Supplementary information

Tuning hydrogels through metal-based gelation triggers

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Contents

Solid phase peptide synthesis of capped dipeptides

Carbazole acetic acid was synthesised as previously reported.[1]

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-Asp(OtBu)-OH (3 equiv., 679 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

Fmoc-Phe-OH (3 equiv., 640 mg) 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).

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

Carbazole acetic acid coupling

The carbazole capping group was introduced as previously reported.[1]

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). A solution of 1:9 dichloromethane: trifluoroacetic acid with one drop of water was then added to the resin, and the resin stirred for 2 hours using an orbital shaker. 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-diphenylalanine-aspartic acid**, **1**: IR: 3292 (m), 3064 (m), 3031 (m), 2928 (m), 1710 (s), 164 (s), 1518 (s), 1450 (m), 1405 (w), 1324 (w), 1224 (s), 1082 (m), 758 (m), 739 (s), 699 (s); ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.61 (s, 1H), 8.42 (d, *J* = 8.1 Hz, 1H), 8.11 (d, *J* = 8.3 Hz, 1H), 7.87 (d, *J* = 7.6 Hz, 2H), 7.61 (t, *J* = 8.2 Hz, 2H), 7.53 (d, J = 4.7 Hz, 1H), 7.40 (td, *J* = 3.9 Hz, 5.3 Hz, 2H), 7.32 – 7.12 (m, 6H), 4.62 – 4.53 (m, 2H), 4.24 – 4.07 (m, 4H), 3.06 (dd, J = 14.3 Hz, 4.2 Hz, 2H), 2.94 – 2.57, m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.22, 171,64, 171,28, 170.82, 155,61, 143,76, 143,69, 140.65, 138,14, 137,47, 129.31, 129,20, 127,98, 127,61, 127.06, 126.25, 126.17, 125.34, 125.23, 120.07, 63.63, 56.06, 53.50, 48.64, 45.52, 37.67, 37.43, 35.96; HR-MS (ESI): calcd for $C_{37}H_{35}N_3O_8$ + Na^+: 672.2301, found 672.2317.

Characterisation data for **carbazole-diphenylalanine-aspartic acid**, **2**: IR: 3276 (m), 3059 (m), 2927 (m), 1723 (m), 1647 (s), 1537, (m), 1486 (m), 1454 (s), 1326 (m), 1255 (w), 1230 (w), 1213 (s), 1155 (m), 848 (m), 747 (s), 721 (s), 700 (m); ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.80 (s, 1H), 8.61 (d, *J* = 8.8 Hz, 1H), 8.54 (d, *J* = 7.8 Hz, 1H), 8.09 (d, *J* = 7.7 Hz, 2H), 7.36 (t, *J* = 7.0 Hz, 2H), 7.29 – 7.21 (m, 6H), 7.17 (t, *J* = 7.44 Hz, 2H), 4,97 (dd, *J* = 47 Hz, 17 Hz, 2H), 4.67 – 4.52 (m, 3H), 3.08 (dd, *J* = 9.8 Hz, 3.9 Hz, 1H), 2.81 – 2.55 (m, 8H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.76, 172.11, 171.37, 167.48, 140.95, 138.06, 129.84, 129.71, 128.53, 126.77, 126.05, 122.55, 120.46, 109.81, 53.98, 49.1938.45, 36.54; HR-MS (ESI): calcd for $C_{36}H_{34}N_4O9$ + H^+: 657.2388, found 657.2320.

Preparation of hydrogels

pH switch

1.5 equivalents of 0.1 M sodium hydroxide was added to the peptide of interest and milliQ water added to make the suspension up to the required concentration. This suspension was sonicated until homogenous, upon which time 3 molar equivalents of glucono-δ-lactone was added to lower the pH, resulting in gelation.

Salt screening

1.5 equivalents of 0.1 M sodium hydroxide was added to the peptide of interest and milliQ water added to make the suspension up to the required concentration. This suspension was sonicated until homogenous, upon which time an equal volume of a metal salt solution of the appropriate concentration (typically 10 equivalents relative to the peptide concentration for sodium, and 1 equivalent for the other divalent and trivalent metals tested). For DMEM triggered hydrogels, an equal volume of DMEM was added to the dissolved peptide solution.

Circular dichroism measurements

CD measurements 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, 1% (w/v) peptide sols were prepared as above and diluted 1:20 (v/v) in water. Temperature was kept constant at 20 °C and all experiments were repeated three times and averaged into a single plot.

Attenuated Total Reflectance-Infrared Spectroscopy measurements

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 1% (w/v) and pressed between the diamond crystal and substrate. All spectra were scanned 16 times over the range of 4000 - 650 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 1% (w/v) peptide solution mixed with either GdL or a salt solution 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. 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.

AFM measurements

Gel samples of **1** and **2** 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. These samples were left to dry in air overnight. Imaging was undertaken on a BrukerMultimode 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.

Small angle neutron scattering measurements and modelling

Hydrogels were prepared using the appropriate method as described above and transferred to a demountable titanium cell of 2 mm path length before being left to set overnight at room temperature. Measurements were performed at detector distances of 2, 12 and 20 m. Isotropic scattering patterns were radially averaged and combined for a q range of 0.003 – 0.7 Å, where $q = 4\pi\sin(\theta)/\lambda$, and λ is neutron wavelength (5 Å) with a wavelength spread, $\Delta\lambda / \lambda$ 10%) and 2θ is the scattering angle. 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.

Data was modelled using SasView,[4] with a flexible cylinder model chosen for each scattering pattern, based upon AFM characterisation data. The scattering length density (SLD) of the peptide was calculated to be 2.1×10^{-6}, with this value and the SLD of the solvent (D_2O) fixed at 6.3×10^{-6} $Å^{-2}$.. Other parameters were allowed to vary freely and following a few optimisation cycles, the background was fixed. After this, multiple different starting points were used for the Kuhn length, radius and length of the cylinder, to ensure that a global, physically realistic minimum was found.

Cell viability measurements

Cytotoxicity measurements were performed using an AlamarBlue colorimetric assay on HeLa cells. Each experiment was repeated three times. To a 96-well plate, 100 µL of gel (triggered using methods described above) was added in triplicate and allowed to set overnight. Surrounding wells were supplemented with water to ensure hydration of the gels. Cells were passaged using standard cell culture procedures. Cells were detached with trypsin and centrifuged (500 rpm for 5 min). The supernatant was removed and the cells resuspended in DMEM at a concentration of 100,000 cells/mL. Cells were seeded atop hydrogels at a concentration of 10,000 cells/well. For glucono-δ-lactone (GdL) cytotoxicity, cells were seeded directly into wells, allowed to adhere overnight then treated with 100 µL of an appropriate concentration of GdL dissolved in DMEM. Control wells included no gel, no cells and a negative control of 10% DMSO. After 48h, DMEM was removed and 100 µL 10% AlamarBlue in DMEM was added to the appropriate wells, followed by incubation for a further 3 h (37 °C, 5.0% CO_2). Fluorescence emission at 590 nm (λ_{ex} = 540 nm) was recorded using a Tecan Infinite 200 Pro Multimode Microplate reader.

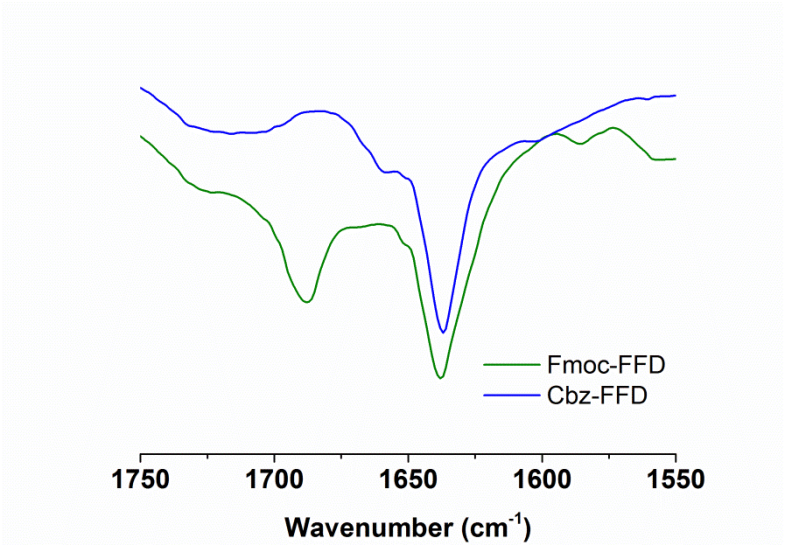


Fig. S1 – Amide I region of the ATR-IR spectrum for pH triggered hydrogels of Fmoc-FFD, **1**, and Cbz-FFD, **2**. Hydrogels were measured at a concentration of 1% (w/v) using deuterated solvents in order to avoid interference from water peaks.

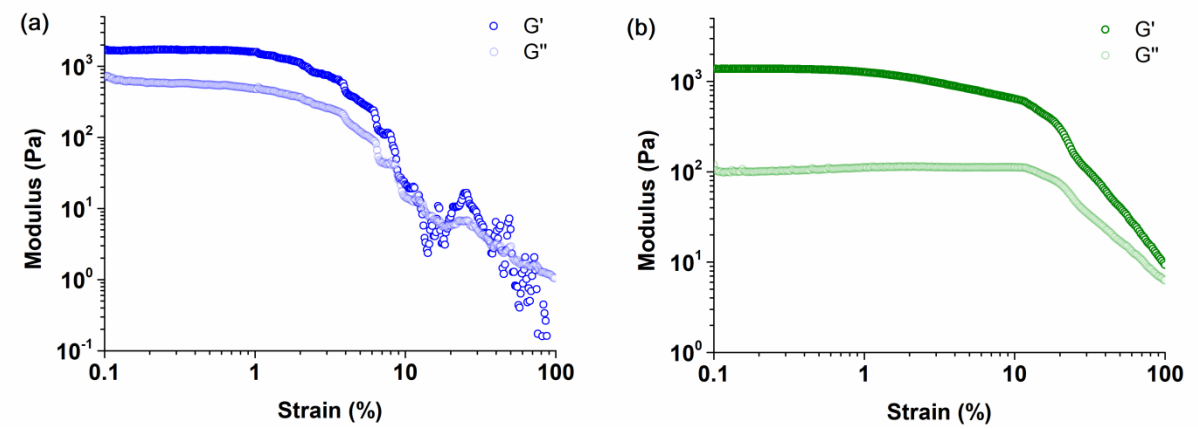
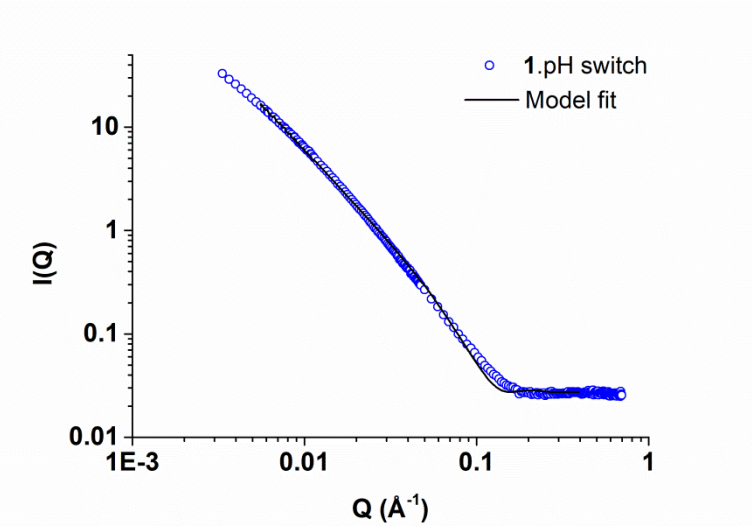


Fig. S2 – Strain sweep rheological measurements for hydrogels formed through a pH switch for (a) **1** and (b) **2**. Hydrogels were measured at a concentration of 1% (w/v), using a constant frequency (1 Hz).



Parameter (Å)	Value
Radius	24.6 ± 0.2
Length	11200 ± 500
Kuhn length	46 ± 3
χ^2	33.6

Fig. S3 – Small angle neutron scattering measurement performed on a 1% (w/v) pH triggered hydrogel of 1 (blue) fitted to a flexible cylinder model (black) and output parameters generated from the model.

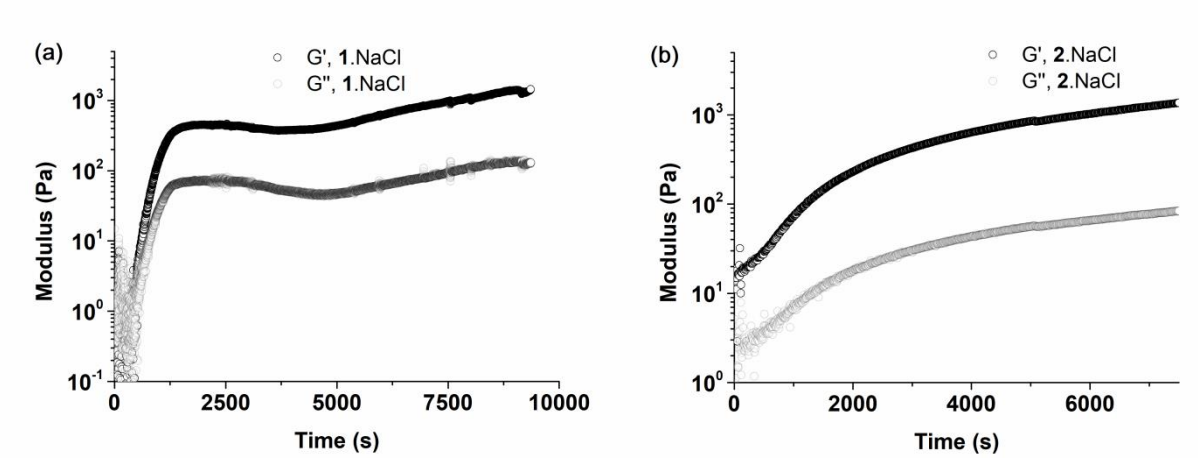


Fig. S4 – Time resolved rheology for hydrogels triggered through addition of NaCl for (a) **1** and (b) **2**. Hydrogels were measured at a concentration of 1% (w/v), using a constant frequency (1 Hz) and strain (0.2%).

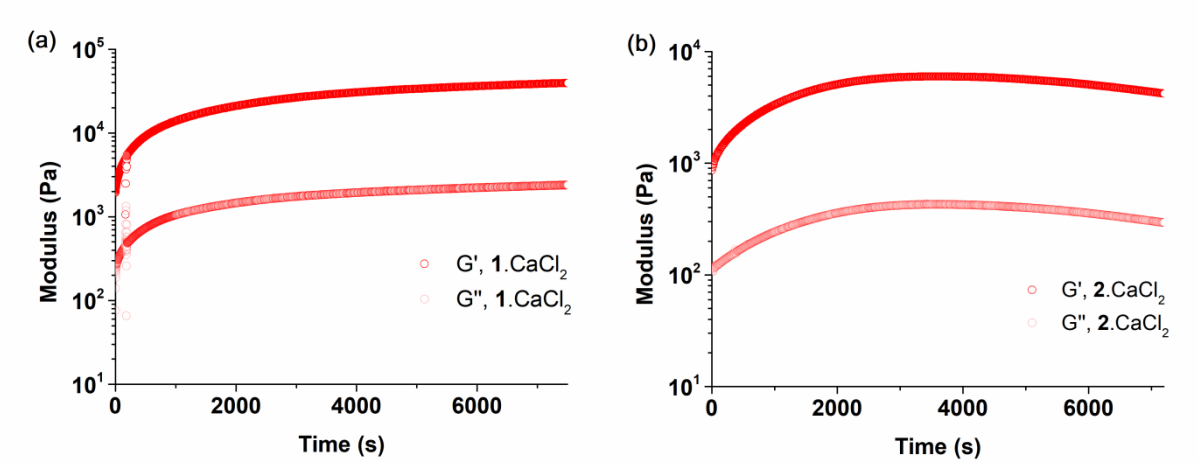


Fig. S5 – Time resolved rheology for hydrogels triggered through addition of $CaCl_2$ for (a) **1** and (b) **2**. Hydrogels were measured at a concentration of 1% (w/v), using a constant frequency (1 Hz) and strain (0.2%).

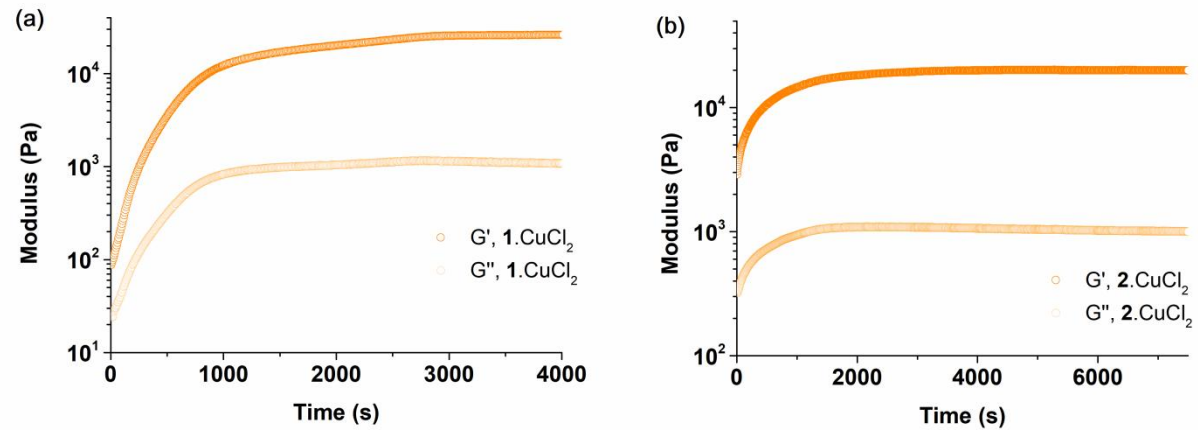


Fig. S6 – Time resolved rheology for hydrogels triggered through addition of $CuCl_2$ for (a) **1** and (b) **2**. Hydrogels were measured at a concentration of 1% (w/v), using a constant frequency (1 Hz) and strain (0.2%).

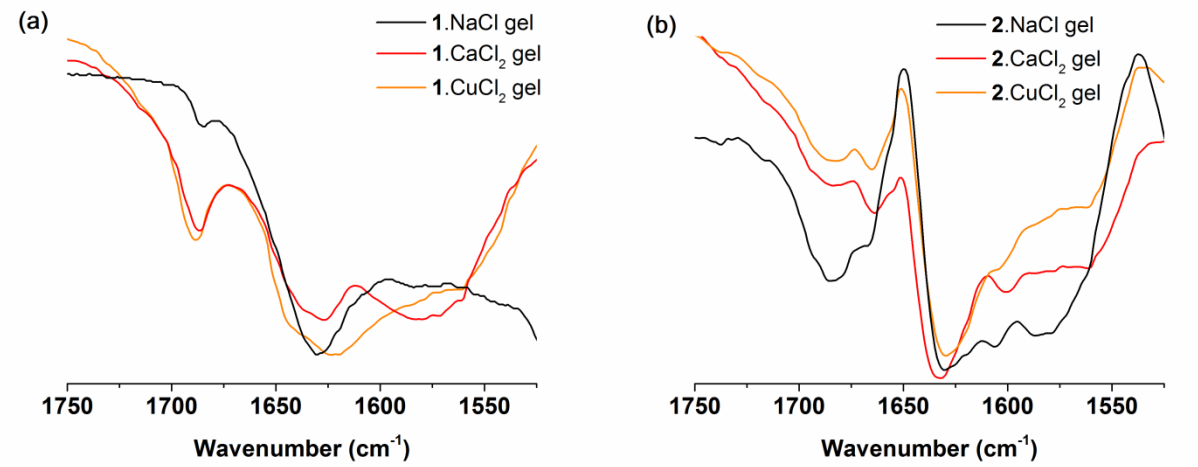


Fig. S7 – Amide I region of ATR-IR spectra for metal-triggered hydrogels of (a) **1** and (b) **2**. Measurements were performed at a concentration of 1% (w/v) using deuterated solvents in order to avoid interference from water peaks.

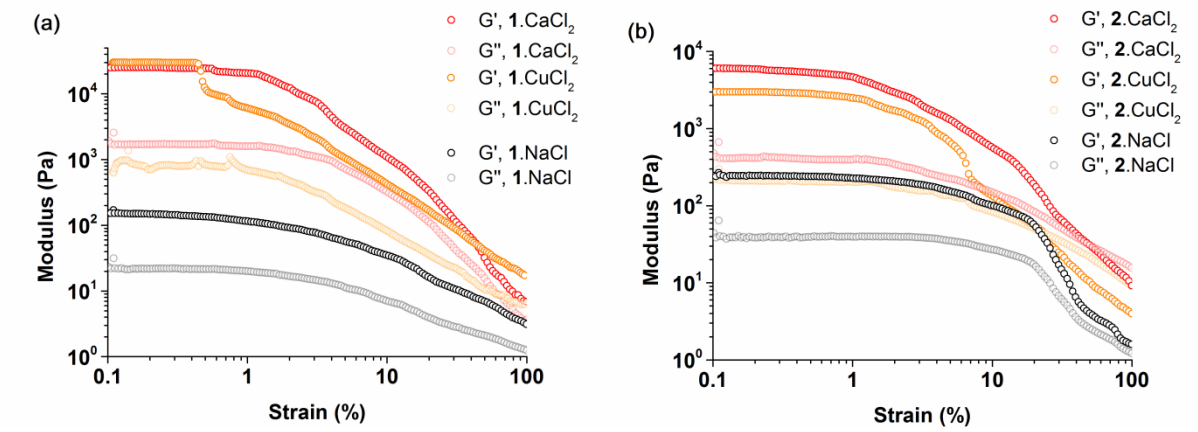
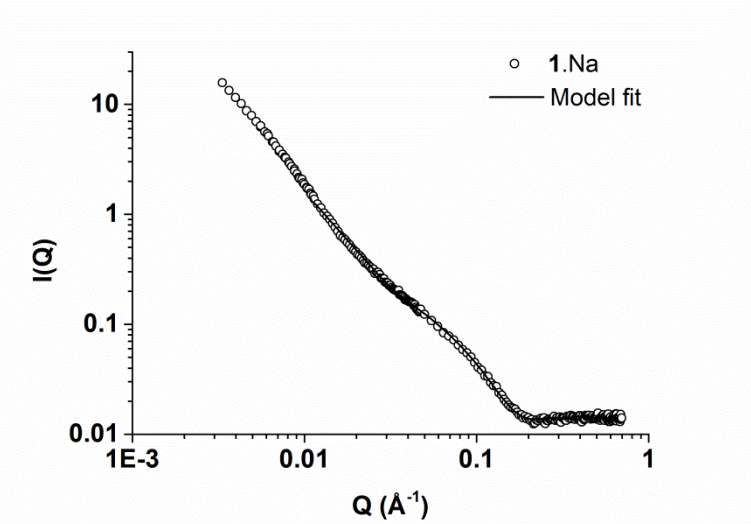
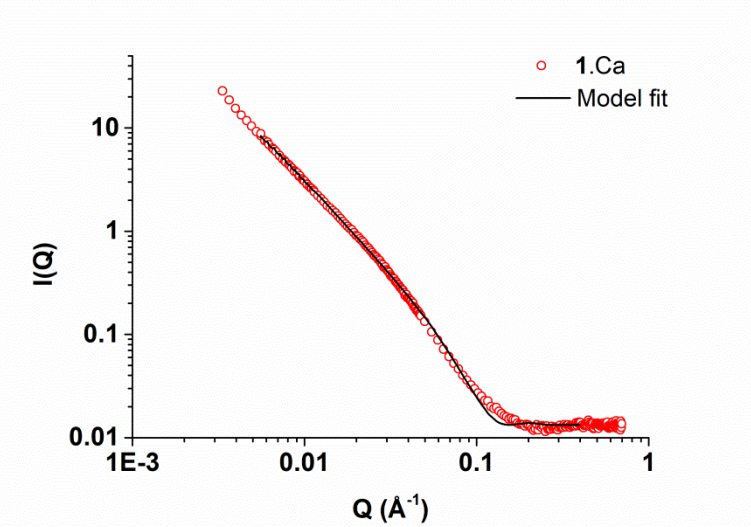


Fig. S8 – Rheological strain sweeps for metal-triggered hydrogels of (a) **1** and (b) **2**. All metal-triggered hydrogels were measured at a concentration of 1% (w/v), at a constant frequency of 1 Hz and temperature of 25 °C.



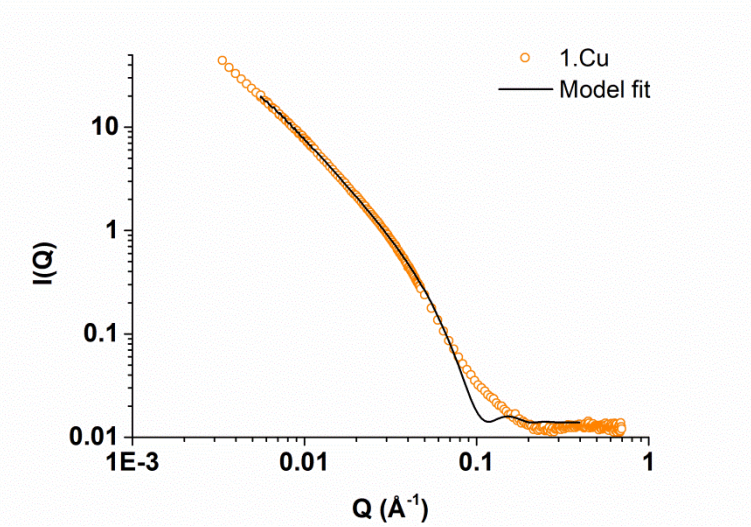
Parameter (Å)	Value
Radius	17.4 ± 0.1
Length	25000 ± 1000
Kuhn length	114 ± 3
χ^2	2.01

Fig. S9 – Small angle neutron scattering measurement performed on a 1% (w/v) hydrogel of **1** (black) triggered through addition of 10 equivalents of NaCl, fitted to a flexible cylinder model (black) and output parameters generated from the model.



Parameter (Å)	Value
Radius	25.1 ± 0.4
Length	9800 ± 750
Kuhn length	46 ± 5
χ^2	9.63

Fig. S10 – Small angle neutron scattering measurement performed on a 1% (w/v) hydrogel of **1** (red) triggered through addition of 1 equivalent of $CaCl_2$, fitted to a flexible cylinder model (black) and output parameters generated from the model.



Parameter (Å)	Value
Radius	33.3 ± 0.3
Length	7240 ± 375
Kuhn length	53 ± 4
χ^2	34.4

Fig. S11 – Small angle neutron scattering measurement performed on a 1% (w/v) hydrogel of **1** (orange) triggered through addition of 1 equivalent of $CuCl_2$, fitted to a flexible cylinder model (black) and output parameters generated from the model.

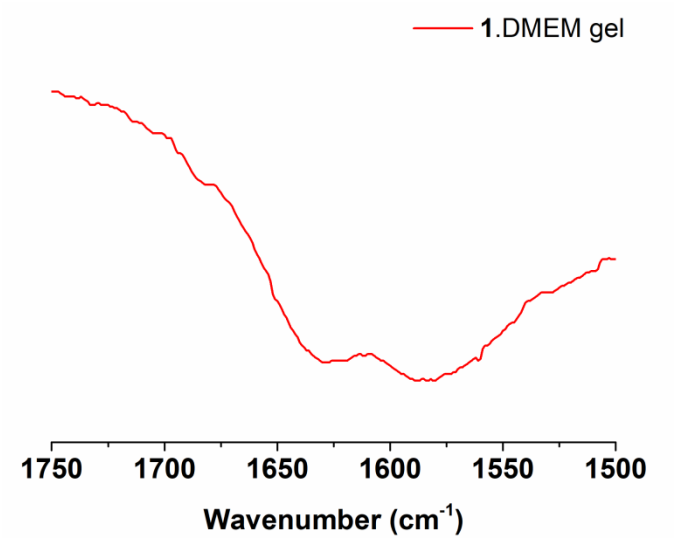


Fig. S12 – Amide I region of ATR-IR spectra for DMEM triggered hydrogel of **1** at a concentration of 1% (w/v). Gelation was performed in deuterated solvents in order to avoid interference from water peaks.

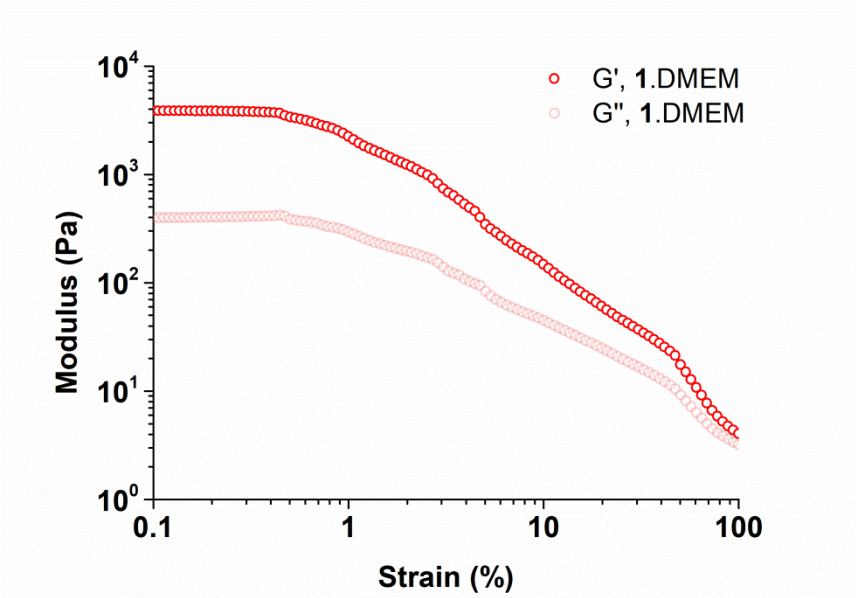


Fig. S13 – Rheological strain sweeps for hydrogels of **1** triggered through the addition of DMEM. Hydrogels were measured at a concentration of 1% (w/v), a constant frequency of 1 Hz and temperature of 25 °C.

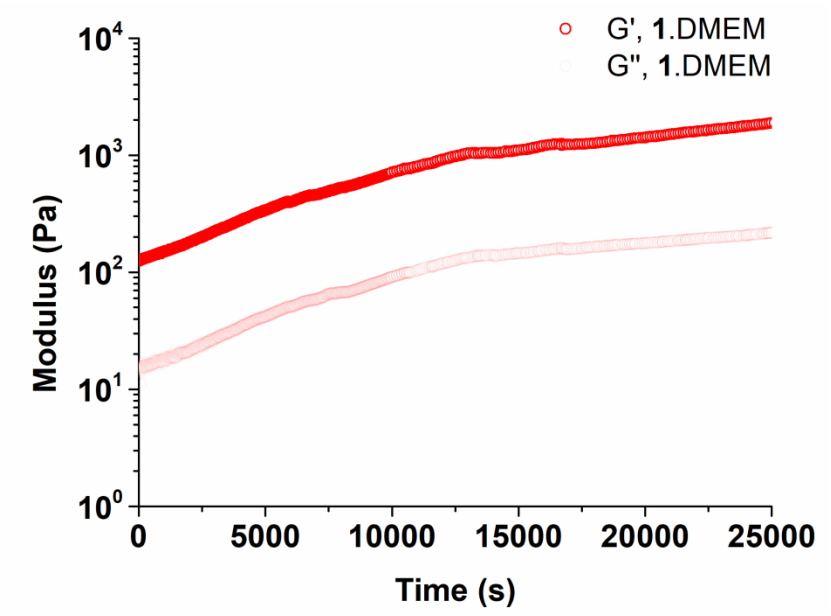
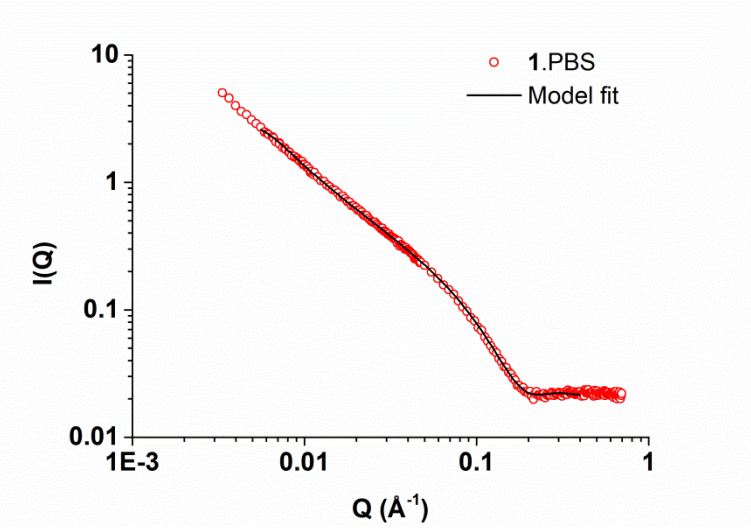


Fig. S14 – Time resolved rheology for hydrogels of **1** triggered through addition of DMEM. Hydrogels were measured at a concentration of 1% (w/v), a constant temperature of 25 °C and at a constant frequency (1 Hz) and strain (0.2%).



Parameter (Å)	Value
Radius	16.9 ± 0.1
Length	1035 ± 55
Kuhn length	490 ± 18
χ^2	0.75

Fig. S15 – Small angle neutron scattering measurement performed on a 1% (w/v) hydrogel of **1** (red) triggered through addition of an equal volume of PBS, fitted to a flexible cylinder model (black) and output parameters generated from the model.

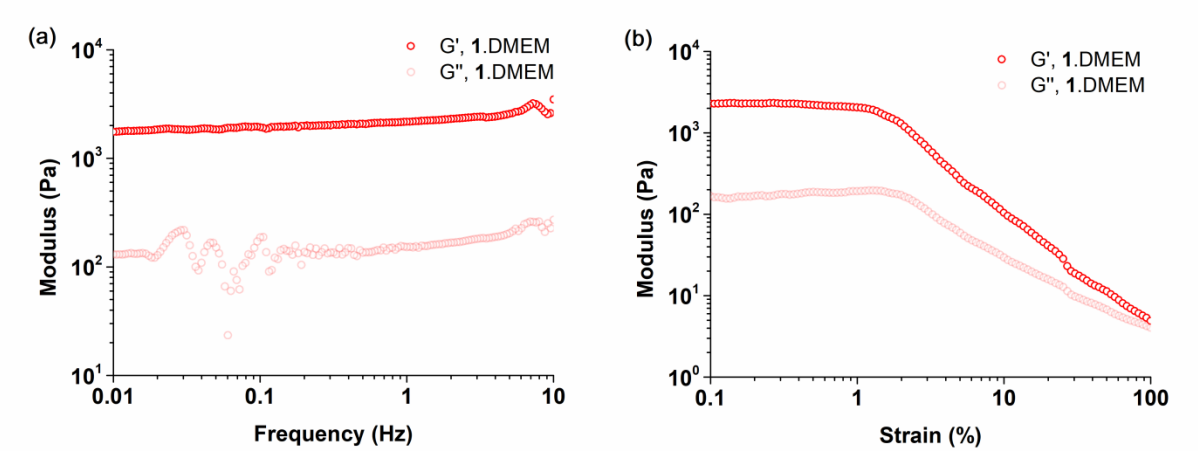


Fig. S16 – Rheological (a) frequency sweep and (b) strain sweep for hydrogels of **1** triggered through addition of DMEM at 37 °C.

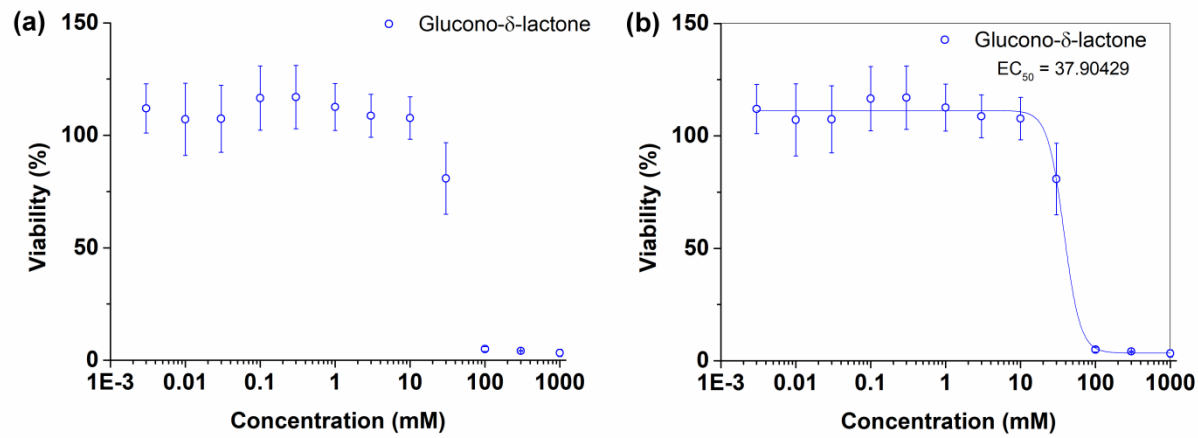


Fig. S17 – Cytotoxicity evaluation of glucono-δ-lactone at different concentrations on HeLa cells after 48h, showing (a) raw data and (b) sigmoidal fitted curved, yielding a value for 50% viability.

Table S1 – Testing metal triggered gelation for Fmoc-FFD, **1**. Key: S – solution, OG – overnight gelation, IG – instant (<1 min) gelation

Metal	1 eq.	2 eq.	5 eq.	10 eq.	50 eq.	100 eq.
Na	S	S	OG	OG	OG	OG
K	S	S	OG	OG	OG	OG
Ca	IG	IG	IG	IG	IG	IG
Mg	IG	IG	IG	IG	IG	IG
Cu	IG	IG	IG	IG	IG	IG
Fe(III)	IG	IG	IG	IG	IG	IG

Table S2 – Testing metal triggered gelation for carbazole-FFD, **2**. Key: S – solution, OG – overnight gelation, IG – instant (<1 min) gelation, P - precipitate

Metal	1 eq.	2 eq.	5 eq.	10 eq.	50 eq.	100 eq.
Na	S	S	S	G	P	P
K	S	S	S	S	P	P
Ca	IG	IG	IG	IG	IG	IG
Mg	IG	IG	IG	IG	IG	IG
Cu	IG	IG	IG	IG	IG	IG
Fe(III)	IG	IG	IG	IG	IG	IG

References

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2. S. R. Kline, Reduction and analysis of SANS and USANS data using IGOR Pro *J. Appl. Crystallogr.*, 2006, **39**, 895-900.
3. E. P. Gilbert, J. C. Schultz and T. J. Noakes, *Physica B*, 2006, **385-386**, 1180-1182.
4. *SasView for Small Angle Scattering Analysis*, http://www.sasview.org/