Supplementary information

Exceptionally strong biocompatible hydrogels through self-assembly of an indole-capped dipeptide

Adam D. Martin,[a] Andrew B. Robinson,[a] Alexander F. Mason,[a] Jonathan P. Wojciechowski,[a] and Pall Thordarson[a]*

[a]School of Chemistry, The Australian Centre for Nanomedicine and the ARC Centre of Excellence for Convergent Bio-Nano Science and Technology, The University of New South Wales, Sydney, 2052, NSW, Australia. E-mail: p.thordarson@unsw.edu.au; Fax: +61-2-9385-6141; Tel: +61-2-9385-4478.

Contents:

Experimental

Materials and methods

Chemicals and solvents, including phosphate buffered saline (PBS) were purchased through Sigma Aldrich and used as supplied, except for Peptide-grade DMF (Auspep). Fmoc-Phe-OH was purchased from ChemImpex and used without further purification. Cell culture reagents including Dulbecco's Modified Eagle Medium (DMEM) and alamarBlue were purchased from Life Technologies.

Synthesis of 1

Initial amino acid loading

2-chlorotrityl chloride resin (100-200 mesh; 1% DVB; 1.41 mmol/g) (400 mg, 0.56 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 (655 mg, 3 equiv.) was dissolved in dry dichloromethane (4 mL) and *N,N*-diisopropylethylamine (DIPEA) (0.8 mL, 8 equiv.) and taken up into the syringe with resin and stirred overnight using an orbital shaker. The resin was then washed with dichloromethane (3 x 4 mL) 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 5 mins, then a fresh aliquot was taken up again and left for 10 min. 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 (655mg, 3 equiv.) was dissolved in a 0.5M solution of 1-hydroxybenzotriazole hydrate (HOBt)/ *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluoro-phosphate (HBTU) (3.4 mL, 3 equiv.) and DIPEA (0.6 mL, 6 equiv.) 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, the indole capping group was introduced using the amino acid coupling procedure described above, employing 297 mg (3 equiv.) of 3-indoleacetic acid.

Cleavage of the peptide

After the final coupling step, the resin was washed with DMF (3 x 4 mL), dichloromethane (3 x 4 mL) and methanol (2 x 4 mL) before being dried under a flow of nitrogen. To the dry

resin was added a 9:1 v/v trifluoroacetic acid/water solution (4 mL) and the resin was then stirred for 3 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 purple residue was lyophilised and purified by semi-preparative HPLC using an acetonitrile/water gradient, giving a white solid (yield = 32%).

IR: 3394 (m), 3284 (m), 1719 (w), 1643 (s), 1531 (s), 1497 (w), 1455 (w), 1420 (w), 1378 (w), 1350 (w), 1278 (w), 1252 (w), 1234 (w), 1218 (m), 741 (s), 698 (m);^{1}H NMR (DMSO-d_6, 400 MHz): 10.79 (s, 1H, N*H*), 8.19 (d, 1H, Ar*H*), 7.99 (d, 1H, Ar*H*), 7.16-7.32 (m, 12H, ArH), 6.99-7.06 (m, 2H, Ar*H*), 6.89 (t, 1H, Ar*H*), 4.46 (m, 2H, CH_2), 2.87-3.09 (m, 4H, CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz): 172.66, 171.10, 170.39, 137.78, 137.63, 136.01, 129.24, 128.12, 127.91, 127.17, 126.33, 126.14, 123.74, 120.83, 118.63, 118.24, 111.13, 108.55, 53.69, 37.49, 36.76; HR-MS (ESI): calcd for $C_{28}H_{27}N_3O_4$ + Na^+: 492.1899, found 492.1894.

Preparation of hydrogels

pH switch: 1 mg of **1** was suspended to 78 µL milliQ H_2O and 22 µL 0.1 M NaOH (1 molar equivalent) added, raising the pH to 9. This suspension was sonicated until homogenous, upon which time 0.8 mg of glucono-δ-lactone (GdL) was added to lower the pH, and gelation was induced after approximately 15 minutes.

PBS: 1 mg of **1** was suspended in 100 µL 1 mM PBS and the solution heated to boiling, which resulted in the dissolution of the gelator molecule. Upon cooling, a clear hydrogel was formed which gradually turned white over the course of 24 hours. Substitution of PBS with milliQ H_2O did not result in dissolution of **1**.

Solvent switch: 1 mg of **1** was dissolved in 20 µL of DMSO, this was followed by the addition of 80 µL milliQ H_2O which immediately resulted in a white suspension, which over time gradually solidified to form a gel.

CD 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) hydrogels were prepared and diluted 1:8 (v/v) in water. Temperature was kept constant at 20 °C and all experiments were repeated three times and averaged into a single plot.

ATR-IR 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 - 600 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) sol onto one of the stainless steel plates, lowering the other plate to the measurement position, and allowing an hour for the gel to form *via* the pH switch method described above. Throughout the casting of the gel onto the rheometer and the subsequent measurements, solvent loss was limited through the use of a solvent trap filled with water and lowering the temperature-controlled solvent trap cover in order to create a closed system within the sample chamber of the rheometer. Frequency sweeps were performed with a log ramp frequency (f) = 0.01 – 10 Hz and constant strain (γ) = 0.5%. Amplitude Sweeps were performed with constant frequency (f) = 1 Hz and log ramp strain (γ) = 0.1 – 100%. A peltier temperature control hood was used to maintain a temperature of 25 °C for frequency and amplitude sweeps. The rheology plots displayed are an average of at least three repeats for each point.

TEM measurements

TEM micrographs were recorded on a JEOL 1400 TEM at 80 kV. A 1% (w/v) sample of **1** was prepared by dissolving in an appropriate amount of milliQ H_2O/0.1 M NaOH, followed by addition of GdL. Samples were prepared for TEM by placing 5 μL of sample onto a formvar-coated copper grid, and excess solution was wicked away after 2 minutes with filter paper. Samples were left to dry at 25 °C for at least 4 hours prior to TEM analysis.

AFM measurements

0.01% (w/v) and 1% (w/v) hydrogels of **1** were prepared using the pH switch method described above. Upon addition of GdL and before gelation, the hydrogel solutions were spin coated onto a freshly cleaved mica substrate for three minutes at 3000 rpm. After spin coating, these samples were left to dry in air overnight. Imaging was undertaken on a BrukerMulitmode 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. BrukerScanasyst-Air probes were used, with a spring constant of 0.4 - 0.8 N/m and a tip radius of 2 nm.

Gel stability measurements

Two 200 μL 1 % (w/v) gel samples of **1** were prepared. One of the samples was layered with 200 μL milliQ water on top of the gel, the other with 200 μL saturated sodium chloride. Upon standing overnight, the hydrogel layered with milliQ water maintained its gel-like behaviour when subjected to the vial inversion test, whereas the hydrogel that had been exposed to the sodium chloride broke apart when subjected to the same test.

Cytotoxicity measurements

Cytotoxicity measurements were performed using and alamarBlue colorimetric assay on HeLa cells. Each experiment was repeated three times. Cells were passaged using standard cell culture procedures. Cells were detached with trypsin and centrifuged (2000 rpm for 2 min). The supernatant was removed and the cells resuspended in PBS. This sample was again centrifuged (2000 rpm for 3 min), the supernatant removed, and the cells resuspended in DMEM (10% FBS) at a concentration of 300,000 cells/mL. Cells were plated in a 96-well plate at a concentration of 30, 000 cells/well and left to adhere for 12 h (37 °C, 5.0% CO_2). Treatments were prepared by dissolving the gelator in dimethyl sulfoxide (DMSO) and diluting the resulting solution with DMEM (10% FBS) such that the final solution was 0.5% w/v sample and 1% v/v DMSO. The sample was serially diluted with DMEM (10% FBS, 1% DMSO) to obtain the appropriate concentrations. DMEM was removed from the wells and the appropriate treatment added in triplicate. Control wells were treated with DMEM (10% FBS, 1% DMSO). These treatments were left to incubate for either 24 h or 48 h (37 °C, 5.0% CO_2). alamarBlue was added to the appropriate wells and the plate left to incubate for a further 3 h (37 °C, 5.0% CO_2).The absorbance at 570 nm and 596 nm was recorded using a Thermo Multiskan Ascent plate reader.

Cell Seeding Experiments

Cell viability in seeding experiments was monitored using an alamarBlue assay on HeLa cells. Hydrogels of **1** were prepared as previously described using the pH switch method. The gel solutions were pipetted into wells of a 96 well plate and incubated for 30 minutes (37 °C, 5.0% CO_2) to set the hydrogels. The resulting hydrogels were then washed with Phosphate Buffered Saline (2x 50 μL) to neutralise residual glucono-δ-lactone. HeLa cells were then passaged following standard procedures and diluted to a total concentration of 5000 cells/mL. To the hydrogels was then added HeLa cells (100 μL) which was allowed to incubate on top of the hydrogels for 24 hours (37 °C, 5.0% CO_2). alamarBlue (20 μL) was then added to each well and the plate was incubated (37 °C, 5.0% CO_2) for 6 hours. Plates were then imaged on a Biorad Ultramark 550, measuring the subtracted absorbance of 575 nm and 590 nm.

Cell viability was calculated assuming the well containing only HeLa cells to have 100% viability. The resulting viability of the samples were calculated using the equation:

$$Cell\ Viability\ (\%) = \frac{Subtraced\ Absorbance\ Average\ Sample}{Subtraced\ Absorbance\ Average\ Control} \times 100$$

After 24 hours, no viable cells were found seeded on top of **1**. This experiment was repeated three times (n=3) with samples in triplicate with similar results.

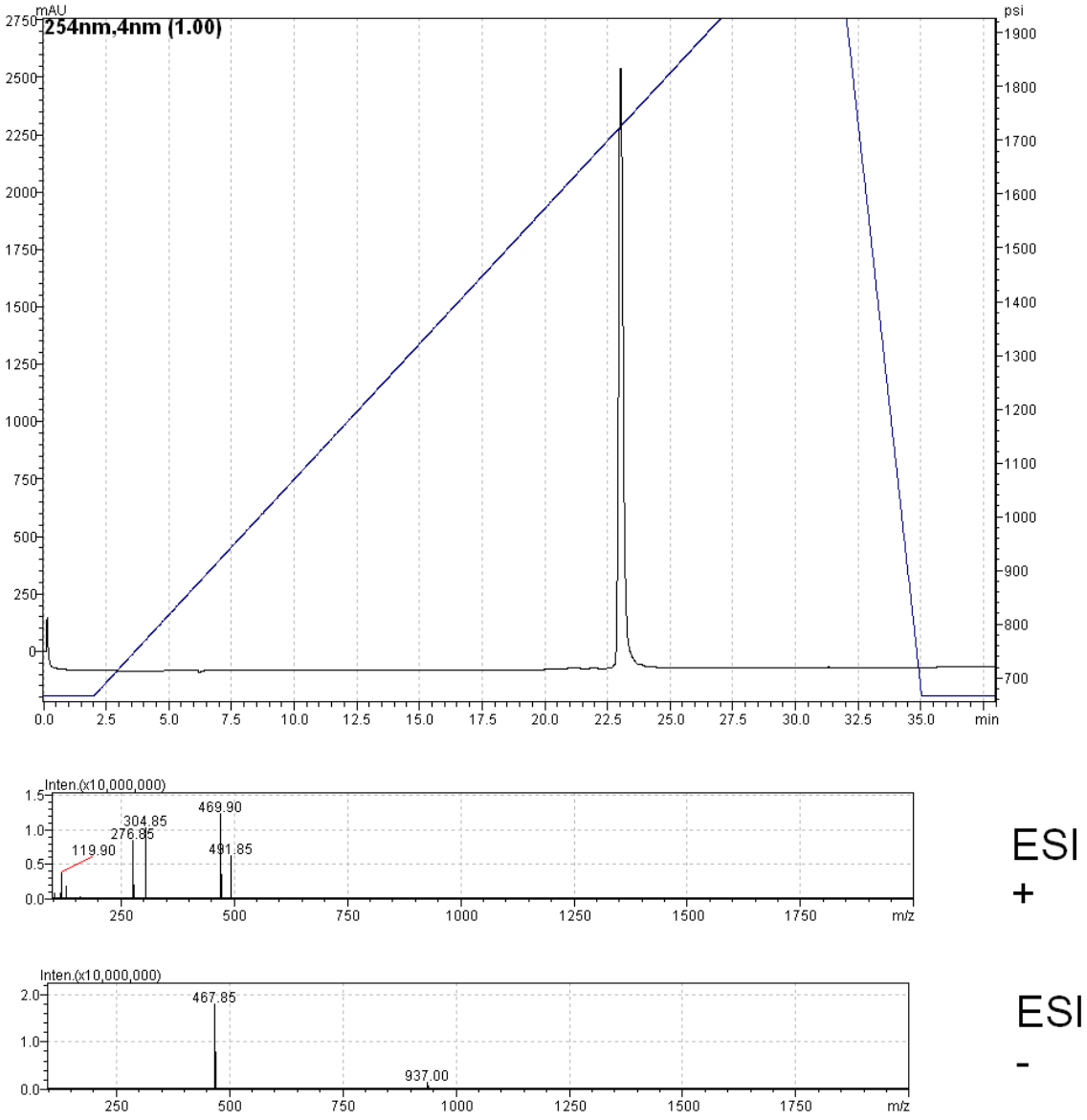

Fig.S1 LC-MS trace of **1** after purification.

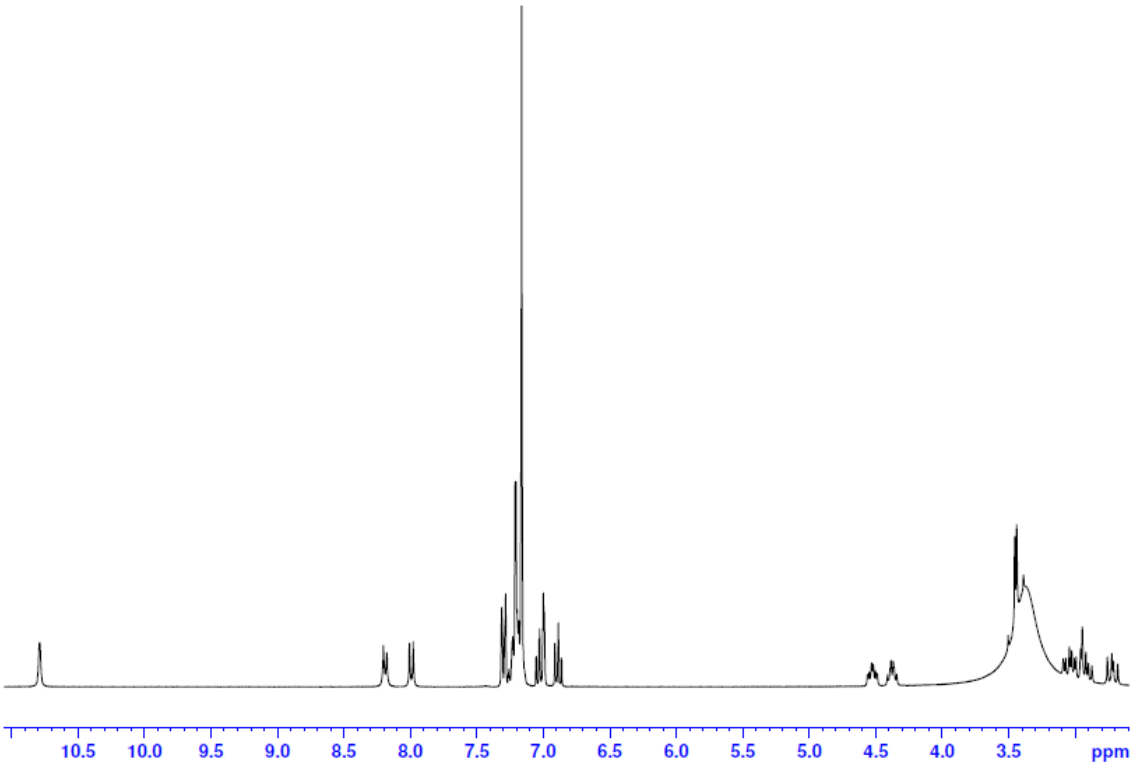

Fig.S2 ^{1}H NMR spectra of **1**.

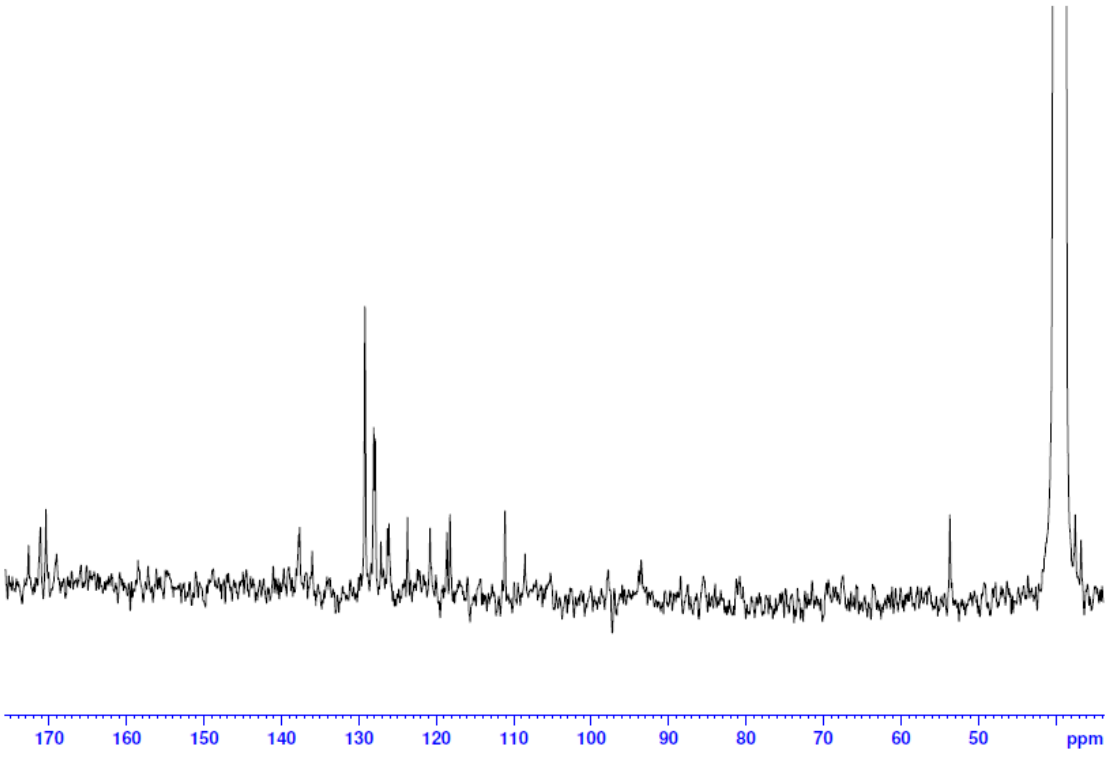

Fig. S3 ^{13}C NMR of **1**

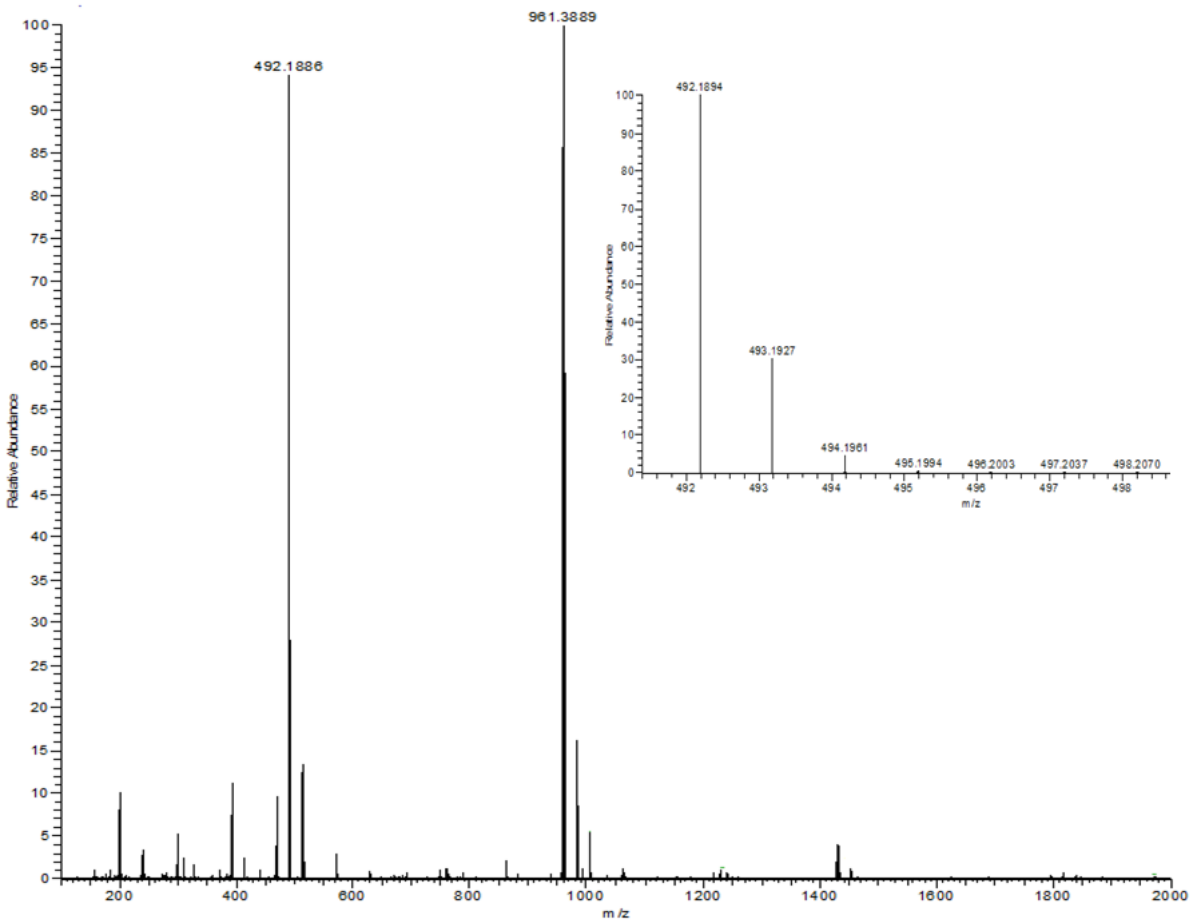

Fig.S4 HR-MS of **1**.

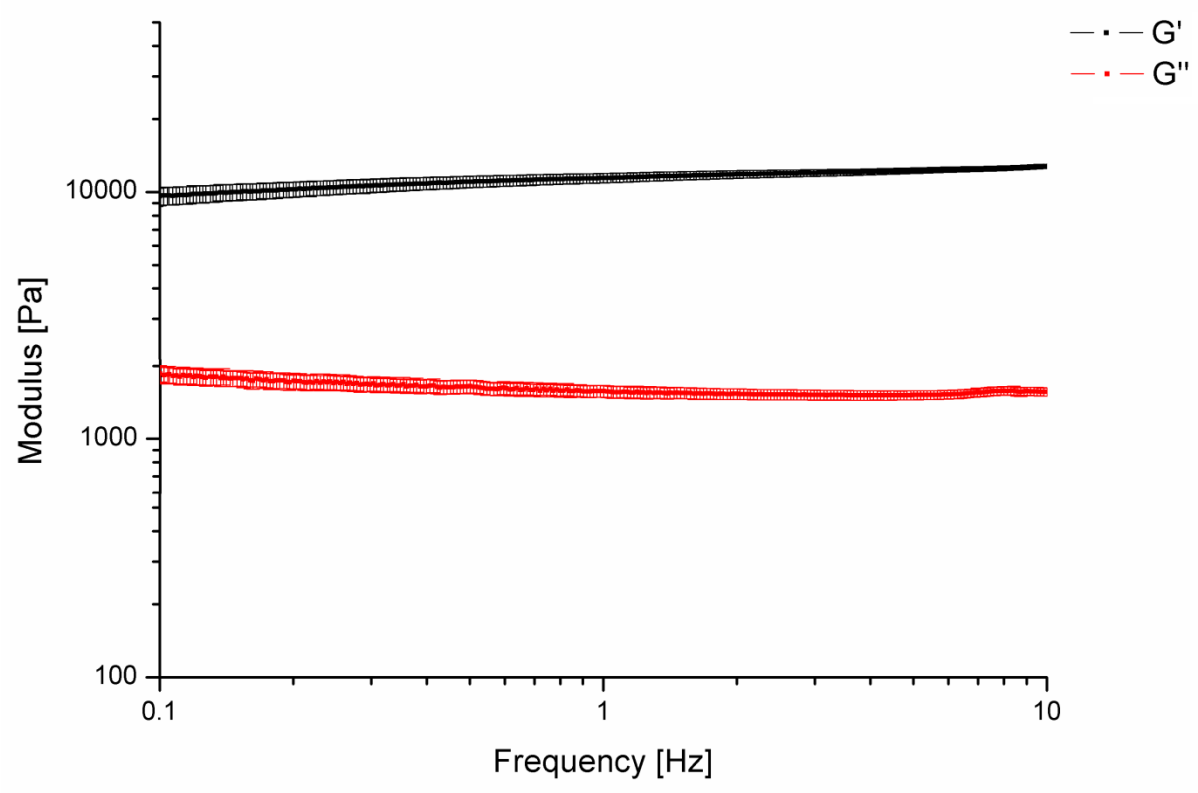

Fig S5 – Rheology of a hydrogel of **1** formed by solvent switching (final concentration of **1** is 1% w/v in Water/DSMO, 80/20, v/v). Measurements are performed at 25 °C.

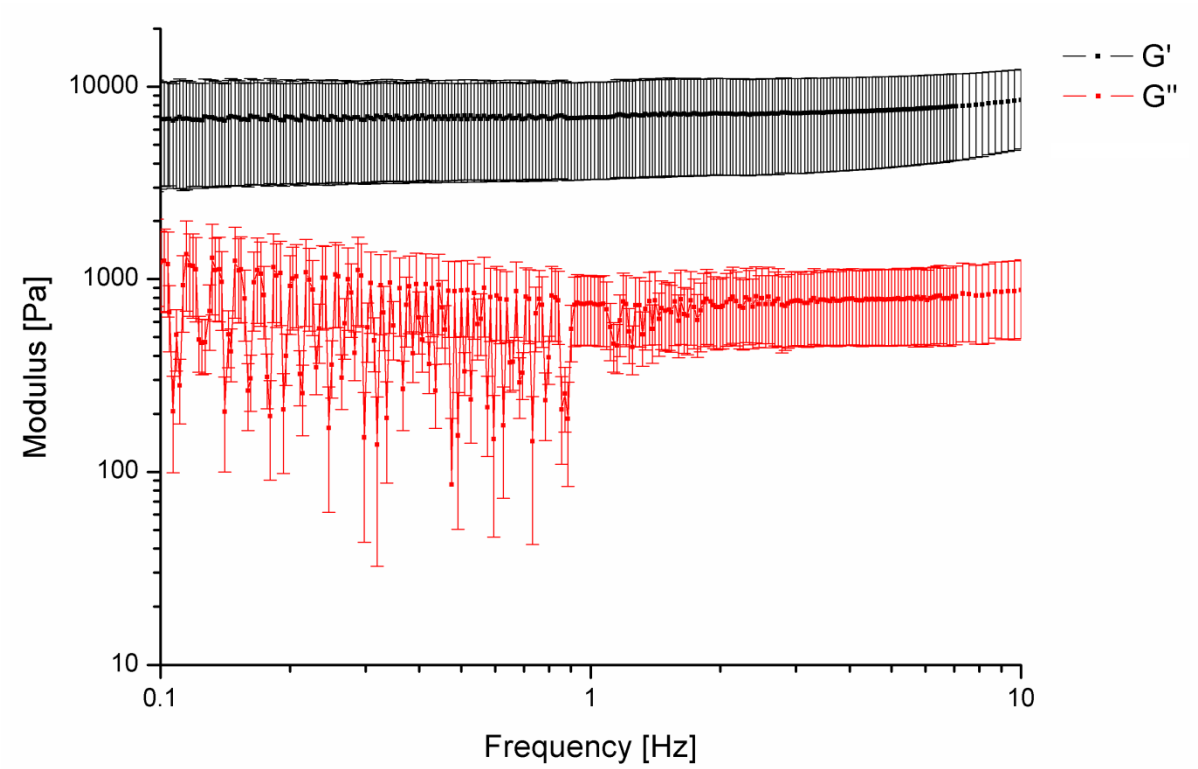

Fig S6 – Rheology of a hydrogel of **1** formed by temperature switching in PBS (final concentration of **1** is 1% w/v in 1 mM PBS). Measurements are performed at 25 °C.