Supplementary information

Biocompatible small peptide super-hydrogelators bearing carbazole functionalities

Adam D. Martin,[a] *Andrew B. Robinson,*[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: adam.martin2@unsw.edu.au, p.thordarson@unsw.edu.au; Fax: +61-2-9385-6141; Tel: +61-2-9385-4478.

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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-Gly-OH and 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 9*H*-carbazole acetic acid

1 g (6 mmol) of carbazole and 2.34 g (7.2 mmol) of Cs_2CO_3 was dissolved in 100 mL of dry acetonitrile. 0.73 mL (6.6 mmol) of ethyl bromoacetate was added and the resulting suspension stirred for 24h. The suspension was filtered and the solvent removed under reduced pressure. The residue was dissolved in dichloromethane (150 mL), washed with water (2 x 100 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the clear oil purified by column chromatography (4:1 hexane:ethyl acetate) to give 1.30 g (86%) ethyl 9*H*-carbazole acetate. The characterisation data matches that previously reported in the literature.[1]

1g of ethyl carbazole acetate was refluxed with 80 mL of a 10% (w/v) solution of NaOH for 5h, cooled, then diluted with 200 mL H_2O. The pH of the solution was adjusted to 5 using dilute HCl and the resulting precipitate filtered. The precipitate was dissolved in dichloromethane (100 mL), washed with water (2 x 100 mL), dried and the solvent removed under reduced pressure to yield 0.85 g (97%) of 9*H*-carbazole acetic acid, which was used in subsequent reactions.

Synthesis of carbazole-capped peptides

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-AA-OH (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 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-AA-OH (655 mg, 3 equiv.) was dissolved in a 0.5 M solution of 1-hydroxybenzotriazole hydrate (HOBt●H_2O)/ *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1H-benzotriazol-1-yl) uroniumhexa-fluorophosphate (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, iterative couplings were performed in order to build up the required peptide sequence.

Carbazole coupling

The carbazole capping group was introduced using a DIC-based coupling, whereby 9*H*-carbazole acetic acid (254 mg, 2 equiv.) and HOBt●H_2O (152 mg, 2 equiv.) were dissolved in 4 mL DMF and added to the resin, followed by *N*,*N*'-diisopropylcarbodiimide (180 µL, 2 equiv.) and the resin stirred for 2 hours using an orbital shaker. Coupling was confirmed through the Kaiser test.

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 green residue was lyophilised and purified by semi-preparative HPLC using an acetonitrile/water gradient, giving a white fluffy solid for both Cbz-Phe-Phe **1** and Cbz-Gly-Phe-Phe **2**.

Characterisation data for Cbz-Phe-Phe **1**: IR: 3379 (m), 3274 (s), 1716 (w), 1647 (s), 1603 (w), 1542 (m),1486 (w), 1461 (w), 1454 (m), 1425 (w), 1363 (w), 1326 (w), 1254 (w), 1229 (w), 1211 (w), 747 (m), 721 (m), 699 (m);^{1}H NMR (DMSO-d_6, 400 MHz): 8.55 (d, 1H, Ar*H*), 8.44 (d, 1H, Ar*H*), 8.10 (d, 2H, Ar*H*), 7.13-7.39 (m, 14H, Ar*H*), 4.93 (q, 2H, CH_2), 4.38-4.66 (m, 2H, C*H*), 2.71-3.12 (m, 4H, CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz): 171.49, 167.38, 140.92, 138.04, 137.99, 129.83, 129.59, 128.65, 128.51, 126.88, 126.76, 126.04, 122.53, 120.46, 119.33, 109.79, 54.02, 45.61, 38.37, 37.13; HR-MS (ESI): calcd for $C_{32}H_{29}N_3O_4$ + Na^+: 542.2105, found 542.2051.

Characterisation data for Cbz-Gly-Phe-Phe **2**: IR: 3391 (w), 3278 (m), 1731 (w), 1644 (s), 1601 (w), 1542 (m), 1486 (w), 1460 (w), 1454 (m), 1423 (w), 1359 (w), 1326 (w), 1262 (w), 1229 (w), 1209 (m), 746 (m), 719 (m), 697 (m);^{1}H NMR (DMSO-d_6, 400 MHz): 8.38 (m, 2H, Ar*H*), 8.12 (m, 2H, Ar*H*), 7.11-7.55 (m, 14H, Ar*H*), 5.06 (m, 2H, CH_2), 4.39-4.62 (m, 2H, C*H*), 3.55-3.82 (M, 2H, CH_2), 2.63-3.15 (m, 4H, CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz): 172.69, 168.05, 167.63, 140.61, 138.68, 137.70, 137.43, 134.24, 129.18, 129.09, 128.19, 127.96, 126.44, 125.63, 122.19, 121.56, 120.07, 118.94, 109.36, 53.50, 37.62, 36.58; HR-MS (ESI): calcd for $C_{34}H_{32}N_4O_5$ + Na^+: 599.2314, found 599.2267.

HPLC purification of peptides

Semi-preparative HPLC was performed using a Grace VisionHT C18 HL 150 mm column, with a particle size of 5 μm. Water containing 0.1% (v/v) formic acid and acetonitrile containing 0.1% (v/v) formic acid were used as the eluents, and flow rates of 5 mL/min were employed. For 1, a gradient from 50 – 80% acetonitrile was used to elute the product, and for 2, a gradient of 50 – 70% acetonitrile was utilised. Neither compound formed a hydrogel at any stage during purification by semi-preparative HPLC.

Preparation of hydrogels

pH switch: 1 molar equivalent of 0.1M NaOH was added to 1 mg of Cbz-Phe-Phe **1** or Cbz-Gly-Phe-Phe **2** and milliQ water added to then make the suspension up to 1% (w/v) . This suspension was sonicated until homogenous, upon which time 2 molar equivalents of glucono-δ-lactone (GdL) was added to lower the pH, resulting in gelation after approximately 60 minutes. For both gelators, the pH of the gel was measured to be pH 6.

PBS: 1 mg of Cbz-Gly-Phe-Phe **2** 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 to room temperature, a clear hydrogel was formed. Substitution of PBS with milliQ H_2O did not result in the dissolution of Cbz-Gly-Phe-Phe **2**.

Solvent switch: Suspensions of Cbz-Gly-Phe-Phe **2** were prepared in the same manner as for the pH switched gels, through the addition of 1 molar equivalent 0.1M NaOH, however instead of dilution to 1% (w/v) with milliQ water; Dulbecco's Modified Eagle Medium (DMEM) was used. No GdL was added, as gelation occurred instantly.

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 with milliQ water. Temperatures were kept constant at 20 °C and all experiments 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 - 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) sol onto one of the stainless steel plates, lowering the other plate to the measurement position, and allowing two hours for the gel to form *via* the pH switch method described above. 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) = 0.5%. Amplitude Sweeps were performed with constant frequency (f) = 1 Hz and log ramp strain (γ) = 0.1 – 100%. 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

0.1% (w/v) hydrogels of **1** and **2** were prepared using the pH switch method described above. Upon addition of GdL and before gelation, 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. For spin coated samples, three drops of 0.5% (w/v) hydrogel solutions of Cbz-Phe-Phe **1** and Cbz-Gly-Phe-Phe **2** prepared using a pH witching method described above were deposited onto a freshly cleaved mica substrate. The sample was then spun at 200 rpm over one minute, before being dried in air overnight.

Imaging was undertaken on a Bruker Mulitmode 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.

Measurement of fibre sizes were performed using the height of the fibres from AFM images, assuming a cylindrical geometry. At least 50 fibres were counted for each sample, with the error representing two times the standard deviation of the average size.

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. IC_{50} values were calculated by fitting a sigmoidal curve to the collected cytotoxicity data, and extrapolating from where this drops below 50%.

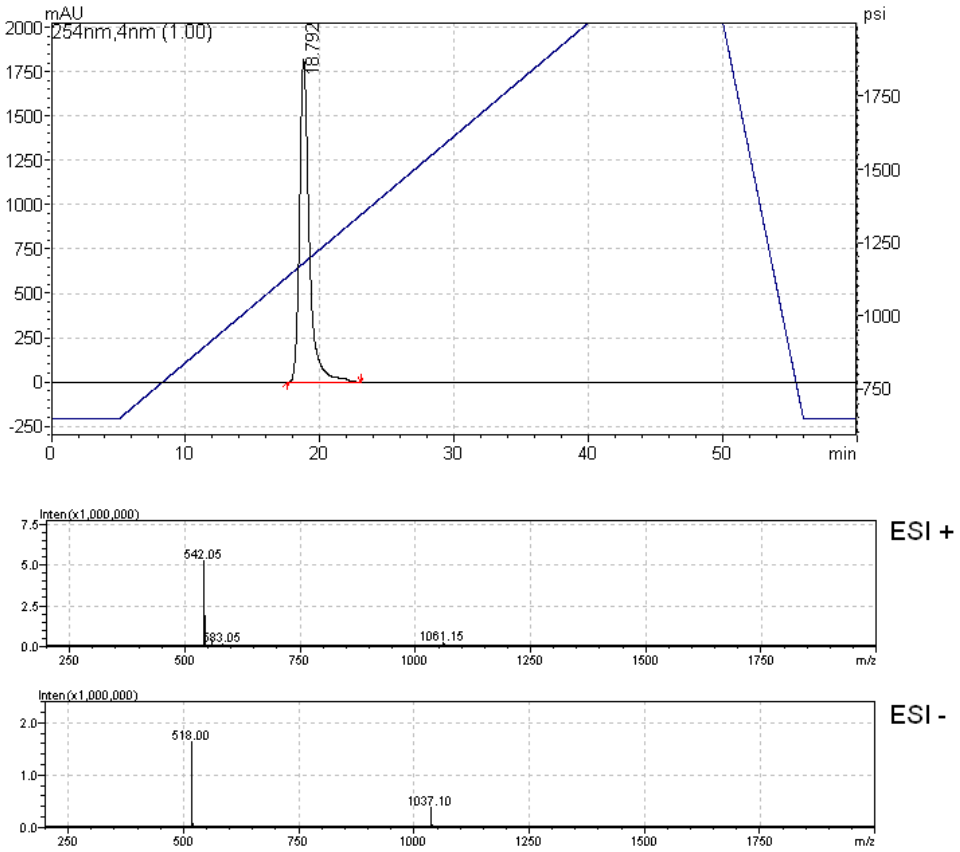

Fig.S1 LC-MS trace of Cbz-Gly-Phe-Phe **1** after purification.

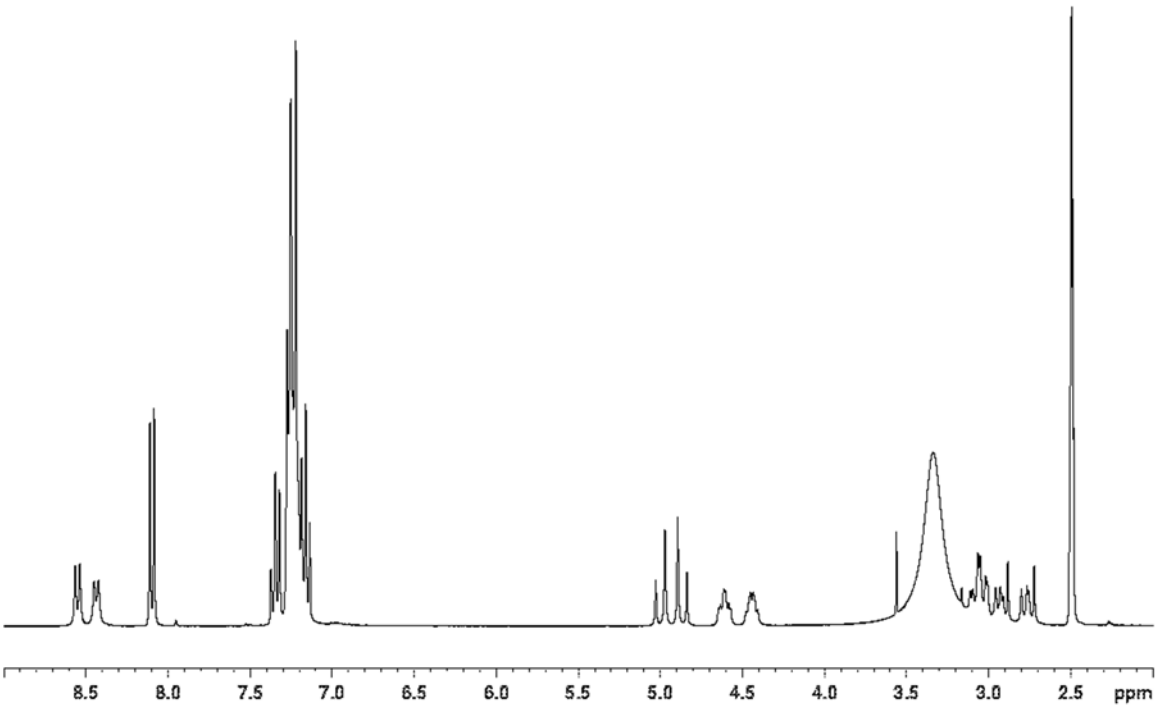

Fig. S2 ^{1}H NMR spectra of Cbz-Phe-Phe **1**.

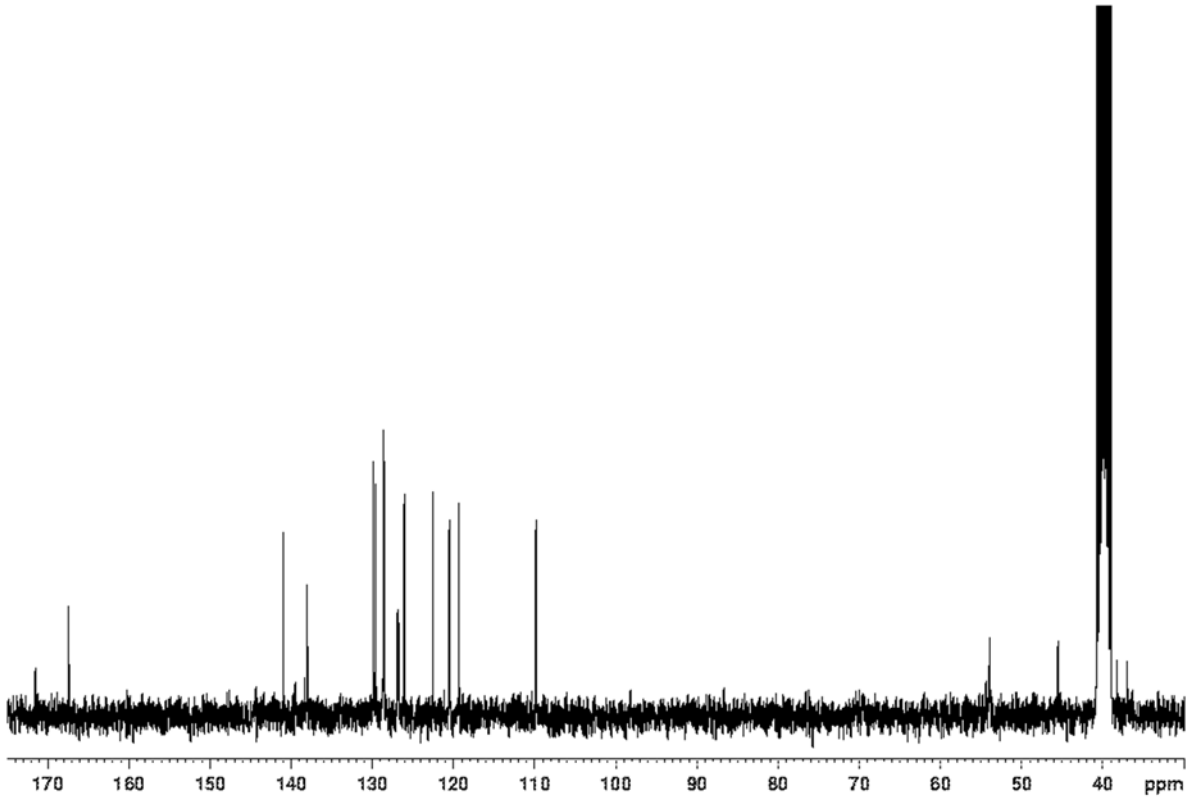

Fig. S3 ^{13}C NMR of Cbz-Phe-Phe **1**.

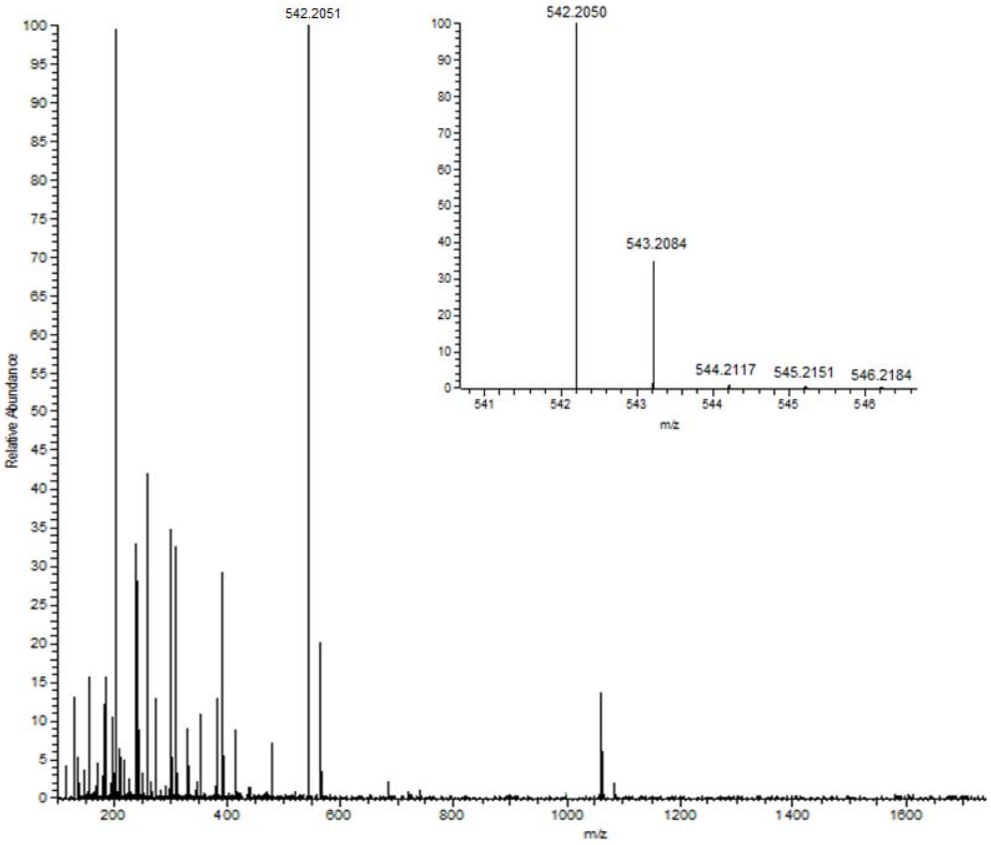

Fig. S4 HR-MS of Cbz-Phe-Phe **1**.

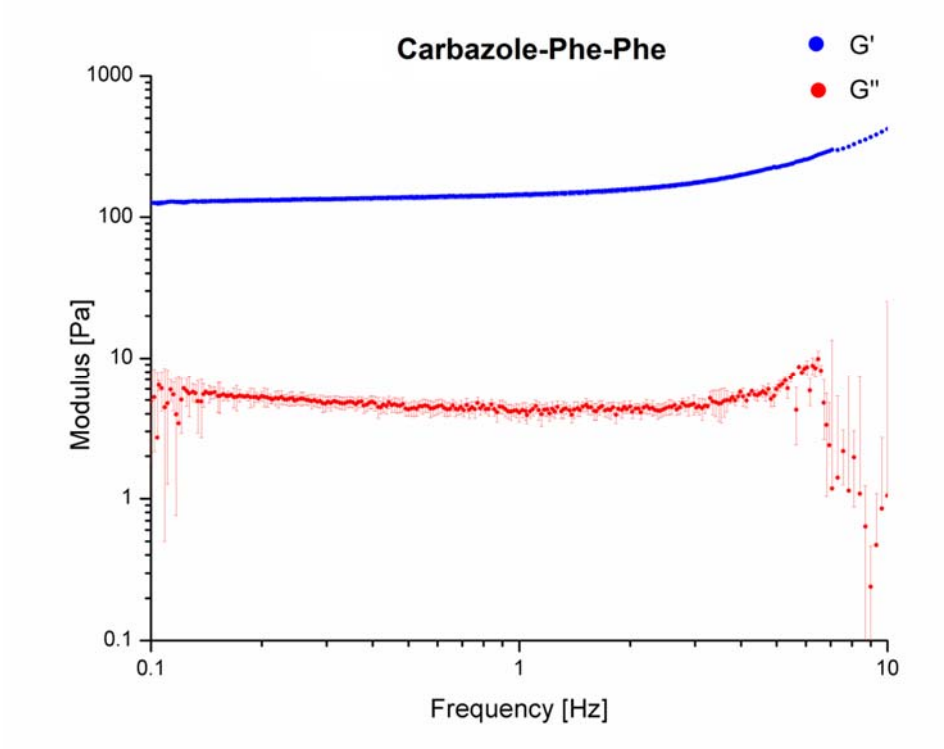

Fig. S5 Rheology of Cbz-Phe-Phe **1** at its minimum gel concentration, 0.03% (w/v).

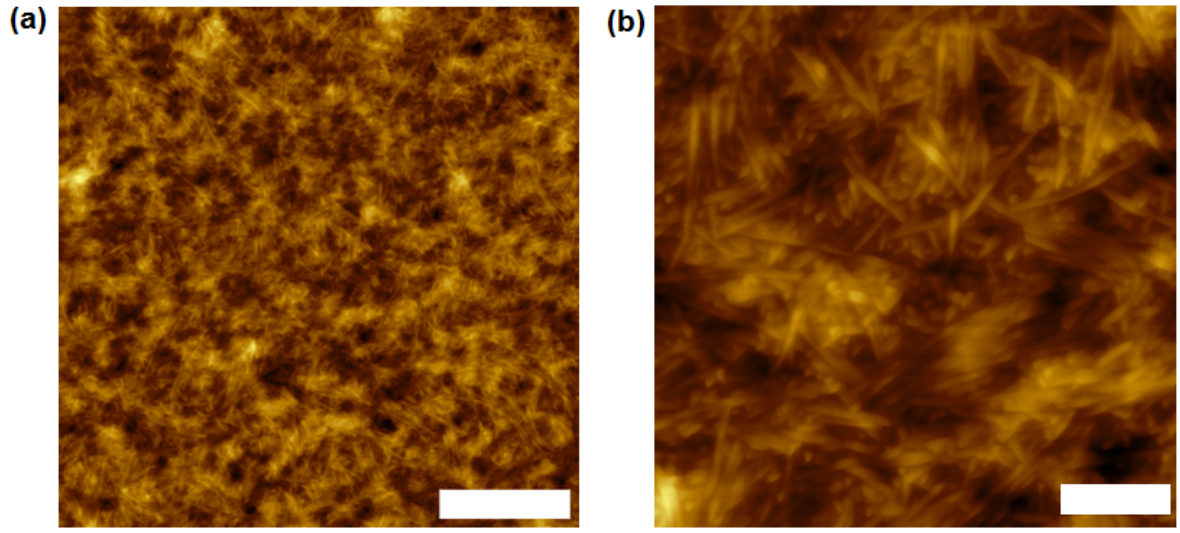

Fig. S6 – AFM of Cbz-Phe-Phe **1** spin coated onto mica at 0.5% (w/v). Scale bars represent 1 μm (a) and 200 nm (b). The dense mat of small nano-fibres is clearly visible.

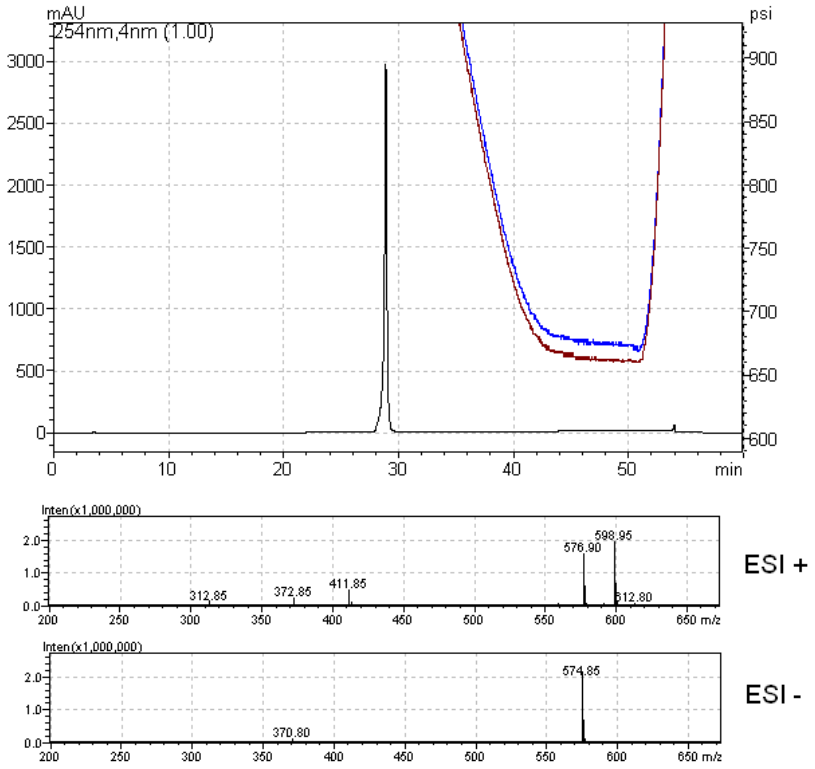

Fig. S7 LC-MS trace of Cbz-Gly-Phe-Phe **2** after purification.

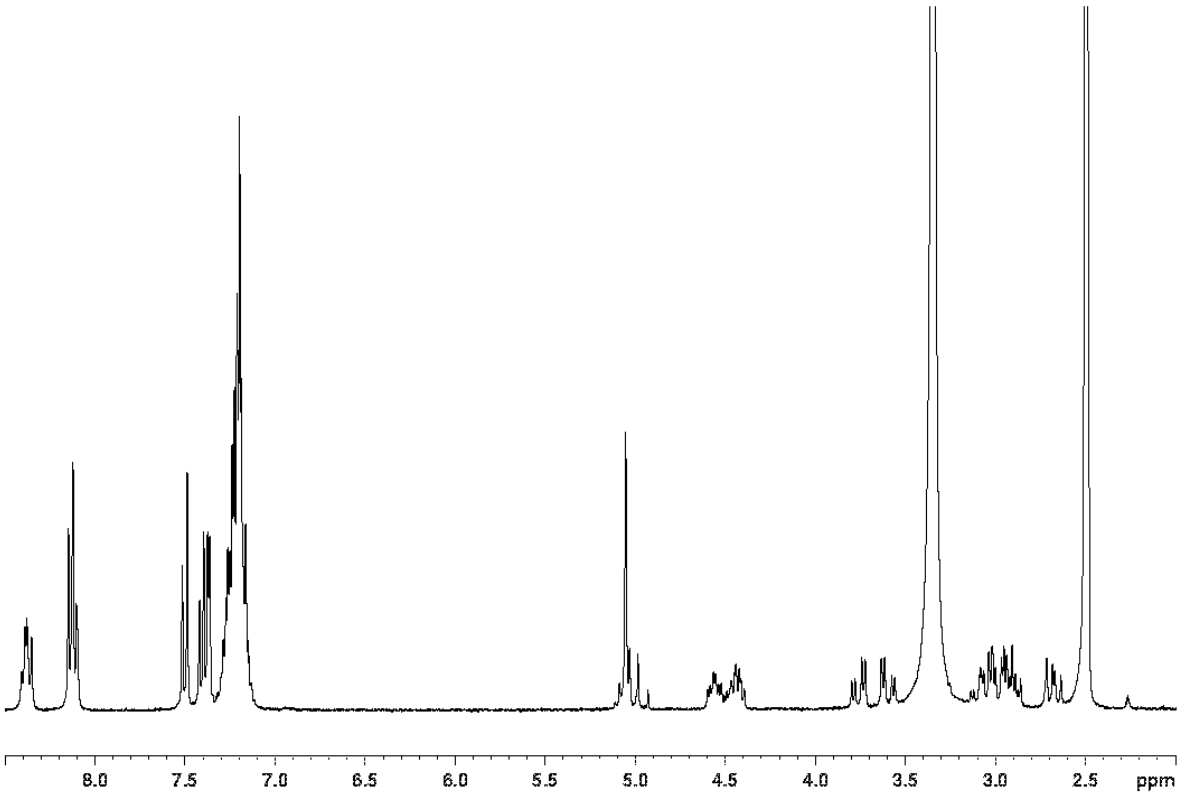

Fig. S8 ^{1}H NMR spectra of Cbz-Gly-Phe-Phe **2**.

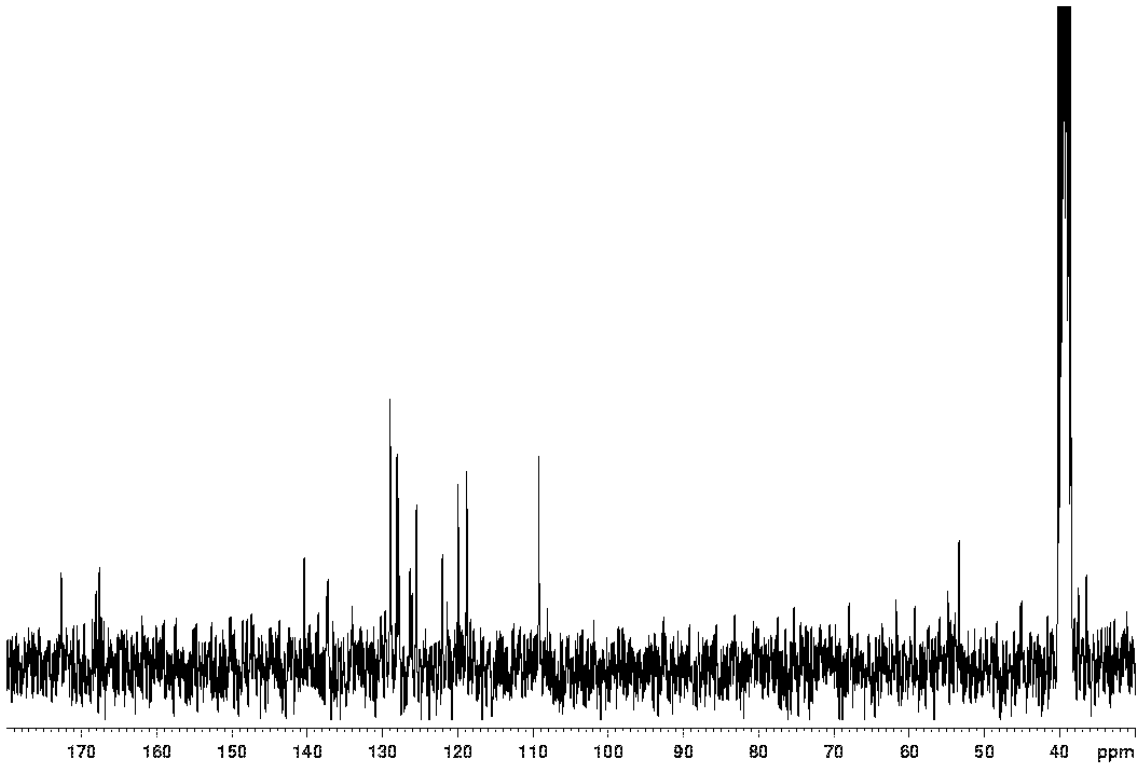

Fig. S9 ^{13}C NMR of Cbz-Gly-Phe-Phe **2**.

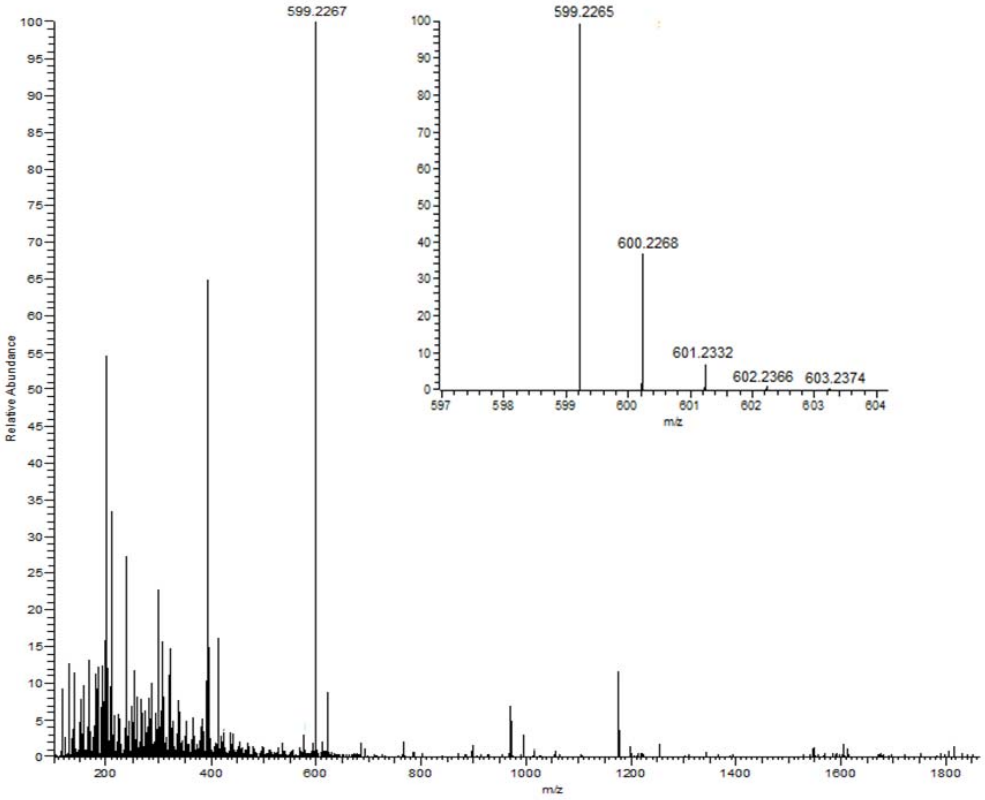

Fig. S10 HR-MS of Cbz-Gly-Phe-Phe **2**.

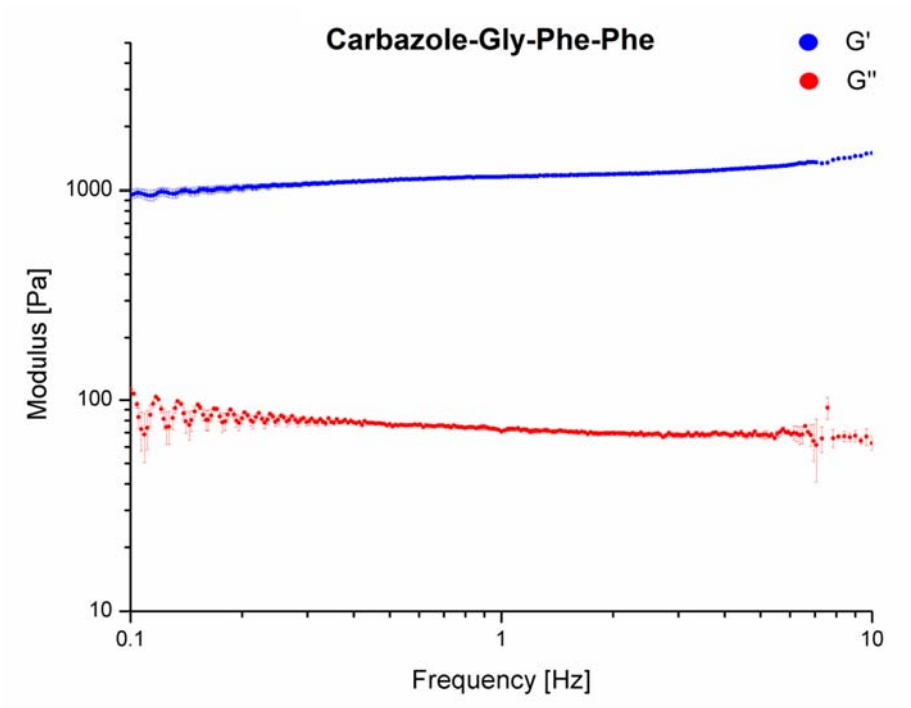

Fig. S11 Rheology of Cbz-Gly-Phe-Phe **2** at its minimum gel concentration, 0.1% (w/v).

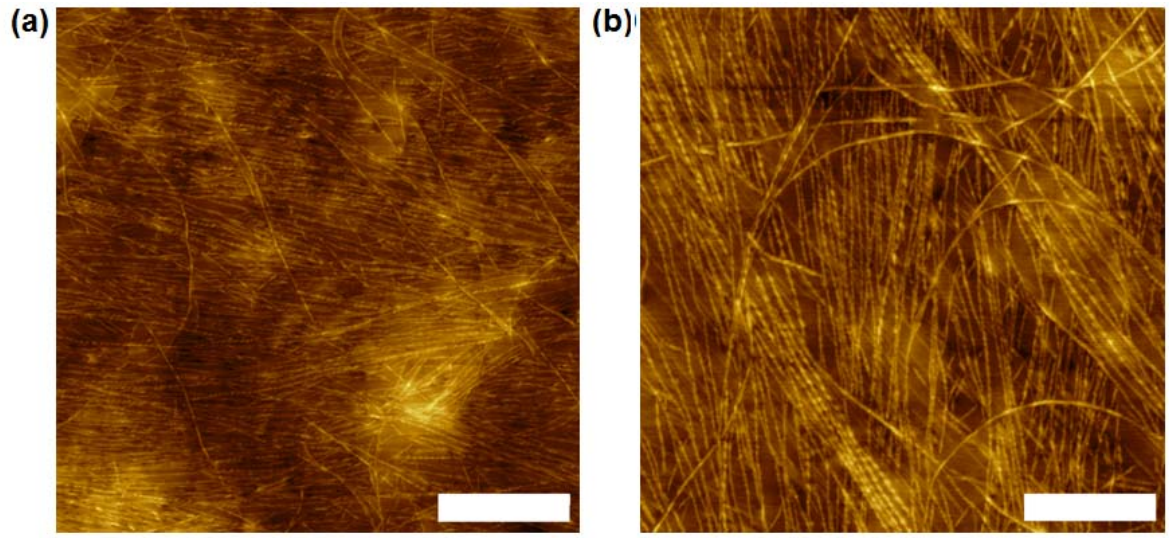

Fig. S12 AFM of Cbz-Gly-Phe-Phe **2** spin coated onto mica at 0.5% (w/v). Scale bars represent 1 µm (a) and 400 nm (b). The dense mat of small nano-fibres is clearly visible.

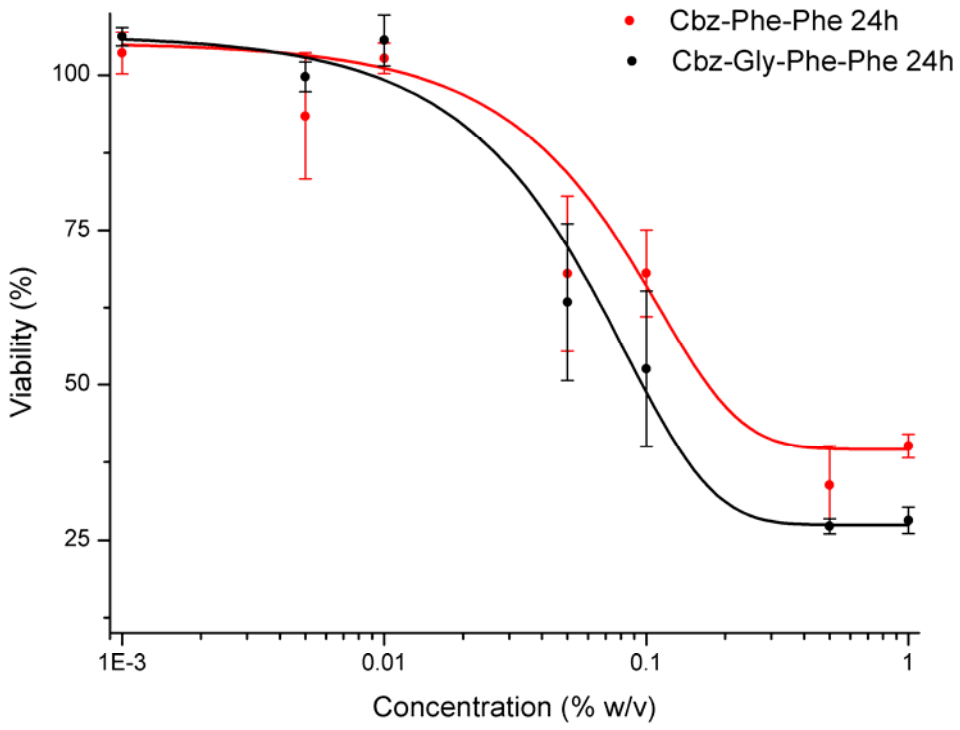

Fig. S13 Cytotoxicity data for Cbz-Phe-Phe **1** and Cbz-Gly-Phe-Phe **2** after 48 h.

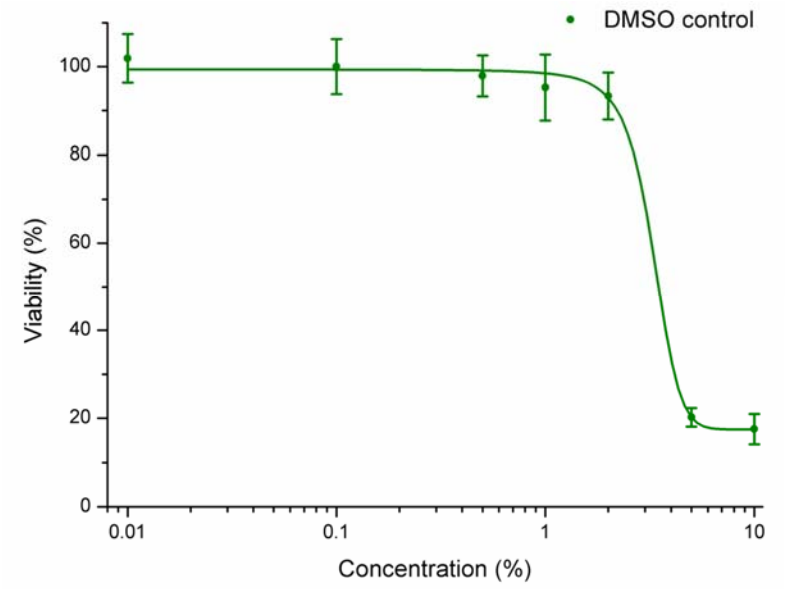

Fig. S14 Cell viability (24 h) control experiments using various concentrations of DMSO.

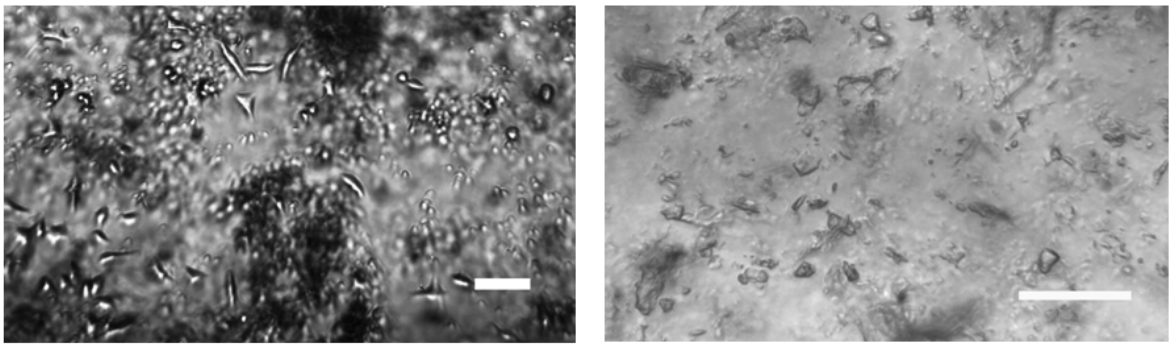

Fig. S15 Images of Cbz-Phe-Phe **1** (left) and Cbz-Gly-Phe-Phe **2** (right) at their minimum gel concentrations with HeLa cells seeded on top. Images were obtained after 24h. Scale bars represent 1 mm.

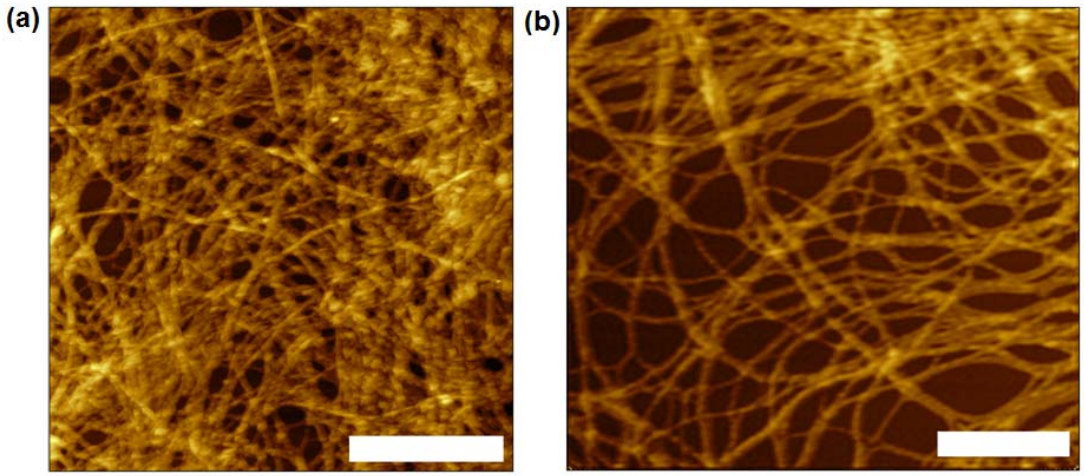

Fig. S16 AFM of Fmoc-Gly-Phe-Phe **2**, spread coated onto a freshly cleaved mica substrate at 0.05% (w/v). Scale bars represent 1 μm (a) and 500 nm (b).

References

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