
Supporting Information

A modular self-assembly approach to functionalised β -sheet peptide hydrogel biomaterials

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S.1. Peptide Synthesis, Purification and Characterisation

Peptides were synthesised on a 0.1 or 0.25 mmol scale, using standard Fmoc-based solid-phase methods on a CEM Liberty microwave automated peptide synthesiser. Synthesis was performed from low-loading Wang resins (*ca.* 0.25 mmol/g), pre-loaded with Fmoc-Glu(tBu) or Fmoc-Lys(Boc) for peptides with an acid C-terminus. Coupling agents used for peptide synthesis were di-*iso*-propylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt). Cleavage of peptides and their conjugates from the resin was achieved through agitation in 95 % trifluoroacetic acid (TFA) for 2-4 h (dependent on sequence) at room temperature, using 2.5 % purified H₂O and 2.5 % triisopropylsilane as scavengers. Following filtration, the crude material was obtained by removing most of the TFA solution under reduced pressure and precipitation from an excess of cold diethyl ether. The residue was collected by centrifugation, further washed in cold diethyl ether, then lyophilised to obtain a low-density powder.

Purification was achieved using reverse-phase HPLC (Agilent 1100 series), using a semi-preparative C-18 column (Agilent Eclipse XDB-C18, 5 μ m, 9.4 mm \times 250 mm) at 20 °C. The solvent system used was CH₃CN and purified water, both containing 0.1 % TFA. Typically a gradient in which the CH₃CN content increased linearly from 5 % to 95 % over 1 h was used (with ten-minute isocratic periods either side of the gradient using the initial and final solvent mixtures). Peptide and peptide-conjugate identities were determined using mass spectrometry, with either a Bruker Ultraflex II TOF/TOF MALDI-TOF (Bruker, Coventry, UK) or a Waters LCT-TOF connected to a Waters Alliance LC (Waters, Herts, UK).

Final purity of all peptides was > 99%, as determined by analytical HPLC on the same system and solvents, but using an analytical C18 column with a flow rate of 2 mL.min⁻¹ (Agilent Eclipse XDB-C18, 5 μ m, 4.6 mm \times 150 mm). A JASCO V-660 UV/VIS detector was used to monitor peptide elution at 230 nm and 280 nm, and dabsyl-conjugate elution monitored at 400 nm. Purified fractions were collected and lyophilised.

Analytical HPLC for p1

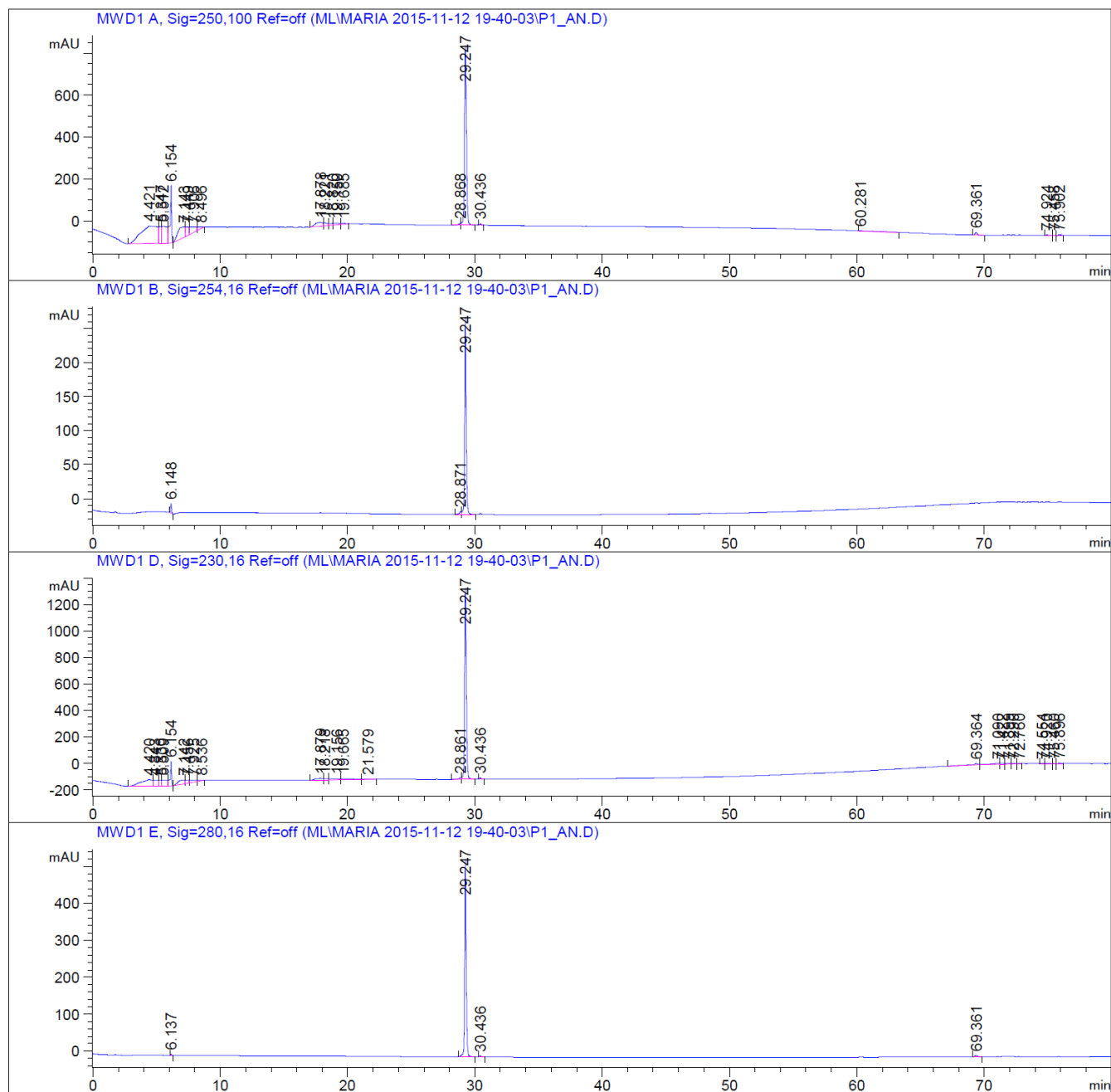


Figure S.1: Analytical HPLC for p1

HR-MS (ESI, m/z): Found 1399.6877, expected for $C_{67}H_{95}N_{14}O_{19}^+$ ($[M+H]^+$); 1399.6892
 Found 700.3489, expected for $C_{67}H_{96}N_{14}O_{19}^{2+}$ ($[M+2H]^{2+}$); 700.3482
 Found 467.2359, expected for $C_{67}H_{97}N_{14}O_{19}^{3+}$ ($[M+3H]^{3+}$); 467.2345

Analytical HPLC for p2

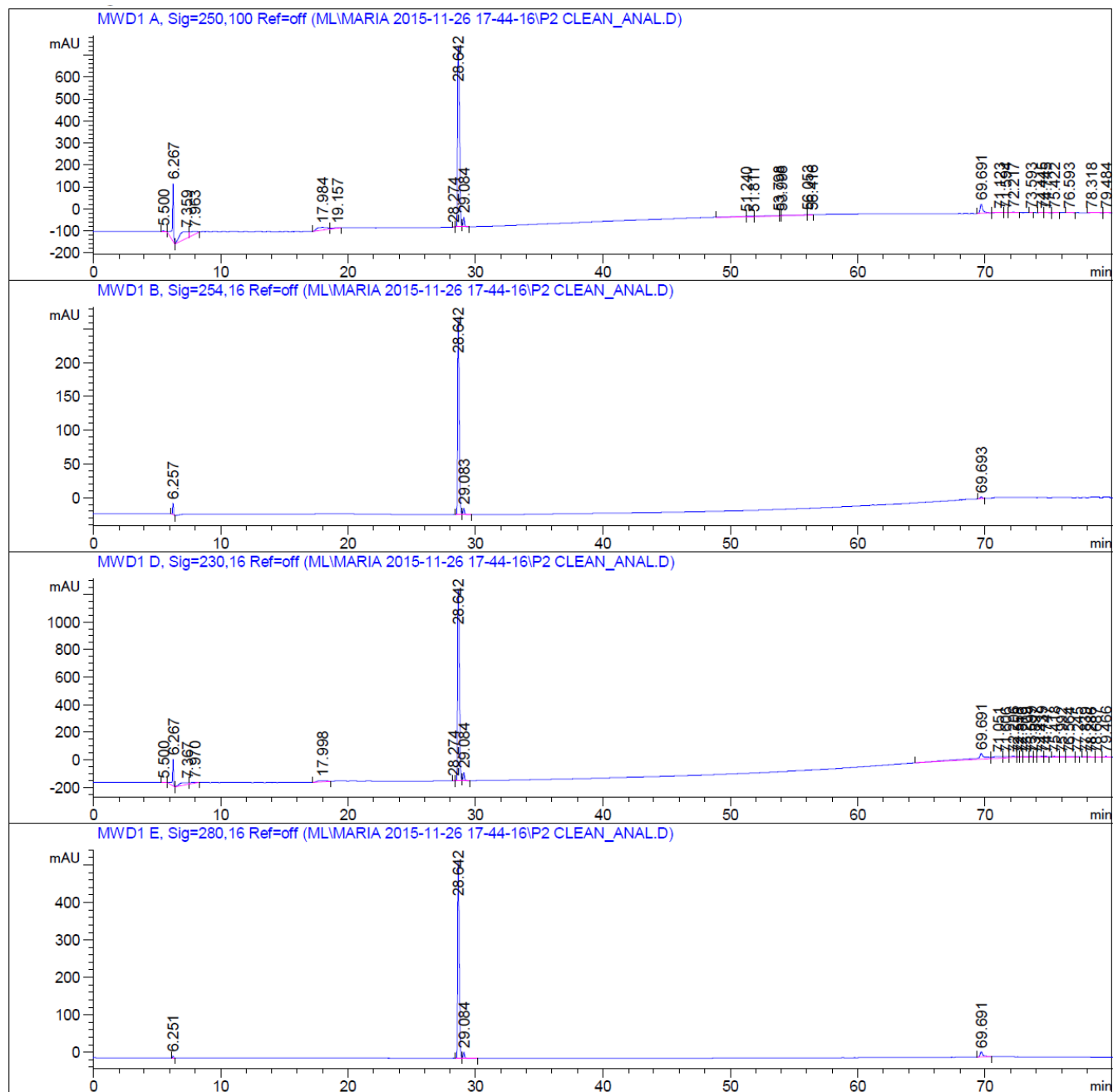


Figure S.2: Analytical HPLC for p2

HR-MS (ESI, m/z): Found, 1398.7446; expected for $C_{68}H_{100}N_{15}O_{17}^+$ ($[M+H]^+$), 1398.7416
 Found, 699.8802; expected for $C_{68}H_{101}N_{15}O_{17}^{2+}$ ($[M+2H]^{2+}$), 699.8744
 Found, 466.9232; expected for $C_{68}H_{102}N_{15}O_{17}^{3+}$ ($[M+3H]^{3+}$), 466.9187

Analytical HPLC for RGD-p1

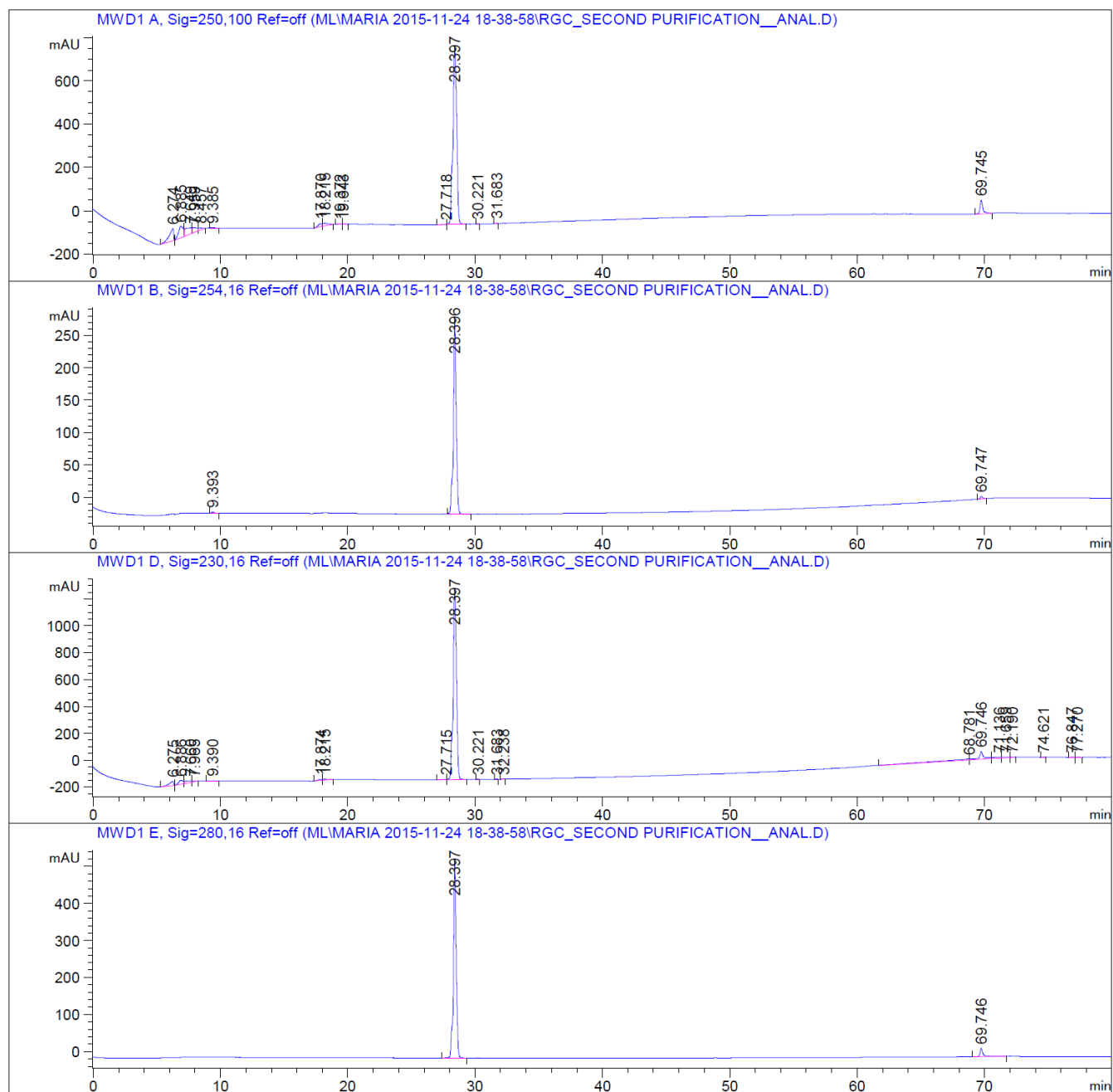


Figure S.3: Analytical HPLC for RGD-p1

S.2. Optical observations, gelation times and stability to DMEM buffer

Gelation times in PBS:

10 mM p1 + 10 mM p2 at 37 °C	20-40 s
10 mM p1 + 10 mM p2 at 20 °C	< 60 s
5 mM p1 + 5 mM p2 at 20 °C	270-300 s
3.3 mM p1 + 3.3 mM p2 at 20 °C	6 min (weak gel) to 10 min (firm gel)
2 mM p1 + 2 mM p2 at 20 °C	≤ 2 h

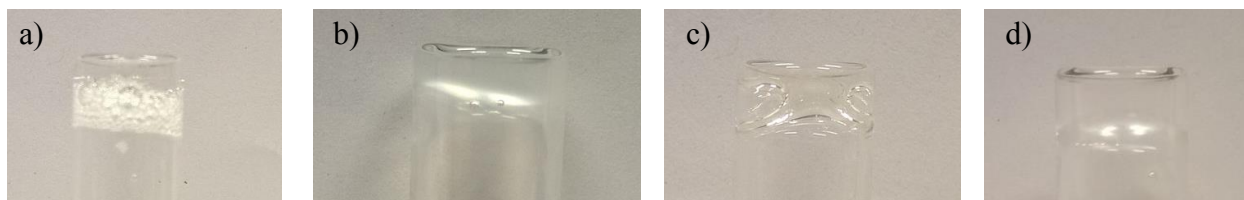


Figure S.4. Images demonstrating the self-supporting and transparent nature of the hydrogel formed by a) 10 mM **p1** and 10 mM **p2** in PBS at 37 °C; b) 10 mM **p1** and 10 mM **p2** in PBS at 20 °C; c) 5 mM **p1** and 5 mM **p2** in PBS at 20 °C. d) 3.3 mM **p1** and 3.3 mM **p2** in PBS at 20 °C.

Gelation times in DMEM at 37 °C:

A thick viscous solution resulted after 10 min, but this did not form a self-supporting gel, even after 1 h.

Stability of standard to **p1** + **p2** gels in DMEM buffer at 37 °C

Conditions analogous to those employed for the cell culture experiments were chosen. Samples of **p1** + **p2** gels were incubated with DMEM buffer at 37 °C for 24 h. Two peptide concentrations were tested, the standard 20 mM total peptide concentration (200 μ L) and a lower 6.7 mM total peptide concentration (300 μ L). After formation of the gels in PBS (colourless) in a small vial, DMEM (red) was added to the top (500 μ L to the standard sample, an analogous 750 μ L to the 6.7 mM peptide gel), and the vials incubated at 37 °C. At set timepoints over 48 h, the vials were removed, inverted and imaged (Figure S.5).

Over this period of time there was a small loss in volume from the gels, but they remained self-supporting. However, the inability to form self-supporting gels in DMEM alone suggested interference from one of the additives present in DMEM, and this may also lead to the gradual weakening observed during cell culture.

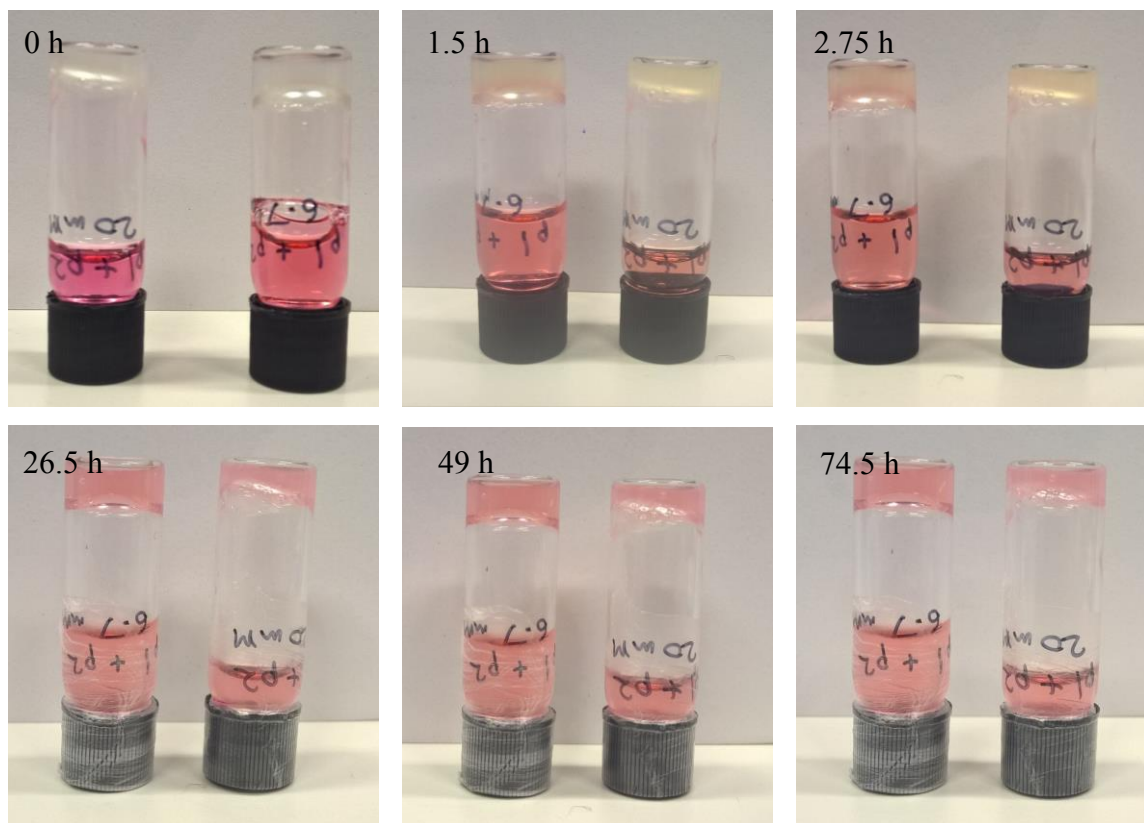


Figure S.5. Images showing the effect of incubating **p1 + p2** gel samples (300 μ L, 6.7 mM total peptide; 200 μ L, 20 mM total peptide) with DMEM buffer (0.75 and 0.5 mL respectively) at 37 $^{\circ}$ C for three days.

S.3. Additional FTIR data

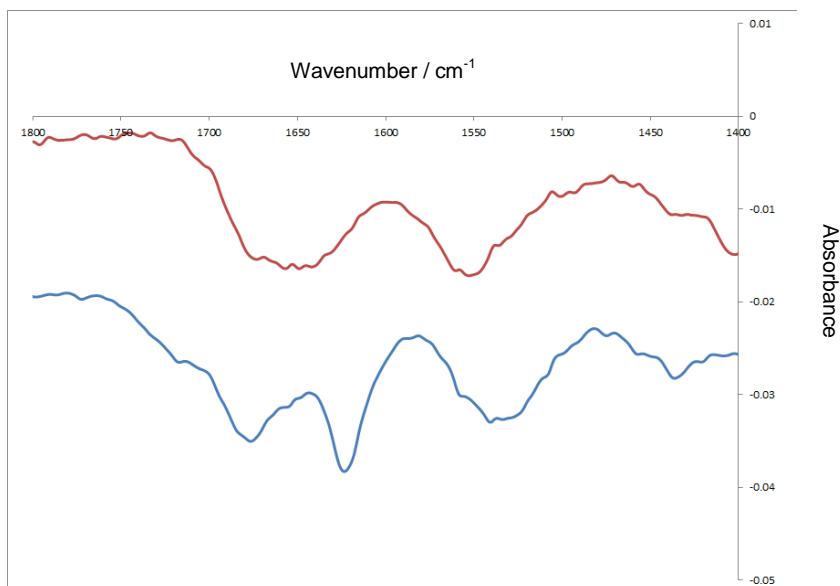


Figure S.6. FTIR spectra demonstrating **p1** (10 mM, blue line) and **p2** (10 mM, red line) adopt a combination of random coil (1654 cm^{-1}) and β -sheet (1625 cm^{-1} , 1684 cm^{-1}) structure in 50 mM NaCl. Peptide concentrations 10 mM, at pH 7, 20°C . Samples pre-incubated for 20 minutes at room temperature before characterisation.

FTIR of either **p1** or **p2** in solution showed a complete lack of secondary structure, independent of pH or concentration up to 0.2 M, with a broad peak observed at 1654 cm^{-1} in all cases (Figure S.7).

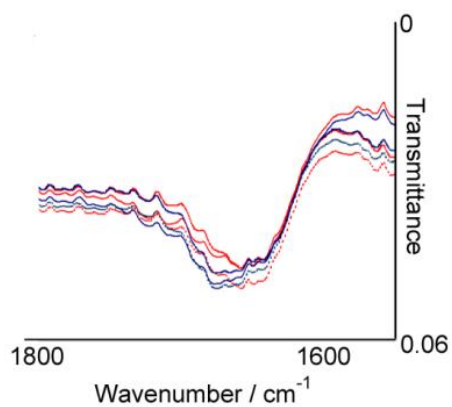


Figure S.7. FTIR spectra of 5 mM **p1** at pH 1 (—), 7 (—) and 14 (—); 5 mM **p2** at pH 1 (---), 7 (---) and 14 (---).

S.4. SEM and TEM analysis

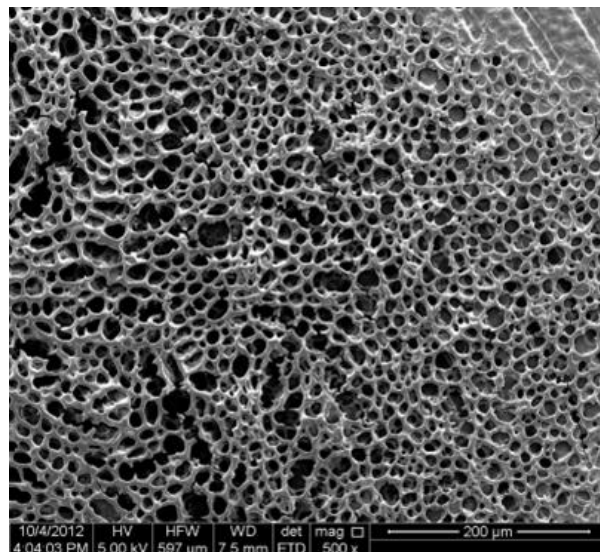


Figure S.8. SEM image showing the porous network formed through lyophilisation of peptide hydrogels. Sample was prepared as follows: A standard hydrogel sample comprising 5 mM **p1** and 5 mM **p2** was prepared at pH 7, leaving overnight at room temperature. On the second day sample was transferred to a polished metal stub, flash-frozen in liquid N₂ and lyophilised overnight to form an aerogel, then sputter-coated with Pt/Pd, and imaged using a Philips XL30 FEGSEM, fitted with a Nordlys II camera (Oxford Instruments, Abingdon, UK).

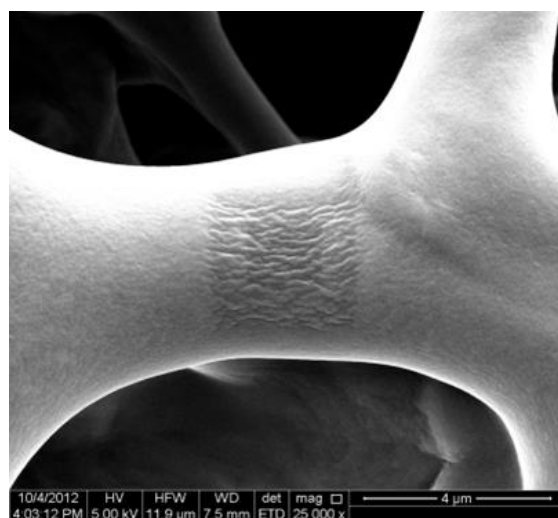


Figure S.9. SEM image showing that the apparent large fibres observed in aerogels formed through the lyophilisation of hydrogel samples comprise smaller, twisted fibres. Smaller, twisted fibres were revealed after surface ablation of the square region revealed the underlying structure of the larger fibres. Sample otherwise prepared as described for Figure S.8.

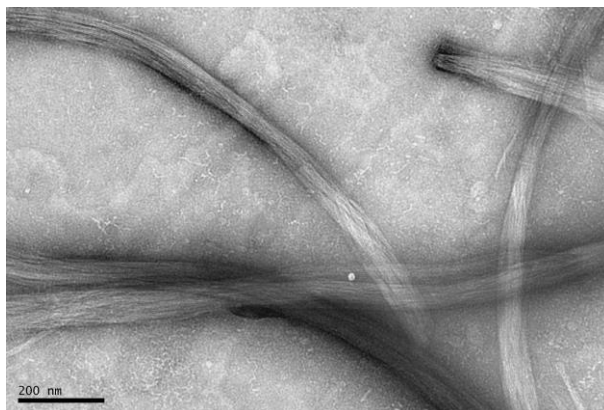


Figure S.10. TEM image of larger fibres observed in hydrogel samples, demonstrating they comprise smaller, twisted fibrils. Diluted gel samples at pH 7 were placed onto glow-discharged 400 mesh carbon coated grids (Agar Scientific, Stansted, UK) for one minute. Grids washed with phosphate buffer of the same pH two times, and negatively stained with freshly-prepared and filtered 2% (w/v) uranyl acetate (Agar Scientific, Stansted, UK) for one minute, blotting at each stage using Whatman filter paper. Samples viewed on a Tecnai Biotwin (FEI, Oregon, USA), under an accelerating voltage of 100 Kv, and imaged with a GATAN Orius CCD (Gatan, Oxford, UK), with a sample increment of 3.5 Å/pixel.

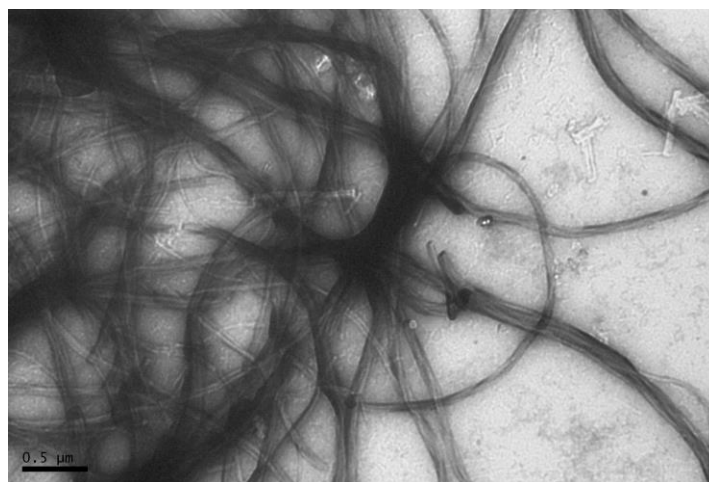


Figure S.11. TEM image showing the dense entangled mats of fibres that form hydrogels. Sample prepared as described for Figure S.10. The region shown was a particularly dense region of the grid, demonstrating the entanglements between fibres that lead to hydrogel formation.

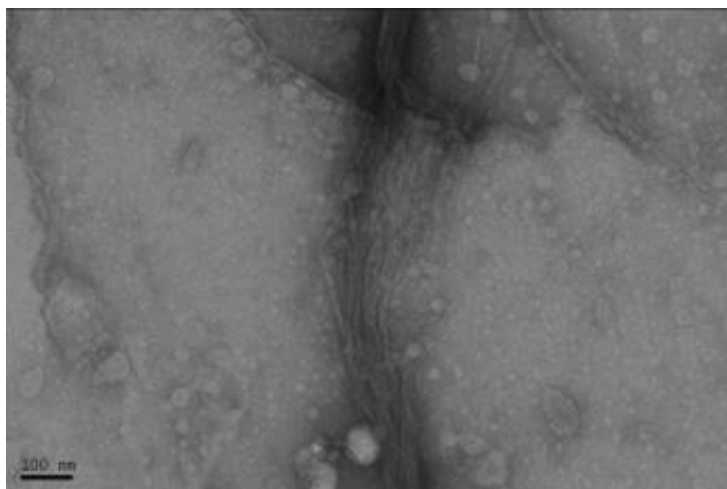


Figure S.12. TEM image showing larger fibres fraying into constituent fibrils. Sample prepared as described in Figure S.10. The majority of observed fibres were intact, but on occasion examples such as the one shown were observed, where mechanical stress, presumably induced by the drying process, led to the fraying of fibres into smaller fibrils.

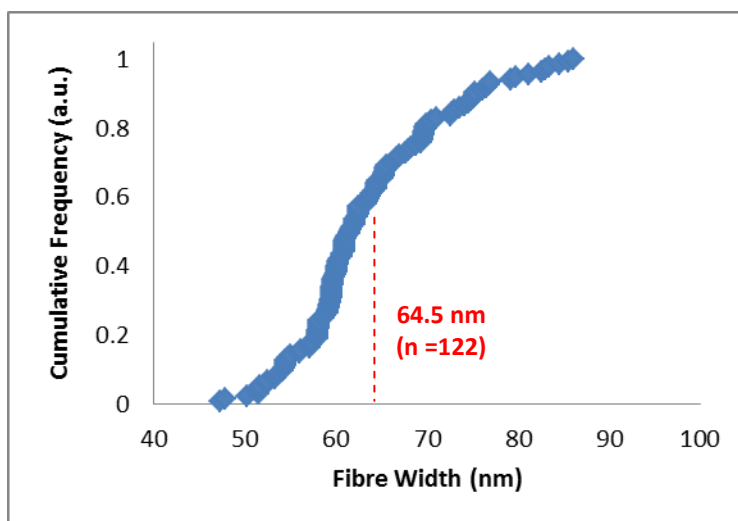


Figure S.13. Cumulative frequency analysis of fibre diameters from TEM images, using ImageJ software, giving an average fibre diameter of 64.5 nm (n=122). Samples prepared as described in Figure S.10.

S.5. SAXS analysis

The Porod regime gives the fractal dimension of the objects and excluded volume. Porod exponents <3 are for “mass fractals” and Porod exponents between 3 and 4 are for “surface fractals”.¹ The porod exponent can be obtained from the slope of a Porod plot, as the slope of the plot at high q values varies with different shapes/fractal dimensions, e.g. a Porod slope between -1 and -3 denotes “mass fractals” and -3 to -4 “surface fractals”. A slope of -1 is obtained for scattering from rigid rods and a slope of -2 indicates disks/lamella or Gaussian polymer chains. A value of $-5/3$ indicates swollen coils, whereas a slope between -2 and -3 denotes some form of branched system or network.^{2,3}

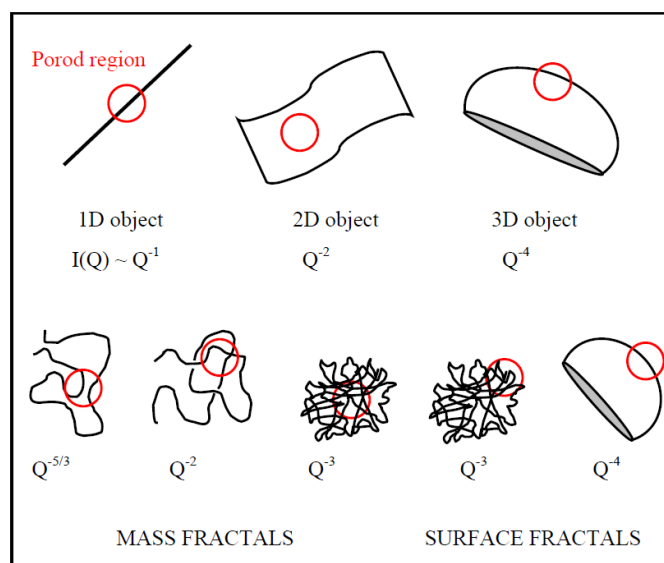


Figure S.14. Porod law behaviours for objects of different shapes.^{2,3}

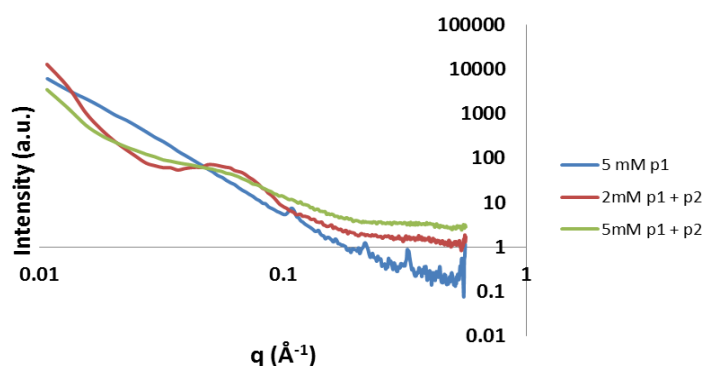


Figure S.15. SAXS measurements showing a feature at 0.06 \AA^{-1} in hydrogel samples that corresponds to the formation of thickened rods, which did not appear in non-hydrogel samples (i.e. 5 mM **p1**). Analysis of this peak gives a fibre diameter of 104.7 nm, similar to the average fibre diameter from analysis of TEM images (64.5 nm, Figure S.13) SAXS measurements were performed using a HECUS instrument with a Xenocs micro-focus copper source with montel optics, using a Pilatus 100k detector (HECUS (Bruker), Coventry, UK). Liquid and hydrogel samples were pipetted into the sample chamber. Hydrogel samples were allowed to reform for 24 h at room temperature within the sample chamber before acquisition.

S.6. Additional rheology data

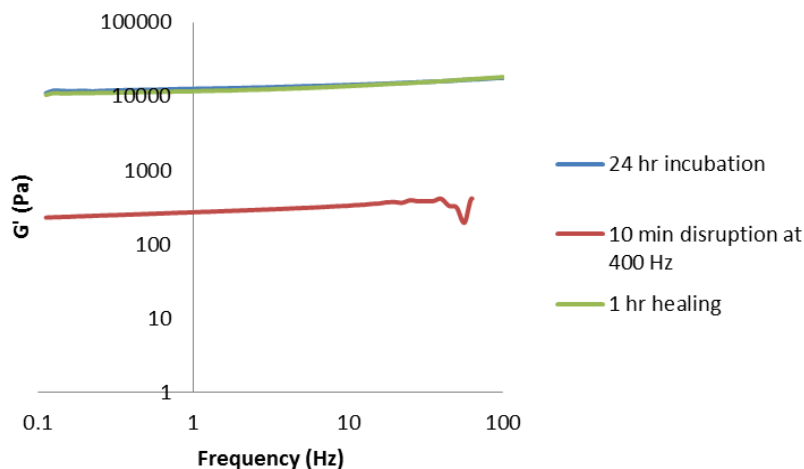


Figure S.16. **p1/p2** hydrogel healing after the application of vigorous shear force. Rheology measurements of hydrogels comprising 10 mM **p1** and 10 mM **p2** at pH 7, after 24 h incubation at room temperature (blue), immediately after application of 400 Hz shear force for 10 minutes to break the hydrogel structure (red), and after 1 h reformation at room temperature (green). Hydrogels demonstrated a recovery of 96 % of original gel strength after 1 h incubation at room temperature, from 2 % immediately after disruption. Hydrogel was incubated for 24 h in a sealed container, and then remained in the sample chamber during all measurements shown.

Time dependence: **p1** + **p2** hydrogels in PBS remained gelled for several weeks, and **p1** + **p2** hydrogels (10 mM total peptide) measured after 10 days indicate that the gels have similar properties after this time.

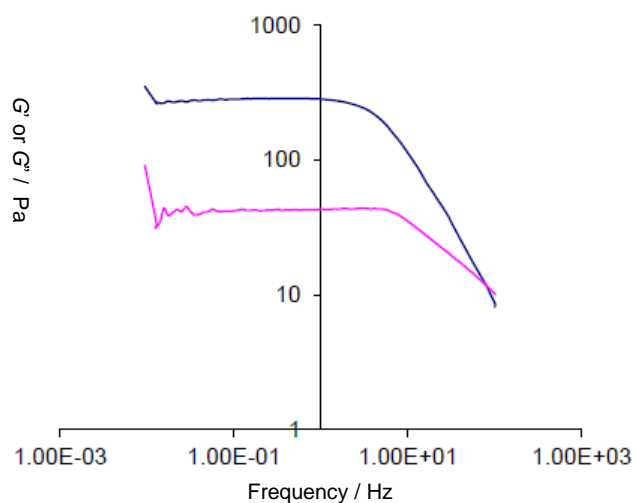


Figure S.17. Rheology measurements of hydrogels comprising 5 mM **p1** and 5 mM **p2** at pH 7 after 10 days incubation at room temperature. G' (blue line), G'' (pink line).

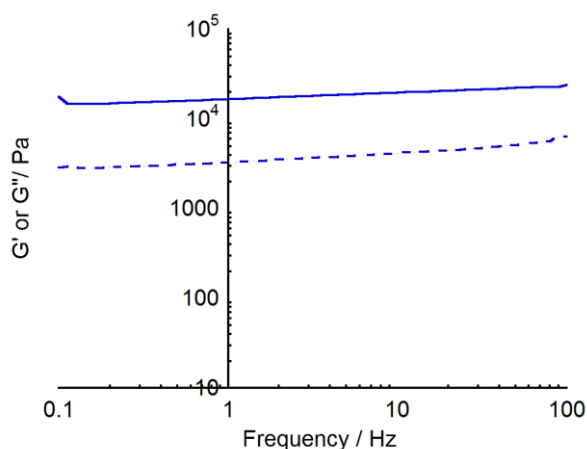


Figure S.18. Rheology measurements of hydrogels comprising 9 mM **p1**, 1 mM **p1-RGD** and 10 mM **p2** at pH 7. G' (—), G'' (---).

S.7. Preliminary studies of the stability of **p1** + **p2** hydrogels to elastase and trypsin; cleavage of trypsin-sensitive dabsylated **p2**.

For enzymatically-released compound studies an orange-coloured derivative of **p2**, dabsyl-FFK-PEG(16)-**p2** (MW 2840 g mol⁻¹), was developed. This peptide is N-terminally modified with dabsyl chloride attached to an trypsin-cleavable FFK sequence, which is separated from the peptide with a flexible PEG linker (RHN(CH₂CH₂O)₁₅(CH)₂CONHR'). Dabsyl-FFK-PEG-**p2**, was synthesized using standard Fmoc-based solid-phase synthesis followed by subsequent on-resin coupling procedures as described in Section S.1. Dabsyl chloride couplings to FFK-PEG-**p2** were pushed to completion using 10 eq. dabsyl chloride, 10 eq. PyBop and 11 eq. DIPEA in DMF with microwave irradiation for 2 h at 75 °C. This derivative was incorporated within gels during their preparation, using 0.5 mM of dabsyl-FFK-PEG-**p2**, 4.5 mM **p2** and 5 mM **p1** (dabsyl-FFK-PEG-**p2** is 5 mol % of total peptide) Gel samples (300 μL) containing this mixture of peptides were prepared at 20 °C, pH 7, and incubated at this temperature for 24 h in a glass vial. Initially, phosphate buffer at pH 7 (300 μL) was placed on top of the gel layer for 10 min to study any leakage. Subsequently a solution of elastase (300 μL, 1 mM) was placed on top of the gel for 10 min, followed by a further wash with phosphate buffer. This was then followed by the addition of a trypsin solution (300 μL, 0.5 mM) top of the gel for 10 min. A final wash was performed using phosphate buffer (300 μL, for 10 min).

The supernatant from each sample was analysed by ESI-MS. These ESI-MS spectra showed no fragments from dabsyl-FFK-PEG-**p2**, **p2** and **p1**, and the gel remained intact (Figure S19a,b). However, the addition of the protease trypsin appeared to release coloured material from the gel, and ESI-MS

spectra of the supernatant showed the appearance of K-PEG-**p2** (m/z 2261), indicating cleavage of dabsyl-FFK-PEG-**p2**. Only intact **p1** or **p2** were observed in the ESI-MS spectra however (no fragments), and the gel appeared to remain self-supporting (Figure S19c). On a blank sample only containing **p2** and **p1**, no degradation was seen, and no peaks other than intact peptides were seen.

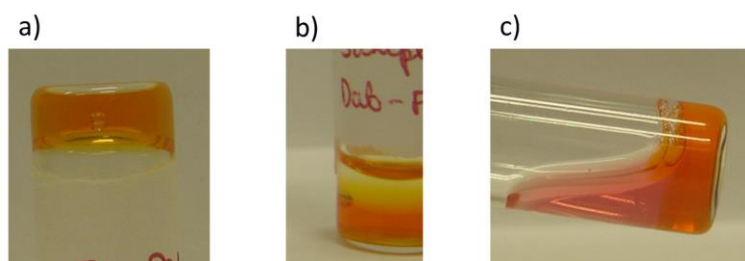


Figure S19. Self-supporting hydrogel comprising 0.5 mM of dabsyl-FFK-PEG-**p2**, 4.5 mM **p2** and 5 mM **p1** after 16 h. (b) After exposure to elastase solution (c) After exposure to trypsin solution.

References

¹ B. Hammouda, *J. Appl. Cryst.* 2010, **43**, 716-719.

² http://www.ncnr.nist.gov/staff/hammouda/distance_learning/chapter_22.pdf, accessed on 5/8/2015

³ http://www.diamond.ac.uk/Home/dms/Events/S4SAS_talks/doutch_colloids.pdf, accessed on 5/8/2015