Supplementary information

Effect of polar amino acid incorporation on Fmoc-diphenylalanine-based tetrapeptides

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Contents:

Solid phase peptide synthesis of capped dipeptides

Initial amino acid loading

2-chlorotrityl chloride resin (100-200 mesh; 1% DVB; 1.1 mmol/g) (500 mg, 0.55 mmol) was weighed into a 10 mL polypropylene syringe equipped with a porous polypropylene frit (Torviq SF-1000), which was used as the reaction vessel. The resin was washed with dichloromethane (3 × 5 mL) before being allowed to swell in dichloromethane (5 mL) for at least 0.5 h prior to the loading of the first amino acid.

A solution of Fmoc-Phe-OH (3 equiv., 640 mg) was dissolved in a mixture of dry dichloromethane (2 mL), *N,N*-dimethylformamide (2 mL) and *N,N*-diisopropylethylamine (DIPEA) (8 equiv., 0.8 mL) and taken up into the syringe with resin and stirred overnight using an orbital shaker. The resin was then washed with dichloromethane (3 x 4mL) and *N,N*-dimethylformamide (DMF) (3 × 4 mL).

N-terminal Fmoc deprotection

A solution of 20% (v/v) piperidine in DMF (2 × 4 mL) was added to the resin once for 1 min, then a fresh aliquot was taken up again and stirred for 10 mins. The solution was subsequently expelled and the resin washed with DMF (5 × 4 mL). The resulting resin-bound amine was used immediately in the next peptide coupling step.

Amino acid coupling

The next amino acid (3 equiv., masses as below) was dissolved in a 0.45 M DMF solution of 1-hydroxybenzotriazole hydrate (HOBt·H_2O)/*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) (3 equiv.) and DIPEA (6 equiv., 0.6 mL) and this coupling solution added to the resin and stirred for 45 mins using an orbital shaker. The solution was expelled and the resin washed with DMF (5 x 4 mL).

Amino acid	Mass used (mg)
Fmoc-Phe-OH	640
Fmoc-Asp(O^{t}bu)-OH	680
Fmoc-Glu(O^{t}bu)-OH	702
Fmoc-Asn(Trt)-OH	984
Fmoc-Gln(Trt)-OH	1008
Fmoc-Arg(Pbf)-OH	1070
Fmoc-Lys(Boc)-OH	773

After another *N*-terminal Fmoc deprotection, iterative couplings were performed in order to build up the required peptide sequence.

Cleavage of the peptide

After the final coupling step, the resin was washed with DMF (3 x 4 mL) and dichloromethane (3 x 4 mL). For all peptides except Fmoc-NNFF, Fmoc-QQFF and Fmoc-RRFF, a solution of 1:9 dichloromethane: trifluoroacetic acid with three drops of water was then added to the resin, and the resin stirred for 2 hours using an orbital shaker. For Fmoc-NNFF and Fmoc-QQFF, the protected peptide was cleaved from the resin using 10% trifluoroacetic acid in dichloromethane and purified using semi-preparative HPLC, before lyophilisation and cleavage using 1:9 dichloromethane: trifluoroacetic acid and three drops of water for 2 hours. For Fmoc-RRFF, a solution of trifluoroacetic acid, water and triisopropylsilane in a 95: 2.5: 2.5 ratio was added to the resin and the resin stirred for 3h. The cleavage solution was then expelled, the resin washed with dichloromethane (2 x 4 mL) and the solvents evaporated under a stream of nitrogen. The resulting residue was lyophilised and purified by semi-preparative HPLC using an acetonitrile/water gradient, giving a white fluffy solid.

Characterisation data for **Fmoc-diaspartic acid-diphenylalanine (Fmoc-DDFF)**: IR: 3281 (m), 3064 (w), 3032 (w), 1703 (s), 1645 (s), 1534 (s), 1498 (m), 1448 (w), 1408 (w), 1260 (m), 1226 (s), 1192 (m), 1084 (w), 1049 (w), 916 (w), 739 (s), 699 (s); ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.06 (br s, 1H), 8.20 (d, *J* = 7.2 Hz, 1H), 7.89 (d, *J* = 6.9 Hz, 1H), 7.76-7.66 (m, 2H), 7.41 (t, *J* = 7.0 Hz, 1H), 7.34-7.14 (m, 6H), 4.51 – 4.34 (m, 2H), 4.24 – 4.07 (m, 4H), 3.07-2.87 (m, 2H), 2.79 – 2.73 (m, 0.5H), 2.65-2.57 (m, 1H), 2.47-2.39 (m, 0.5H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.08, 172.30, 172.25, 171.49, 171.05, 170.53, 156.31, 152.12, 151.75, 148.32, 148.16, 144.29, 141.19, 137.98, 137.84, 129.68, 129.61, 128.68, 128.46, 128.11, 127.57, 126.92, 125.81, 120.58, 54.18, 54.03, 50.07, 47.08, 37.79, 37.25, 36.37; HR-MS (ESI): calcd for $C_{41}H_{40}N_4O_{11}$ + Na^+: 787.2634, found 787.2679.

Characterisation data for **Fmoc-diglutamic acid-diphenylalanine (Fmoc-EEFF)**: IR: 3278 (m), 3065 (w), 3032 (w), 1697 (s), 1665 (m), 1636 (s), 1537 (s), 1498 (m), 1447 (m), 1404 (w), 1402 (w), 1286 (m), 1264 (s), 1219 (s), 1216 (s), 1104 (w), 1086 (w), 1041 (w), 914 (w), 785 (w), 760 (m), 741 (s), 697 (s); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.31 (d, *J* = 7.7 Hz, 1H), 7.99 (d, *J* = 8.2 Hz, 1H), 7.93–7.86 (m, 3H), 7.71 (t, *J* = 7.9 Hz, 2H), 7.56 (d, *J* = 8.0 Hz, 1H), 7.45–7.38 (m, 2H), 7.36–7.30 (m, 2H), 7.29–7.12 (m, 10H), 4.58–4.42 (m, 2H), 4.32–4.18 (m, 4H), 4.06–3.97 (m, 1H), 3.10–2.87 (m, 4H), 2.79–2.70 (m, 1H), 2.28–2.12 (m, 4H), 1.92–1.62 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.50, 174.44, 173.12, 171.77, 171.34, 171.14, 156.43, 144.36, 144.18, 144.16, 137.95, 137.76, 129.58, 129.55, 128.66, 128.44, 128.12, 127.57, 126.91, 126.69, 125.77, 125.75, 120.58, 66.16, 54.34, 53.93, 53.86, 52.11, 47.11, 37.91, 37.16, 30.73, 30.46, 28.07, 27.68; HR-MS (ESI): calcd for $C_{43}H_{44}N_4O_{11}$ + Na^+: 793.3004, found 793.3087.

Characterisation data for **Fmoc-diasparagine-diphenylalanine (Fmoc-NNFF)**: IR: 3284 (m), 3067 (w), 1697 (m), 1643 (s), 1537 (s), 1442 (m), 1387 (w). 1321 (m), 1265 (s), 1200 (m), 910 (w), 740 (s), 696 (s); 1H NMR (400 MHz, DMSO-d_6) δ 8.14 (d, *J* = 7.5 Hz, 1H), 8.07 (d, *J* = 7.5 Hz, 1H), 7.95 (d, *J* = 8.1 Hz, 1H), 7.82 (d, *J* = 7.8 Hz, 2H), 7.67–7.60 (m, 2H), 7.50 (d, *J* = 7.8 Hz, 1H), 7.38–7.30 (m, 3H), 7.28–7.25 (m, 2H), 7.25–7.22 (m, 1H), 7.21–7.18 (m, 2H), 7.18–7.16 (m, 2H), 7.16–7.10 (m, 6H), 7.09–7.03 (m, 1H), 6.92 (s, 1H),

6.84 (s, 1H), 4.43–4.24 (m, 4H), 4.23–4.10 (m, 3H), 3.04–2.82 (m, 3H), 2.76–2.65 (m, 1H), 2.50–2.46 (m, 1H), 2.40–2.24 (m, 3H). 13C NMR (126 MHz, DMSO-d_6) δ 173.03, 172.11, 171.82, 171.19, 171.02, 156.21, 144.31, 144.25, 144.22, 141.14, 138.35, 137.97, 129.61, 129.57, 129.50, 128.83, 128.69, 128.51, 128.23, 128.11, 128.00, 127.60, 127.11, 126.89, 126.72, 126.62, 125.83, 120.56, 66.35, 56.23, 54.43, 54.16, 52.05, 50.41, 47.04, 37.95, 37.36, 37.24; HR-MS (ESI): calcd for $C_{41}H_{42}N_6O_9$ + Na^+: 763.3022, found 763.3097.

Characterisation data for **Fmoc-diglutamine-diphenylalanine (Fmoc-QQFF)**: IR: 3303 (m), 3066 (w), 1661 (s), 1637 (s), 1542 (s), 1444 (s), 1334 (w), 1270 (m), 1151 (m), 1010 (m), 755 (s), 698 (s); 1H NMR (400 MHz, DMSO-d6) δ 8.33 (d, *J* = 8.2 Hz, 1H), 7.98 (d, *J* = 7.9 Hz, 2H), 7.90 (d, *J* = 7.5 Hz, 2H), 7.72 (t, *J* = 7.5 Hz, 2H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.37–7.28 (m, 4H), 7.28–7.24 (m, 4H), 7.23–7.19 (m, 6H), 7.15–7.11 (m, 1H), 6.78 (d, *J* = 8.8 Hz, 2H), 4.57–4.40 (m, 2H), 4.29–4.18 (m, 4H), 4.01–3.93 (m, 1H), 3.11–2.88 (m, 3H), 2.81–2.68 (m, 1H), 2.18–1.95 (m, 4H), 1.91–1.60 (m, 4H). 13C NMR (126 MHz, DMSO-d6) δ 174.40, 174.29, 173.12, 171.96, 171.43, 171.36, 156.45, 144.35, 144.25, 144.22, 141.16, 137.96, 137.79, 129.57, 129.50, 128.82, 128.68, 128.47, 128.23, 128.12, 128.00, 127.58, 127.11, 126.92, 126.72, 125.80, 120.57, 66.22, 56.23, 54.87, 53.93, 52.51, 47.09, 37.95, 37.16, 32.06, 31.89, 28.70, 28.25; HR-MS (ESI): calcd for $C_{43}H_{46}N_6O_9$ + H^+: 791.3345, found 791.3408.

Characterisation data for **Fmoc-diarginine -diphenylalanine (Fmoc-RRFF)**: IR: 3288 (m), 3189 (m), 3066 (m), 2947 (w), 1664 (s), 1516 (s), 1451 (m), 1325 (w), 1254 (m), 1193 (s), 1182 (s), 1132 (s), 1032 (w), 837 (w), 801 (m), 759 (w), 741 (m), 721 (m), 699 (m); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.53 (s, 1H), 8.39 (d, *J* = 8.2 Hz, 1H), 8.22 (s, 1H), 7.87 (d, *J* = 7.9 Hz, 1H), 7.83 (d, *J* = 7.5 Hz, 2H), 7.64 (d, *J* = 7.5 Hz, 2H), 7.50 (d, *J* = 7.9 Hz, 1H), 7.41–7.32 (m, 3H), 7.30–7.22 (m, 3H), 7.20–7.13 (m, 7H), 7.13–7.06 (m, 8H), 4.27–4.18 (m, 2H), 4.17–4.08 (m, 3H), 4.03–3.96 (m, 1H), 3.93–3.86 (m, 1H), 3.09–3.02 (m, 1H), 3.00–2.90 (m, 4H), 2.90–2.81 (m, 2H), 2.72–2.63 (m, 1H), 1.65–1.49 (m, 2H), 1.46–1.11 (m, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.66, 171.86, 170.74, 163.72, 157.41, 157.28, 156.45, 144.27, 144.21, 144.18, 138.73, 138.38, 130.13, 129.38, 128.62, 128.29, 128.16, 127.55, 126.77, 126.46, 125.77, 125.73, 120.62, 66.11, 56.09, 55.51, 54.43, 52.96, 47.09, 41.14, 40.83, 37.99, 37.53, 30.50, 29.13, 25.19, 24.65; HR-MS (ESI): calcd for $C_{45}H_{54}N_{10}O_7$ - H^+: 845.4220, found 845.4105.

Characterisation data for **Fmoc-dilysine -diphenylalanine (Fmoc-KKFF)**: IR: 3286 (m), 3063 (m), 3034 (w), 2944 (m), 1663 (s), 1635 (s), 1524 (s), 1450 (m), 1412 (w), 1395 (m), 1334 (w), 1254 (m), 1201 (s), 1182 (s), 1130 (s), 1082 (w), 1032 (w), 837 (w), 800 (w), 757 (w), 739 (m), 722 (m), 697 (m); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.35 (d, *J* = 8.3 Hz, 1H), 7.90-7.87 (m, 4H), 7.72-7.69 (m, 3H), 7.42 (t, *J* = 7.8 Hz, 3H), 7.33 (t, *J* = 7.4 Hz, 2H), 7.25-7.15 (m, 10H), 4.37-4.13 (m, 6H), 3.96-3.91 (m, 1H), 3.09-2.89 (m, 4H), 2.76 – 2.64 (m, 5H), 1.58-1.17 (m, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.99, 170.92, 155.95, 143.79, 140.73, 138.25, 137.97, 129.53, 128.91, 128.07, 127.67, 127.07, 125.99, 125.28, 120.15, 65.54, 54.90, 54.16, 46.67, 37.47, 37.33, 32.77, 31.31, 27.12, 26.58, 22.45, 22.30; HR-MS (ESI): calcd for $C_{45}H_{54}N_6O_7$ + H^+: 791.4120, found 791.4196.

Preparation of hydrogels

pH switch

For Fmoc-DDFF, Fmoc-EEFF, Fmoc-NNFF and Fmoc-QQFF, 3.5 equivalents of 0.1 M aqueous sodium hydroxide was added to the peptide and milliQ water added to make the suspension up to the required concentration. This suspension was sonicated until homogenous, upon which time 4.5 molar equivalents of glucono-δ-lactone was added to lower the pH, resulting in gelation.

Salt screening

Fmoc-RRFF and Fmoc-KKFF were dissolved in milliQ water at the appropriate concentration, before an equal volume of Dulbecco's Modified Eagle Medium (DMEM) was added, to give the desired hydrogel concentration. For AFM, CD and FTIR experiments, gelation was triggered through the addition of PBS (or deuterated PBS for ATR-IR experiments) to avoid interference from components of DMEM.

AFM measurements

Gel samples were prepared according to the appropriate gelation trigger described above and one drop of the hydrogel solutions was cast onto a freshly cleaved mica substrate, followed by spreading of the drop over the mica using a glass slide, with the excess liquid wicked away using capillary action. Samples were cast at 2x below their minimum gel concentration and left to dry in air overnight. Imaging was undertaken on a Bruker Multimode 8 atomic force microscope in Scanasyst mode in air, whereby the imaging parameters are constantly optimised through the force curves that are collected, preventing damage of soft samples. Bruker Scanasyst-Air probes were used, with a spring constant of 0.4 - 0.8 N/m and a tip radius of 2 nm.

Circular dichroism measurements

CD measurements for Fmoc-DDFF, Fmoc-EEFF, Fmoc-RRFF and Fmoc-KKFF 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. CD measurements for Fmoc-NNFF and Fmoc-QQFF were collected on a Jasco J-1500 spectrophotometer, with data collected between 180 – 500 nm with a bandwidth of 2 nm, digital integration time (D.I.T.) of 2 seconds, scan speed of 100 nm/min and data pitch of 0.1 nm. In a typical experiment, 1% (w/v) peptide sols or hydrogels were prepared as above and diluted as necessary in water. Temperature was kept constant at 25 °C and all experiments were repeated at least three times and averaged into a single plot.

Attenuated Total Reflectance-Infrared Spectroscopy measurements

For Fmoc-DDFF, Fmoc-EEFF, Fmoc-RRFF and Fmoc-KKFF, 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. For Fmoc-NNFF and Fmoc-QQFF, measurements were made on a JASCO FT/IR 4700 spectrophotometer fitted with a PIKE MIRacleTM Single Reflection ATR accessory and ZnSe crystal plate. Hydrogels were prepared at 1% (w/v) in D_2O and pressed between the diamond crystal and substrate. 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}.

Rheology measurements

Rheological measurements were performed on an Anton Paar MCR 302 rheometer using a 25 mm stainless steel parallel plate geometry configuration and analysed using RheoPlus v3.61 software. Typical rheology measurements involved casting 550 μL of a peptide hydrogel at the desired concentration, using the appropriate trigger described above, onto one of the stainless steel plates, lowering the other plate to the measurement position, and monitoring the storage and loss moduli over time. Once the storage modulus had plateaued, frequency sweep measurements were commenced. A Peltier temperature control hood and solvent trap was used to reduce evaporation and maintain a temperature of 25 °C for frequency and amplitude sweeps. Frequency sweeps were performed with a log ramp frequency (f) = 0.01 – 10 Hz and constant strain (γ) = 0.2%. Strain sweeps were performed with a log ramp strain (γ) = 0.1 – 100% at a constant frequency (f) = 1 Hz. Time resolved rheology was performed at constant frequency (f) = 1 Hz and strain (γ) = 0.2%. The rheology plots displayed are an average of at least three repeats for each point and error bars denote two standard deviations from the log-averaged mean.

pK_a measurements

Anionic and neutral polar tetrapeptides Fmoc-DDFF, Fmoc-EEFF, Fmoc-NNFF and Fmoc-QQFF were dissolved at a concentration of 0.1% (w/v) through the addition of MilliQ water and 3.5 equivalents of 0.1 M NaOH. 0.1 M HCl was added in 50 μL aliquots, and the pH allowed to stabilise for several minutes before a reading was taken. For cationic tetrapeptides Fmoc-RRFF and Fmoc-KKFF, 3.5 equivalents of 0.1 M HCl and MilliQ water were added to dissolve the peptide at 0.1% (w/v) and 0.1 M NaOH added in 50 μL aliquots. Each titration was repeated three times and averaged into a single plot. Measurements were recorded on a Hannah Edge HI2002 pH probe. pK_a for Fmoc-DDFF and Fmoc-EEFF was determined to be the "buffering plateau" seen in Fig. S11, and pK_b was calculated by determining the volume at which the equivalence point occurs through plotting the derivative of the graph, finding the pH at half of this volume and subtracting from 14.

Zeta potential measurements

The zeta potential of peptide samples were measured using a Malvern Instruments Zetasizer NanoZS, equipped with a He-Ne laser beam with a wavelength of 633 nm and scattering angle of 12°. Measurements were performed in folded capillary cells (Malvern Instruments, DTS1070) using peptide sols prepared at 0.5% (w/v) (*i.e.* Fmoc-DDFF, Fmoc-EEFF, Fmoc-QQFF, Fmoc-NNFF dissolved in basic water and Fmoc-RRFF, Fmoc-KKFF dissolved in acidic water) and hydrogels prepared at 1% (w/v) (using a GdL trigger for Fmoc-DDFF,

Fmoc-EEFF, Fmoc-NNFF and Fmoc-QQFF, and NaOH for Fmoc-RRFF and Fmoc-KKFF to avoid interference from buffer ions) which had then been dispersed in an equal volume of MilliQ water.

Dye incubation measurements

Hydrogels were prepared using the appropriate method as described above. For the anionic Fmoc-DDFF, the peptide was dissolved in aqueous sodium hydroxide (0.1 M) and MilliQ water at a concentration of 1% (w/v) before the addition of glucono-δ-lactone. Before gelation occurred, an equal volume of 100 μM dye solution was added, giving a final concentration of 50 μM dye and 0.5% (w/v) hydrogel. For the cationic Fmoc-KKFF, the peptide was dissolved in a 100 μM solution of dye, before an equal volume of 1x PBS (pH 7.4), giving final concentrations of 50 μM dye and 0.5% w/v) hydrogel. The gels were left to set overnight.

The next day, 1 mL 1x PBS (pH 7.4) was added to the gels and 500 μL aliquots removed at 1, 2, 4, 6, 8, 12 and 24 hours. After each aliquot was removed, an equal volume of 1x PBS was added to the gel. Absorbance measurements were recorded on a Varian Cary 50 Bio UV-Visible spectrophotometer.

Cell viability measurements

Cytotoxicity measurements were performed using an Alamar Blue colorimetric assay on HEK-293T cells. Each experiment was repeated at least three times. Cells were passaged using standard cell culture procedures. Cells were detached with trypsin and centrifuged (1000 rpm for 3 min). The supernatant was removed and the cells resuspended in Dulbecco's Modified Eagle Medium (DMEM) at a concentration of 100,000 cells/mL. Cells were seeded at a concentration of 10,000 cells/well.

For solution cytotoxicity measurements, cells were seeded into a 96 well plate and incubated overnight to attach to the substrate. Peptides were dissolved in DMSO and then diluted in DMEM to their desired concentration, with a final DMSO concentration of 1% (v/v). Media was then aspirated from the cells and replaced with 100 μL of the dissolved peptide solution in triplicate. Cells were then incubated for 24 hours and 10 μL Alamar Blue added to the wells, followed by incubation for 4 hours. Control wells included no cells, no treatment and a negative control of 20% (v/v) DMSO. The absorbance at 570 nm and 596 nm was recorded using a BioRad Benchmark plate reader.

For contact cytotoxicity measurements, to a 96-well plate, 100 μL of gel was added in triplicate with their respective triggers and allowed to set overnight. Surrounding wells were supplemented with water to ensure hydration of the gels. Gels triggered with GdL were then incubated for 24 hours with PBS in order to buffer the gels and minimise the effects of any excess GdL. Cells were then seeded atop the hydrogels and incubated for 24 hours, before 10 μL Alamar Blue was added to the wells, followed by further incubation for 4 hours. Control wells included cell-free gels, no hydrogels and a negative control of 20% (v/v) DMSO. The absorbance at 570 nm and 596 nm was recorded using a BioRad Benchmark plate reader.

Tethered bilayer lipid membrane experiments

Formation of tethered bilayer lipid membranes (tBLMs) was achieved using pre-prepared tethered benzyl-disulfide (tetra-ethyleneglycol) n = 2 C_{20}-phytanyl tethers (DLP) and benzyl-disulfide-tetra-ethyleneglycol-OH spacers (TEGOH) in the ratio of 1:9, as described previously (SDx Tethered Membranes Pty. Ltd., Australia).[1] To the tethering chemistry first layer was added 8 μL of a 3 mM solution of a mobile lipid-phase mixture of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) lipids (Sigma-Aldrich) dissolved in ethanol. After a 2 min incubation, a rapid solvent exchange was undertaken with a buffer consisting of 100 mM NaCl and 10 mM tris at pH 7.2 to form the self-assembled tBLM. Swept frequency AC electrical impedance spectroscopy using a TethaQuick electrical impedance spectrometer (SDx Tethered Membranes Pty. Ltd., Australia) was used to determine membrane conduction. For this, phase and impedance magnitude data was collected using a 50 mV peak-peak AC excitation (2000 Hz – 0.1 Hz). An equivalent circuit consisting of a Constant Phase Element (CPE) in series with a Resistor/Capacitor (RC) element representing the lipid membrane, and an extra resistor in series representing the NaCl/tris buffer, was used to fit the data. The CPE in this circuit represents the imperfect capacitance created by the chemically coated gold tethering electrode.[2] Data were fitted using a proprietary adaptation of a Levenberg–Marquardt fitting routine. Peptides were initially dissolved in water (for polar cationic tetrapeptides) or basic water (for polar anionic/neutral tetrapeptides) at a concentration of 1% (w/v) (approximately 10 mM) and diluted to the appropriate concentration using the NaCl/tris buffer described above.

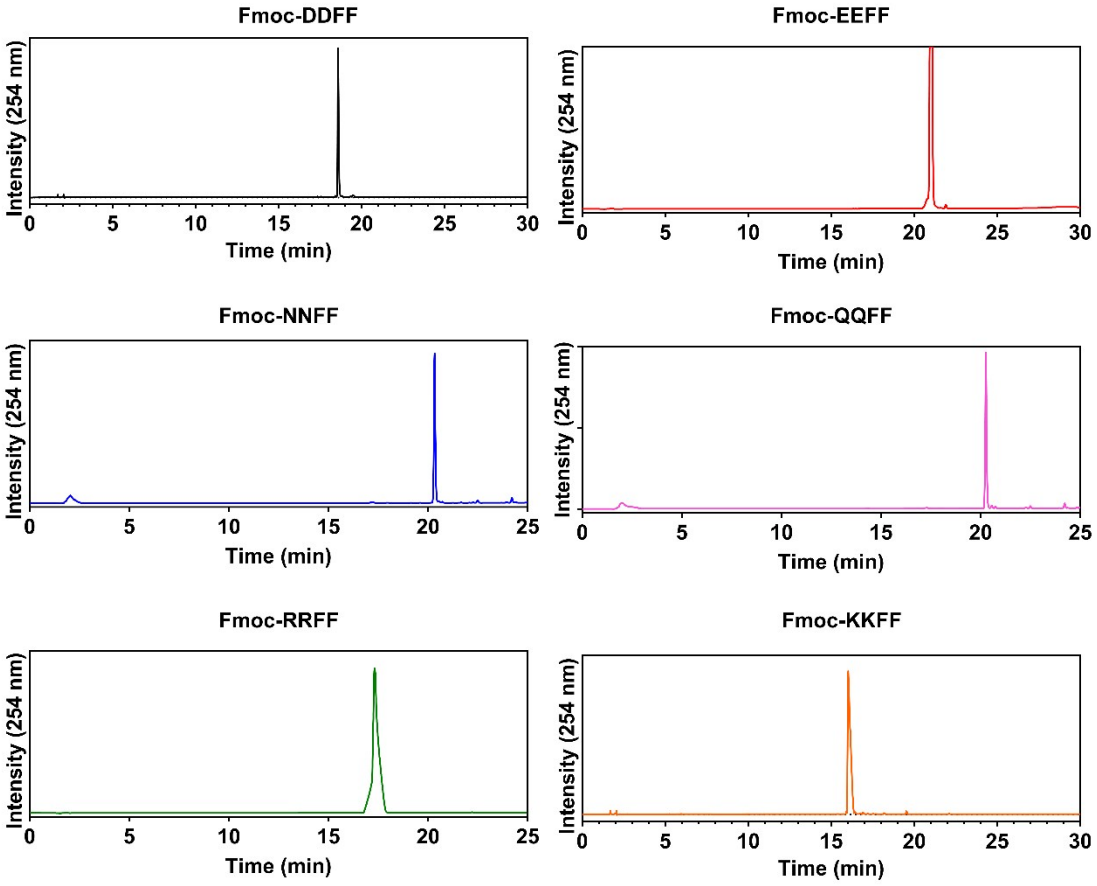

Fig. S1 – Analytical HPLC traces of polar tetrapeptides. Chromatrograms were performed at room temperature using a 5-95% acetonitrile gradient and a Waters XBridge BEH130 C_{18} 5 µm 4.6 × 150 mm column.

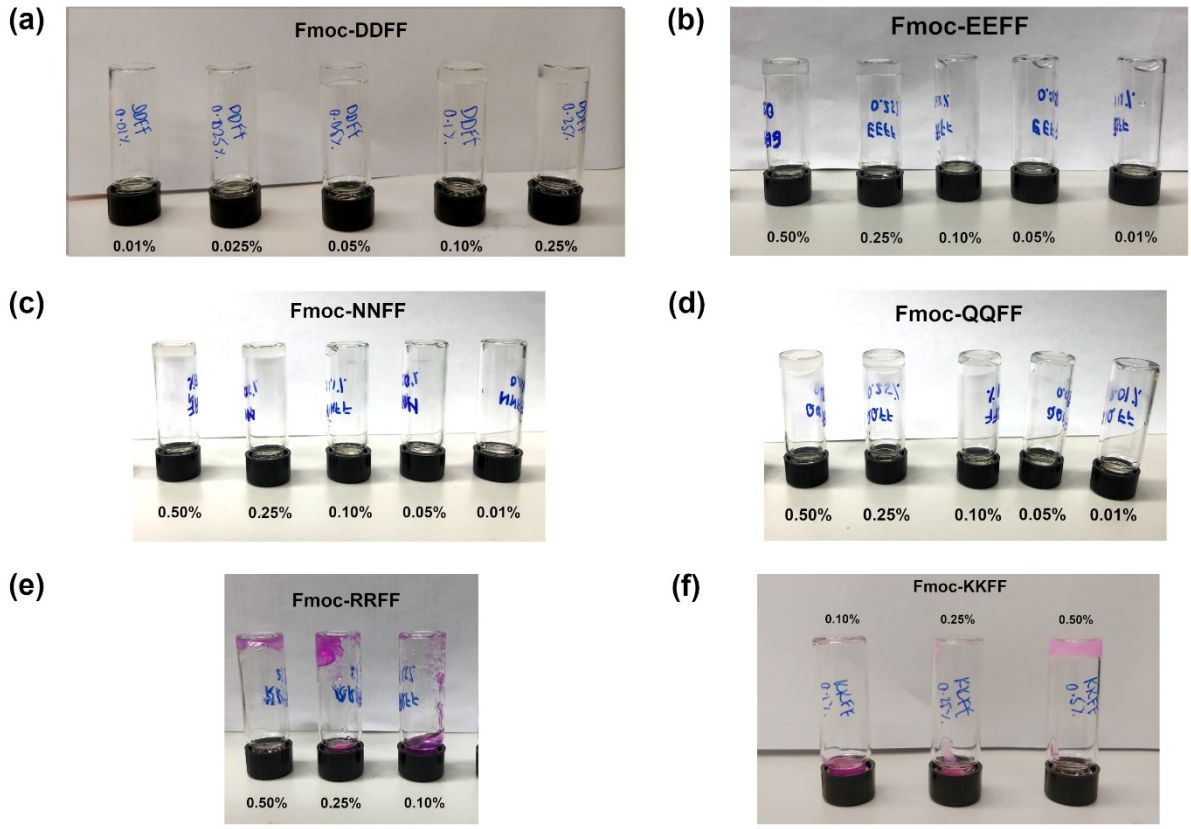

Fig. S2 – Minimum gel concentration tests for (a) Fmoc-DDFF, (b) Fmoc-EEFF, (c) Fmoc-NNFF, (d) Fmoc-QQFF, which were performed using the pH switch method described above. Minimum gel concentration tests for (e) Fmoc-RRFF and (f) Fmoc-KKFF were carried out via the aforementioned salt screening method.

Table S1. Fibre diameters measured for each polar tetrapeptide. A minimum of 20 nanofibres were measured across multiple AFM images.

Peptide	Fibre diameter (nm)
Fmoc-DDFF	5.5 ± 2.2
Fmoc-EEFF	6.5 ± 1.1
Fmoc-NNFF	2.8 ± 1.1
Fmoc-QQFF	6.4 ± 1.5
Fmoc-RRFF	6.1 ± 1.8
Fmoc-KKFF	4 ± 1.3

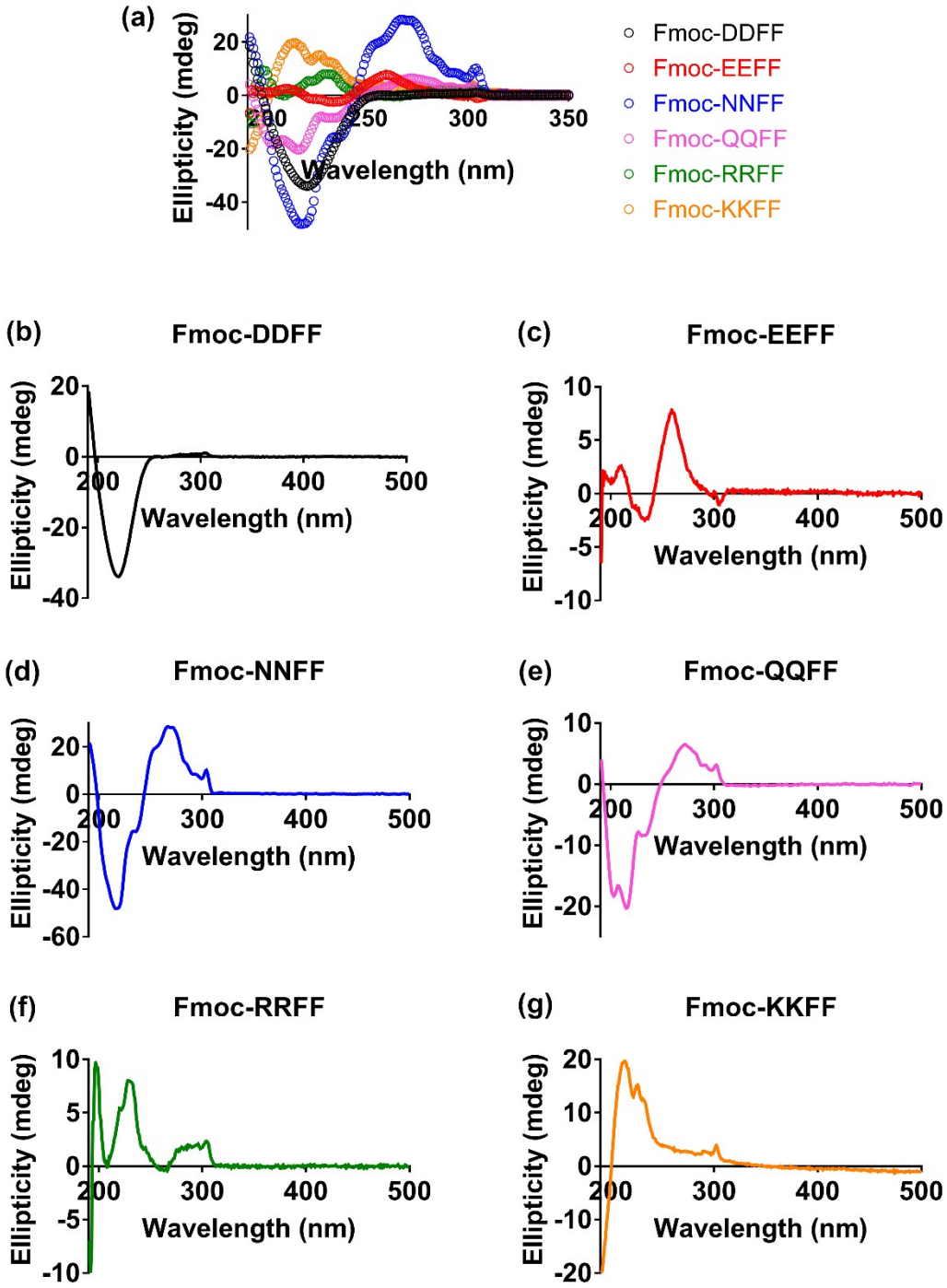

Fig. S3 – (a) overlaid and (b-g) individual CD spectra of polar tetrapeptide hydrogels formed using either pH switch method (Fmoc-DDFF, Fmoc-EEFF, Fmoc-NNFF, Fmoc-QQFF) or salt screen method (Fmoc-RRFF, Fmoc-KKFF) at 1% (w/v) and dispersed in MilliQ water to achieved a final concentration of 0.125% (w/v).

Table S2 – Peaks observed in CD spectra between 190 nm and 250 nm for each polar tetrapeptide and the associated sign (positive/negative) for each peak.

	Peak 1	Value	Peak 2	Value
Fmoc-DDFF	220	-		
Fmoc-EEFF	210	+	233	-
Fmoc-NNFF	217	-	235	-
Fmoc-QQFF	215	-	230	-
Fmoc-RRFF	220	+	228	+
Fmoc-KKFF	214	+	226	+

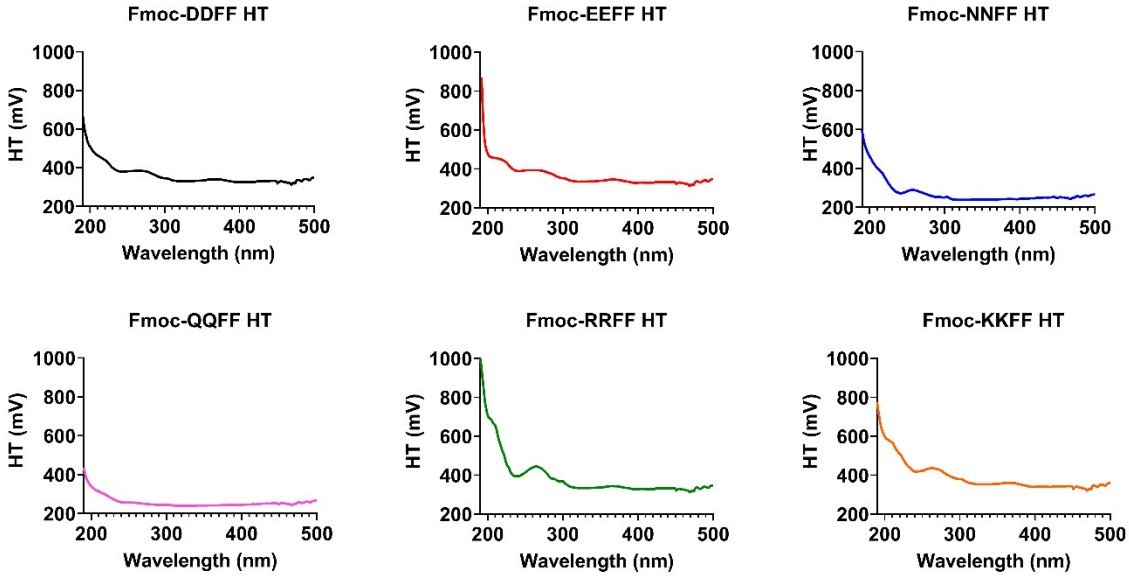

Fig. S4 – Individual HT spectra of polar tetrapeptide hydrogels formed using either pH switch method (Fmoc-DDFF, Fmoc-EEFF, Fmoc-NNFF, Fmoc-QQFF) or salt screen method (Fmoc-RRFF, Fmoc-KKFF) at 1% (w/v) and dispersed in MilliQ water to achieved a final concentration of 0.125% (w/v).

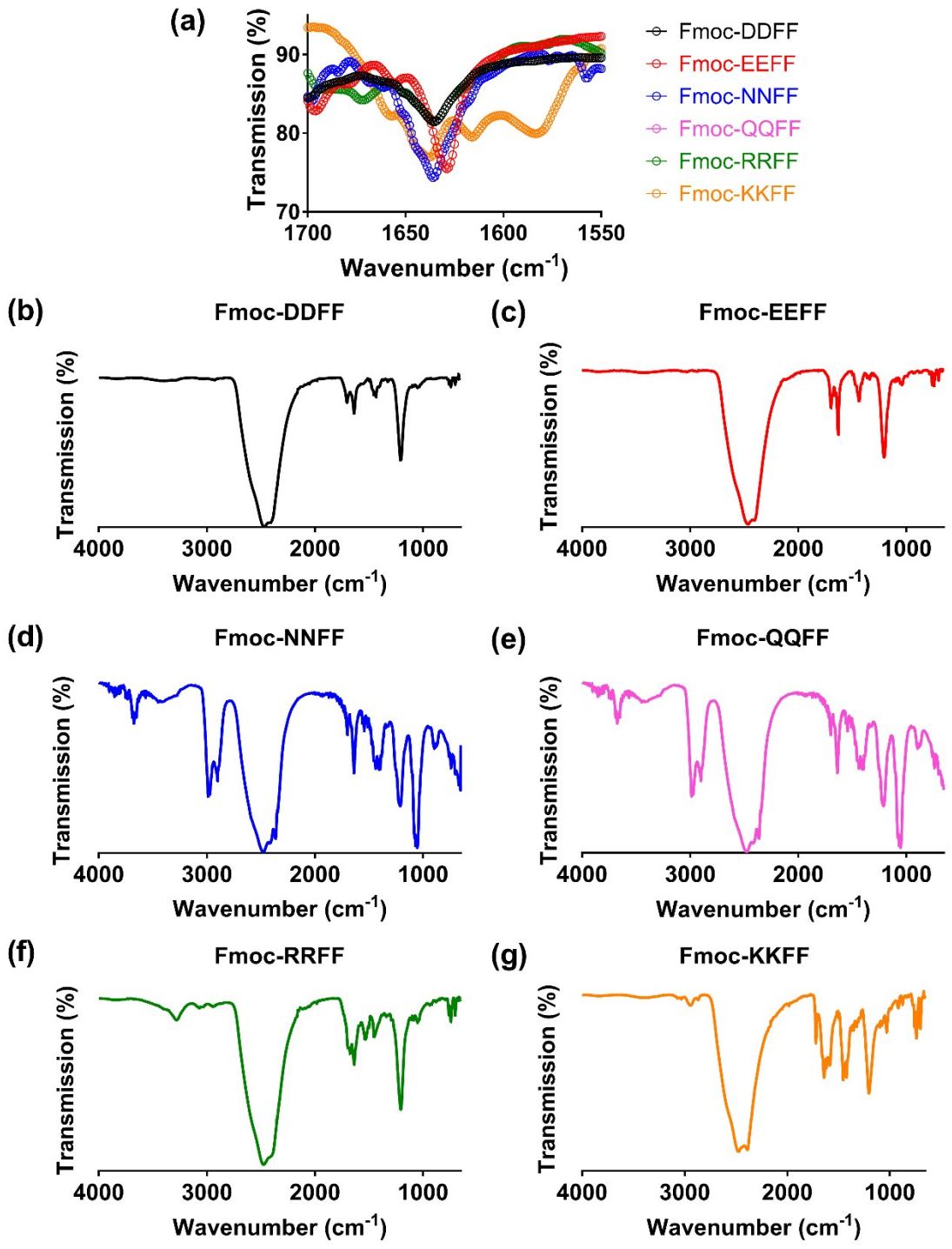

Fig. S5 – (a) Amide I region in the ATR-IR spectra of polar tetrapeptide hydrogels, full ATR-IR spectra of tetrapeptide hydrogels of (b) Fmoc-DDFF, (c) Fmoc-EEFF, (d) Fmoc-NNFF, (e) Fmoc-QQFF, (f) Fmoc-RRFF and (g) Fmoc-KKFF. Hydrogels were prepared at 1% (w/v) using the appropriate methodology in D_2O to avoid water O-H stretching vibrations at 1640 cm^{-1} which interfere with the Amide I region of the peptides.

Table S3 – Peaks observed in ATR-IR spectra between 1600 cm^{-1} and 1700 cm^{-1} (Amide I region) for each polar tetrapeptide.

	Peak 1	Peak 2	Peak 3	Value
Fmoc-DDFF	1636			
Fmoc-EEFF	1629	1655	1680	1696
Fmoc-NNFF	1636	1661	1685	
Fmoc-QQFF	1636	1661	1685	
Fmoc-RRFF	1635	1672	1683	
Fmoc-KKFF	1616	1638	1657	

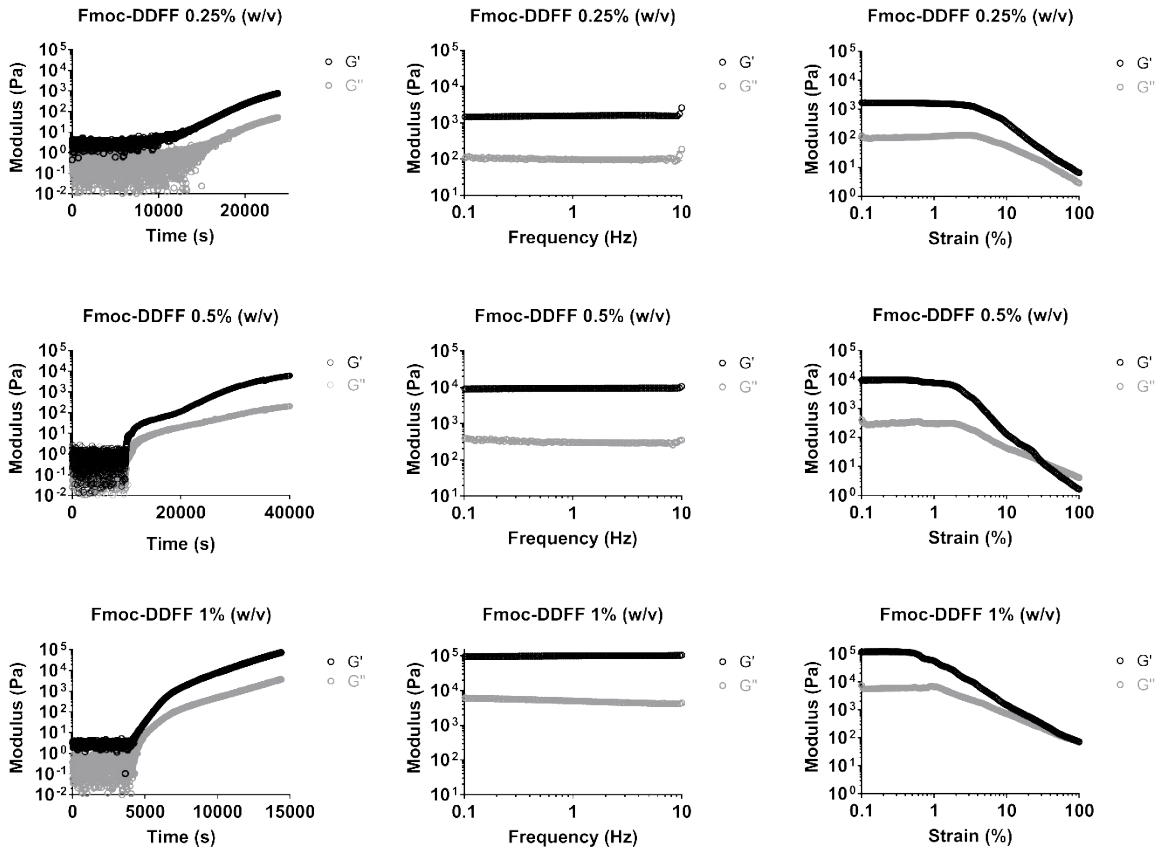

Fig. S6 – Rheological characterisation of Fmoc-DDFF at (top) 0.25% (w/v), (middle) 0.5% (w/v) and (bottom) 1% (w/v), reflecting the concentrations used in contact cytotoxicity measurements. Measurements consist of (left column) time resolved rheology (f = 1 Hz and γ = 0.2%), (middle column) frequency sweep (f = 0.1-10 Hz and γ = 0.2%) and (right column) strain sweep (f = 1 Hz and γ = 0.1-100%). All measurements were performed at 25 °C.

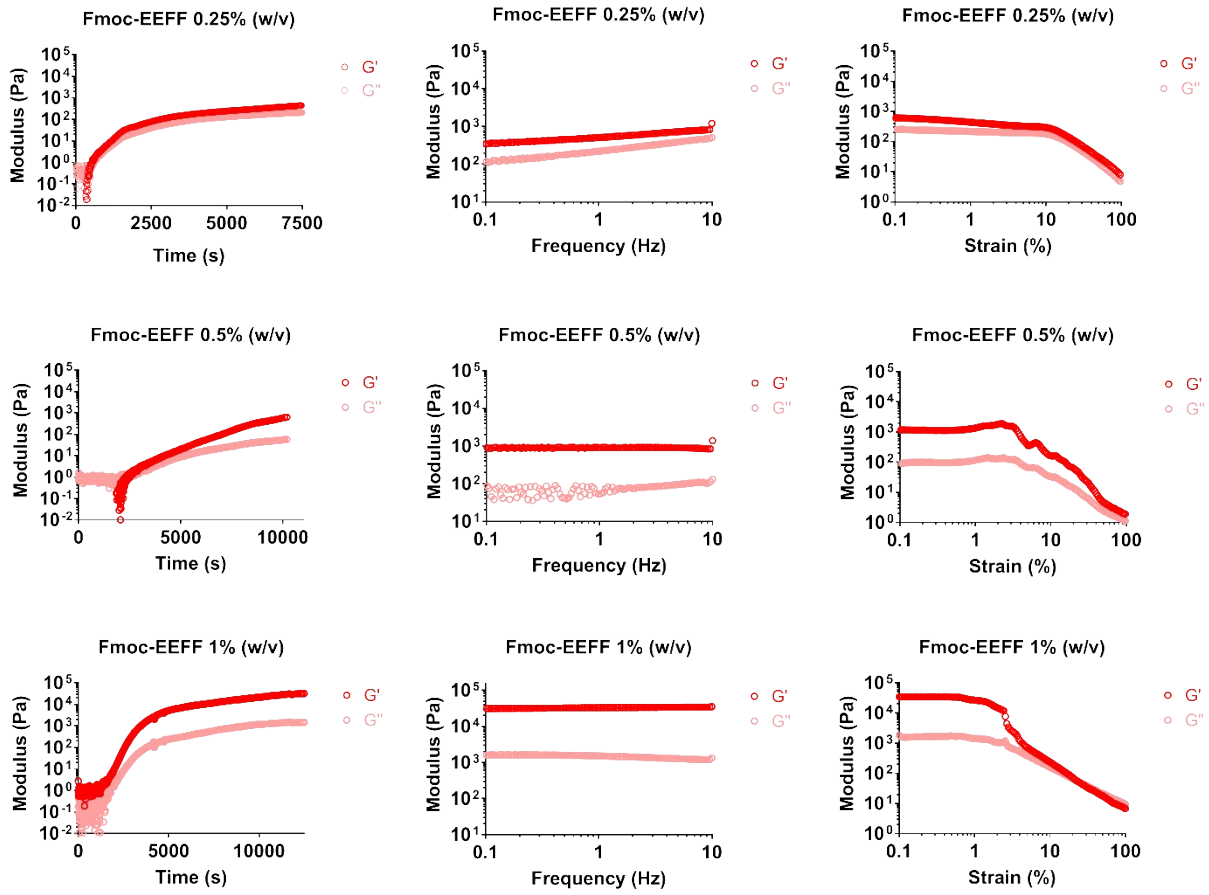

Fig. S7 – Rheological characterisation of Fmoc-EEFF at (top) 0.25% (w/v), (middle) 0.5% (w/v) and (bottom) 1% (w/v), reflecting the concentrations used in contact cytotoxicity measurements. Measurements consist of (left column) time resolved rheology (f = 1 Hz and γ = 0.2%), (middle column) frequency sweep (f = 0.1-10 Hz and γ = 0.2%) and (right column) strain sweep (f = 1 Hz and γ = 0.1-100%). All measurements were performed at 25 °C.

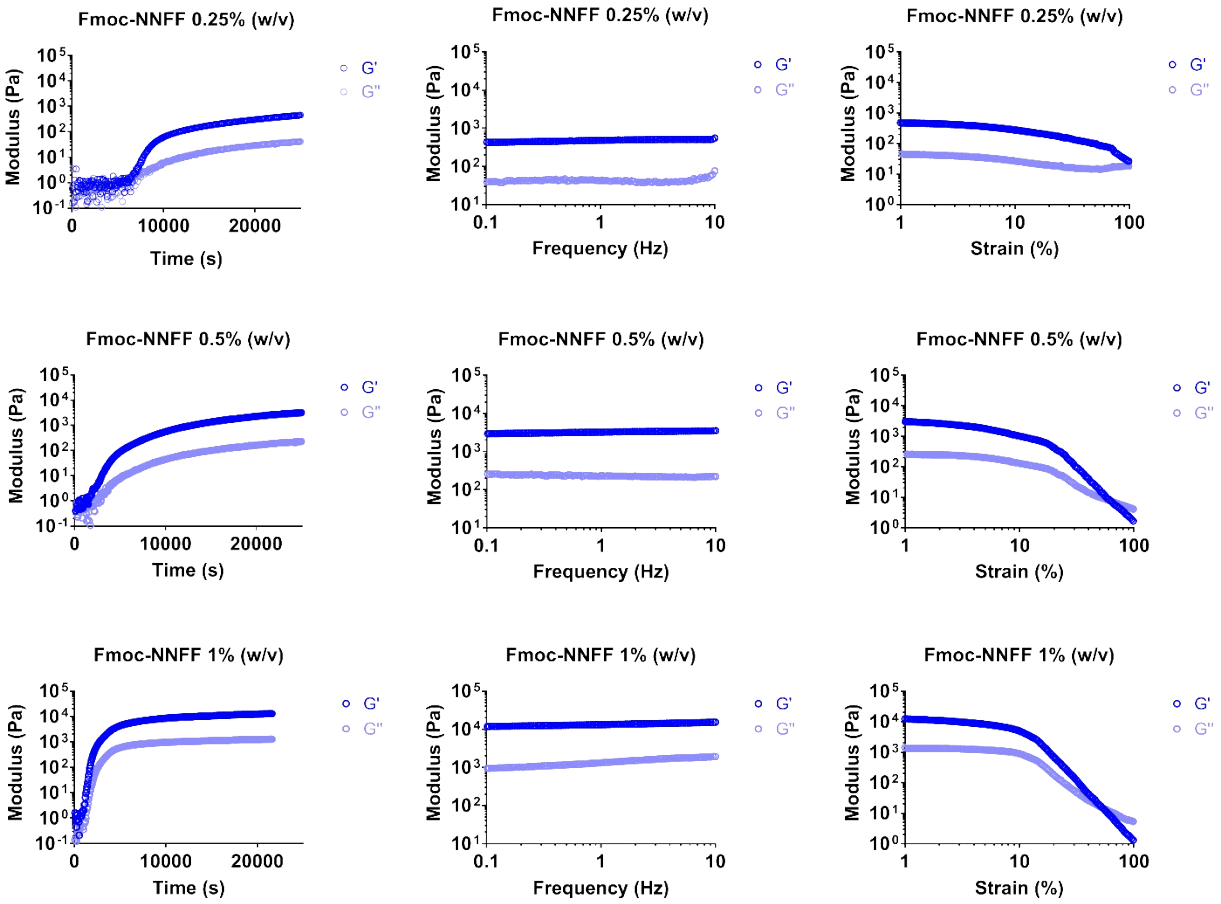

Fig. S8 – Rheological characterisation of Fmoc-NNFF at (top) 0.25% (w/v), (middle) 0.5% (w/v) and (bottom) 1% (w/v), reflecting the concentrations used in contact cytotoxicity measurements. Measurements consist of (left column) time resolved rheology (f = 1 Hz and γ = 0.2%), (middle column) frequency sweep (f = 0.1-10 Hz and γ = 0.2%) and (right column) strain sweep (f = 1 Hz and γ = 1-100%). All measurements were performed at 25 °C.

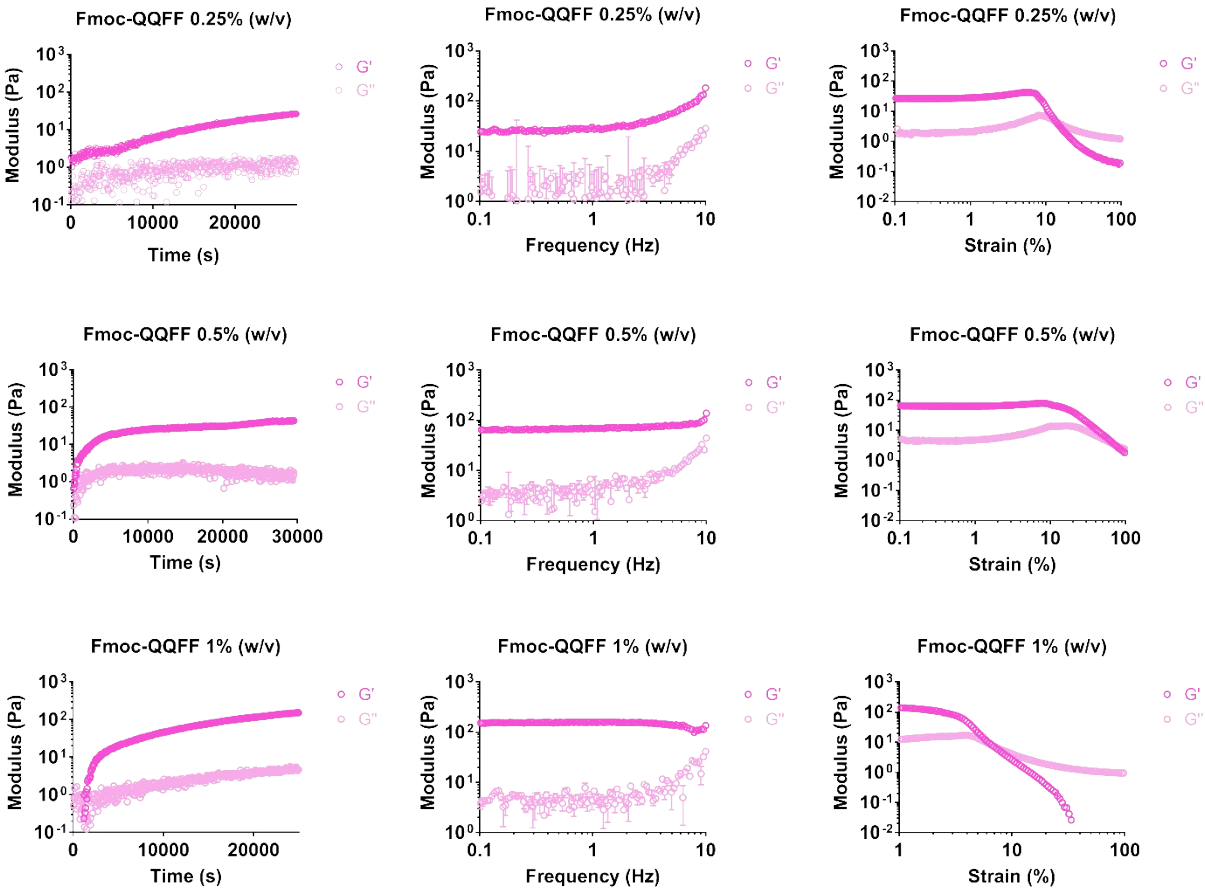

Fig. S9 – Rheological characterisation of Fmoc-QQFF at (top) 0.25% (w/v), (middle) 0.5% (w/v) and (bottom) 1% (w/v), reflecting the concentrations used in contact cytotoxicity measurements. Measurements consist of (left column) time resolved rheology (f = 1 Hz and γ = 0.2%), (middle column) frequency sweep (f = 0.1-10 Hz and γ = 0.2%) and (right column) strain sweep (f = 1 Hz and γ = 0.1-100%). All measurements were performed at 25 °C.

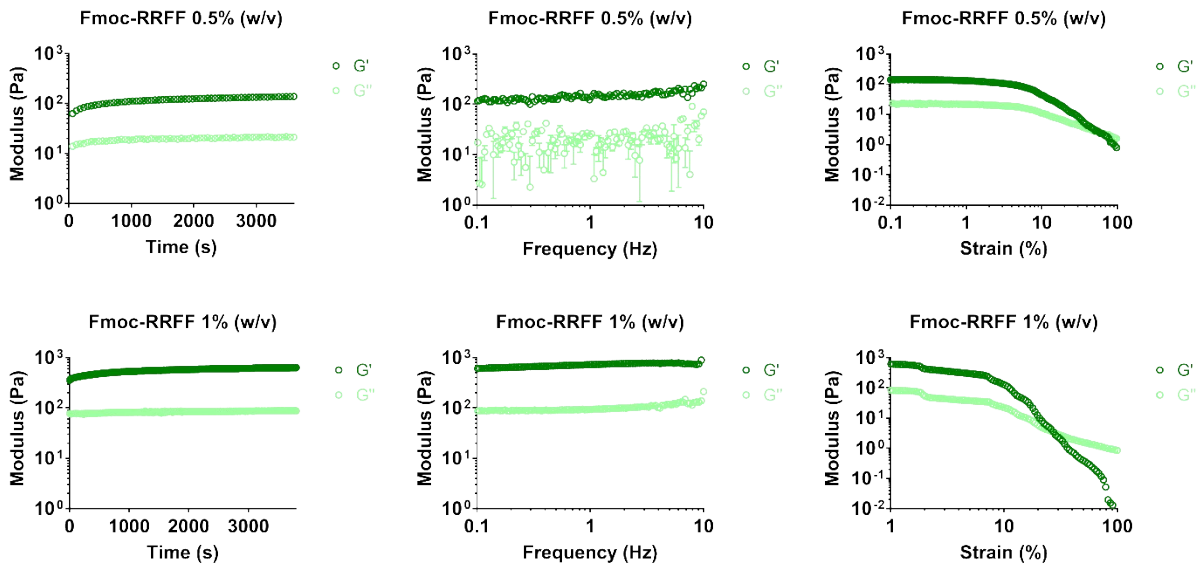

Fig. S10 – Rheological characterisation of Fmoc-RRFF at (top) 0.5% (w/v) and (bottom) 1% (w/v), reflecting the concentrations used in contact cytotoxicity measurements. Measurements consist of (left column) time resolved rheology (f = 1 Hz and γ = 0.2%), (middle column) frequency sweep (f = 0.1-10 Hz and γ = 0.2%) and (right column) strain sweep (f = 1 Hz and γ = 0.1-100%). All measurements were performed at 25 °C.

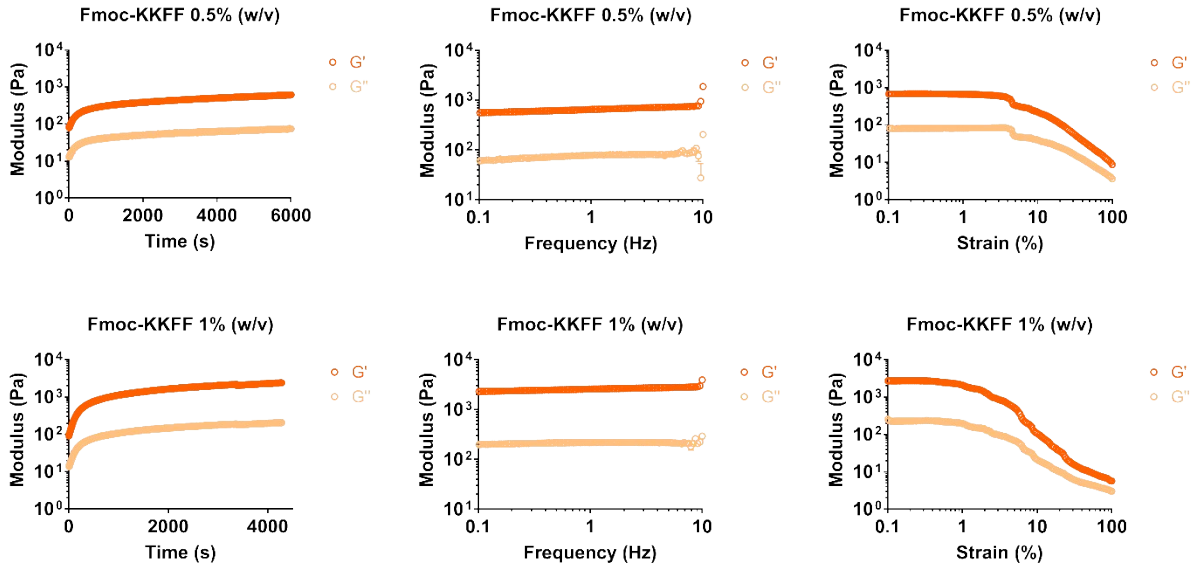

Fig. S11 – Rheological characterisation of Fmoc-KKFF at (top) 0.5% (w/v) and (bottom) 1% (w/v), reflecting the concentrations used in contact cytotoxicity measurements. Measurements consist of (left column) time resolved rheology (f = 1 Hz and γ = 0.2%), (middle column) frequency sweep (f = 0.1-10 Hz and γ = 0.2%) and (right column) strain sweep (f = 1 Hz and γ = 0.1-100%). All measurements were performed at 25 °C.

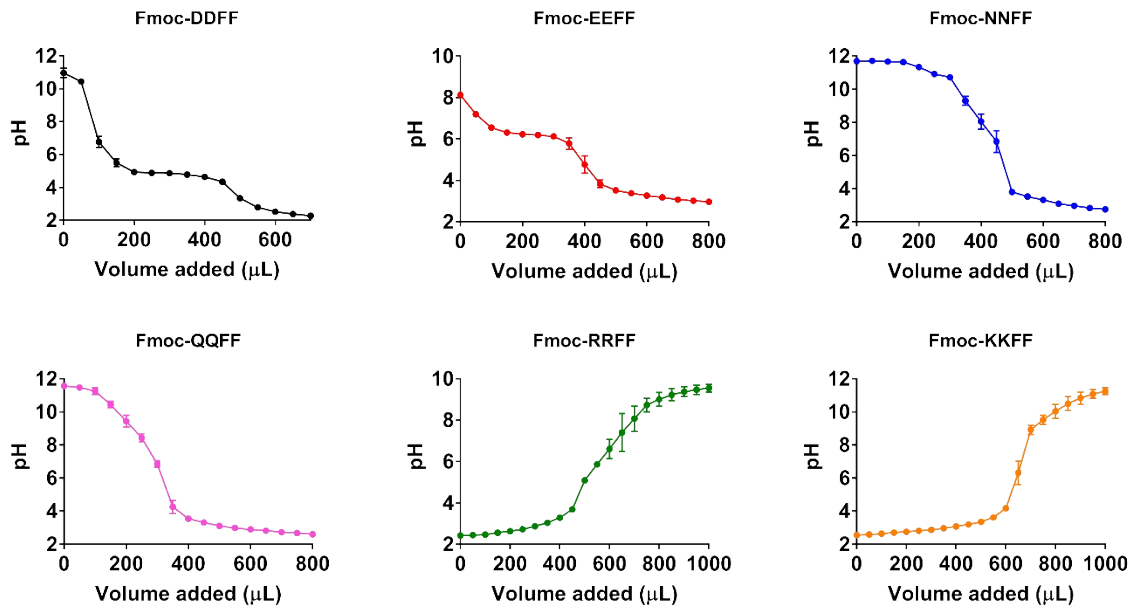

Fig. S12 – pK_a and pK_b determination for polar tetrapeptides. Polar anionic and neutral tetrapeptides were dissolved using 0.1 M NaOH and MilliQ water and 50 µL aliquots of 0.1 M HCl added. Polar cationic peptides were dissolved using 0.1 M HCl and MilliQ water and 50 µL aliquots of 0.1 M NaOH added. Experiments were repeated at least three times. Equivalence points were determined through plotting the first derivative of the graph.

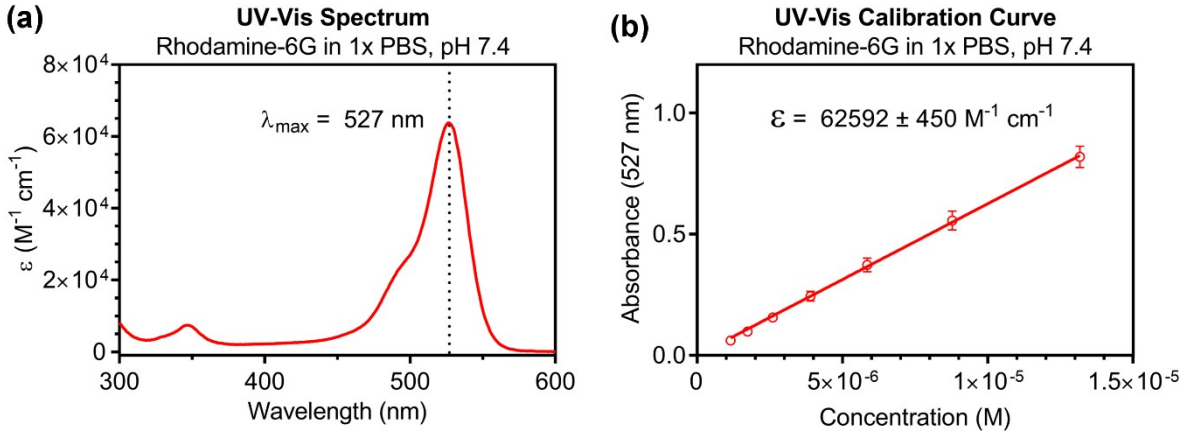

Fig. S13 – UV-Visible spectrum of (a) Rhodamine 6G and (b) associated calibration curve with calculated extinction coefficient.

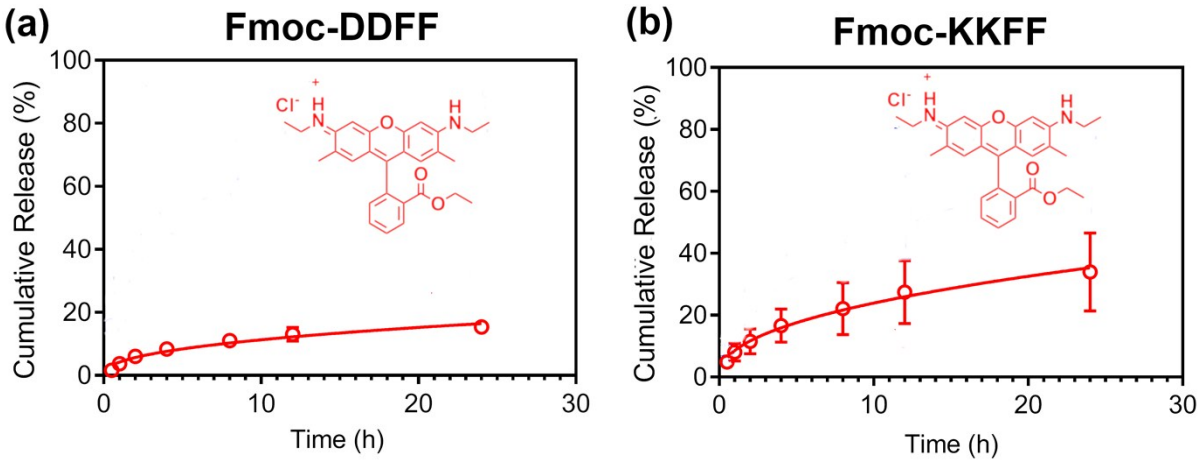

Fig. S14 – Release of positively charged Rhodamine 6G fluorophore (inset) from hydrogels of (a) negatively charged Fmoc-DDFF and (b) positively charged Fmoc-KKFF. Hydrogels were prepared at 1% (w/v) and total release after 24 h was approximately 17% for Fmoc-DDFF and 31% for Fmoc-KKFF, suggesting that peptide charge is conserved in the gel state.

References

1. C. G. Cranfield, S. Carne, B. Martinac. B. Cornell, *Methods in Molecular Biology (Methods and Protocols)*, **1232**, Humana Press, New York, 2015.

2. T. Berry, D. Dutta, R. Chen, A. Leong, H. Wang, W. A. Donald, M. Parviz, B. Cornell, M. Wilcox, N. Kumar, C. G. Cranfield, *Langmuir*, 2018, **34**,11586-11592.