

Supporting information

Design of a hydrophobic tripeptide that self-assembles into amphiphilic superstructures forming a hydrogel biomaterial

A. V. Vargiu^a, D. Iglesias^b, K. E. Styan^c, L. J. Waddington^c, C. D. Easton^c and S. Marchesan.^{b*}

^a University of Cagliari, Department of Physics, S.P. Monserrato-Sestu Km. 0.700, 09042 Monserrato (CA), Italy

^b University of Trieste, Chemical and Pharmaceutical Sc. Dep., Via L. Giorgieri 1, 34127 Trieste, Italy.

^c CSIRO Manufacturing, Clayton, VIC 3168, Australia

* corresponding author. email: smarchesan@units.it

Table of Contents

| | |
|--|-----|
| 1. Materials and Methods..... | S2 |
| 2. Rheometric data for Phe- ^D Leu-Phe hydrogel..... | S7 |
| 3. DSC data for Phe- ^D Leu-Phe hydrogel | S7 |
| 4. Molecular dynamics simulations | S8 |
| 5. FT-IR and CD spectra for Phe- ^D Leu-Phe hydrogel | S9 |
| 6. Cytotoxicity assay for the gelling tripeptide in solution..... | S9 |
| 7. Cryo-TEM imaging of Phe- ^D Leu-Phe hydrogel..... | S11 |
| 8. Spectroscopic identification of Phe- ^D Leu-Phe..... | S11 |
| 9. XRD spectrum of Phe- ^D Leu-Phe hydrogels..... | S13 |

1. Materials and Methods.

Materials. Phe-Wang resin, O-Benzotriazole-N,N,N,N'-tetramethyl-uronium-hexafluorophosphate (HBTU), and Fmoc protected D-leucine and L-phenylalanine were purchased from GL Biochem (Shanghai) Ltd. All solvents were purchased of analytical grade from Merck. Piperidine, trifluoroacetic acid (TFA), diisopropyl ethyl amine (DIPEA), triisopropyl silane (TIPS) were from Acros. Sodium dihydrogen phosphate and disodium hydrogen phosphate were from BDH AnalaR. High purity Milli-Q-water (MQ water) with a resistivity greater than 18 M Ω cm was obtained from an in-line Millipore RiOs/Origin system. Silicon wafers (M.M.R.C Pty Ltd., Australia) were cleaned by ultrasonication (1 h) in a surfactant solution of 2% ethanol with 2% RBS 35 (Pierce, USA) followed by rinsing with copious amounts of MQ water and dried with nitrogen, then cleaned for 1h in a UV/ozone ProcleanerTM (Bioforce Nanosciences). ¹H-NMR spectra were recorded at 400 MHz and ¹³C-NMR spectra were recorded at 100 MHz on a Bruker Instrument Biospin Av400H. Chemical shifts are reported in ppm relative to TMS. Low resolution ESI-MS spectra were acquired with a Shimadzu LCMS-2010EV mass spectrometer using a cone voltage of 50V and the source was maintained at 80°C. The solvent used was methanol containing 0.1% formic acid with a flow rate of 0.1ml/min.

Peptide synthesis and characterization. Tripeptides were synthesized and purified using the standard Fmoc solid phase peptide synthesis with HBTU activation, following the same procedure described previously, employing reverse-phase HPLC (Agilent Technologies) with a gradient consisting of acetonitrile (AcN)– water with 0.1% TFA using the following program (10 ml/min flow): t = 0–3 min, 25% AcN; t = 15 min, 55% AcN; t = 16–20 min, 95% AcN. The compounds were freeze-dried; their purity was verified by HPLC using the same equipment and gradient as above, but on an analytical C-18 column (Luna, 5 mm, 100 Å, 150 x 4.60 mm, Phenomenex; gradient 5–95% AcN over 15 min; 1 ml/min flow).

Peptide gelation. Typically, 4.2 mg of peptide was added to 300 μ l of 0.1 M sodium phosphate buffer (pH 11.8) and dissolved with the aid of sonication for 5 min in a water bath at room temperature, then diluted 1:1 with another 300 μ l of 0.1 M sodium phosphate buffer (pH 5.6–5.7) to yield a final pH of 7.4. A supramolecular hydrogel formed immediately. All buffer solutions were filtered (0.2 μ m) prior to use.

Differential Scanning Calorimetry (DSC). DSC data were collected on a Q100 calorimeter (TA Instruments). The hydrogel samples were prepared directly in the DSC pans by dissolving the peptide in the alkaline buffer (1.3 mg in 100 μ l), then transferring 10 μ l of the peptide solution in

the DSC aluminium pan, followed by another 10 μl of the second buffer. Pans were closed with their lids, and measurements started after 15 min at room temperature. DSC scans started with an isotherm at 20 $^{\circ}\text{C}$ for 5 min, followed by a 10 $^{\circ}\text{C min}^{-1}$ ramp up to 200 $^{\circ}\text{C}$ (*i.e.*, below peptide decomposition temperature). Measurements were repeated in triplicates.

Rheometry. Dynamic time sweep rheological analysis was conducted on an Ares rheometer (TA Instruments, USA) with a 25 mm aluminium parallel plate geometry. A Peltier temperature controller was connected to the rheometer to maintain a temperature of 25 $^{\circ}\text{C}$. Gel samples were analysed after 1 h from preparation with a gap of 300 μm . Strain sweeps were recorded at a frequency of 10 rad s^{-1} and frequency sweeps at a strain of 0.3%.

Fourier-transformed infrared spectroscopy (FT-IR). FT-IR spectra were collected on a Nicolet 6700 FT-IR spectrometer in ATR mode. A portion of the gel was transferred onto a clean piece of silicon wafer (1 cm x 1 cm), and then gently spread over the surface by pressing a coverslip on top; the coverslip was immediately removed and samples were dried under vacuum for 24 h. Dried samples on the silicon wafers were placed directly onto the ATR crystal. Scans were between 1800 and 1500 cm^{-1} with 80 accumulations at a resolution of 0.4 cm^{-1} .

Circular dichroism (CD) spectroscopy. The secondary structure of peptides was analysed using a 0.1 cm quartz cell on a Jasco J815 Spectropolarimeter, with 1 s integrations and a step size of 1 nm with a bandwidth of 1 nm over a range of wavelengths from 200 to 280 nm. Peptide samples were freshly prepared by mixing the two precursor solutions directly in the CD cell. Spectra were recorded after 15 min. Measurements were repeated at least 5 times, and to reduce the noise near 200 nm, their average was plotted.

Thioflavin T (ThT) confocal fluorescence microscopy. Gel precursor solutions were prepared as indicated above and 12.5 μl of each were immediately placed on wells of a “ μ -Slide Angiogenesis” uncoated (Ibidi, Germany). 25 μl of a solution of ThT (200 μM in 50 mM glycine–NaOH, pH 7.5, 0.2 μm -filtered) were placed on top. After 15 min, the slides were imaged using a Leica SP5 microscope (63x water immersion objective, NA 1.2, ex. 458 nm, em. 468–600 nm). Samples treated and stained using an identical protocol but without the peptide were used as controls and did not reveal any fluorescent structure (data not shown).

Cryo-transmission electron microscopy (cryo-TEM). A locally built vitrification system was used to prepare the hydrogels for imaging in a thin layer of vitrified ice using cryo-TEM. The ambient temperature and humidity were 22 $^{\circ}\text{C}$ and 80%, respectively. 200-mesh copper grids coated with perforated carbon films (Lacey carbon films: ProSciTech, Qld, Australia) were glow discharged in

nitrogen for 5 s immediately before use. Hydrogels were prepared as described above. At the time of analysis, hydrogels were disrupted by tapping against the glass vial. Approximately 4 μ L aliquots of sample were pipetted onto each grid prior to plunging. After 30 s adsorption the grid was blotted manually using Whatman 541 filter paper, for approximately 6–10 s. The grid was then plunged into liquid ethane cooled by liquid nitrogen. Frozen grids were stored in liquid nitrogen until required. The samples were examined under low dose conditions using a Gatan 626 cryoholder (Gatan, Pleasanton, CA, USA) and Tecnai 12 TEM (FEI, Eindhoven, The Netherlands) at an operating voltage of 120 kV. Images were recorded using a FEI Eagle 4kx 4k CCD camera (FEI, Eindhoven, The Netherlands) at magnifications in the range 15000–30000x.

TEM with negative staining. Carbon-coated 300-mesh copper grids were glow-discharged in nitrogen to render the carbon film hydrophilic. A 4 μ L aliquot of the sample was pipetted onto each grid. After 30 s adsorption the excess was drawn off using Whatman 541 filter paper, followed by staining with 2% aqueous potassium phosphotungstate at pH 7.2, for 10 seconds. Grids were air-dried until needed. Samples were examined using a Tecnai 12 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) at an operating voltage of 120 kV. Images were recorded with a MegaView III camera (Olympus, Tokyo) at magnifications in the range 30000–60000x.

Atomic force microscopy (AFM). An Asylum Research MFP-3D atomic force microscope (Santa Barbara, CA, USA) was used to measure surface topography in tapping mode employing ultrasharp silicon nitride tips (NSC15 noncontact silicon cantilevers, MikroMasch, Spain). The tips used in this study had a typical force constant of 40 N m⁻¹ and a resonant frequency of 320 kHz. Typical scan settings involved the use of an applied piezo deflection voltage of 0.6–0.7 V at a scan rate of 0.7 Hz. All images were processed (1st order flattening algorithm). The samples were prepared in a glass vial as described above for the hydrogelation test and a small amount (~30 μ l) was spread onto a clean square of silicon wafer (1 cm x 1 cm) by gently pressing a glass coverslip on top. The samples were then dried at room temperature for 24 h.

X-ray fiber diffraction (XRD). A Bruker D8 Advance X-ray diffractometer with CuK α radiation (40 kV, 40 mA) equipped with a LynxEye silicon strip detector was employed to determine the X-ray diffraction (XRD) patterns. Each sample was scanned over the 2-theta range 1–60° with a stepsize of 0.02° and a count time of 1.6 s per step. An airscatter slit was used to reduce the beam intensity at low angles. The samples were mounted on zero background plates consisting of a silicon wafer located in a standard Bruker specimen holder, and were dried overnight in air.

Molecular modelling. Model structures of zwitterionic tripeptide Phe-^DLeu-Phe were generated using MarvinSketch¹, and their antiparallel beta-sheet structures were built in an incremental fashion as follows. First, we manually assembled a five-strand antiparallel sheet using the software VMD 1.9.1.² Then, molecular optimization of atomic structures was performed using the AMBER15 package.³ The parm99SB-ILDN⁴ force field was used to model the peptides, while solvation was taken into account through an implicit water model.⁵ Structural relaxation was carried out in multi-step fashion: first, a restrained optimization was performed for up to 25.000 steps while applying restraints (with force constant $k = 1 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$) to all non-hydrogenous atoms of the system. Second, five optimization steps (each involving up to 50.000 steps) were performed by applying the same restraints only to backbone atoms. Third, five optimization steps (each involving up to 50.000 steps) were performed by applying the same restraints only to C α atoms. At each cycle of restrained optimization the reference structure to which additional forces were applied was the relaxed one from previous step. Finally, up to 50.000 cycles of unrestrained optimization were performed.

To further investigate self-aggregation propensity and secondary structure preference a solution of Phe – ^DLeu – Phe, we also performed MD simulations with several tripeptides in the presence of explicit water molecules. Namely, we arranged 216 tripeptides on a cubic 6x6x6 grid in a way that each peptide center of mass was placed on a point of the grid at reciprocal distance of 15 Å. Initial orientations of peptides were randomized, and the system was solvated with ~40.000 explicit water molecules (TIP3P model⁶). The total number of atoms amounted to ~125.000, and the initial size of the box was 116 x 118 x 119 Å, corresponding to a concentration of tripeptides $c \sim 0.2 \text{ M}$. The system was relaxed by performing up to 50.000 steps of restrained optimization, applying restraints of force constant $k = 1 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ on all non-hydrogenous atoms of the peptides. The same restraints were then applied on backbone atoms only, and in a third run to C α atoms. At each cycle of restrained optimization the reference structure to which additional forces were applied was the relaxed one from previous step. Finally, up to 100.000 cycles of unrestrained optimization were performed. System was then heated up to 300 K in 1 ns via constant-pressure-temperature (NTP) MD simulations, followed by an equilibration phase of 10 ns. Starting from the equilibrated structure, three independent NTP MD simulations each of 200 ns in length were performed. In all runs a time step of 2 fs was used. Pressure and temperature were regulated at 1 atm and 310 K (after the equilibration phase) using the isotropic Berendsen barostat⁷ and the Langevin thermostat⁸, respectively. Periodic boundary conditions were employed. Electrostatic interactions were evaluated using the Particle Mesh Ewald scheme with a cutoff of 9.0 Å for the short-range evaluation

in direct space. The same cutoff was used for Lennard-Jones interactions (with a continuum model correction for energy and pressure). Structural analyses were performed using the VMD software through ad hoc scripts using the tcl language.

References:

1. Marvin 2.5.9, 2012, ChemAxon (<http://www.chemaxon.com>).
2. W. Humphrey, A. Dalke and K. Schulten, *J. Mol. Graphics*, 1996, 14, 27.
3. D. A. Case, *et al.* AMBER 2012, University of California, San Francisco, 2012.
4. Lindorff-Larsen K, *et al.* *Proteins* 2010, 78 (8), 1950.
5. A. Onufriev, *et al.* *Proteins* 2004, 55, 383.
6. W. D. Jorgensen and J. D. Madura, *J. Am. Chem. Soc.* 1983, 105, 1407.
7. H. J. C. Berendsen, *et al.* *J. Chem. Phys.* 1984, 3684.
8. S. E. Feller, *et al.* *J. Chem. Phys.* 1995, 13, 4613.

Cell assays. The tripeptide in solution was assessed for cytotoxicity in accordance with ISO 10993, and tripeptide gels were also probed for their ability to support cell viability and proliferation. For cytotoxicity studies, L929 mouse fibroblast cells were seeded at 10000 cells per well of a 96-well tissue culture plate in 100 ml of media (MEM + GlutaMAX (GIBCO)), supplemented with 1 v% NEAA (non-essential amino acids, GIBCO), 2 v% anti-anti (antimycotic– antibiotic, GIBCO), and 10 v% FBS (fetal bovine serum, SAFC Biosciences) and cultured overnight at 37 °C, 5% CO₂. The tripeptide was dissolved in the media at the highest concentration possible without occurrence of precipitation or gelation (i.e., 1 mg ml⁻¹) and 1:1 serial dilutions down to <8 µg ml⁻¹ were prepared. The tripeptide solutions were sterile-filtered and 100 µl was applied to monolayers that were then cultured further for 24 h. Cells were imaged using an inverted microscope (Olympus IX71) before quantitation by reduction of resazurin (120 µl of a 1:9 solution of PrestoBlues in media for 1.5 h) and 100 µl assayed for fluorescence on a Pherastar fluorometer (ex. 540–20 nm, em. 590–20 nm). Media without tripeptide as a negative control and 5 v% dimethylsulphoxide (DMSO) in media as a positive control, were included. Monolayers remained subconfluent throughout. For gel studies, gel precursor solutions were prepared as indicated previously and 15 µl of each were mixed directly in triplicate wells of a ‘µ-Slide angiogenesis’ uncoated (Ibidi, Germany, through DKSH Australia). Gels with lower peptide concentration were not tested to avoid premature gel dissolution during the assay. After 24 h, gels were pre-treated with 30 µl of media for 1 h. L929 cells were added to the gels (10000 cells per cm² in 30 µl media), and cultured at 37 °C, 5% CO₂ for up to 72 h, by handling the slides according to the manufacturers’s instructions, including the addition of a few drops of

deionised water in the empty space between the wells to minimise sample evaporation. Every 24 h, cells were either stained for viability or, if continuing to a later assay time point, had 30 μ l of media exchanged for fresh media. Cell viability was investigated using the LIVE/DEADs assay (Invitrogen), according to the manufacturers's instructions. Cells were imaged using an inverted microscope (Nikon Eclipse TE2000-U) for calcein (ex. 465–495 nm, em. 515–555 nm) and ethidium (ex. 510–560 nm, em. 4590 nm).

2. Rheometry data for Phe-^DLeu-Phe hydrogel.

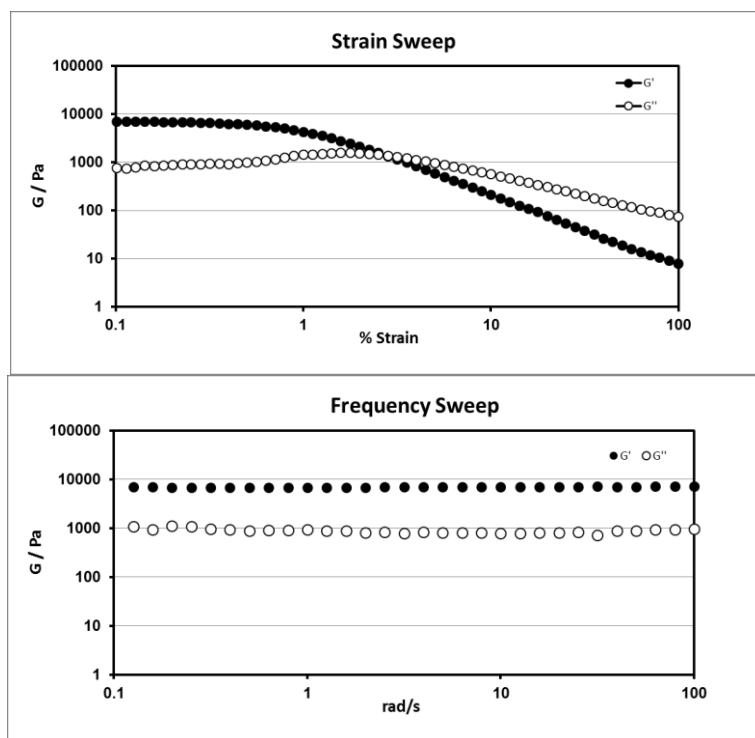


Fig. S1. Strain sweep (top) and frequency sweep (bottom) data for Phe-^DLeu-Phe hydrogel.

3. DSC data.

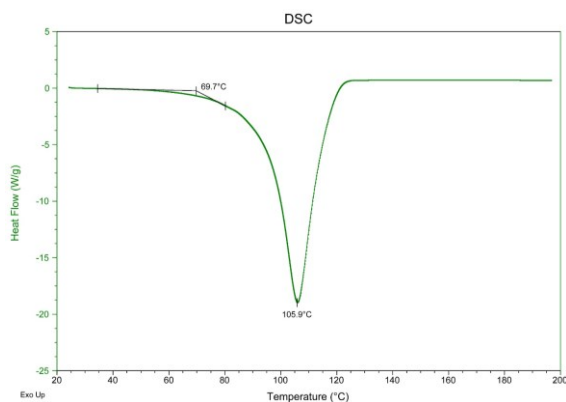


Fig. S2. Typical DSC scan profile for the tripeptide hydrogel.

4. Molecular dynamics simulations.

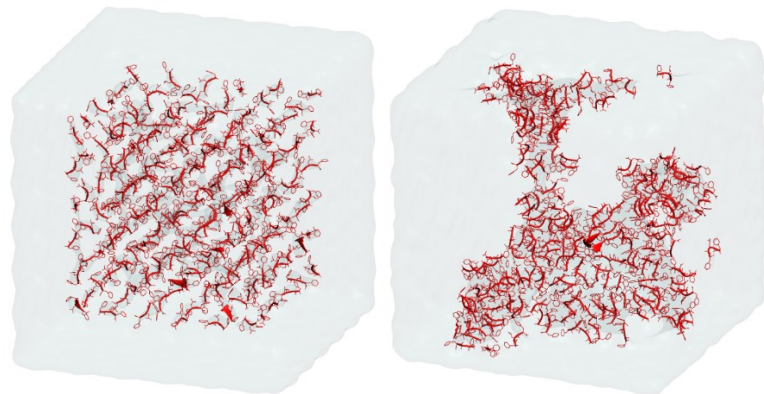


Fig. S3. Molecular dynamics simulations of hundreds of tripeptides (*left*) in explicit water (grey box) reveal propensity for the peptide (backbone in red cartoon superposed to non-hydrogenous atoms as sticks) to form aggregates (*right*).

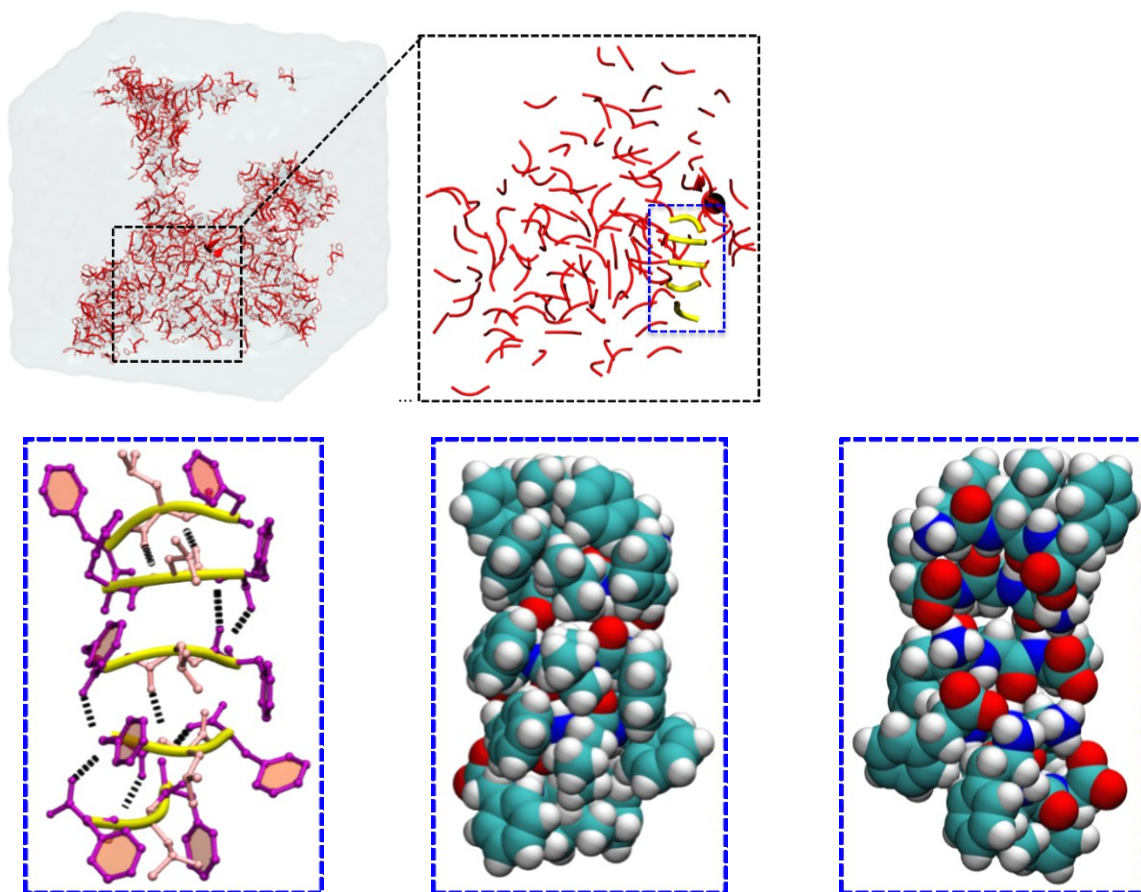


Fig. S4. Enlargement of tripeptide aggregates (black box, top) highlights preservation of tripeptide antiparallel beta-sheet conformation (as an example, see peptide backbone as yellow sticks in blue box, top right). Zooming (bottom) reveals a similar network of H-bonds as the one found by modeling a single sheet in implicit solvent. Also in this case the amphiphilic nature of the sheet is preserved (hydrophobic surface is shown at bottom, centre, and after rotation of 180° the hydrophilic surface shown at bottom, right).

5. FT-IR and CD data for Phe-^DLeu-Phe hydrogel

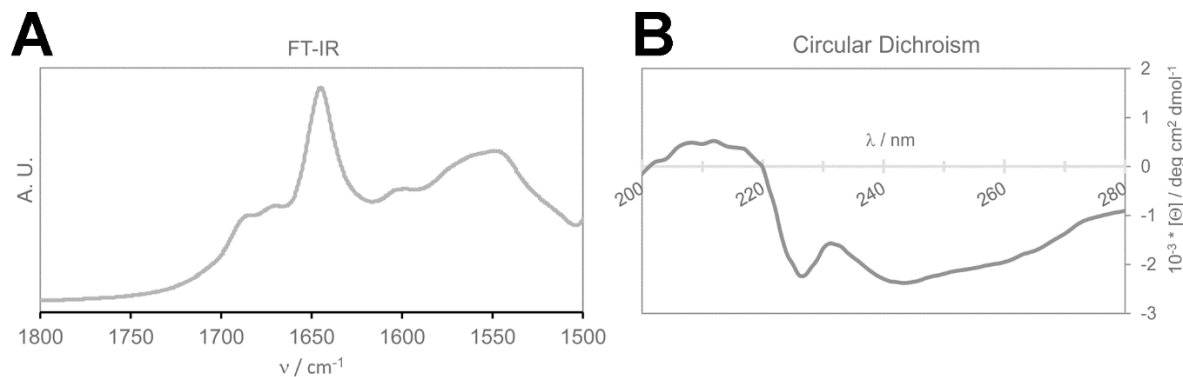


Fig. S5. A) FT-IR and (B) CD spectra of the tripeptide hydrogel.

6. Cytotoxicity assay for the gelling tripeptide in solution

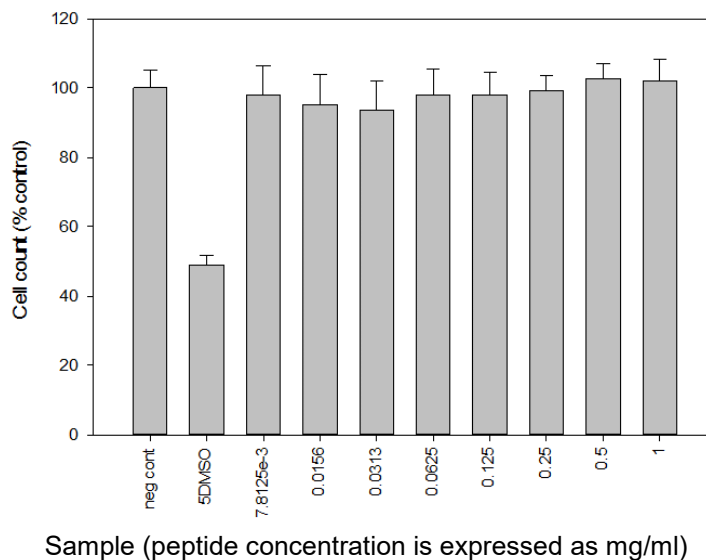


Fig. S6. Cytotoxicity assay according to ISO 10993 shows that the gelling tripeptide in solution is non-toxic to fibroblast cells. Average values \pm SD are shown. Neg cont = negative control. 5DMSO = 5% DMSO (positive control).

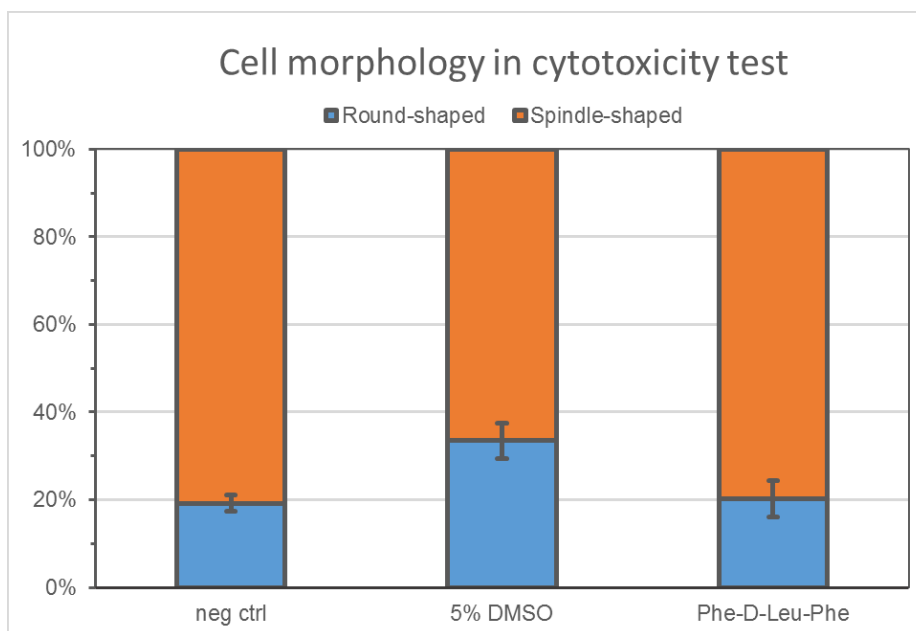


Fig. S7. Quantification of round-shaped versus spindle-shaped cells as visualized by BF microscopy in the cytotoxicity test. Measurements were done on microscopy images ($n = 6$) showing an average of >500 cells each. Average values \pm SD are shown. No significant difference was seen between the negative control and samples treated with the peptide at the concentration of 1mg/ml (peptide solubility limit). Samples with 5% DMSO were the only ones with significantly lower numbers of cells per surface area.

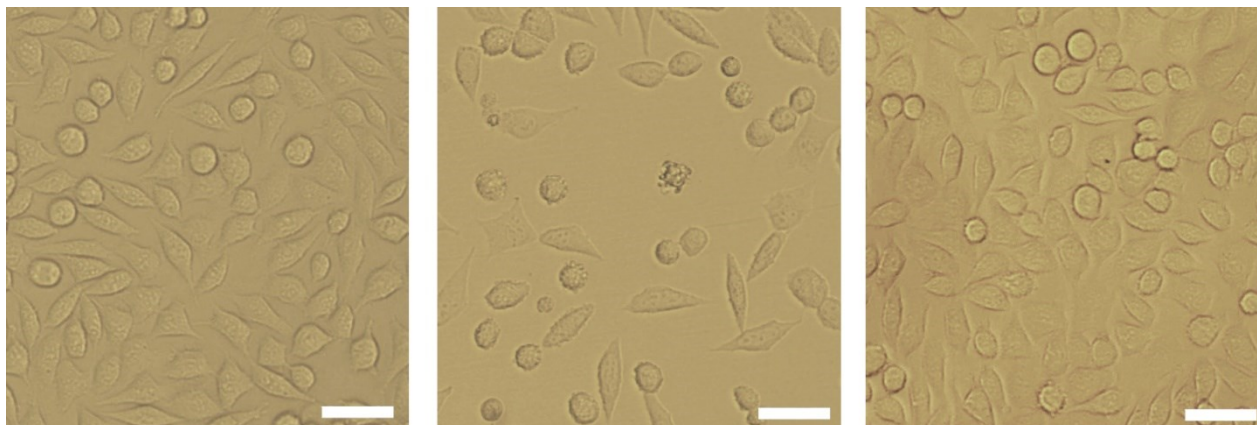


Fig. S8. Representative BF images of the cytotoxicity assay quantified above. From left to right: negative control, 5% DMSO, and peptide sample at the concentration of 1 mg/ml. Scale bar = 50 μm .

7. Cryo-TEM imaging of Phe-^DLeu-Phe hydrogel

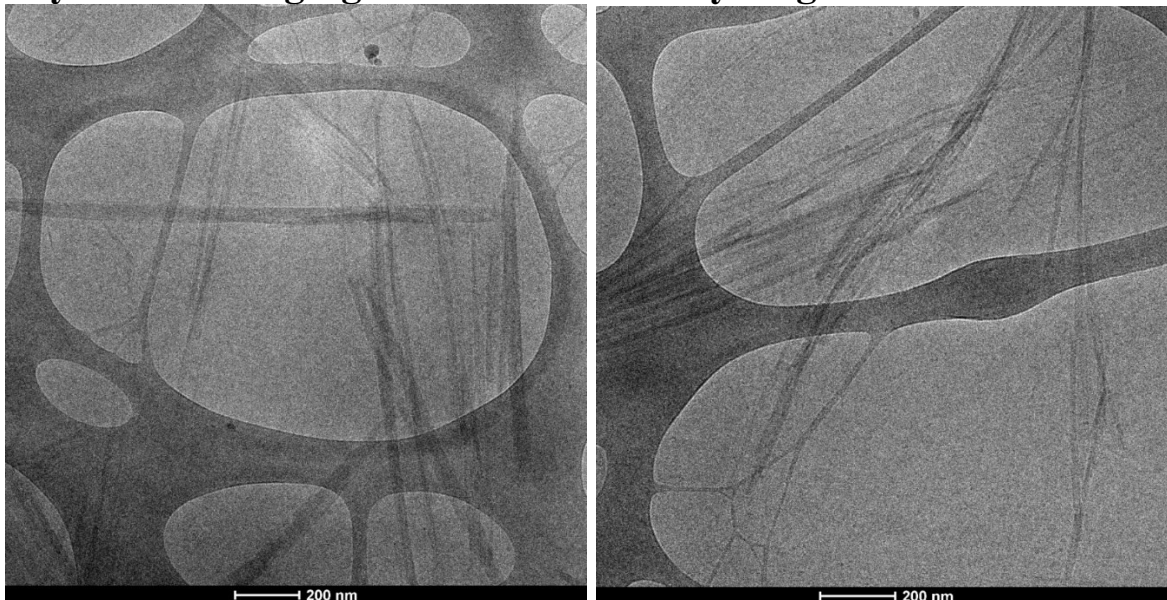
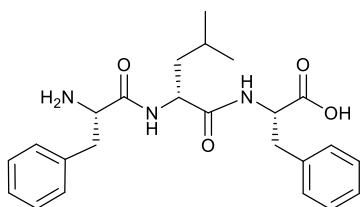


Fig. S9. Cryo-TEM images of the tripeptide hydrogel.

8. Spectroscopic data for Phe-^DLeu-Phe



¹H-NMR (400 MHz, DMSO, TMS): δ 8.45 (d, J = 8 Hz, 1H, NH), 8.43 (d, J = 8 Hz, 1H, NH), 7.27-7.11 (m, 10H, Ar), 4.40 (m, 1H, α CH), 4.27 (m, 1H, α CH), 4.05 (m, 1H, α CH), 3.06 (dd, J = 4 and 16 Hz, 1H, β CH), 2.96 (m, 2H, 2 x β CH), 2.76 (dd, J = 12 Hz, 1H, β CH), 0.89 (m, 3H, 2 x β CH, γ CH₃), 0.60 (d, 3H, δ CH₃), 0.60 (d, 3H, δ CH₃). ¹³C-NMR (100MHz, DMSO, TMS): δ (ppm) 173.5, 171.4, 168.1 (3 x CO); 138.2, 135.4, 129.9, 129.7, 128.8, 128.5, 127.4, 126.7 (Ar); 53.9, 53.7, 51.2, (3 x α C); 42.0, 37.8, 37.5 (3 x β C); 24.0, 23.4, 22.0 (γ C, 2 x δ C). MS (ESI): m/z 426.1 (M+H)⁺, C₂₄H₃₁N₃O₄ requires 425.2.

Fig. S10. ¹H-NMR (400 MHz, DMSO, TMS)

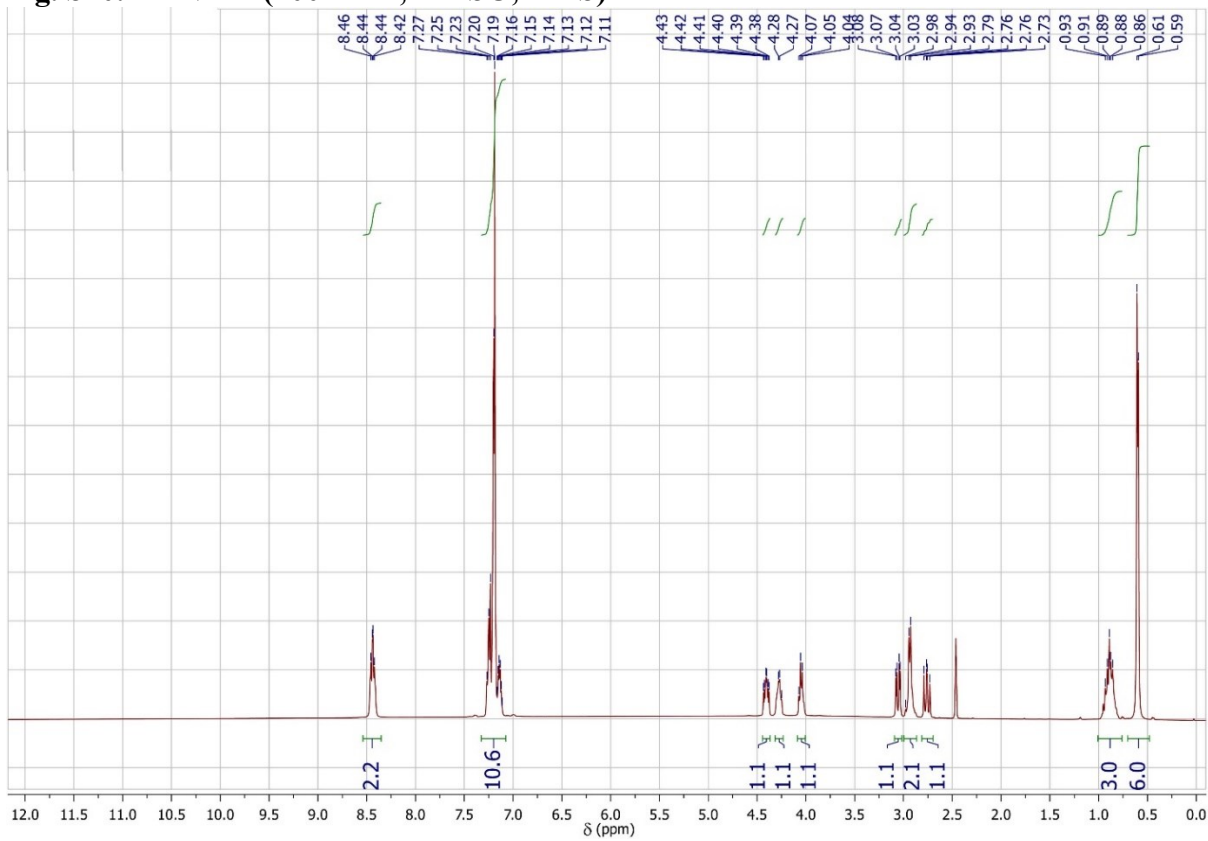
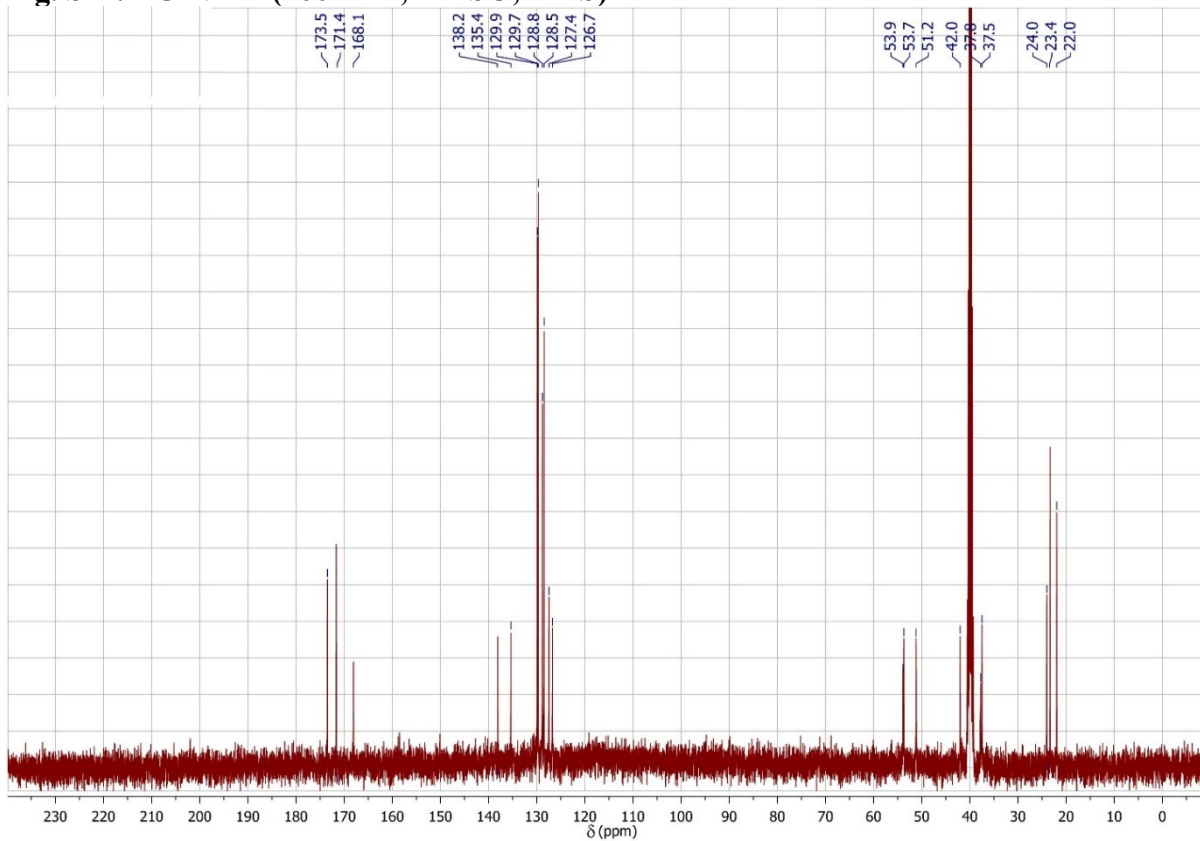


Fig. S11. ¹³C-NMR (100MHz, DMSO, TMS)



9. XRD spectra of Phe-^DLeu-Phe hydrogel

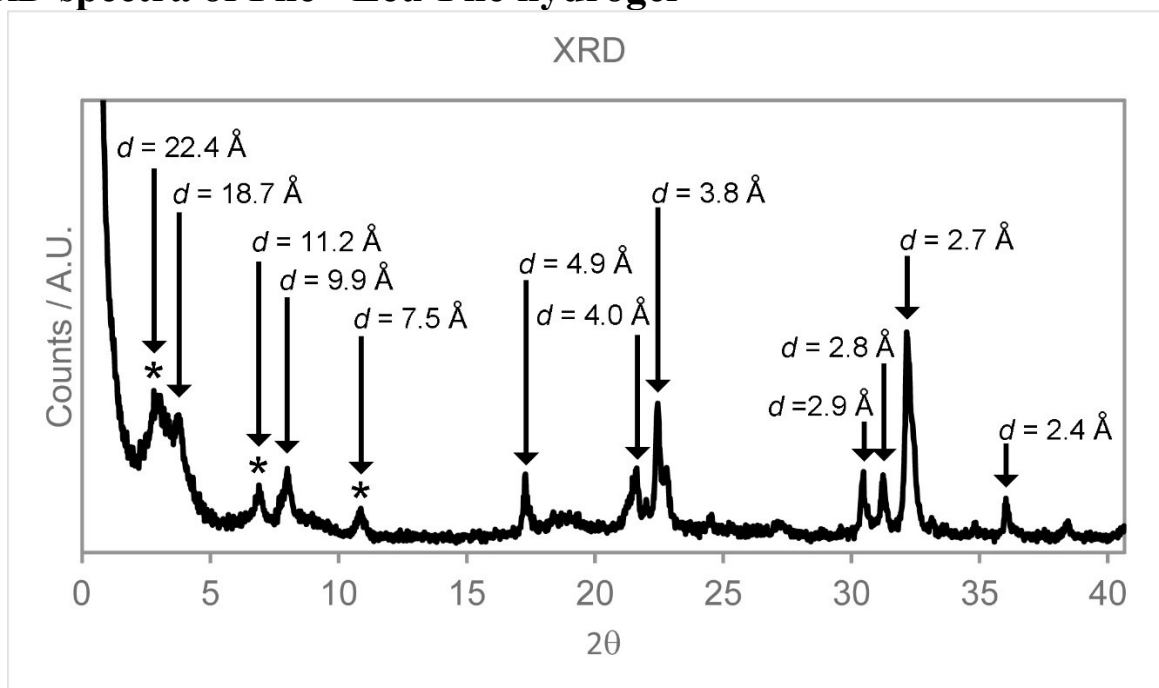


Fig. S12. XRD data. Typical distances observed for amyloids: 9.9 Å for every other strand distance (for anti-parallel beta-sheets), 4.9 Å inter-strand distance. Stars denote higher order reflections: 22.4 Å (n=1), 11.2 Å (n=2), 7.5 Å (n=3).