for crude GRGDS-DBCO reaction mixture synthesised in anhydrous DMSO at 8.64 min retention time. GRGDS-DBCO linker: m/z: calculated for $C_{49}H_{68}N_{10}O_{17}$ 1068.48, found 1069.69 ($[M + H]^+$).

1.2 Aqueous methodology

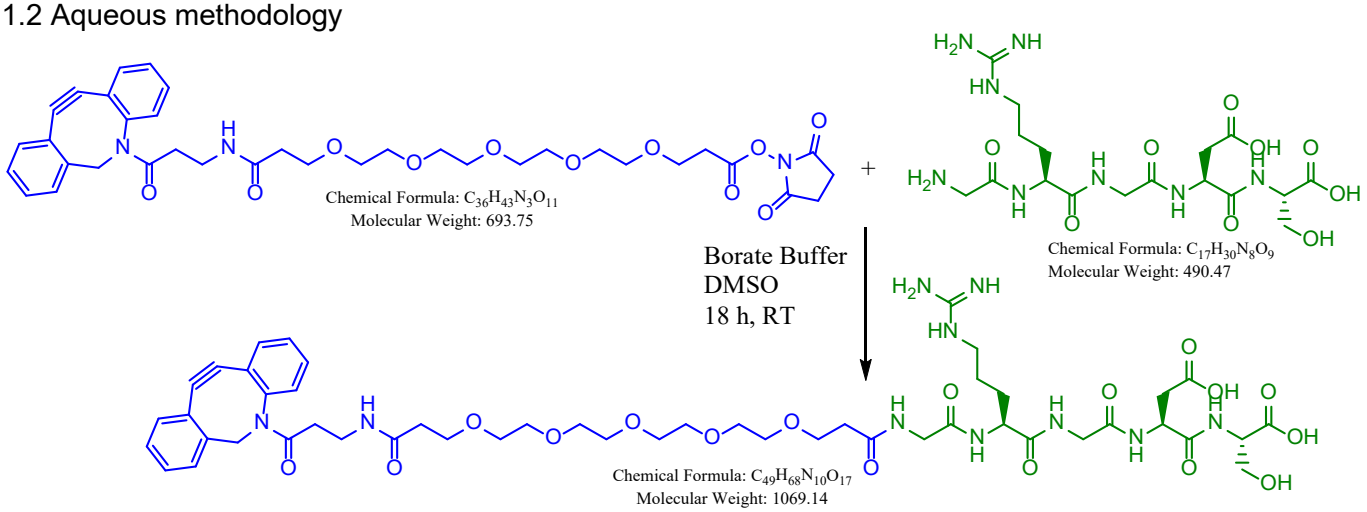

Figure S4: GRGDS-DBCO peptide-linker synthesis scheme in the commonly reported aqueous conditions

GRGDS (0.009 g, 0.019 mmol) was dissolved in borate buffer (1.50 mL, 6 mg mL^{-1}) and combined with a solution of DBCO-PEG5-NHS (0.010 g, 0.015 mmol) dissolved in DMSO (1.66 mL, 6 mg mL^{-1}). The reaction mixture was stirred at room temperature for 17 h protected from light. The efficacy of the peptide-linker synthesis and the ratio of product to starting material at 17 h was determined by liquid chromatography - mass spectrometry (LC-MS) with a Dionex HPLC module equipped with a 4.6 x 50 mm C18 Kintetex column and UV-Vis detector connected to a ThermoFisher TSQ Quantum Ultra QqQ MS. The crude mixture was separated through the C18 column with an eluent system starting at 95% A: 5% B and moving through a gradient to 100% B (A = 0.2% formic acid in H_2O, B = 0.2% formic acid 80% MeCN in H_2O). m/z: calculated for $C_{49}H_{68}N_{10}O_{17}$ 1068.48, found 1069.52 ($[M + H]^+$).

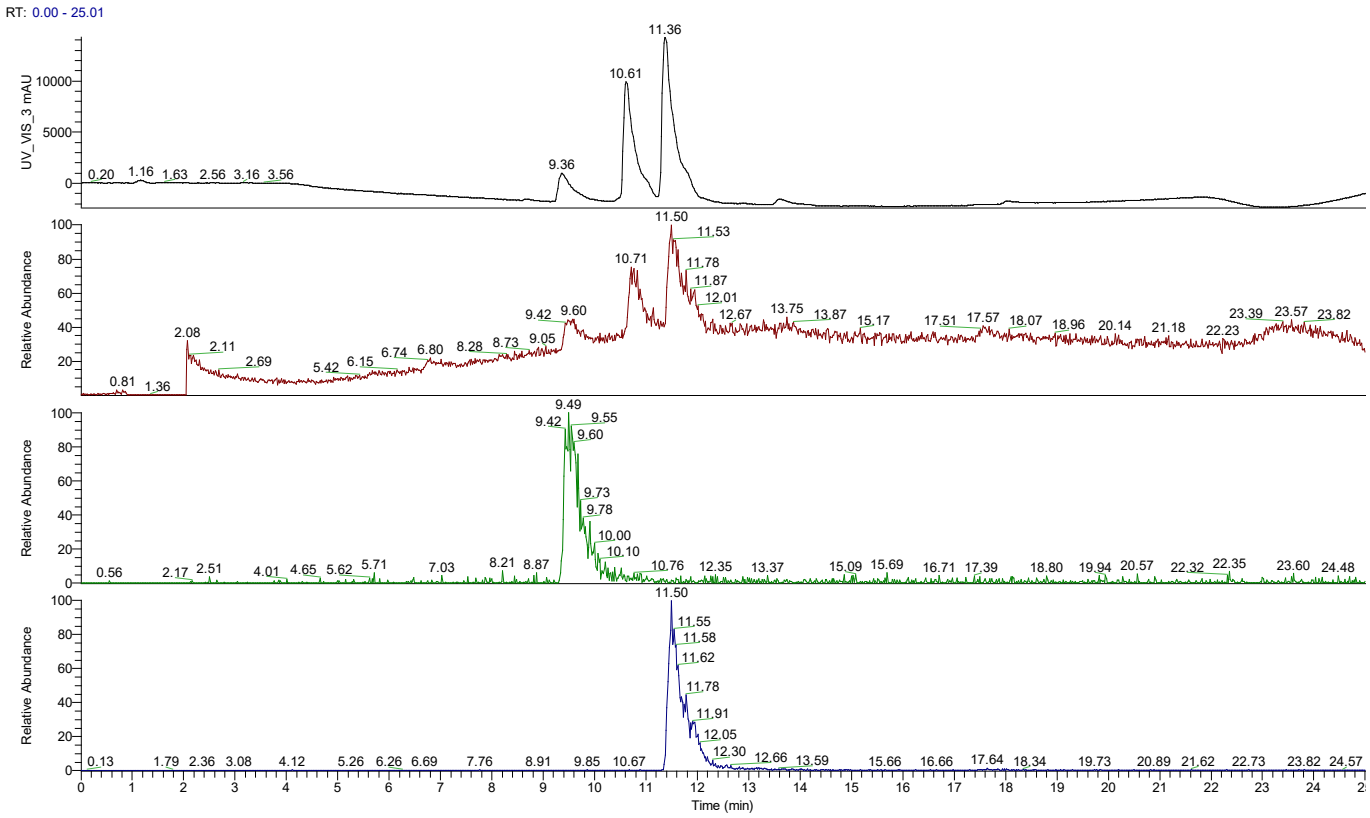

Figure S5: HPLC chromatogram of the crude GRGDS-DBCO reaction mixture synthesised via the aqueous methodology with borate buffer. UV-Vis detector channel 310 nm, total ion count chromatogram, ion count chromatogram for product (mass range 1068-1070 m/z) and, ion count chromatogram for unreacted reagent (mass range 693-695 m/z), top to bottom respectively. Eluent system; A = 0.2% formic acid in H_2O, B = 0.2% formic acid 80% MeCN in H_2O. Gradient eluent starting at 95% A 5% B to 100% B over 25 minutes.

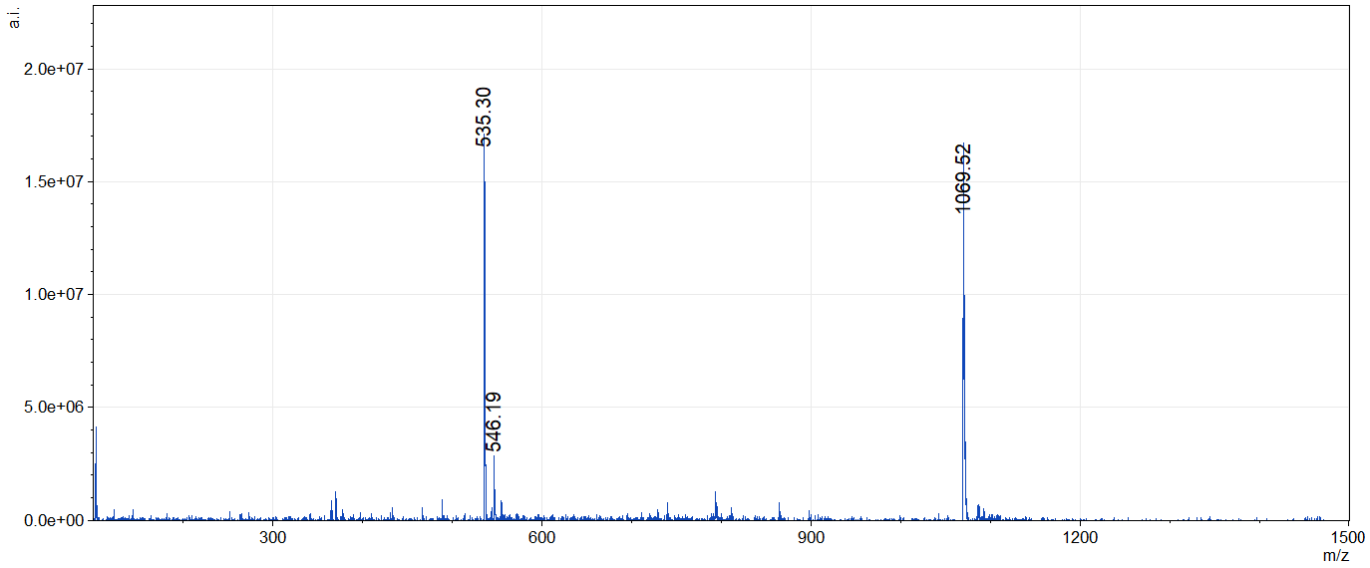

Figure S6: Mass spectra snapshot of HPLC chromatogram for crude GRGDS-DBCO reaction mixture synthesised in borate buffer at 9.5 min retention time. GRGDS-DBCO linker: m/z: calculated for $C_{49}H_{68}N_{10}O_{17}$ 1068.48, found 1069.52 ($[M + H]^+$).

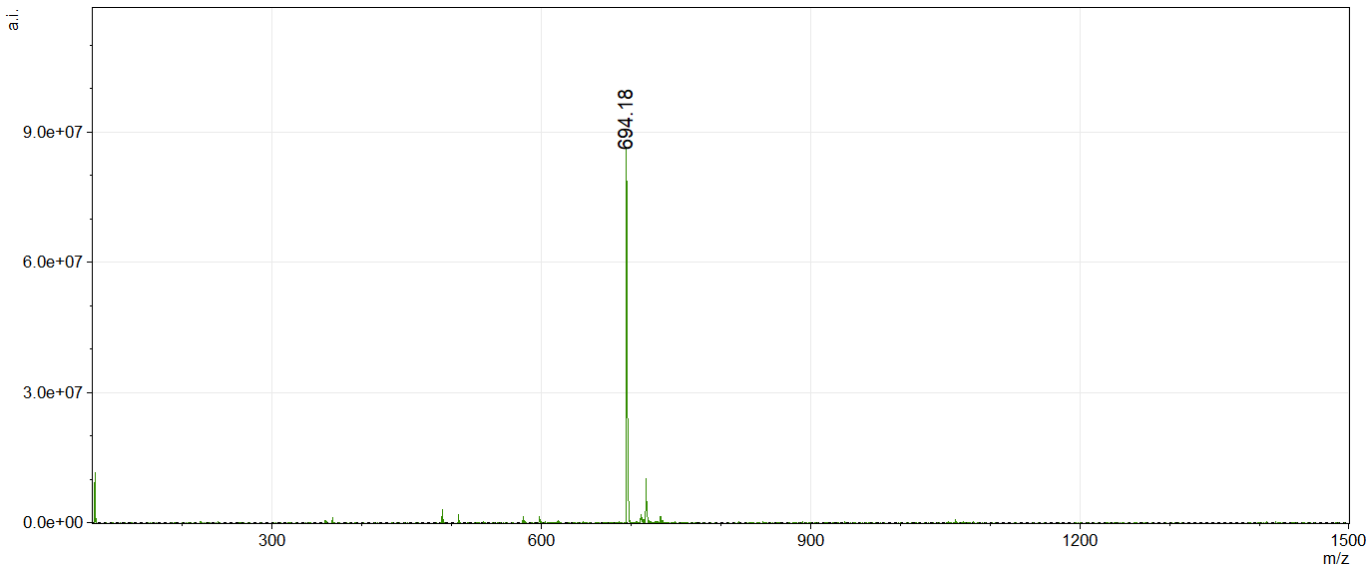

Figure S7: Mass spectra snapshot of HPLC chromatogram for crude GRGDS-DBCO reaction mixture synthesised in borate buffer at 11.5 min retention time. DBCO-PEG5-NHS: m/z: calculated for $C_{36}H_{43}N_3O_{11}$ 693.29, found 694.18 ($[M + H]^+$).

Synthesis and Characterisation of Polyisocyanides.

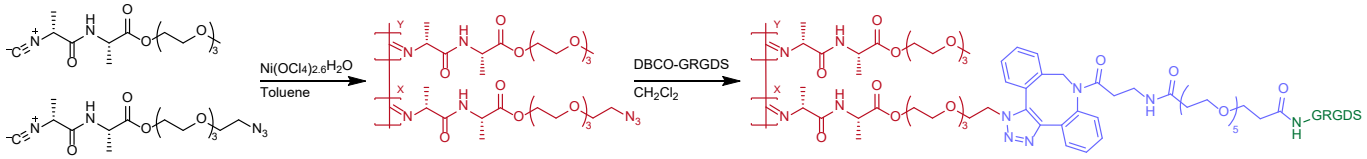

Figure S8: Synthesis scheme for peptide-decorated polyisocyanide polymers

All polymers were synthesised by loading the methoxy isocyanide monomer and azide isocyanide monomer as required, into a dried 100 mL round bottom flask with a B29 size socket. The flask was equipped with a sufficiently large magnetic stir bar and vacuum-dried prior dissolving in toluene. An inert atmosphere was generated through 3 successive vacuum/argon cycles before the isocyanide monomers were dissolved in anhydrous toluene. A stock solution of $Ni(ClO_4)_2.6H_2O$ was prepared in a 10 mL volumetric flask with 1 mL of anhydrous EtOH and 9 mL of anhydrous toluene. The appropriate amount of $Ni(ClO_4)_2 \cdot 6H_2O$ catalyst was combined with the monomer solution to initiate the polymerisation using a 500 µL gas-tight syringe. The polymerisation mixture was stirred under an argon atmosphere for approximately 48 h. IR analysis confirmed the completion of the polymerisation through consumption of the isocyanide peak (2142 cm^{-1}). Once the polymerization was confirmed complete, the solvent was removed *in vacuo*. The crude polymer was redissolved in CH_2Cl_2 and precipitated three times against cold diisopropyl ether collected over a sintered funnel. Purified polymer was dried on a rotary evaporator then stored under reduced pressure overnight, yielding a beige fibrous solid.

PIC-0: 2-(2-(2-methoxyethoxy)ethoxy)ethyl ((R)-2 isocyanopropanoyl)-L-alaninate (1.16 g, $3.68x10^{-3}$ mol) was dissolved in anhydrous toluene (30 mL) before adding the $Ni(ClO_4)_2 \cdot 6H_2O$ catalyst solution ($2.09x10^{-6}$ mol). Yield 1.00 g (86.2%).

PIC-0.5: Isocyanide monomers 2-(2-(2-methoxyethoxy)ethoxy)ethyl ((R)-2 isocyanopropanoyl)-L-alaninate (1.18 g, $3.73x10^{-3}$ mol) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl ((R)-2-isocyanopropanoyl)-L-alaninate (0.007 g, $1.86x10^{-5}$ mol) were combined with anhydrous toluene (30 mL) and $Ni(ClO_4)_2 \cdot 6H_2O$ catalyst solution ($2.78x10^{-6}$ mol). Yield 1.00 g (84.4%).

PIC-1.0: Isocyanide monomers 2-(2-(2-methoxyethoxy)ethoxy)ethyl ((R)-2 isocyanopropanoyl)-L-alaninate (1.12 g, $3.55x10^{-3}$ mol) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl ((R)-2-isocyanopropanoyl)-L-alaninate (0.015 g, $4.03x10^{-5}$ mol) were combined with anhydrous toluene (30 mL) and $Ni(ClO_4)_2 \cdot 6H_2O$ catalyst solution ($1.80x10^{-6}$ mol). Yield 0.866 g (76.4%).

PIC-3.0: Isocyanide monomers 2-(2-(2-methoxyethoxy)ethoxy)ethyl ((R)-2 isocyanopropanoyl)-L-alaninate (1.11 g, $3.50x10^{-3}$ mol) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl ((R)-2-isocyanopropanoyl)-L-alaninate (0.04 g, $1.08x10^{-4}$ mol) were combined with anhydrous toluene (30 mL) and $Ni(ClO_4)_2 \cdot 6H_2O$ catalyst solution ($1.81x10^{-6}$ mol). Yield 0.974 g (84.6%).

PIC-6.0: Isocyanide monomers 2-(2-(2-methoxyethoxy)ethoxy)ethyl ((R)-2 isocyanopropanoyl)-L-alaninate (1.13 g, $3.56x10^{-3}$ mol) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl ((R)-2-isocyanopropanoyl)-L-alaninate (0.084 g, $2.26x10^{-4}$ mol) were combined with anhydrous toluene (30 mL) and $Ni(ClO_4)_2 \cdot 6H_2O$ catalyst solution ($1.89x10^{-6}$ mol). Yield 0.933 g (77.0%).

Molecular Weight Characterisation of Polyisocyanides

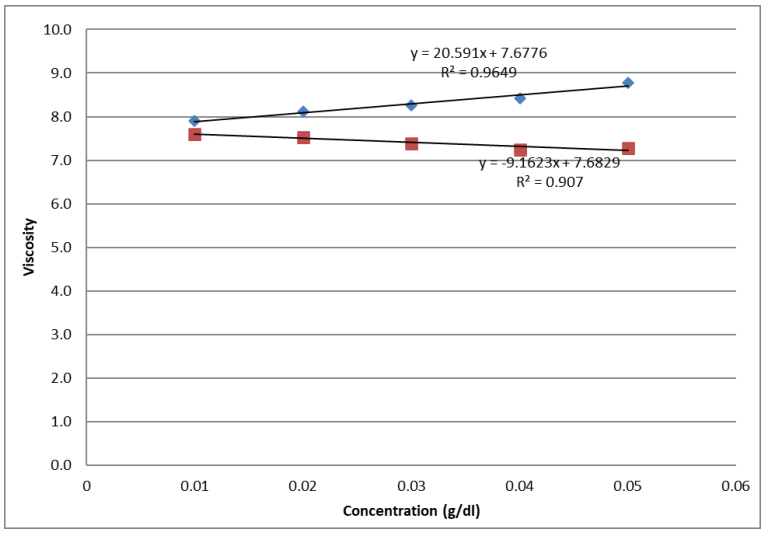

Figure S9: PIC-0 intrinsic viscosity determined from the reduced viscosity (blue) and the inherent viscosity (red) as a function of polymer concentration in acetonitrile.

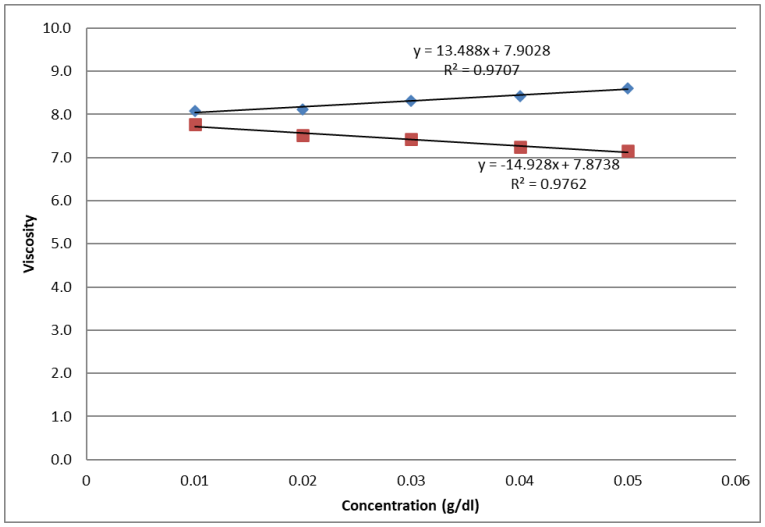

Figure S10: PIC-0.5 intrinsic viscosity determined from the reduced viscosity (blue) and the inherent viscosity (red) as a function of polymer concentration in acetonitrile.

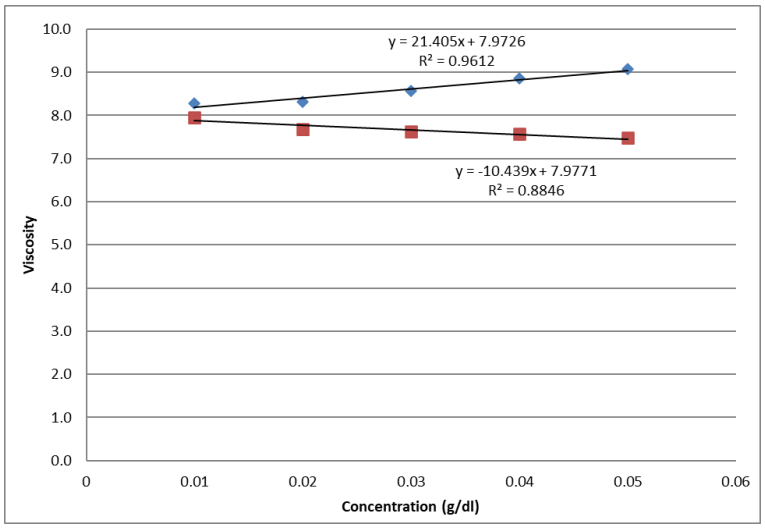

Figure S11: PIC-1.0 intrinsic viscosity determined from the reduced viscosity (blue) and the inherent viscosity (red) as a function of polymer concentration in acetonitrile.

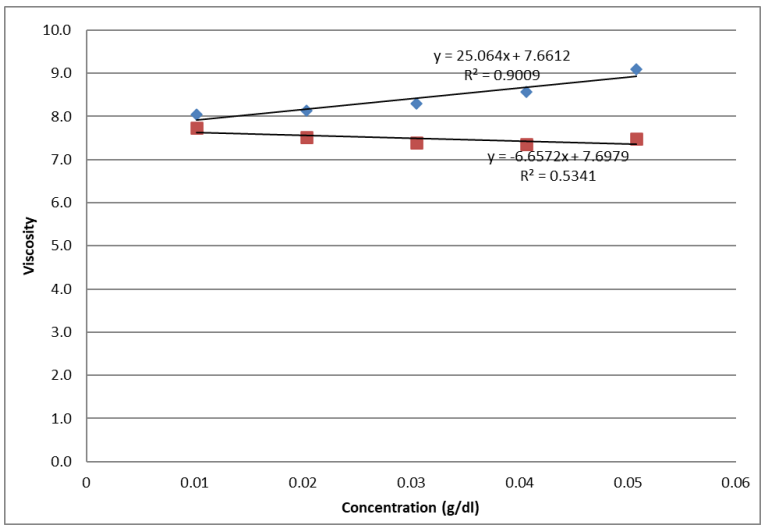

Figure S12: PIC-3.0 intrinsic viscosity determined from the reduced viscosity (blue) and the inherent viscosity (red) as a function of polymer concentration in acetonitrile.

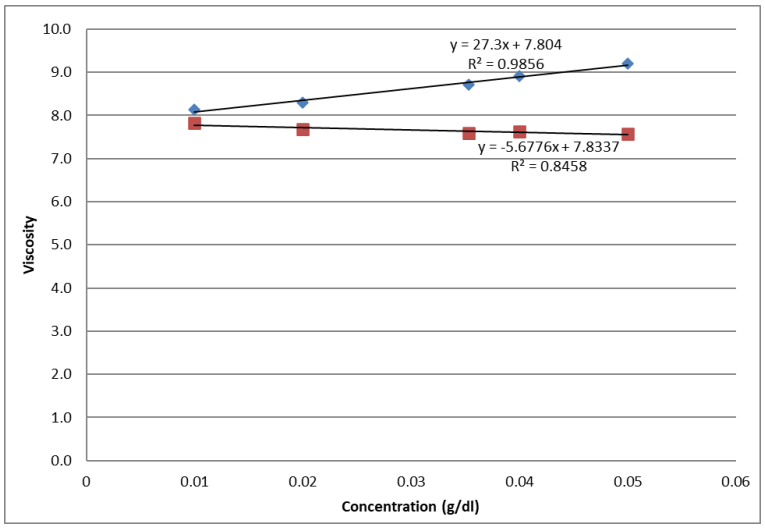

Figure S13: PIC-6.0 intrinsic viscosity determined from the reduced viscosity (blue) and the inherent viscosity (red) as a function of polymer concentration in acetonitrile.

Calculating the polymer lengths from Mv

Polymer length (L) was calculated using the linear relationship between Mv and polymer length as previously defined by Jaspers *et. al.* (*Nat. Commun.* 2014 5:5808 doi: 10.1038/ncomms6808).

Y = 0.3962x + -0.1943

PIC-0	L = (0.3962*368) + -0.1943	L = 145.60
PIC-0.5	L = (0.3962*373) + -0.1943	L = 147.58
PIC-1.0	L = (0.3962*375) + -0.1943	L = 148.38
PIC-3.0	L = (0.3962*368) + -0.1943	L = 145.60
PIC-6.0	L = (0.3962*371) + -0.1943	L = 146.79

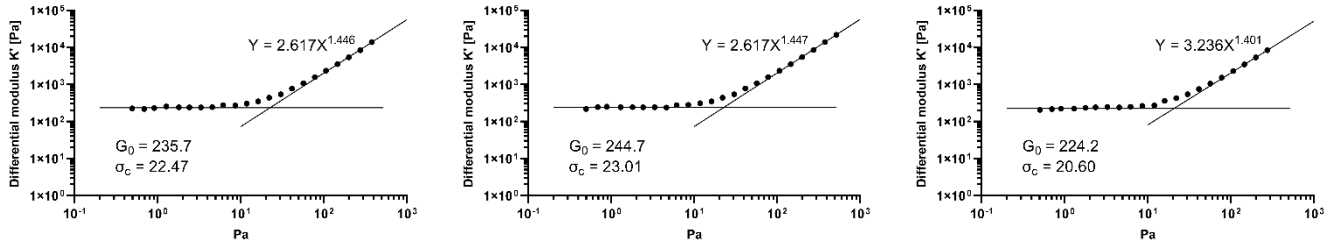

Figure S14: PIC-0 rheology analysis in water at 6.32 ± 0.225 mM (2 mg/mL).

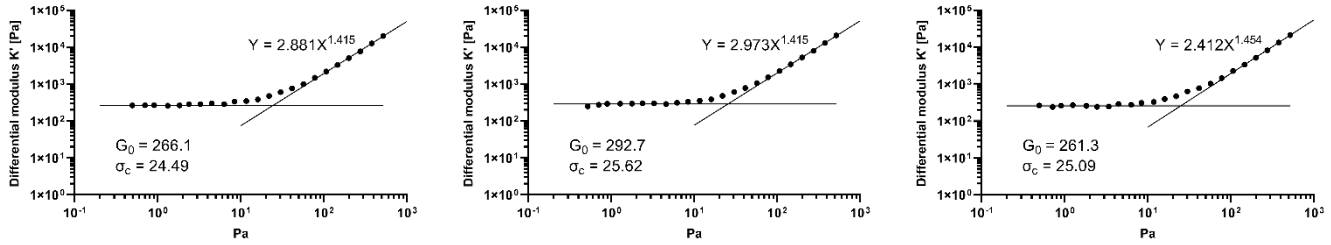

Figure S15: PIC-0.5 rheology analysis in water at 6.32 ± 0.225 mM.

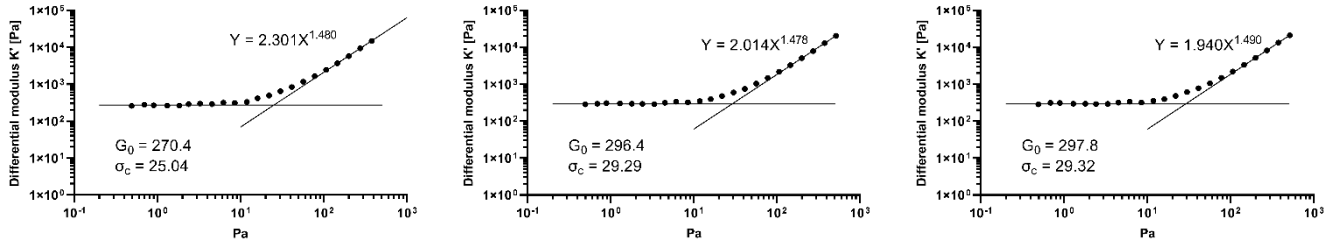

Figure S16: PIC-1.0 rheology analysis in water at 6.32 ± 0.225 mM.

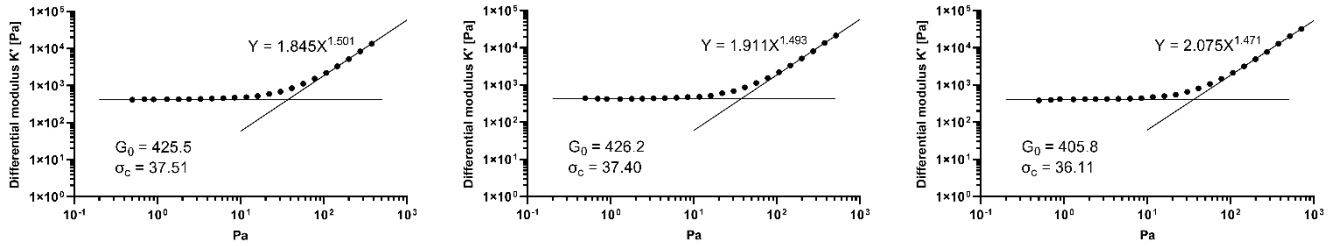

Figure S17: PIC-3.0 rheology analysis in water at 6.32 ± 0.225 mM.

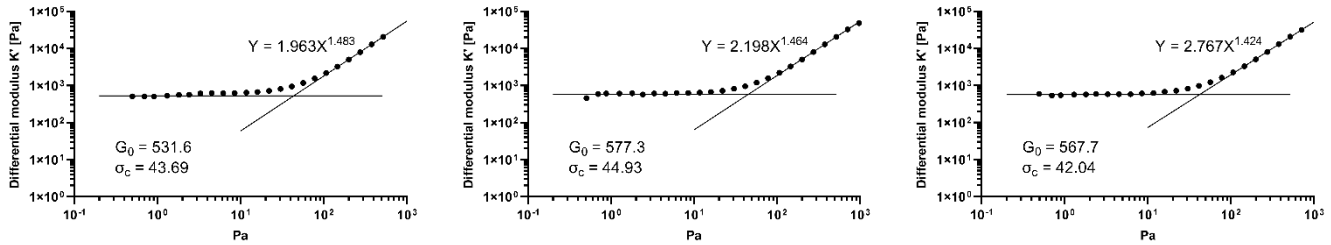

Figure S18: PIC-6.0 rheology analysis in water at 6.32 ± 0.225 mM.

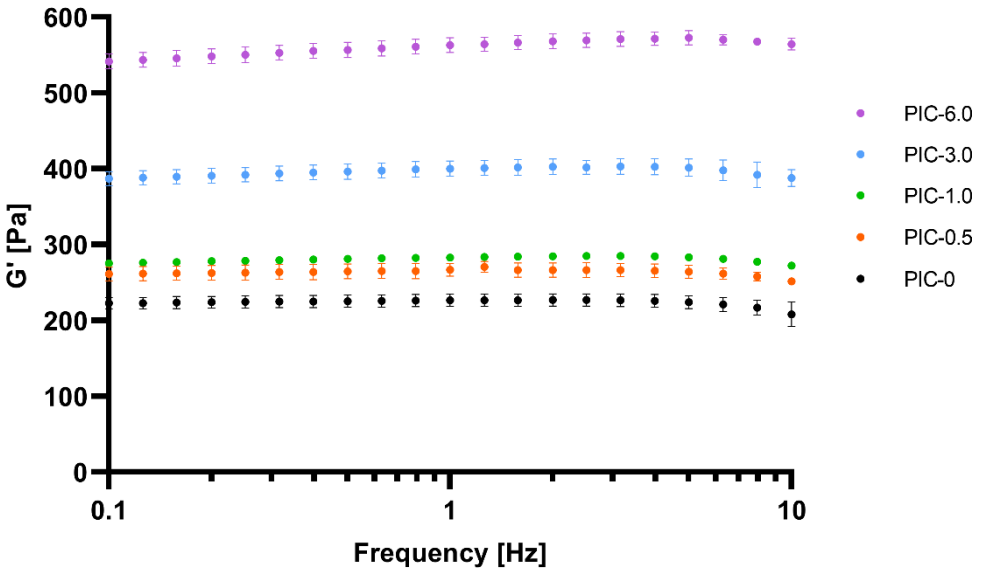

Figure S19: Frequency sweep between 0.1 and 10 Hz for PIC-0 and PIC(methoxy-co-azide) polymers with increasing azide percentage.

Post-polymerisation Peptide Addition.

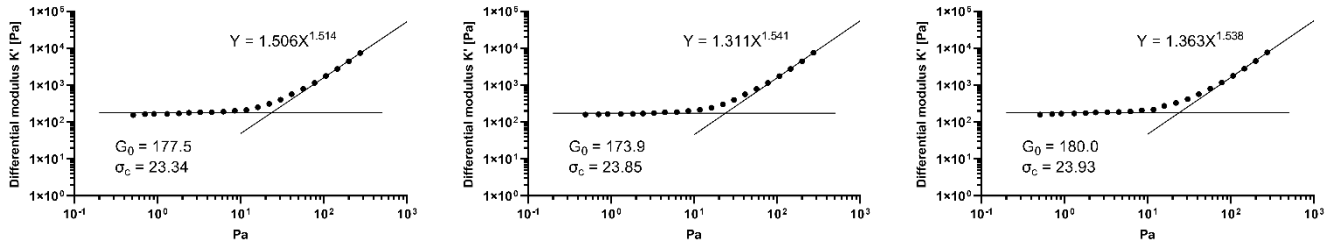

Figure S20: PIC-0.5 GRGDS rheology analysis in water at 6.32 ± 0.225 mM.

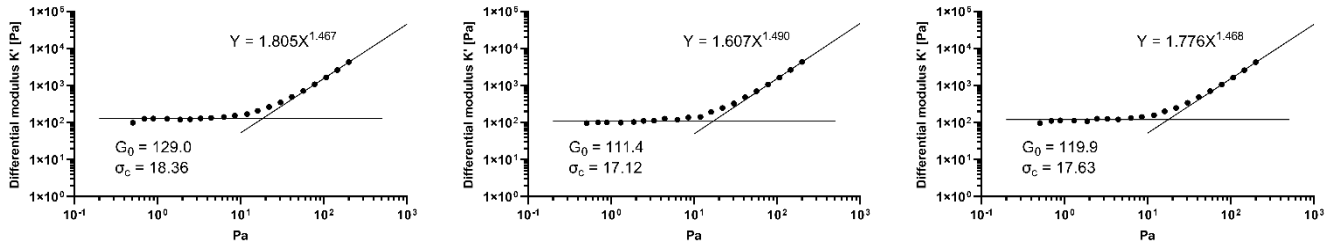

Figure S21: PIC-1.0 GRGDS rheology analysis in water at 6.32 ± 0.225 mM.

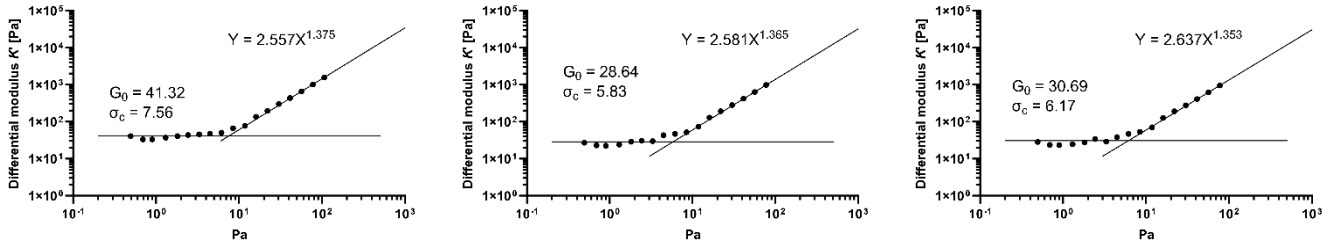

Figure S22: PIC-3.0 GRGDS rheology analysis in water at 6.32 ± 0.225 mM.

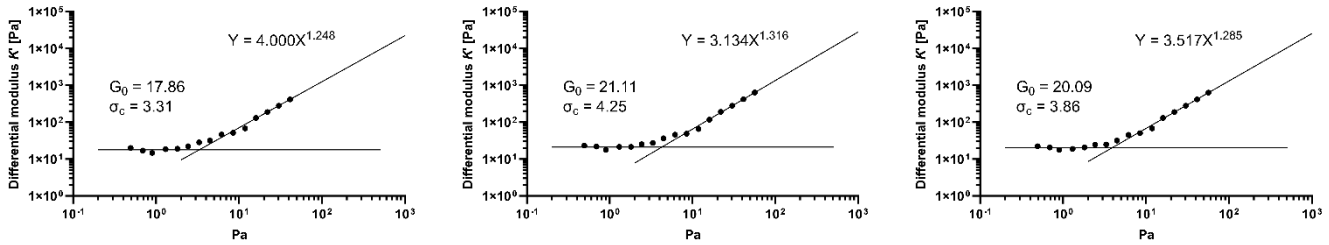

Figure S23: PIC-6.0 GRGDS rheology analysis in water at 6.32 ± 0.225 mM.

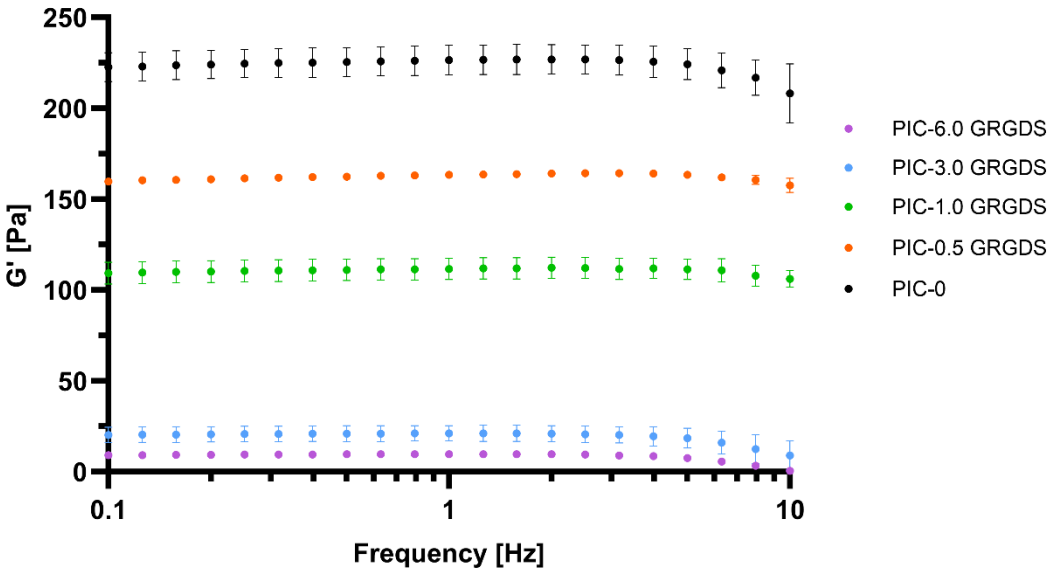

Figure S24: Frequency sweep between 0.1 and 10 Hz of peptide functionalised PIC with increasing ligand density.

Table S1: Stress relaxation half-life data for PIC-based hydrogels PIC-0 and polymer-peptide conjugates (PIC GRGDS 0.5 – 6%).

GRGDS Ligand Density	Stress relaxation half-life ($T_{1/2}$) at 5% constant strain (s)	Stress relaxation half-life ($T_{1/2}$) at 20% constant strain (s)
0	8690	1151
0.5	3860	617
1	3424	486
3	649.5	433
6	621.5	349

Small Angle Neutron Scattering (SANS) Plot

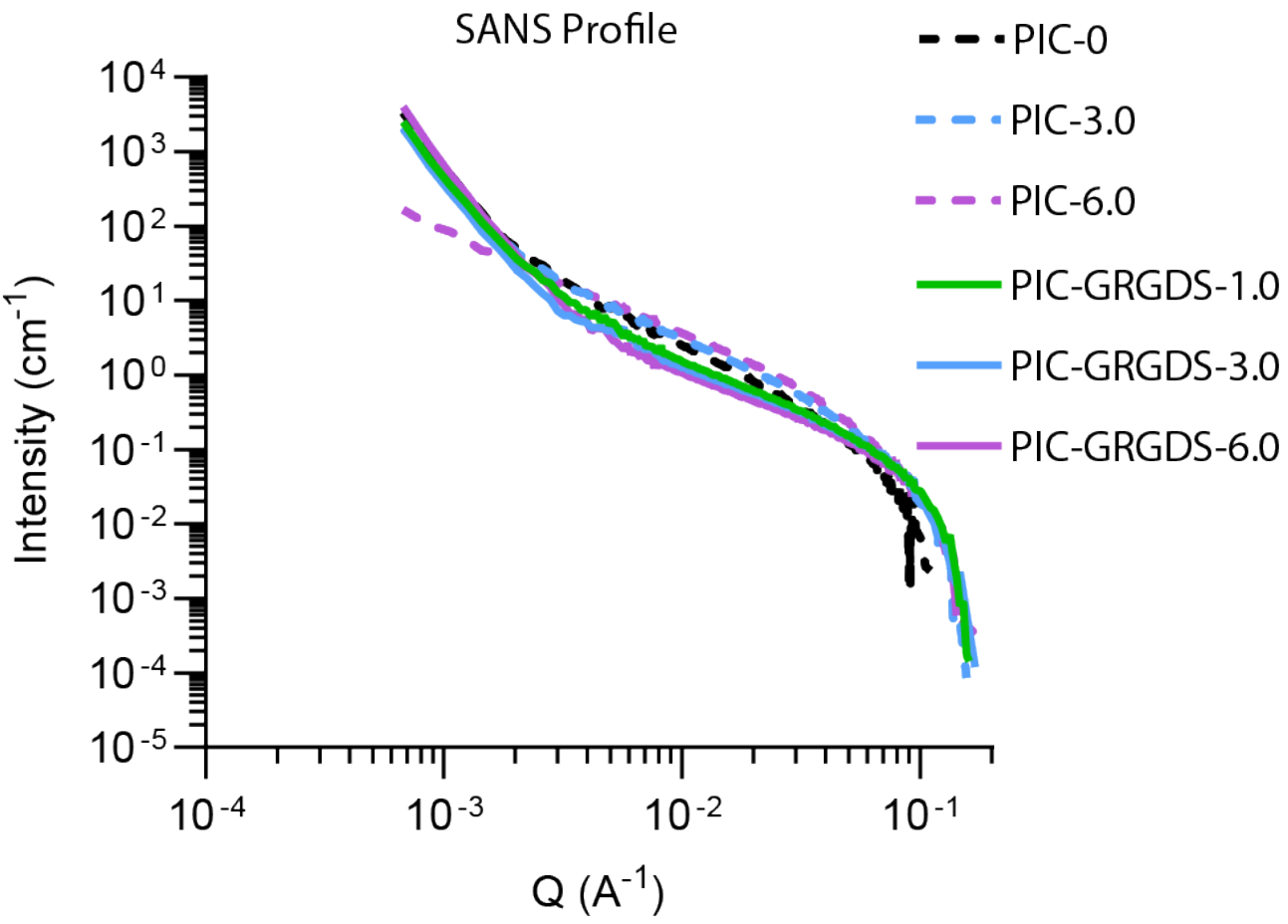

Figure S25: SANS/USANS scattering plot of PIC hydrogels 2 mg mL^{-1} D_2O.

3D cultured fibroblasts at 12 hours

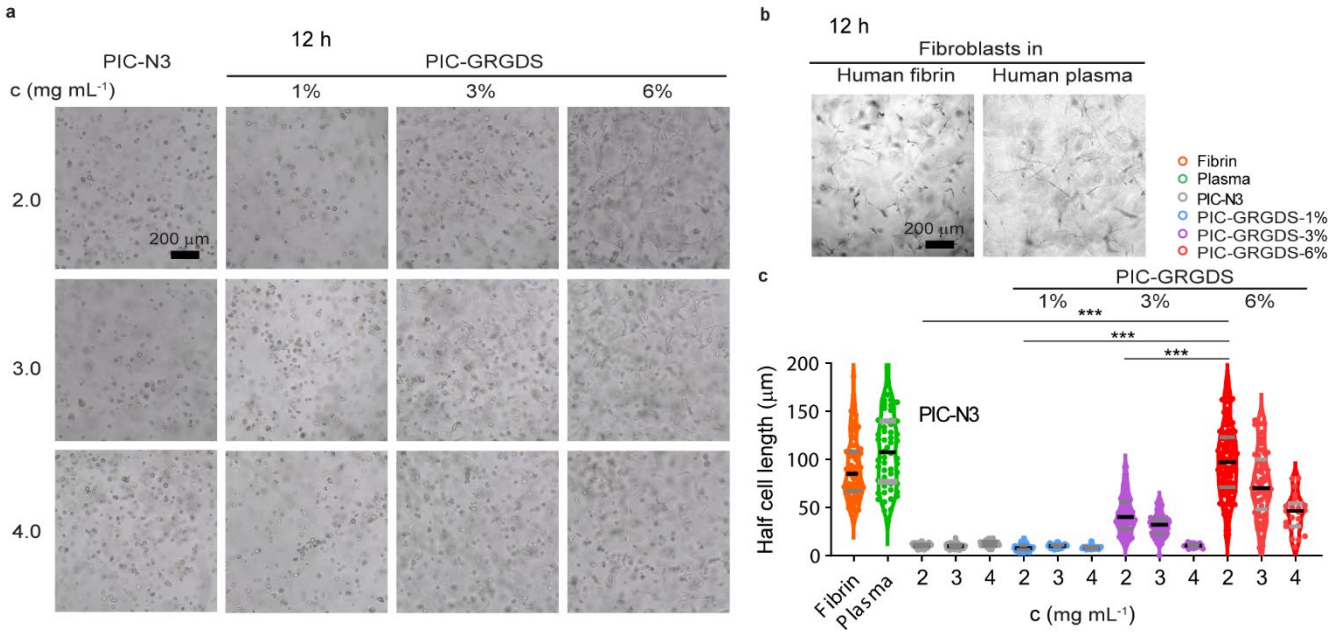

Figure S26: Early morphological change of fibroblasts in different matrices. Phage-contrast microscopic images of fibroblasts cultured in 3D PIC-based matrices (a) or in natural human fibrin network or plasma (b) at 12 hours. Scale bar, 200 μm. Quantified cell morphological changes analysed by half of the cell length (c). One-way ANOVA statistical analysis was performed. *** = $p < 0.001$.