Supporting Information

Non-reversible Heat-induced Gelation of a Biocompatible Fmoc-hexapeptide in Water

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Experimental Procedures

Materials

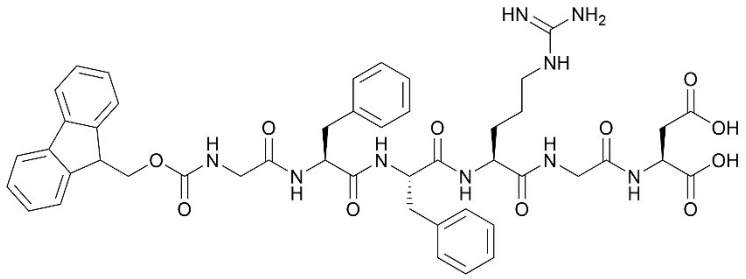
All chemicals unless otherwise specified were used as received. Fmoc amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), hydroxybenzotriazole hydrate (HOBt•H_2O) and 2-chlorotrityl chloride resin was obtained from Chem-Impex International. Trifluoroacetic acid, triisopropylsilane, formic acid, *N,N*-diisopropylethylamine (DIPEA), dichloromethane (DCM) and HPLC grade acetonitrile was obtained from Merck. Peptide synthesis grade *N,N*-dimethylformamide (DMF) was obtained from Auspep. Milli-Q water was used for hydrogel experiments with a resistivity = 18.2 MΩ•cm at 298 K. Dulbeccos Modified Eagle Medium (DMEM) used to make the hydrogels was obtained from Gibco (Reference number: 11960-044).

Peptide Synthesis

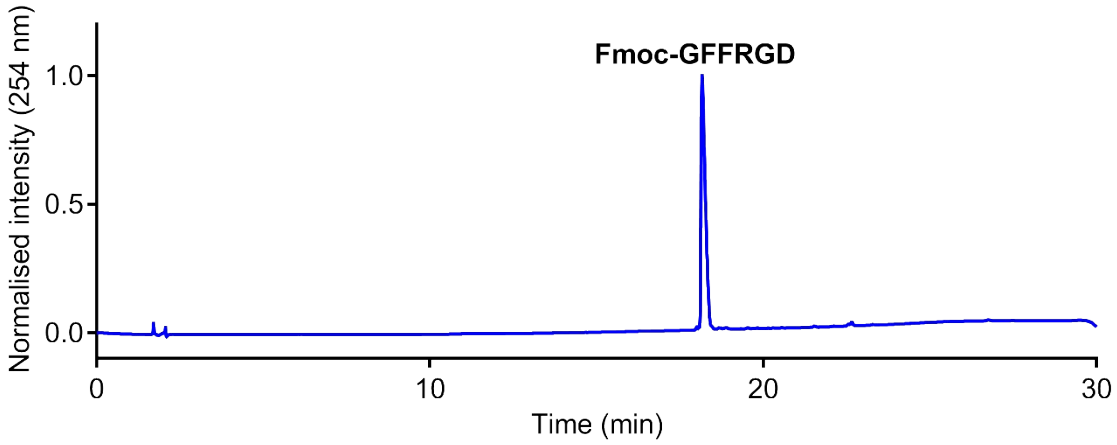
To synthesise the N-terminal capped hexapeptides, Fmoc-based solid phase peptide synthesis (SPPS) was used. In a typical synthesis, C-terminal amino acids were loaded onto the 2-chlorotritylchloride resin (1.1 mmol/g) by reacting overnight at room temperature in a mixture containing Fmoc-Asp(OtBu)-COOH (3 eq.) and DIPEA (6 eq.) in 1:1 DMF:DCM (v/v, 5 mL) on an orbital shaker. The solution was expelled and the resin washed with dichloromethane (5x5 mL) then a mixture containing 9:1:0.5 DCM:methanol:DIPEA (v/v, 5 mL) was added to the resin for 30 minutes. The solution as expelled and the resin washed with DCM (5x5 mL) and DMF (5x5 mL). Fmoc deprotections were performed using 20% piperidine in DMF (v/v) for 1 minute, expelling the solution, then reacting for a further 10 minutes before expelling and washing the resin with DMF (5x5 mL). Amide couplings were achieved using a mixture of Fmoc-amino acid (3 eq.) and DIPEA (6 eq.) dissolved in a 0.45 M solution of HBTU and HOBt in DMF. This coupling solution was added to the resin for 30 minutes before being expelled and washing the resin with DMF (5x5 mL). Complete couplings were checked using a Kaiser test before proceeding to the next amino acid. Before cleavage of the peptides from the resin, the resin was washed with DMF (5x5 mL) and DCM (5x5 mL) then dried with a gentle stream of N_2. The peptide was cleaved from the resin and the amino acid protecting groups removed using a cleavage solution containing trifluoroacetic acid, water and triisopropylsilane (95:2.5:2.5, v/v/v). This solution was added to the resin and shaken for 3 hours. The cleavage solution was expelled into a round bottom flask and concentrated by rotary evaporation. The peptides were dissolved in water/acetonitrile (30:70, v/v) and purified immediately by semi-preparative

HPLC. Semi-preparative HPLC methods are described in more detail in the experimental section. After purification and lyophilisation, the N-terminal capped hexapeptides were obtained in respective isolated yields of 46-47%.

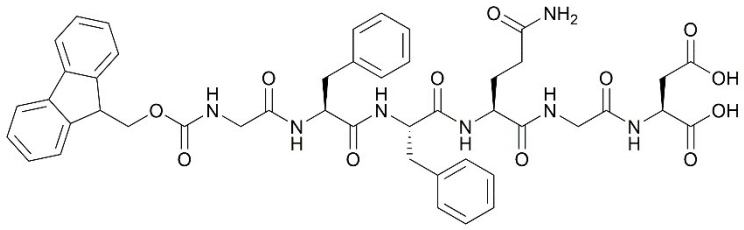
Fmoc-GFFRGD



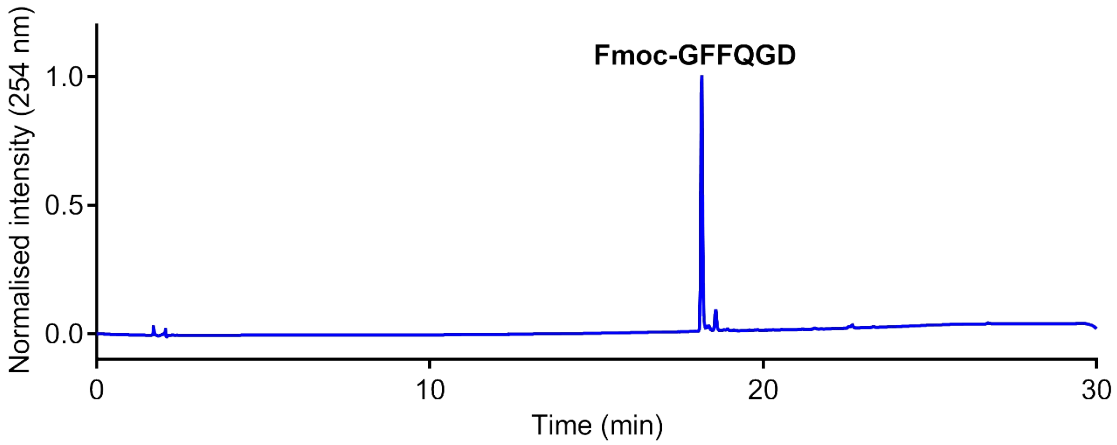
In a typical synthesis starting with 0.458 g of 2-chlorotritylchloride resin, (1.1 mmol/g loading) 0.221 g of **Fmoc-GFFRGD** was isolated after semi-preparative HPLC. (isolated yield: 47%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (s, 1H), 8.49 (s, 1H), 8.19 (d, *J* = 8.0 Hz, 1H), 8.13 (d, *J* = 7.4 Hz, 1H), 8.00 – 7.93 (m, 2H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.70 (d, *J* = 7.4 Hz, 2H), 7.50 (t, *J* = 5.9 Hz, 1H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.32 (t, *J* = 7.4 Hz, 2H), 7.28 – 7.08 (m, 10H), 4.53 (m, 2H), 4.33 – 3.89 (m, 3H), 3.98 – 3.57 (m, 2H), 3.65 – 3.44 (m, 2H), 3.23 – 2.90 (m, 4H), 2.76 (m, 2H), 2.59 – 2.35 (m, 3H), 1.68 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.74, 173.42, 171.69, 170.85, 170.82, 168.83, 168.06, 157.12, 156.45, 143.84, 140.72, 137.65, 129.22, 128.09, 127.99, 127.66, 127.11, 126.28, 126.24, 125.28, 120.13, 65.81, 53.75, 53.66, 52.37, 48.97, 46.61, 43.31, 42.11, 40.71, 37.57, 37.41, 30.08, 24.69. UV-Vis (MeOH) λ_{max}/nm (ε/M^{-1} cm^{-1}) 300 (7.39 × 103), 289 (6.29 × 103), 276 (1.57 × 104), 272 (1.82 × 104), 265 (2.48 × 104), 254 (1.95 × 104). HRMS (ESI$^+$) *m/z*: $[M+H]^+$ calcd for $C_{47}H_{54}N_9O_{11}$: 920.3937, found: 920.3940.



Fmoc-GFFQGD



In a typical synthesis starting with 0.558 g of 2-chlorotritylchloride resin, (1.1 mmol/g loading) 0.253 g of Fmoc-GFFQGD was isolated after semi-preparative HPLC. (isolated yield: 46%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (br s, 2H), 8.14 (d, *J* = 7.4 Hz, 1H), 8.01 (br s, 1H), 7.95 (d, *J* = 8.1 Hz, 1H), 7.89 (d, *J* = 7.4 Hz, 2H), 7.70 (d, *J* = 7.3 Hz, 2H), 7.47 (t, *J* = 5.5 Hz, 1H), 7.41 (t, *J* = 7.3 Hz, 2H), 7.32 (t, *J* = 7.2 Hz, 2H), 7.27 – 7.07 (m, 10H), 6.78 (s, 1H), 4.65 – 4.44 (m, 3H), 4.32 – 4.15 (m, 4H), 3.81 – 3.66 (m, 2H), 3.65 – 3.44 (m, 3H), 3.10 – 2.65 (m, 4H), 2.59 (dd, *J* = 16.7, 6.6 Hz, 2H), 2.12 (t, *J* = 7.6 Hz, 2H), 1.96 – 1.71 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.85, 172.24, 171.63, 171.22, 170.92, 170.87, 168.81, 168.47, 156.40, 143.83, 140.69, 137.69, 137.61, 129.21, 128.07, 127.96, 127.62, 127.08, 126.24, 126.19, 125.26, 120.09, 65.77, 53.89, 53.58, 52.35, 48.54, 46.59, 43.24, 41.59, 37.51, 37.24, 36.11, 31.39, 28.04. UV-Vis (MeOH) λ_{max}/nm (ε/M^{-1} cm^{-1}) 300 (5.37 × 103), 289 (4.98 × 103), 276 (1.20 × 104), 272 (1.39 × 104), 265 (1.86 × 104), 254 (1.52 × 104). HRMS (ESI^{+}) *m/z*: $[M+Na]^{+}$ calcd for $C_{46}H_{49}N_7O_{12}Na$: 914.3337, found: 914.3339.

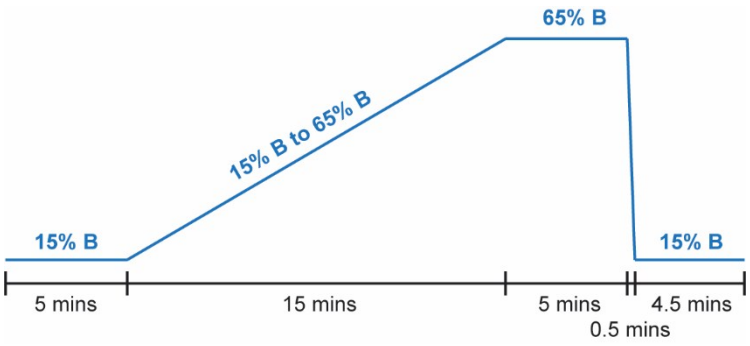


LC-MS of the pre-gel solutions

LCMS samples were prepared by dissolving peptide gelators in 0.1 M NaOH as described above. This pre-gel solution was left at room temperature for 4 hours. A 20 μL aliquot of pre-gel solution was diluted into 1 mL of water. Chromatography traces were recorded using a Shimadzu Prominence Ultra Performance Liquid Chromatography system. The column used was a Restek Viva C4 column (5 μm, 100 x 2.1 mm) at a flow rate of 0.2 mL/min. 20 μL of diluted sample was injected for each chromatogram.

Semi-preparative HPLC

Semi-preparative HPLC was performed on a Shimadzu Prominence LC-20A HPLC system using a Grace® VisionHT™ C18-HL C18 5 μm, 150 x 200 mm preparative HPLC column at a flow rate of 5 mL/min. The mobile phase consisted of A: miliQ water with 0.1% formic acid and B: acetonitrile with 0.1% formic acid.



Hydrogel preparation

In a typical experiment to make a 0.5% (w/v) hydrogel, N-terminal hexapeptide was suspended in milliQ water with 2 equiv. of 0.1 M aqueous sodium hydroxide. The mixture was vortexed for 2 minutes to give a transparent solution was obtained (pH 9). The basic pre-gel mixture was mixed 1:1 with DMEM (pH 7.3) and then heated at 37 °C overnight to yield transparent hydrogels. In the case of Fmoc-GFFQGD, hydrogels were formed within minutes of mixing.

Rheology

Rheological measurements were performed on an Antor Paar MCR 302 rheometer fitted with a ridged 25 mm diameter parallel plate (model PP25/P2) and equipped with a Peltier-temperature-controlled hood (model H-PTD 200). Measurements were performed at various temperatures as specified on the figure caption, with gel samples covered with mineral oil to prevent sample evaporation. Time-resolved rheology was performed with a constant strain = 0.1 % and constant frequency = 1 Hz. Frequency sweeps using a constant strain = 0.1% and log ramp of frequency (0.1 – 100 Hz). Strain

sweeps were conducted using a constant frequency = 1 Hz and a log ramp of strain (0.01 – 100%). The temperature ramp was conducted using a constant frequency = 1 Hz and constant strain = 0.5%. The sample was heated from 20 °C to 60 °C at a rate of 0.0167 °C/s and then cooled from 60 °C to 20 °C rate of 0.0167 °C/s. For viscosity measurements the samples prepared and tested as pre-gel solutions were prepared at twice the concentration of the corresponding gels. Peptide gelators were prepared as described above. The viscosity was tested using a 25 mm diameter stainless steel parallel plate at a gap height of 0.5 mm. The shear rate was set at a logarithmic ramp to 1 to 10 000 /s.

Atomic Force Microscopy (AFM)

AFM measurements of Fmoc-GFFRGD pre-gel mixtures and hydrogels were performed on a Bruker Mulitmode 8 Atomic Force Microscope in ScanAsyst mode in air, whereby the imaging parameters are constantly optimized through the force curves that are collected, preventing damage of soft samples. Bruker ScanAsyst-Fluid+ probes were used, with a spring constant of 0.7 N/m. To prepare AFM samples, 0.5% (w/v) pre-gel mixtures or hydrogels were diluted 1/20 in milliQ water and vortexed for 30 seconds. A 10 μL of this solution was then placed on freshly cleaved mica and dried with a stream of N_2. Samples were allowed to dry overnight in the fume hood before imaging. Images were processed and analysed using Gwyddion 2.55.[1]

SANS measurements

Hydrogels were prepared according to the above method, except using D_2O, 0.1 M NaOD and deuterated DMEM (i.e. DMEM which had been lyophilised and reconstituted in the same volume of D_2O). Hydrogels were prepared according to the above method at 0.5% (w/v) and transferred as quickly as possible to a demountable titanium cell with quartz windows of 1 mm path length. Measurements were performed at 25 °C or 37 °C as noted, at a detector distances of 2 m and 14 m, which allowed for a q range of 0.008 – 0.41 Å to be covered. Neutrons of wavelength 5 Å with a wavelength spread characterised by a full width half maximum of 12% were used. The scattered data was corrected for the background, empty cell scattering and the sensitivity of the individual detector pixels. The data was reduced using IgorPro software[2] employing NIST macros specific to QUOKKA[3] to an absolute intensity scale and modelled using SasView.[4]

Circular Dichroism

CD measurements for were performed using a ChirascanPlus CD spectrometer, with data collected between wavelengths of 180 – 500 nm with a bandwidth of 1 nm, sample ratio of 0.1 s/point and step

of 1 nm. In a typical experiment, 0.5% (w/v) peptide sols or hydrogels were prepared as above and diluted as necessary in a 1:1 water DMEM mixture such that the HT values did not exceed 1000. Variable temperature experiments were performed at 3 °C intervals, with the sample given five minutes to equilibrate prior to measuring.

Fourier Transform Infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) measurements were made on a Perkin Elmer Spotlight 400 FT-IR spectrophotometer equipped with a diamond crystal attenuated total reflectance (ATR) accessory. Hydrogels were prepared at 0.5% (w/v) using deuterated solvents (D_2O, NaOD and DMEM which had been reconstituted in D_2O) and pressed between the diamond crystal and substrate. Measurements were performed at 25 °C and 37 °C using a temperature-controlled stage, with samples given 90 seconds to equilibrate before measurements were recorded. All spectra were scanned at least 16 times over the range of 4000 - 650 cm^{-1} and were acquired at a resolution of 4 cm^{-1}.

pH Measurements

Samples for pH measurements were prepared by dissolving 20 mg of peptide in 1 molar equivalent of 0.1 M sodium hydroxide and diluted to 1 mL using water to give a concentration of 2% w/v. This was serially diluted at a 1:1 ratio to give 1% w/v and 0.5% w/v. The pH was measured with a Sper Scientific IC860031 Benchtop Meter using an Orion™ PerpHecT™ ROSS™ Combination pH Micro Electrode. Once pH measurements were recorded, the samples were diluted with a volume equivalent of DMEM to give 1%, 0.5%, and 0.25% gels which were left overnight before the final pH was recorded.

Biological Studies

The L929 mouse fibroblasts (Cat. No. 85011425, Sigma, Sydney) where modified with a nuclear H2B-GFP (Histone 2B – green fluorescent protein) introduced into cells by electroporation. The L929 cells were cultured in T75 cell culture flasks (75 cm^2 area). Media was aspirated from the flask and the surface washed with phosphate buffer saline (5 mL). The phosphate buffer saline was removed and trypsin (3 mL) was applied. The flask was incubated for 3 min at 37 °C with 5% CO_2. The trypsin was neutralised with 6 mL of Dulbecco's Modified Eagle Medium (DMEM) and the cell suspension was centrifuged (1200 rpm, 3 min). The media was aspirated, and the pellet was resuspended in DMEM. The cells were stained with Trypan Blue and counted. 500,000 cells were reseeded into a T75 flask. Cells were passaged every 2-3 days.

i) AlamarBlue™ contact viability assays

Fmoc-GFFRGD was prepared as an initial solution of 2% w/v by dissolving Fmoc-GFFRGD (2 mg, 0.0022 mmol) in water (78 μL) and 1 molar equivalent of 0.1 M sodium hydroxide (22 μL). To this gelator solution was added 100 μL of DMEM to give a 1% w/v Fmoc-GFFRGD gel. 50 μL of gel was added to a 96 well plate and allowed to set overnight in an incubator (37 °C, 5% CO_2).

10,000 L929 cells were seeded on top of each treatment at a density of 100,000 cells/mL. Three wells containing no gel were used as controls with the same density of cells. The plate was incubated for 48 hours (37 °C, 5% CO_2). After incubation, the DMEM was aspirated and replaced with 10% AlamarBlueTM in DMEM. This was incubated for an additional 3 hours at 37 °C with 5% CO_2 and the plate was then read for absorbance at 571 nm and 596 nm.

ii) 2D live cell imaging

2D cell imaging experiments were performed using PDMS inserts containing 15 μL wells. The PDMS inserts were placed in the centre of the well of a 6 well plate. To the small 15 μL wells was added Fmoc-GFFRGD gels prepared as described above and allowed to set overnight. To the top of each insert was added 25,000 cells at a density of 100,000 cells/mL.

The plate was transferred to imaging stage of an Olympus IX83 system equipped with a high performance Peltier cooled digital 16 bit scientific CMOS (sCOMS) camera (Olympus Corporation, Shinjuku, Tokyo, Japan) which includes temperature and carbon dioxide regulation (37 °C, 5% CO_2). Olympus CellSens ® software was used to control the microscope to capture images. The microscope was set to 10x zoom and images were taken over 48 hours.

iii) 3D z-stack imaging

3D cell imaging was performed using a 24 well plate. A 2% w/v Fmoc-GFFRGD solution was prepared by dissolving Fmoc-GFFRGD (5 mg, 0.0054 mmol) in water (196 μL) and 1 molar equivalent of 0.1 M sodium hydroxide (54 μL). To the gelator solution was added 250 μL cell suspension containing L929 at a density of 400,000 cells/mL. 250 μL of the cell encapsulated mixture was then transferred to a 24 well plate and incubated for 48 hours. Images were obtained using an Olympus IX83 system equipped with a high performance Peltier cooled digital 16 bit scientific CMOS (sCOMS) camera (Olympus Corporation, Shinjuku, Tokyo, Japan) which includes temperature and carbon dioxide regulation (37 °C, 5% CO_2). The microscope was set to 10x zoom and images (1383x1383 μm) were taken with a 4 μm distance between each stack (total of 56 stacks, full z-height = 224 μm).

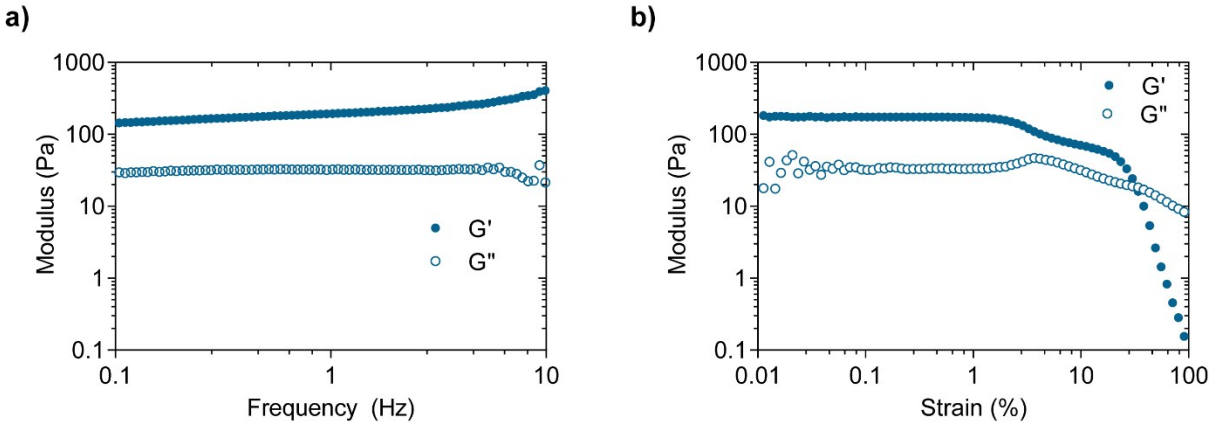


Figure S1. Rheological characterization for Fmoc-GFFRGD. (a) Frequency and (b) amplitude sweep for Fmoc-GFFRGD at 0.5% (w/v). Temperature = 37 °C.

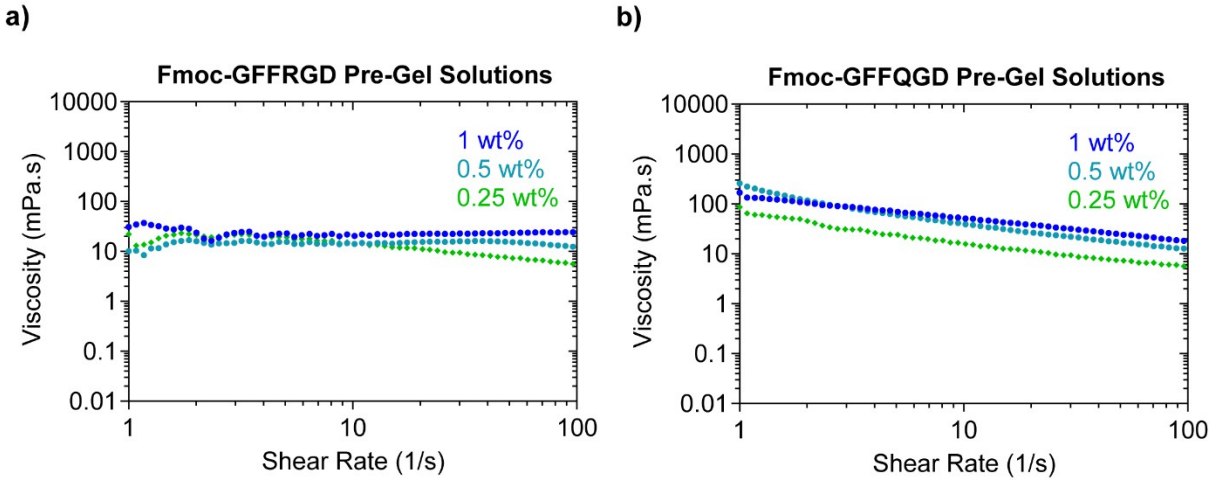


Figure S2. Viscosity measurements of Fmoc-GFFRGD and Fmoc-GFFQGD pre-gel solutions. (a) Fmoc-GFFRGD at 1.0, 0.5 and 0.25 wt% (b) Fmoc-GFFQGD at 1.0, 0.5 and 0.25 wt%. Concentrations represent the final concentration of the pre-gel solutions after being mixed 1:1 with DMEM. Actual concentration of the pre-gel solution is twice of the hydrogel.

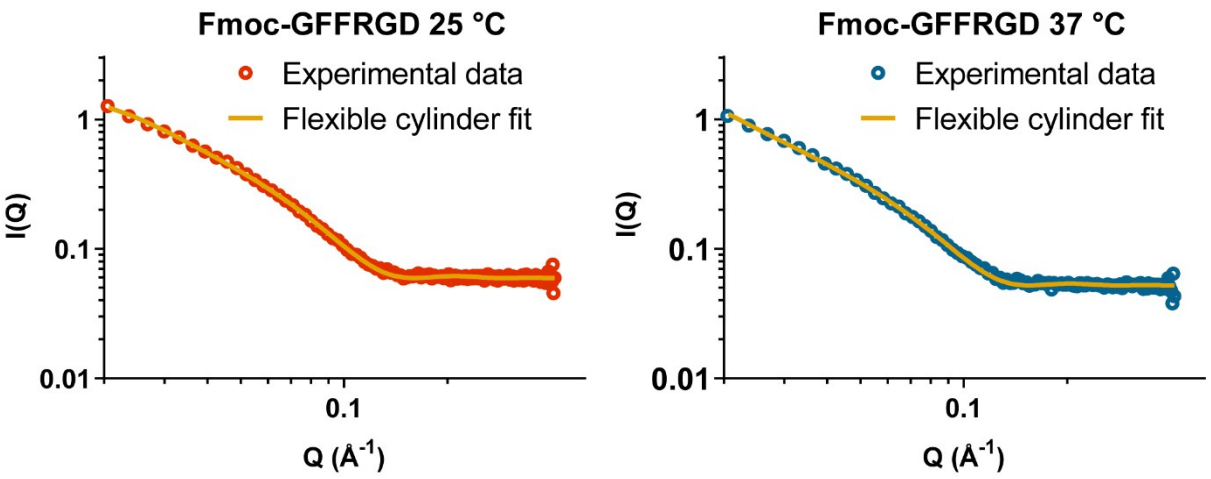


Figure S3. SANS data for Fmoc-GFFRGD at 25 °C and 37 °C after being mixed with DMEM. Data was fit to a flexible cylinder model. Concentration = 0.5% (w/v).

Sample	Cylinder Length (nm)	Kuhn Length (nm)	Radius (nm)	χ^2
25 °C Fmoc-GFFRGD	23.4 ± 2.5	14.2 ± 1.2	2.4 ± 0.02	1.14
37 ° C Fmoc-GFFRGD	25.6 ± 2.6	14.6 ± 1.2	2.47 ± 0.02	1.24

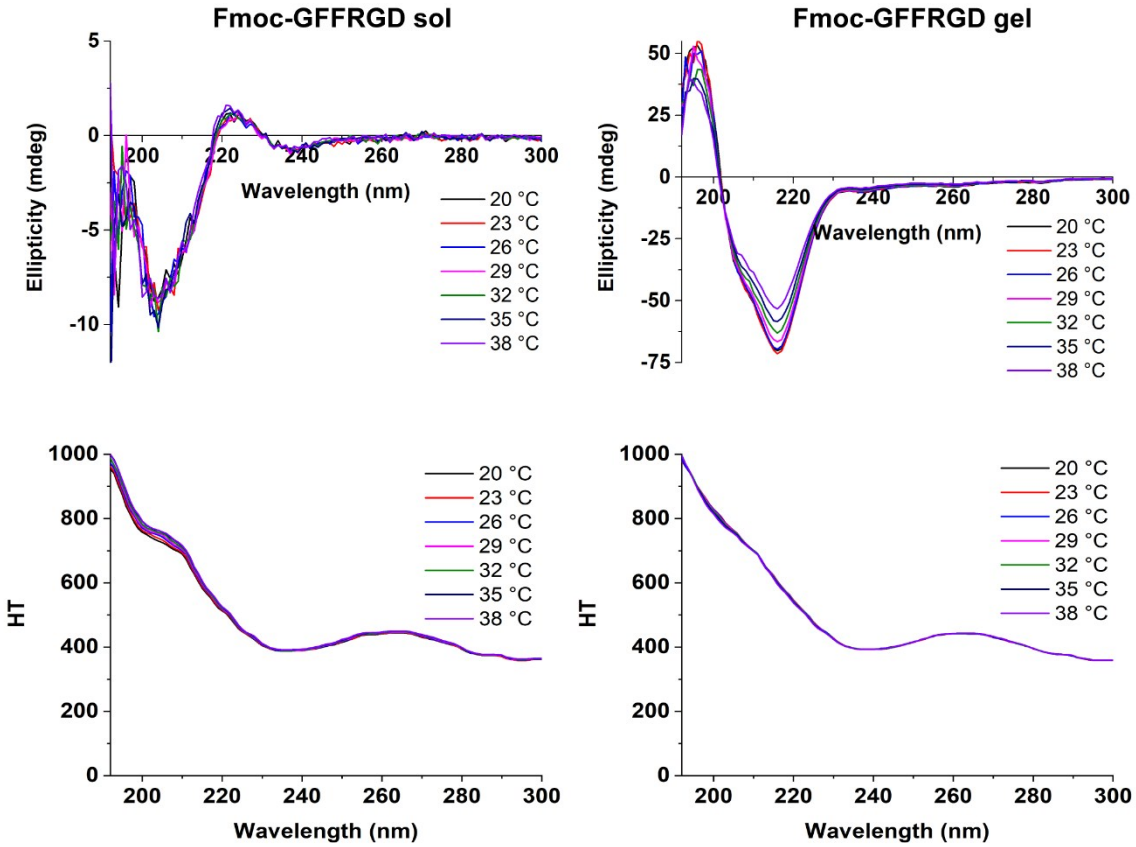


Figure S4. Temperature dependent circular dichroism of Fmoc-GFFRGD in solution before gelation and after gelation.

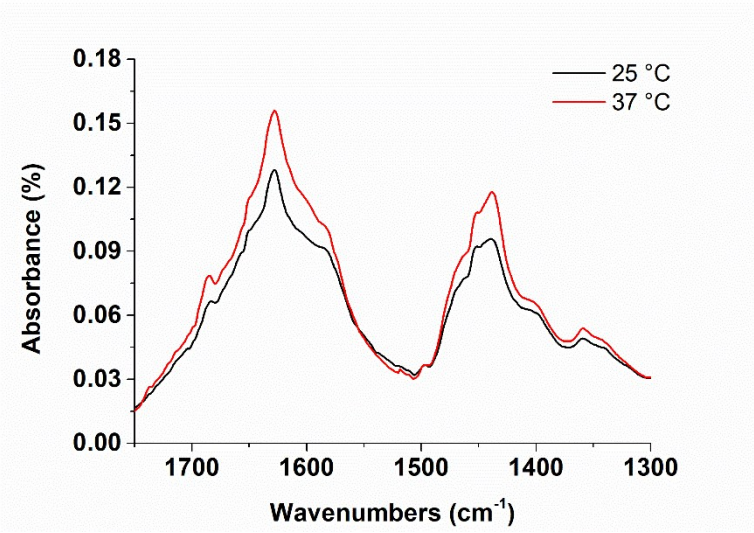


Figure S5. FTIR of Fmoc-GFFRGD after being mixed with DMEM at 25 °C and 37 °C.

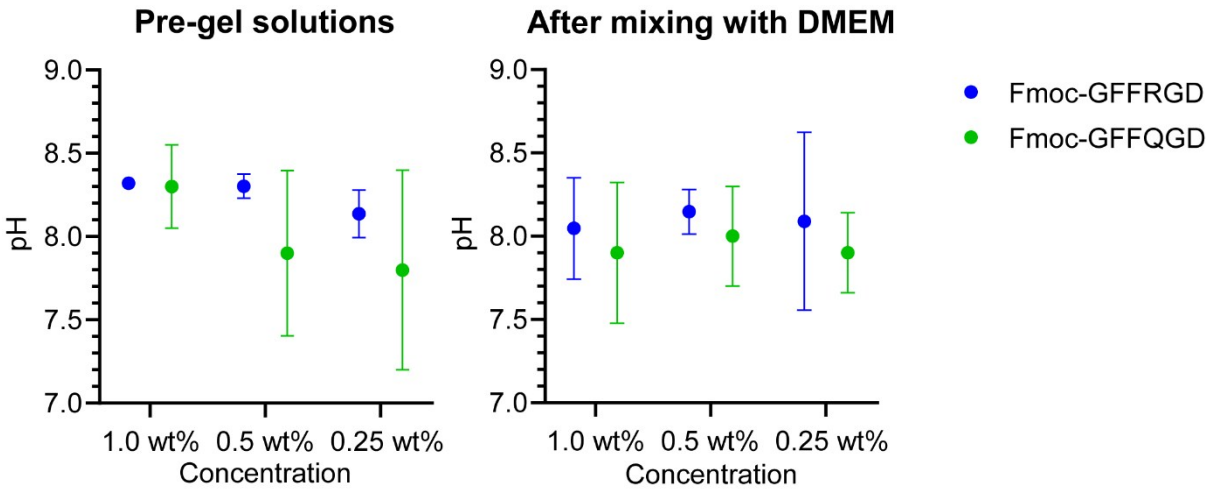


Figure S6. pH Measurements of Fmoc-GFFRGD and Fmoc-GFFQGD. The pH measurements were performed on pre-gel mixtures and after mixing with DMEM. The pH measurements show that the final pH of the Fmoc-GFFRGD and Fmoc-GFFQGD solutions after mixing with DMEM are comparable.

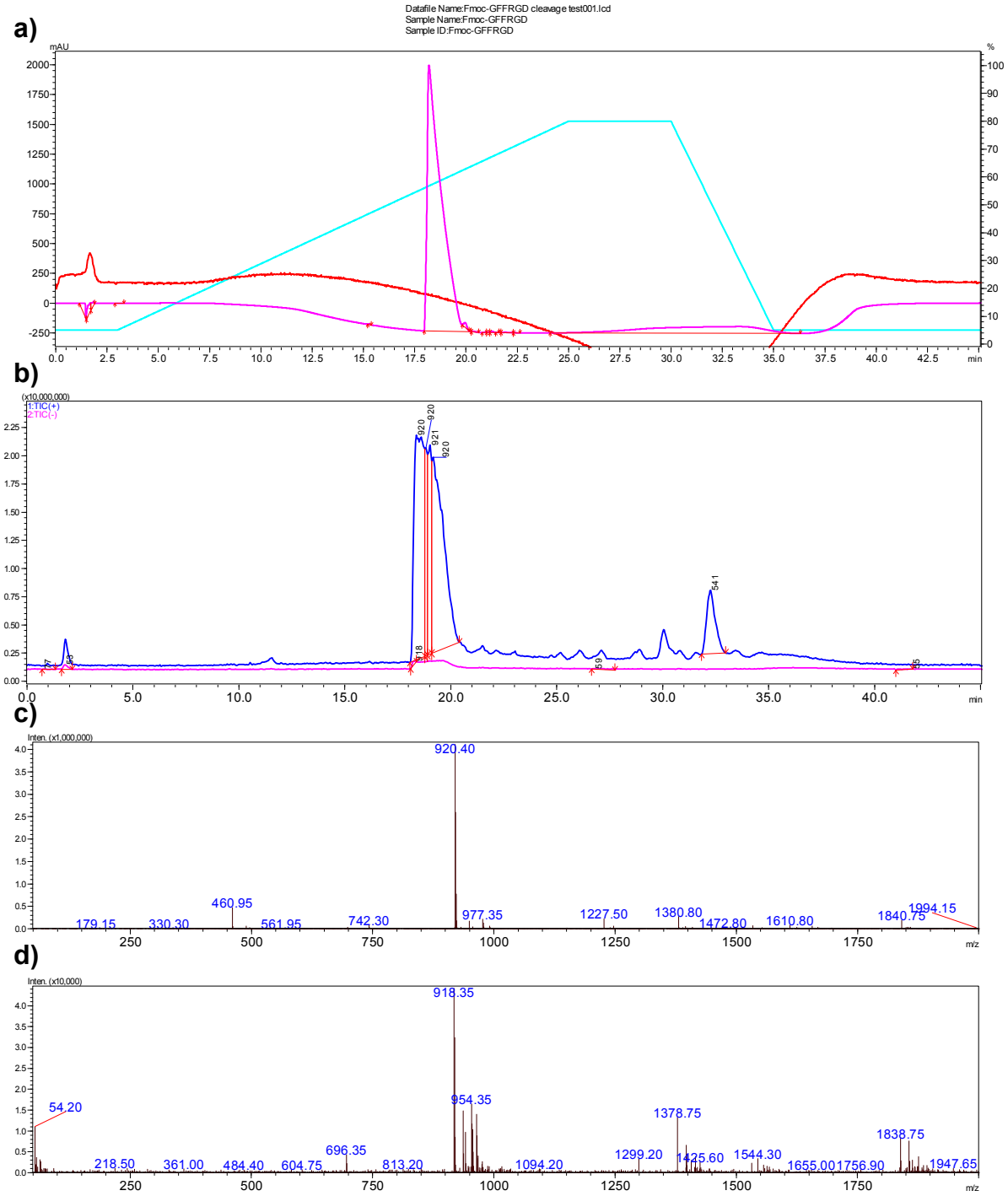


Figure S7. LC-MS analysis of Fmoc-GFFRGD solutions after addition of 0.1 M NaOH solution shows Fmoc group is not removed. a) HPLC trace at 254 nm. b) TIC for positive mode ESI-MS in blue and negative mode ESI-MS in pink. c) Positive mode ESI-MS shows a peak which can be assigned to $[M+H]^+$ for Fmoc-GFFRGD, calculated = 920.40, found 920.40. d) Negative mode ESI-MS shows peaks which can be assigned to $[M-H]^-$ for Fmoc-GFFRGD, calculated = 918.38, found 918.35 and $[M+Cl^-]^-$ for Fmoc-GFFRGD, calculated = 954.36, found 954.35. NB there is a small peak in the negative mode ESI-MS at 696.35 which can be assigned to $[M-H]^-$ for the cleaved product, NH_2-GFFRGD however, this can be attributed to cleavage during ESI-MS which is sometimes observed for Fmoc-protected peptides[5] as there is no change in retention time and observation of the related $[M+H]^+$ adduct in the positive mode ESI-MS.

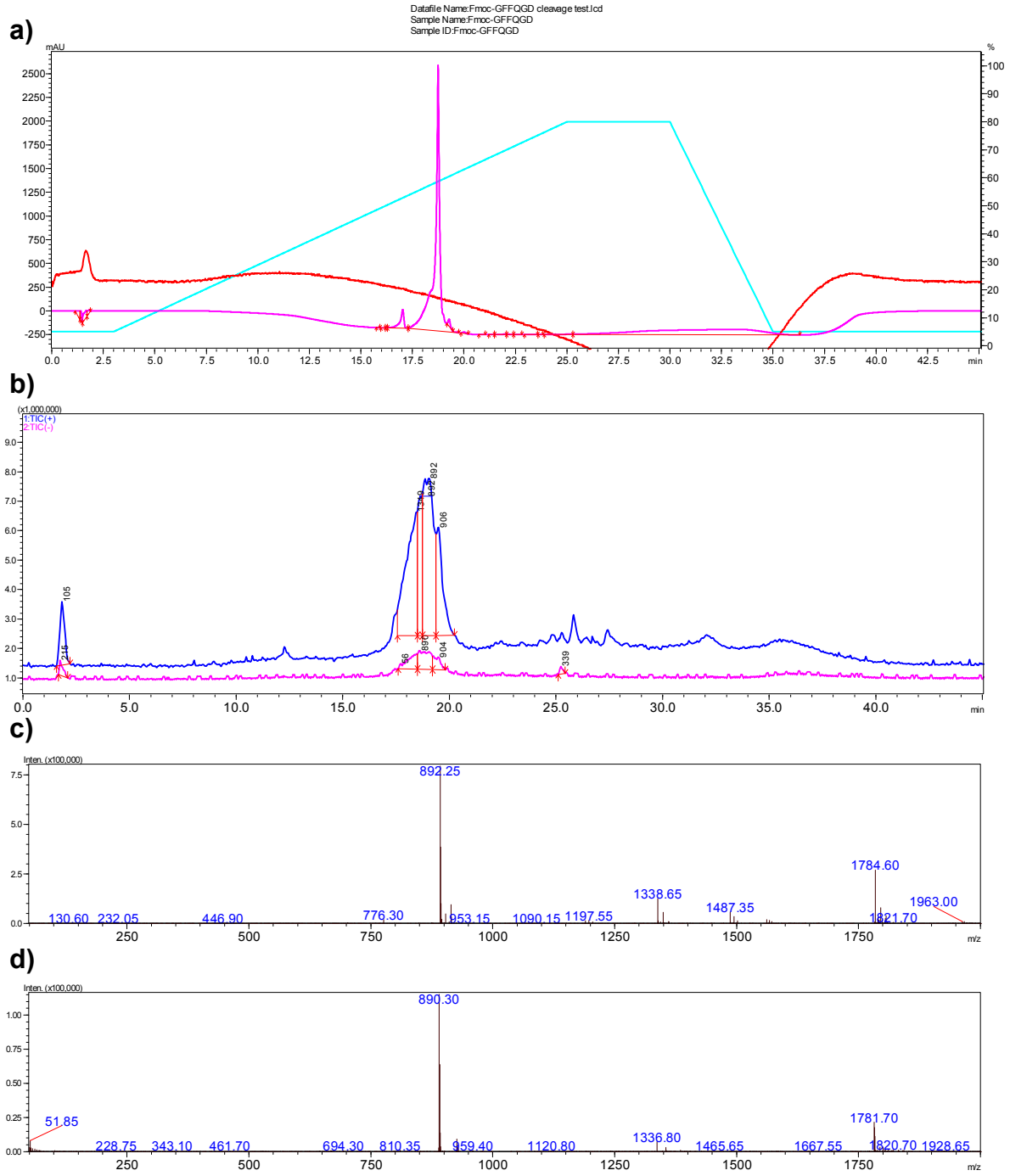


Figure S8. LC-MS analysis of Fmoc-GFFQGD solutions after addition of 0.1 M NaOH solution shows Fmoc group is not removed. a) HPLC trace at 254 nm. b) TIC for positive mode ESI-MS in blue and negative mode ESI-MS in pink. c) Positive mode ESI-MS shows a peak which can be assigned to $[M+H]^+$ for Fmoc-GFFQGD, calculated = 892.34, found 892.25. d) Negative mode ESI-MS shows a peak which can be assigned to $[M-H]^-$ for Fmoc-GFFQGD, calculated = 890.33, found 890.33.

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