Supporting Information

Bioadhesive supramolecular hydrogel from unprotected, short D,L-peptides with Phe-Phe and Leu-Asp-Val motifs

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1. Materials and Methods

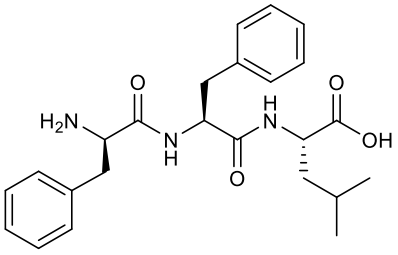
2-chlorotrytil resin, *O*-Benzotriazole-*N,N,N,N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU), and Fmoc protected amino acids were purchased from GL Biochem (Shanghai) Ltd. All solvents were purchased of analytical grade from Merck. Piperidine, trifluoroacetic acid (TFA), *N*,*N*-diisopropyl ethyl 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. ^{1}H-NMR spectra were recorded at 400 MHz and ^{13}C-NMR spectra were recorded at 100 MHz on a Varian Innova Instrument with chemical shift reported as ppm (in DMSO or MeCN with tetramethylsilane as internal standard). ESI-MS spectra were recorded on an Agilent 6120 single quadrupole LC-MS system. Oscillatory rheometry was performed on a Malvern Kinexus Ultra Plus Rheometer (Alfatest, Milan, Italy) equipped with a 20-mm stainless steel parallel plate geometry at the temperature of 25 °C in a Peltier system (Alfatest, Milan, Italy). Hydrogels were assembled *in situ* and measurements were taken immediately with a gap set to 1.00 mm. Kinetics were studied over 60 minutes at a set frequency of 1 Hz and a set stress of 5 Pa. Stress sweeps were measured with a controlled frequency of 1 Hz. CryoTEM and TEM analysis was performed as previously described (S. Marchesan, *et al. J. Mater. Chem. B* **2015**, *3*, 8123). AFM analysis was performed as previously described. (A. M. Garcia, *et al., Chem* **2018**, *4*, 1862). FT-IR analysis (150 scans, 2 cm^{-1} resolution) was performed on a System 2000 (Perkin-Elmer) using a KBr pellet with the hydrogel sample previously dried overnight.

2. Peptide synthesis

The peptide was synthesised using standard Fmoc solid phase peptide synthesis with HBTU activation. Briefly, Fmoc-amino acid deprotection was performed in a sintered funnel, with continuous stirring, in 20% piperidine in *N,N*-dimethylformamide (DMF) for

20 minutes (2 x 10 minutes) until both bromophenol blue and acetaldehyde/chloranil tests were positive. HBTU activation was performed with 2.5 equiv. of Fmoc-amino acid, 2.0 equiv. of HBTU and 2.0 equiv. of HOAt in DMF (4 mL for every equiv. of resin), with DIPEA (2 mL of a 1 M solution in DMF for every equiv. of resin). Coupling was performed at room temperature for 1.5 h in a sintered funnel with continuous stirring, and completeness was monitored by both bromophenol blue and acetaldehyde/chloranil tests after thorough washes with DMF and DCM. Final cleavage was obtained using a mixture of TFA/DCM/TIPS/water (47.5:47.5:2.5:2.5). The crude peptide was too hydrophobic to be precipitated in cold ether, thus the majority of TFA was evaporated under argon flow, and the remaining oil was dissolved in a mixture of acetonitrile/water and then purified by reverse-phase HPLC (Agilent Technologies). The HPLC Agilent 1260 Infinity system was equipped with a preparative gradient pump (1311B), semipreparative C-18 column (Kinetex, 5 microns, 100 Å, 250 x 10 mm, Phenomenex), autosampler (G1329B), Photodiode Array detector (G1315C). The gradient used consisted of acetonitrile (MeCN) / water with 0.1% TFA with the following program: t = 0-2 min. 25% MeCN; t = 16 min. 95% MeCN; t = 20 min. 95% MeCN. The compound was then freeze-dried to yield the corresponding peptide as a white fluffy powder. Peptide identity was verified by ESI-MS, ^{1}H-NMR and ^{13}C-NMR.

3. ^{D}Phe-Phe-Leu spectroscopic data



^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.85 (d, J = 7.2 Hz, 1H, NH), 8.52 (d, J = 6.0 Hz, 1H, NH), 7.92 (s, 3H, NH_3^+), 7.31 – 6.89 (m, 10H, Ar), 4.71 (m, 1H, αCH), 4.23 (m, 1H, αCH), 3.98 (m, 1H, αCH), 3.08 (dd, J = 11.2 Hz and 3.2 Hz, 1H, βCH_2), 2.77 (dd, J = 11.2 Hz and 3.2 Hz, 1H, βCH_2), 2.70 (dd, J = 11.2 Hz and 8.6 Hz, 1H, βCH_2), 2.48 (dd,

J = 11.2 Hz and 8.6 Hz, 1H, βCH$_2$), 1.63 (m, 1H, γCH) 1.55 (m, 2H, βCH$_2$), 0.91 (d, *J* = 6.5 Hz, 3H, CH$_3$) 0.85 (d, *J* = 6.5 Hz, 3H, CH$_3$). **^{13}C NMR** (100 MHz, DMSO-*d*$_6$) δ (ppm): 174.3, 171.4, 168.0 (3 x CO); 137.9, 135.0, 129.9, 129.9, 128.2, 128.5, 127.5, 127.0 (Ar); 54.2, 53.6, 50.8 (3 x αC); 39.5, 38.7, 37.3 (3 x βC), 24.8, 23.3, 21.8 (γC, 2 x δC).
MS (ESI): m/z 426.2 $(M+H)^+$ $C_{24}H_{32}N_5O_4$ requires 426.2

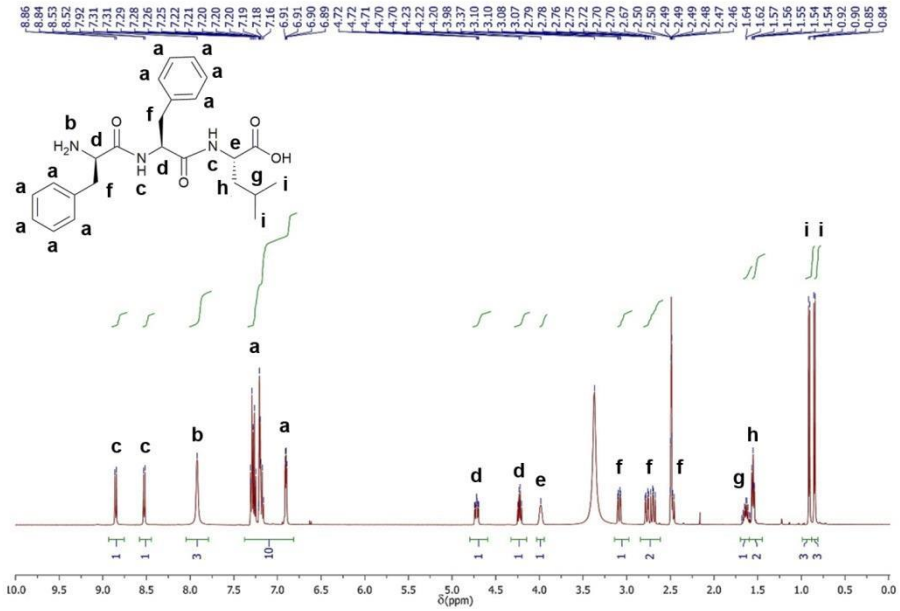


Fig. S1. ^{1}H-NMR spectrum of ^{D}Phe-Phe-Leu.

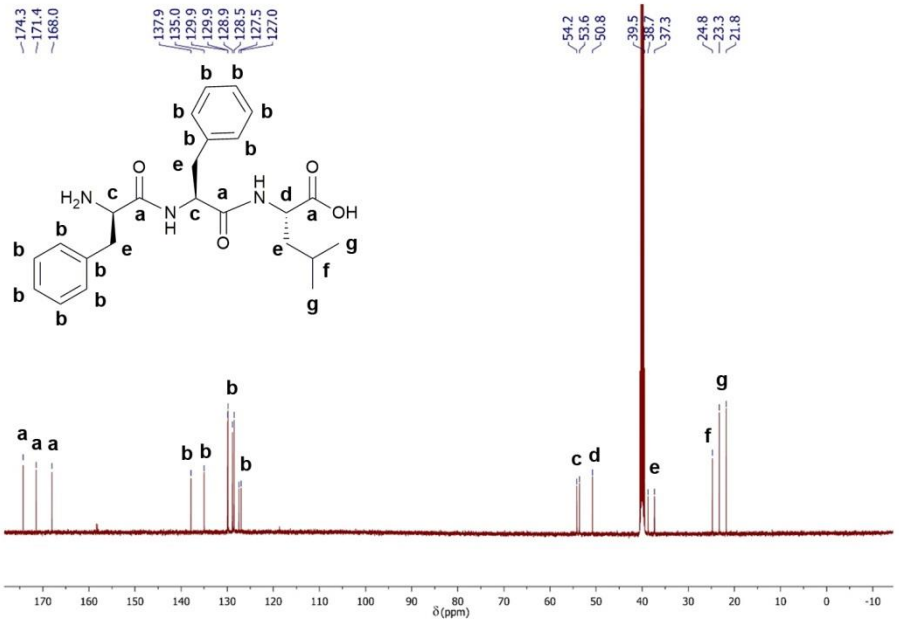


Fig. S2. ^{13}C-NMR spectrum of ^{D}Phe-Phe-Leu.

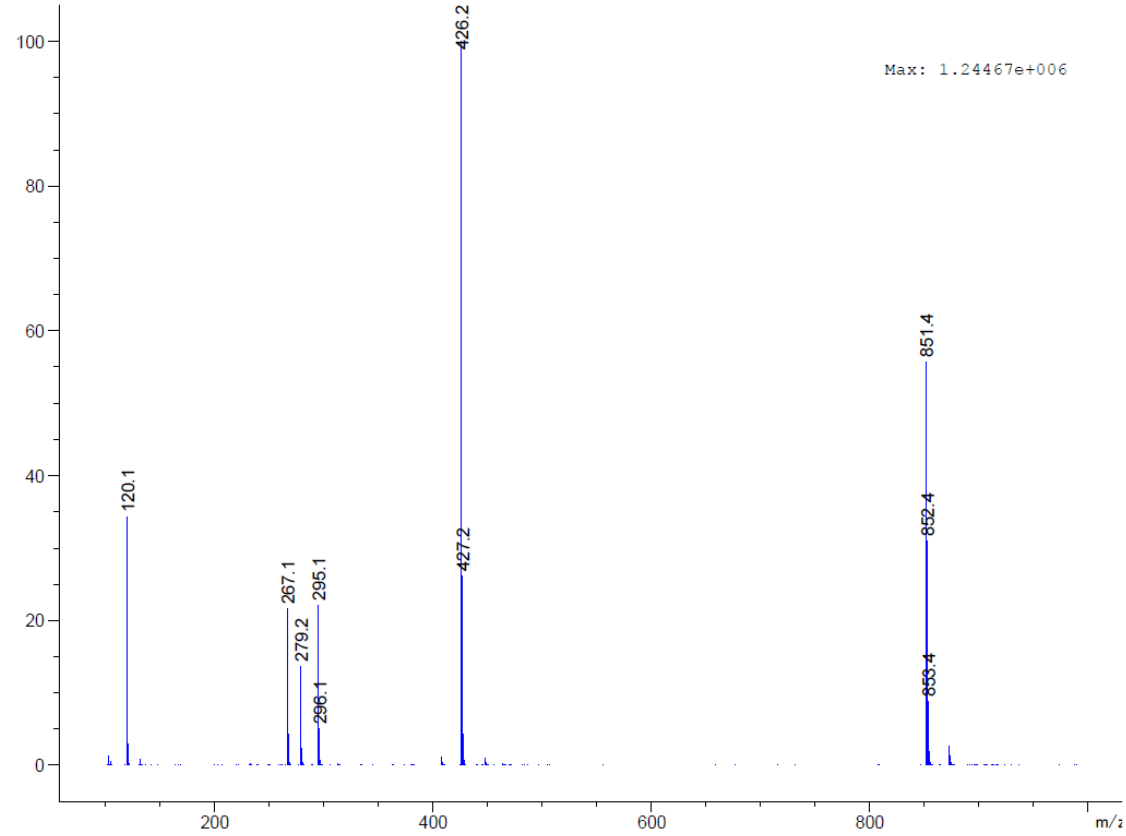
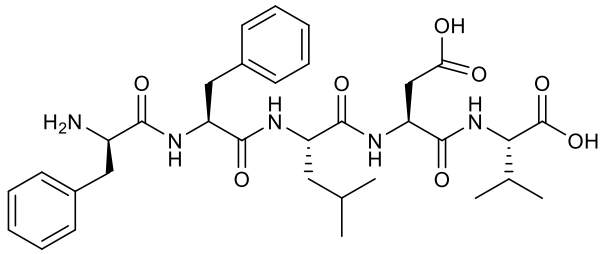


Fig. S3. ESI-MS spectrum of ^{D}Phe-Phe-Leu (positive ion mode).

4. ^{D}Phe-Phe-Leu-Asp-Val spectroscopic data



^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.78 (d, J = 8.4 Hz, 1H, NH), 8.41 (d, J = 8.4 Hz, 1H, NH), 8.30 (d, J = 7.6 Hz, 1H, NH), 7.93 (s, 3H, NH_3^+), 7.65 (d, J = 8.8 Hz, 1H, NH), 7.25 – 6.90 (m, 10H, Ar), 4.72 (m, 1H, αCH), 4.62 (m, 1H, αCH), 4.35 (m, 1H, αCH), 4.12 (dd, J = 8.4 Hz and 5.2 Hz, 1H, αCH), 4.00 (m, 1H, αCH), 3.06 (dd, J = 13.6 Hz and 3.6 Hz, 1H, βCH), 2.76 (dd, J = 13.6 Hz and 4.2 Hz, 1H, βCH), 2.72 – 2.66 (m, 2H, βCH), 2.53 – 2.45 (m, 2H, βCH), 2.03 (m, 1H, βCH) 1.58 (m, 1H, γCH), 1.46 (m, 2H, βCH), 0.88 – 0.83 (m, 12H, γCH and δCH). **^{13}C NMR** (100 MHz, DMSO-d_6) δ (ppm): 173.1, 172.4, 172.1, 171.0, 168.0 (5 x CO); 137.9, 135.0, 129.9, 129.9, 128.9, 128.5, 127.5, 126.9 (Ar); 57.5, 54.2, 53.6, 51.3, 49.8 (5 x αC); 41.7, 38.8, 37.3, 36.2, 30.5 (5 x βC), 24.6, 23.5, 22.1 (3 x γC), 19.4, 18.1 (2 x δC).
MS (ESI): m/z 640.3 $(M+H)^+$ $C_{33}H_{46}N_5O_8$ requires 640.3

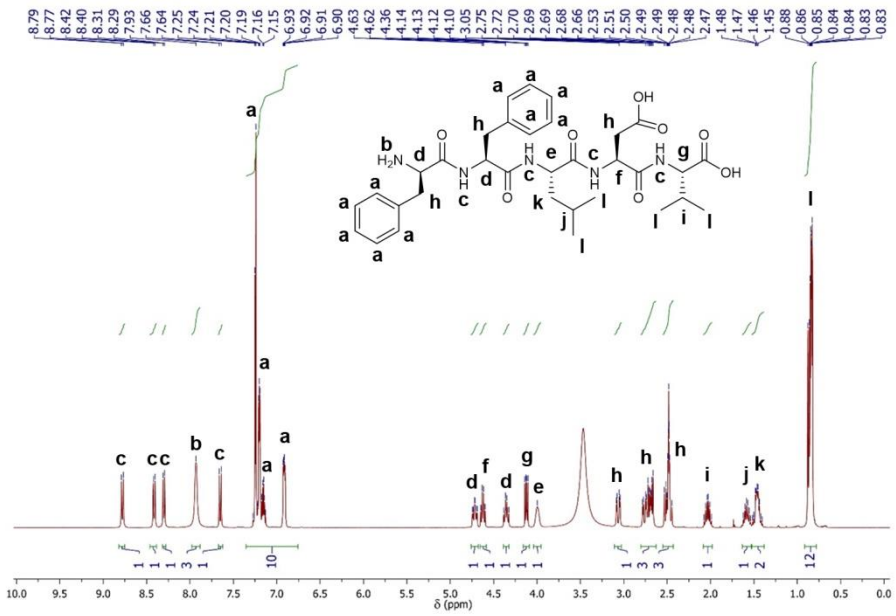


Fig. S4. ^{1}H-NMR spectrum of ^{D}Phe-Phe-Leu-Asp-Val.

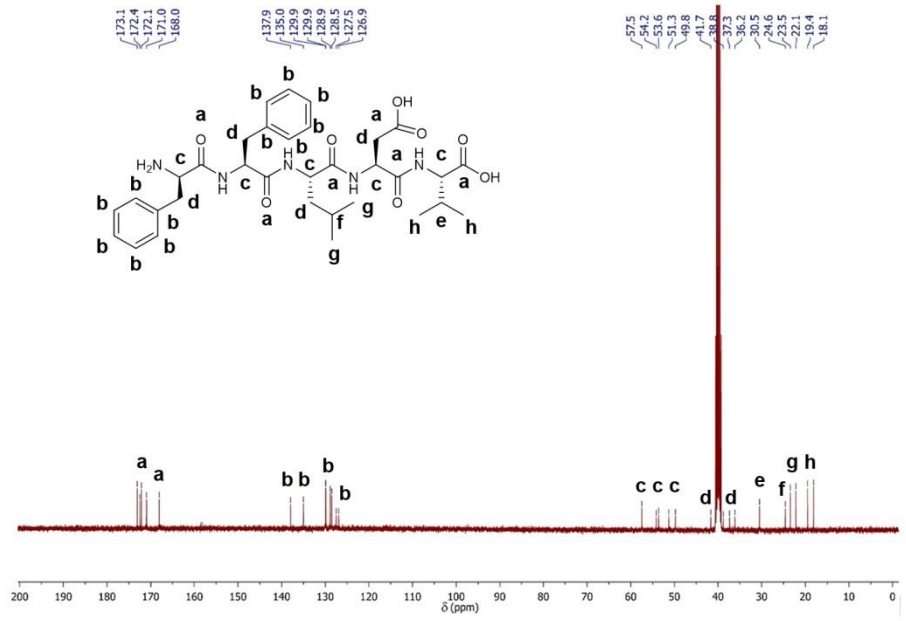


Fig. S5. ^{13}C-NMR spectrum of ^{D}Phe-Phe-Leu-Asp-Val.

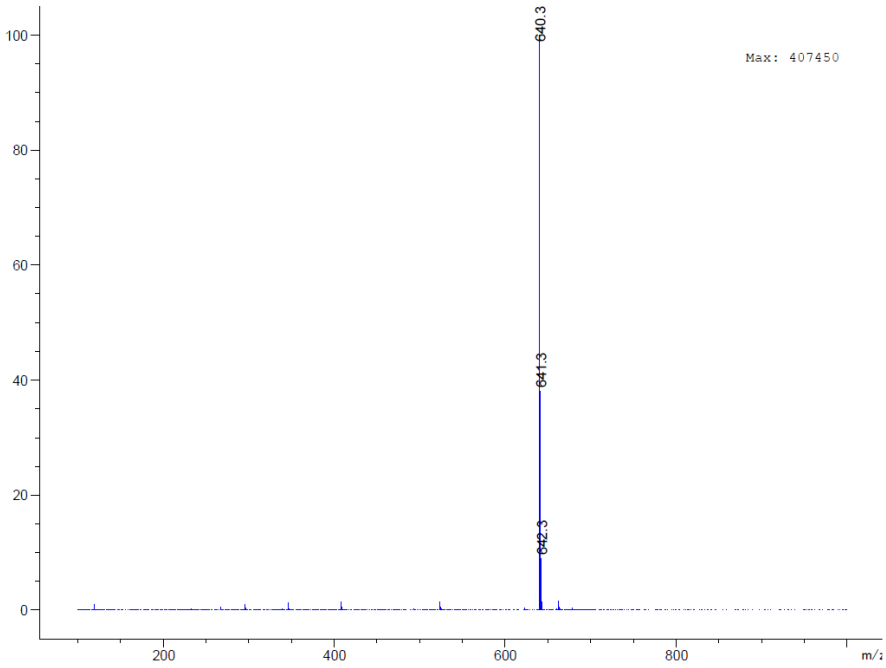


Fig. S6. ESI-MS spectrum of ^{D}Phe-Phe-Leu-Asp-Val (positive ion mode).

5. Peptide gelation protocol

The tripeptide was dissolved at 0.5 wt. % in sodium phosphate buffer (0.1 M pH 11.8), then diluted with an equal volume of sodium phosphate buffer (0.1 M pH 5.8) to reach a final concentration of 0.25 wt. % (5.88 mM), getting a final pH of 7.3 ± 0.1. All buffer solutions were filtered (0.2 µm) prior to use.

Peptide gels containing both the tripeptide and the pentapeptide were formed following the same protocol, but varying concentrations; gels could be obtained with a ratio tripeptide: pentapeptide = 4:1 or higher, *e.g.*, 10 mM : 1 mM (6:1), 15 mM : 3 mM (5:1), 12 mM : 3 mM (4:1). The pentapeptide gels on its own with MGC 50 mM.

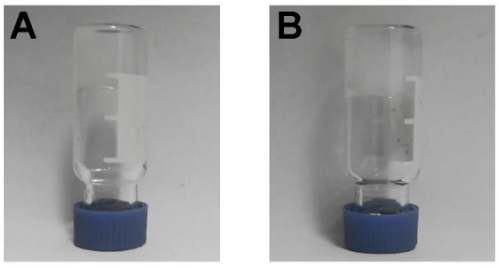


Fig. S7. Photographs of (A) 15 mM tripeptide gel and (B) two-component hydrogel (15 mM of tripeptide and 1.5 mM of pentapeptide).

6. FT-IR spectra

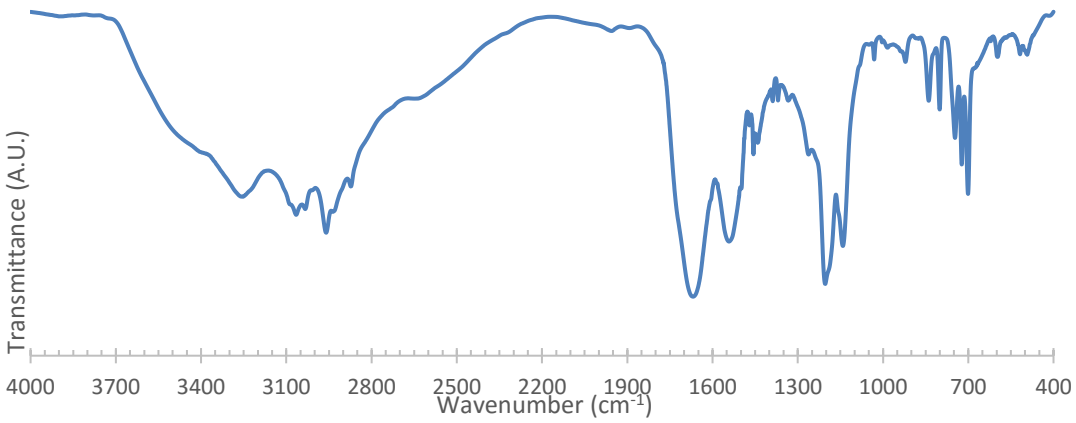


Fig. S8. FT-IR spectrum of ^{D}Phe-Phe-Leu powder.

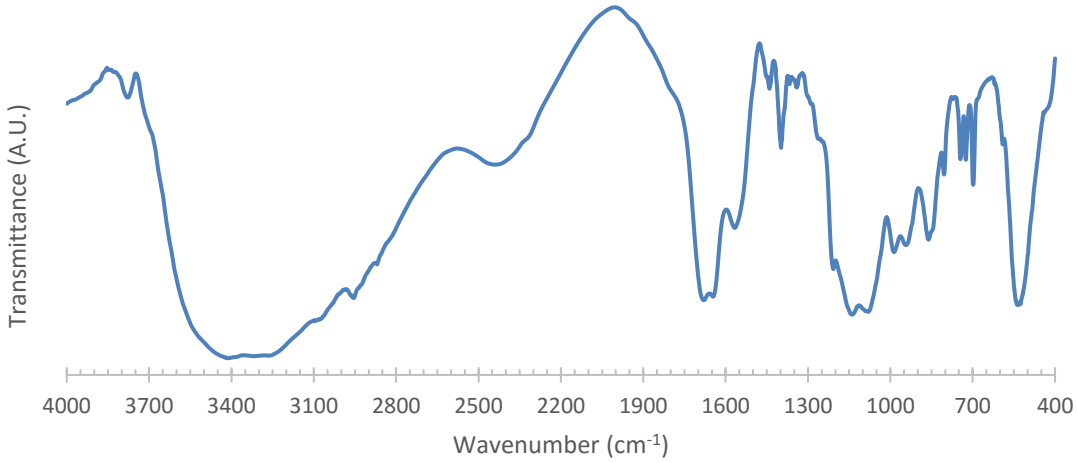


Fig. S9. FT-IR spectrum of ^{D}Phe-Phe-Leu xerogel.

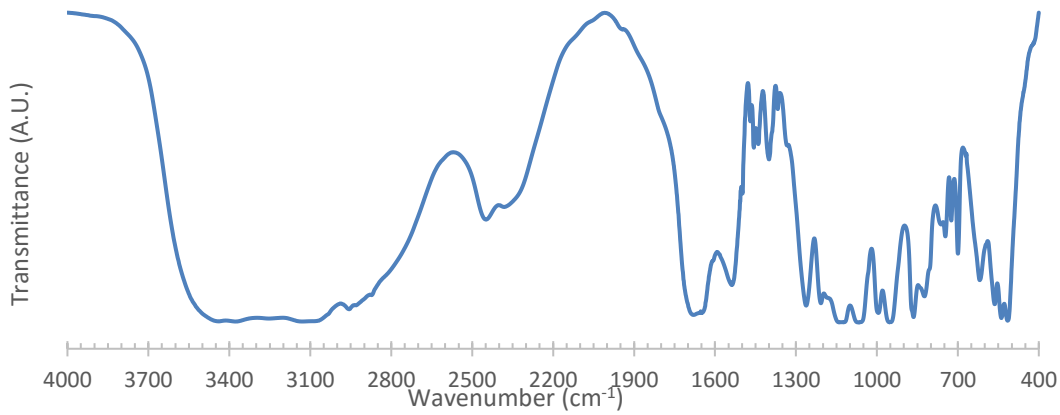


Fig. S10. FT-IR spectrum of the two-component hydrogel (15 mM ^{D}Phe-Phe-Leu and 1.5 mM ^{D}Phe-Phe-Leu-Asp-Val) after 1 hour of self-assembly.

7. Oscillatory rheology

Oscillatory rheometry was performed on a Malvern Kinexus Ultra Plus Rheometer (Alfatest, Milan, Italy) equipped with a 20-mm stainless steel parallel plate geometry at the temperature of 25 °C in a Peltier system (Alfatest, Milan, Italy). Hydrogels were assembled *in situ* and measurements were taken immediately with a gap set to 1.00 mm. Kinetics were studied over 60 minutes at a set frequency of 1 Hz and a set stress of 5 Pa. Stress sweeps were measured with a controlled frequency of 1 Hz.

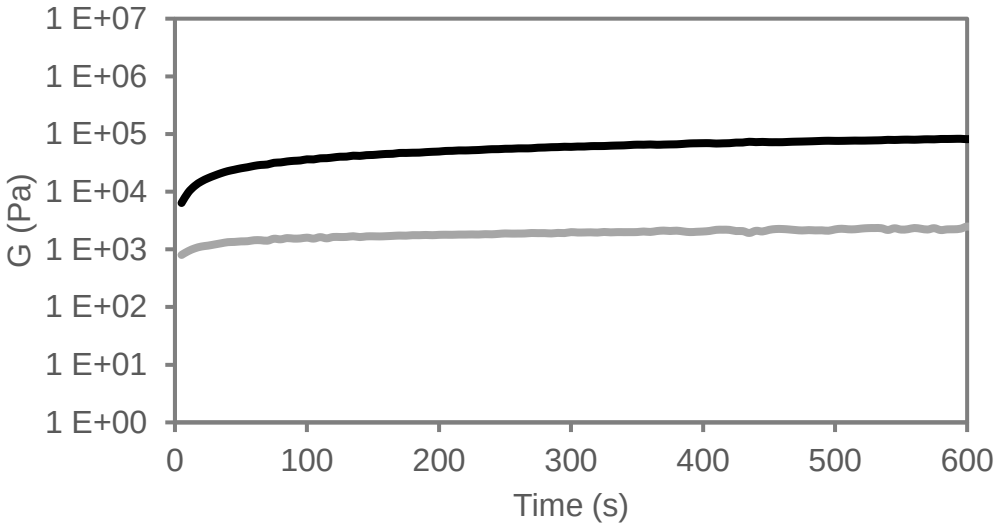


Fig. S11. Gelation kinetics reached a plateau within 10 minutes for ^{D}Phe-Phe-Leu at 15 mM. G' = black line, G" = grey line.

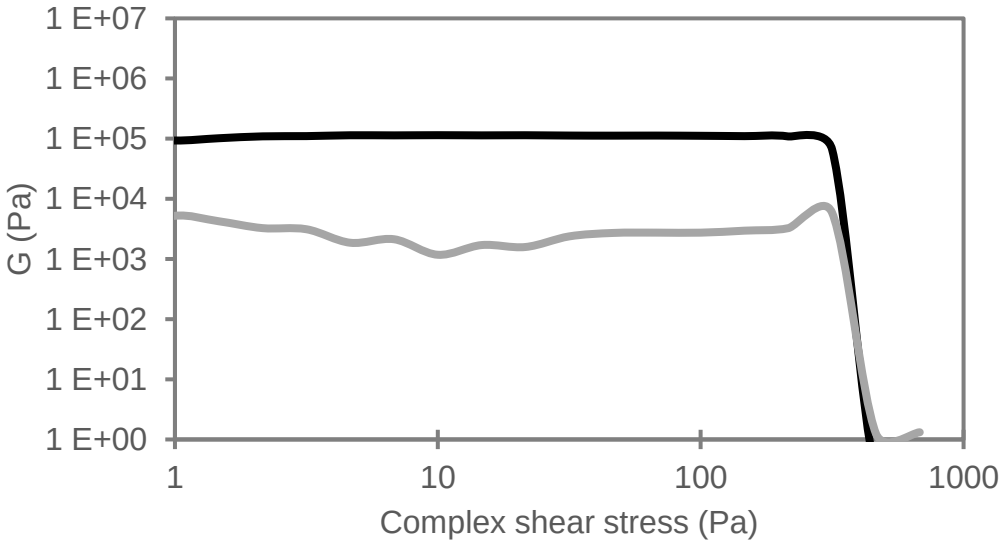


Fig. S12. Stress sweep for 15 mM ^{D}Phe-Phe-Leu hydrogel. G' = black line, G" = grey line.

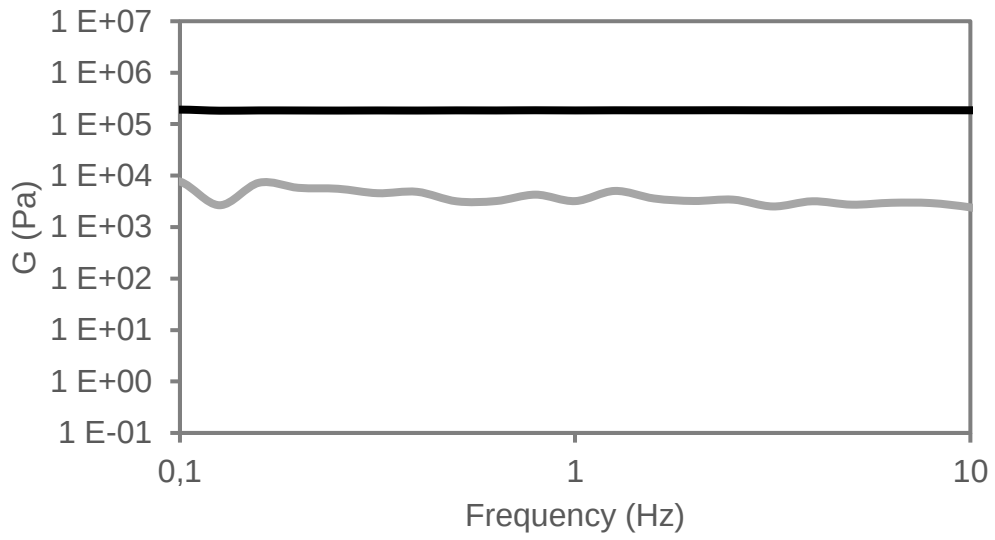


Fig. S13. Frequency sweep for 15 mM ^{D}Phe-Phe-Leu hydrogel. G' = black line, G" = grey line.

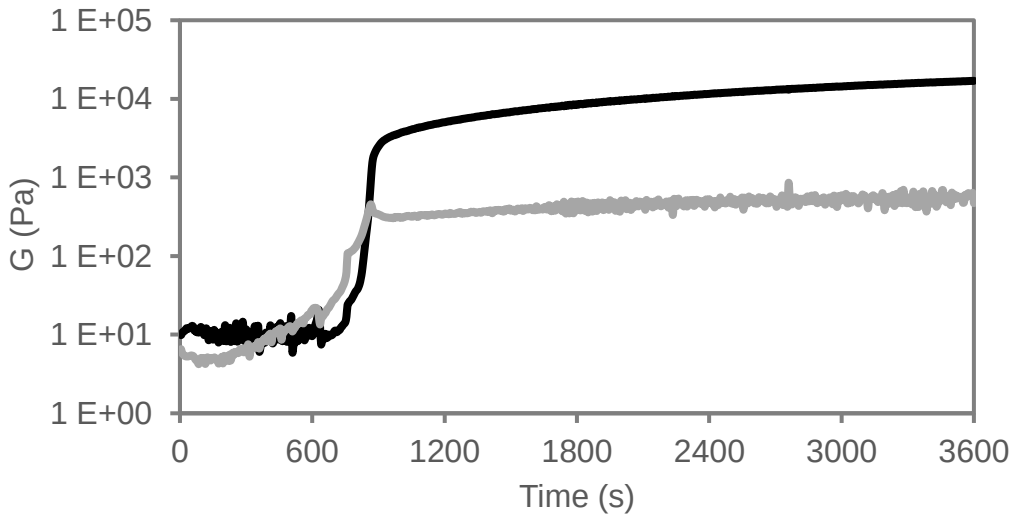


Fig. S14. Time sweep for the two-component hydrogel (15 mM ^{D}Phe-Phe-Leu and 1.5 mM ^{D}Phe-Phe-Leu-Asp-Val) over 1 hour of assembly. G' = black line, G" = grey line.

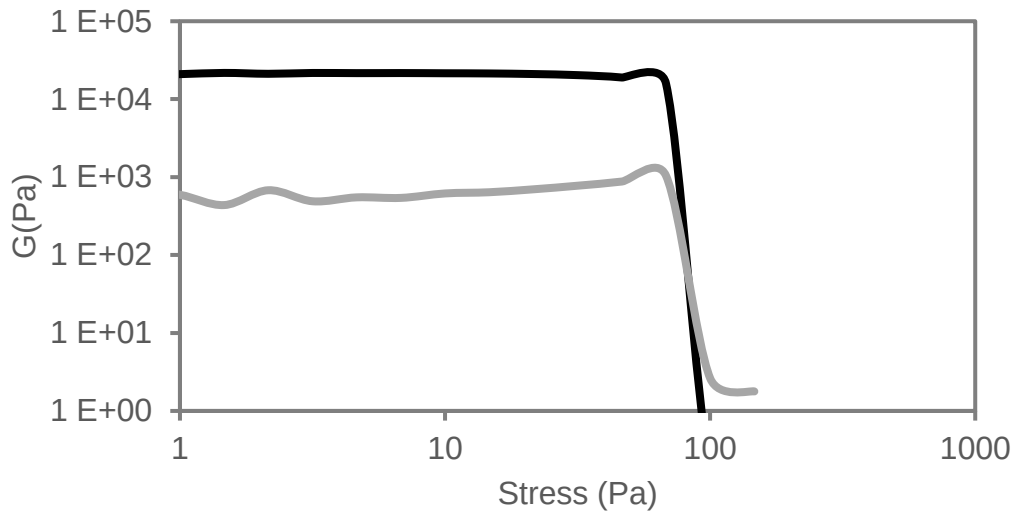


Fig. S15. Stress sweep for the two-component hydrogel (15 mM ^{D}Phe-Phe-Leu and 1.5 mM ^{D}Phe-Phe-Leu-Asp-Val). G' = black line, G" = grey line.

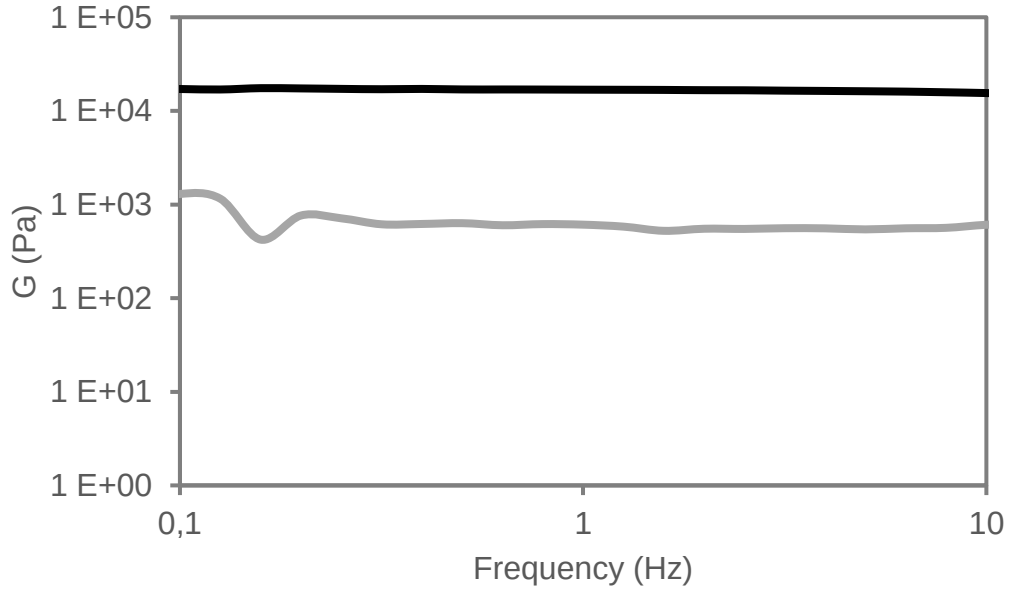


Fig. S16. Frequency sweep for the two-component hydrogel (15 mM ^{D}Phe-Phe-Leu and 1.5 mM ^{D}Phe-Phe-Leu-Asp-Val). G' = black, G" = grey.

8. Thioflavin T (ThT) fluorescence assay

Gel precursor solution (0.15 ml) was prepared as described above and immediately put on wells of Greiner 96 U Bottom Transparent Polystyrene. After 1 hours, 30 μl of a solution of Thioflavin T (24 μM in 20 mM glycine-NaOH pH 7.5, filtered with a 0.2 μm

filter) were added in the wells. After 15 minutes, the fluorescence emission was analysed using a Tecan Infinite M1000 pro, selecting an excitation wavelength of 446 nm and an emission wavelength of 490 nm, with a bandwidth of 20 nm. Each condition was repeated twice in triplicate. Average and standard deviations were calculated and plotted with Excel. Results are shown in Figure S17.

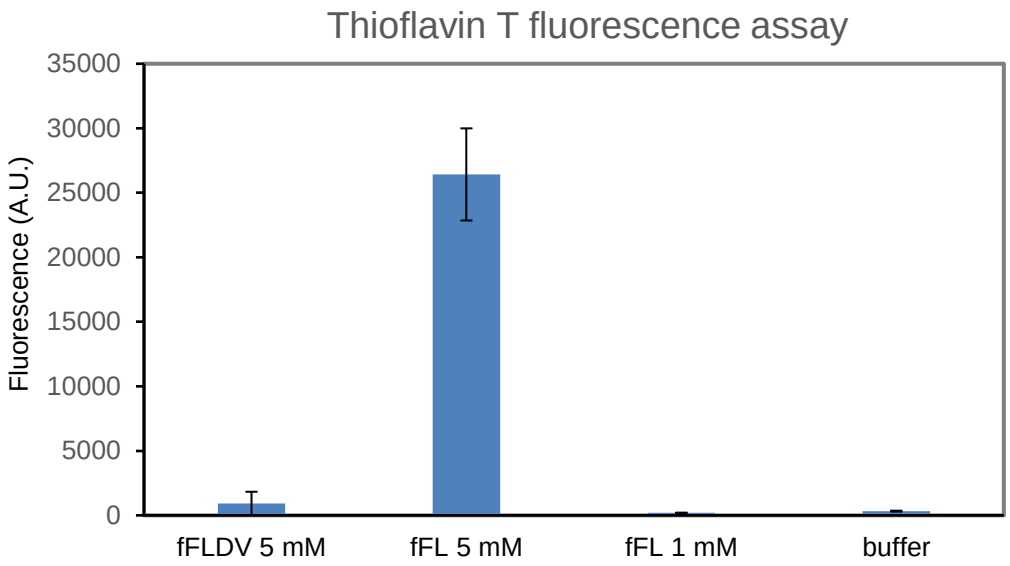


Fig. S17. ThT assay for ^{D}Phe-Phe-Leu (fFL) and ^{D}Phe-Phe-Leu-Asp-Val (fFLDV).

9. Circular Dichroism (CD)

A 0.1 mm quartz cell was used on a Jasco J-815 Spectropolarimeter, with 1 s integrations, 1 accumulation and a step size of 1 nm with a bandwidth of 1 nm over a range of wavelengths from 190 to 280 nm at 25 °C (Peltier). Samples were freshly prepared directly in the CD cell and the spectra immediately recorded.

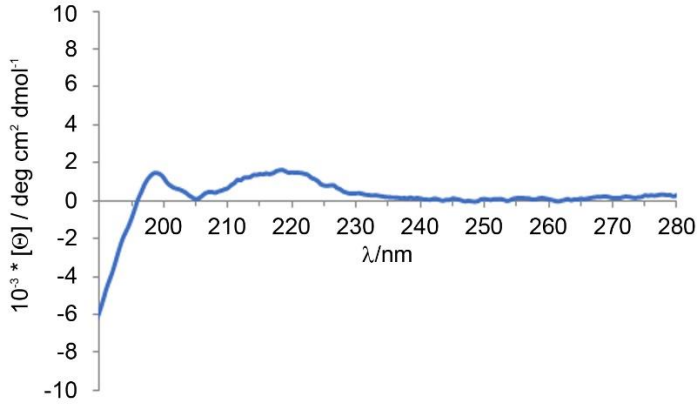


Fig. S18. CD spectrum of ^{D}Phe-Phe-Leu at 0.5 wt. % (11.8 mM) in the alkaline buffer.

10. TEM and AFM images

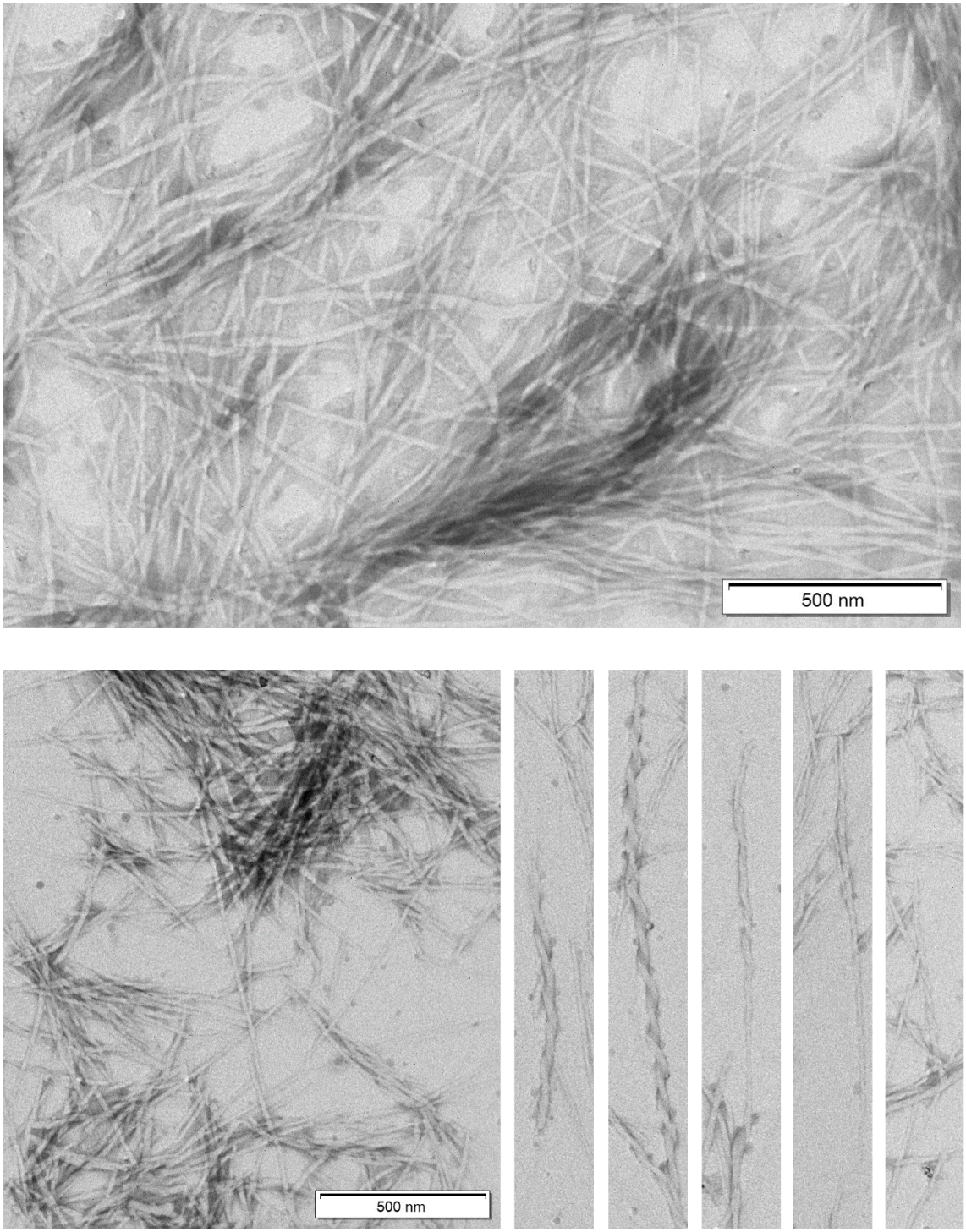


Fig. S19. TEM images of the two-component hydrogel (15 mM ^{D}Phe-Phe-Leu and 1.5 mM ^{D}Phe-Phe-Leu-Asp-Val) after 1 hour of self-assembly, with details showing twisted fibers.

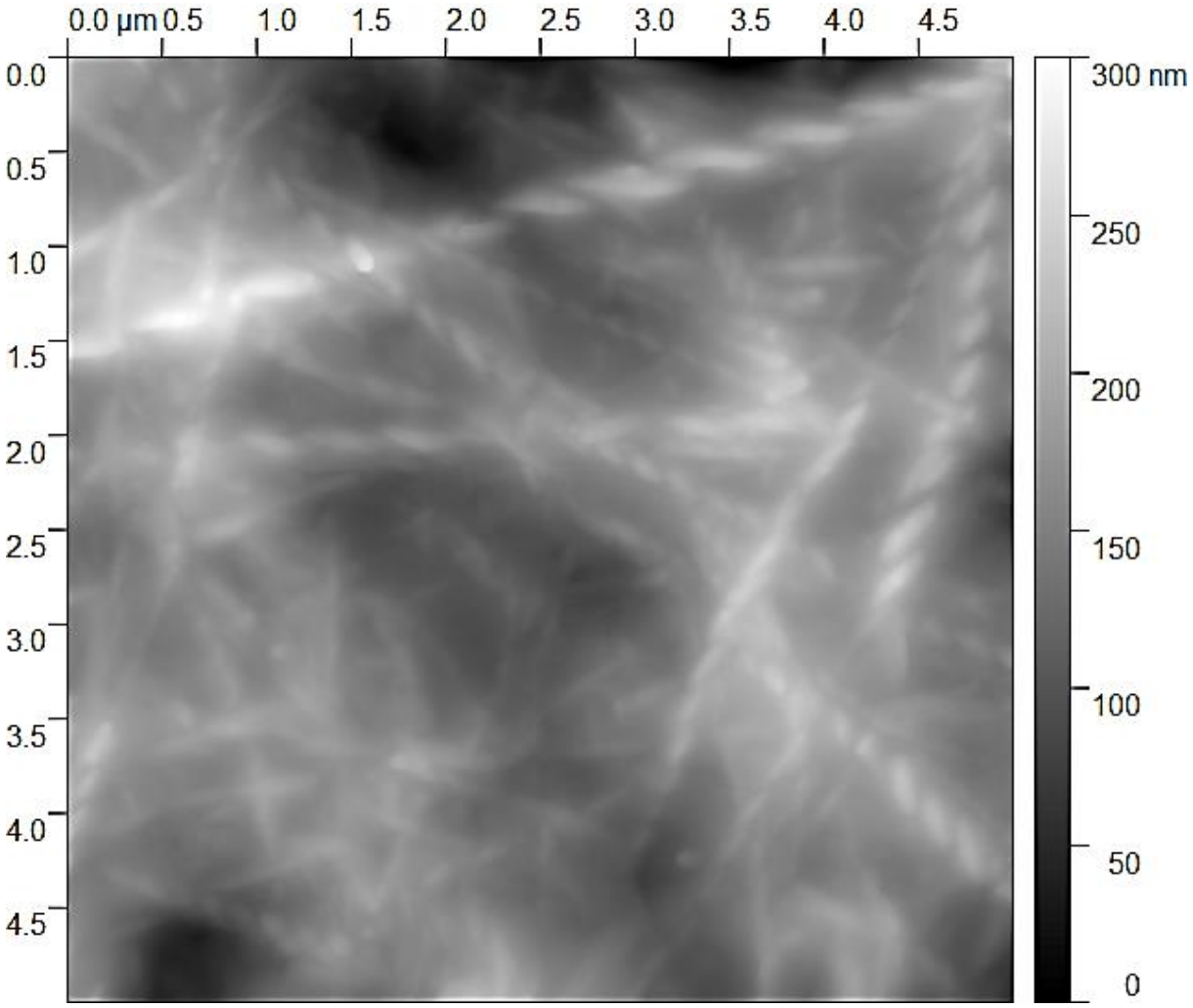


Fig. S20. AFM image of ^{D}Phe-Phe-Leu gel shows right-handed twisted fibers.

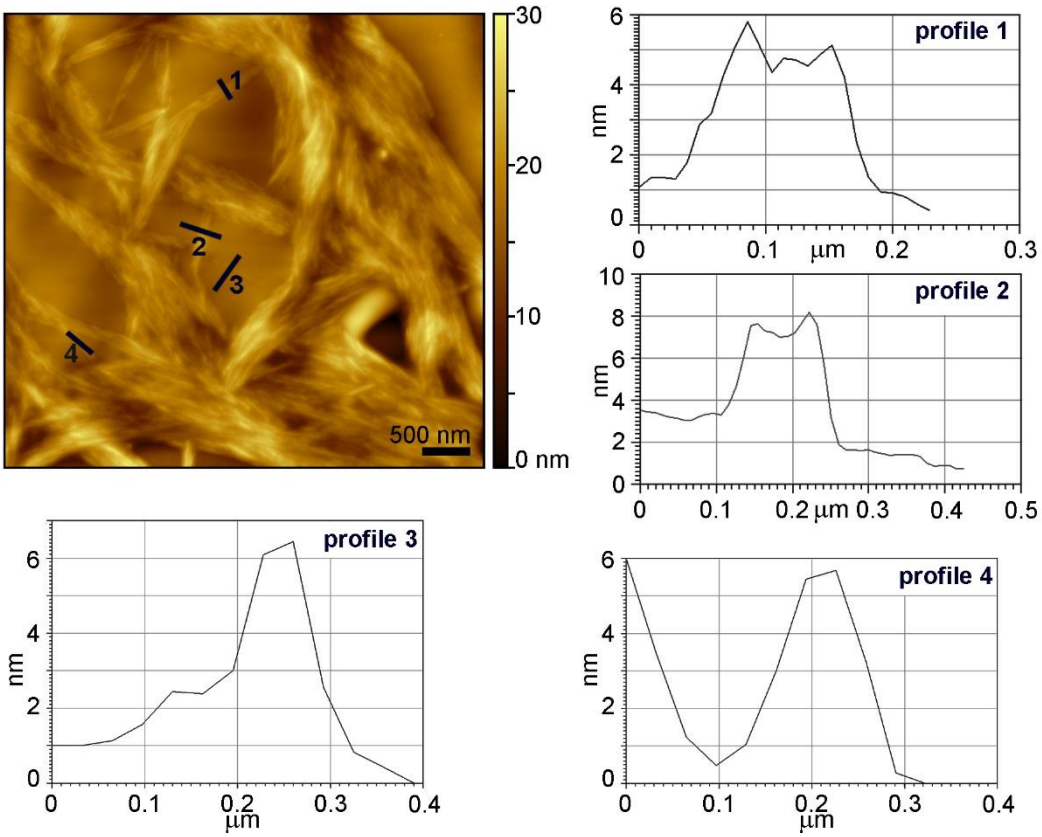


Fig. S21. AFM image of the two-component hydrogel (15 mM ^{D}Phe-Phe-Leu and 1.5 mM ^{D}Phe-Phe-Leu-Asp-Val) with height profiles showing fibril diameter of 5.1± 0.9 nm.

11. Cell assays

Hydrogels were prepared at a final concentration of 15 mM fFL and 1.5 mM fFLDV (other concentrations tested, i.e., 15:3, 12:3 and 20:3 mM led to similar results) following the procedure indicated above (page 2 ESI). 20 µl of each hydrogel with or without the LDV motif were carefully formed in the microwells of a µ-slide Angiogenesis uncoated slide (Ibidi, Madison, WI) and were left to settle at room temperature for 24 h. Solutions were filtered 0.2 µm prior to use. L929 mouse fibroblasts were cultured in DMEM medium containing 10% foetal bovine serum, glutamax (Invitrogen) and penicillin/streptomycin (Lonza). Culture media was added on top of the gels and plates incubated for 30 min at +37°C. The solution was then replaced with 15,000 L929 cells per well in culture media. As controls, cells were grown on chambers coated with bovine serum albumin (BSA). Cells were cultured at +37°C in a humidified incubator for up to 48 h, replacing 15 µl of media every 24 h. To avoid evaporation, drops of PBS was added between the

microwells. Cell viability was analysed by the LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen, Thermo Fisher Scientific) after 24 h or 48 h of culture. 15 µl of the media was replaced, wells carefully washed with PBS and 5µl live/dead solution prepared according to the manufacturers' instructions using 2 µM of calcein AM and 4 µM of Ethidium homodimer. 1. Following 30 min incubation in +37°C, cells were imaged on the EVOS fluorescence microscope (EVOS FL Microscope, Thermo Fischer Scientific). Each condition was repeated four times as triplicates. Fluorescence brightfield images were taken after 48 h. Representative images of cells after the live/dead assay at 48 h are shown and the amount of live and dead cells quantified from 100 cells in triplicate (Fig. S22). Cell spreading surface area was quantified using ImageJ. Binding of L929 cells to the peptides were analysed also by coating wells with 500 µM of peptide for 1h at RT and unbound peptide washed off. Cells were added to the wells and brightfield images of adhering cells taken after 2h (Fig. S23). Cells were washed with PBS and the live/cell assay performed as above (Fig. S24). Cell adhesion and spreading on the peptides were also assessed by iCELLigence (ACEA Biosciences), which measures changes in impedance. 0.05 mM, 0.5 mM or 5mM of fFLDV or fFL peptide were used to coat the iCELLigence wells for 1h at RT and unbound peptide washed off. As control wells were coated with BSA or left uncoated. 50.000 cells were added to iCELLigence wells in DMEM medium containing 10% foetal bovine serum, glutamax and penicillin/streptomycin. ICELLigence measurements were taken every 5 mins for 150 mins. In another experiment, wells were coated with 0.5 mM fFLDV and L929 cells pre-treated with 10 µg/ml anti-β_1 antibody B0550531 or 1mM Mn^{2+} for integrin activation for 20 min or left untreated. ICELLigence assay was performed as above.

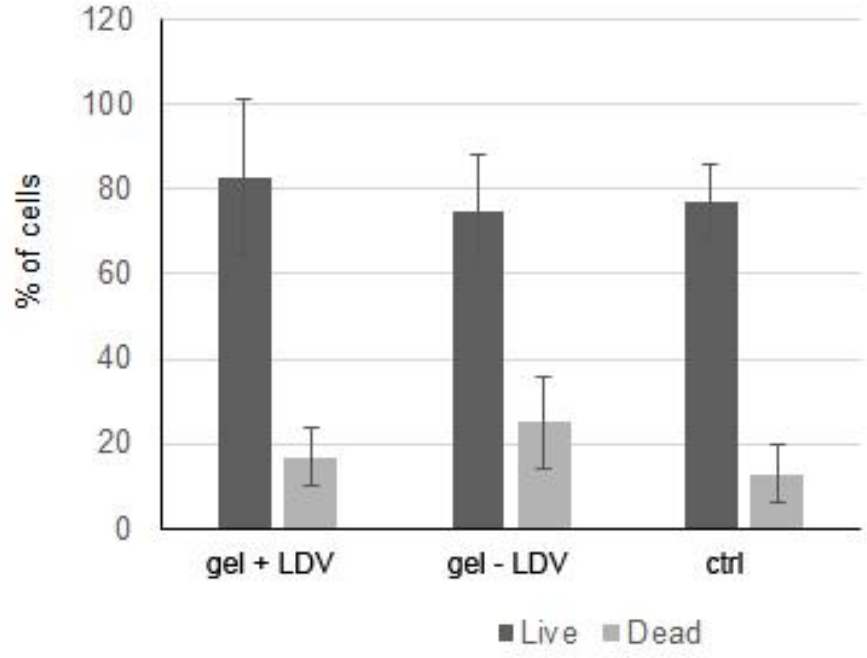


Fig. S22. The amount of live and dead cells was quantified from 100 cells in triplicate, after cell culture for 48h on the hydrogels or control.

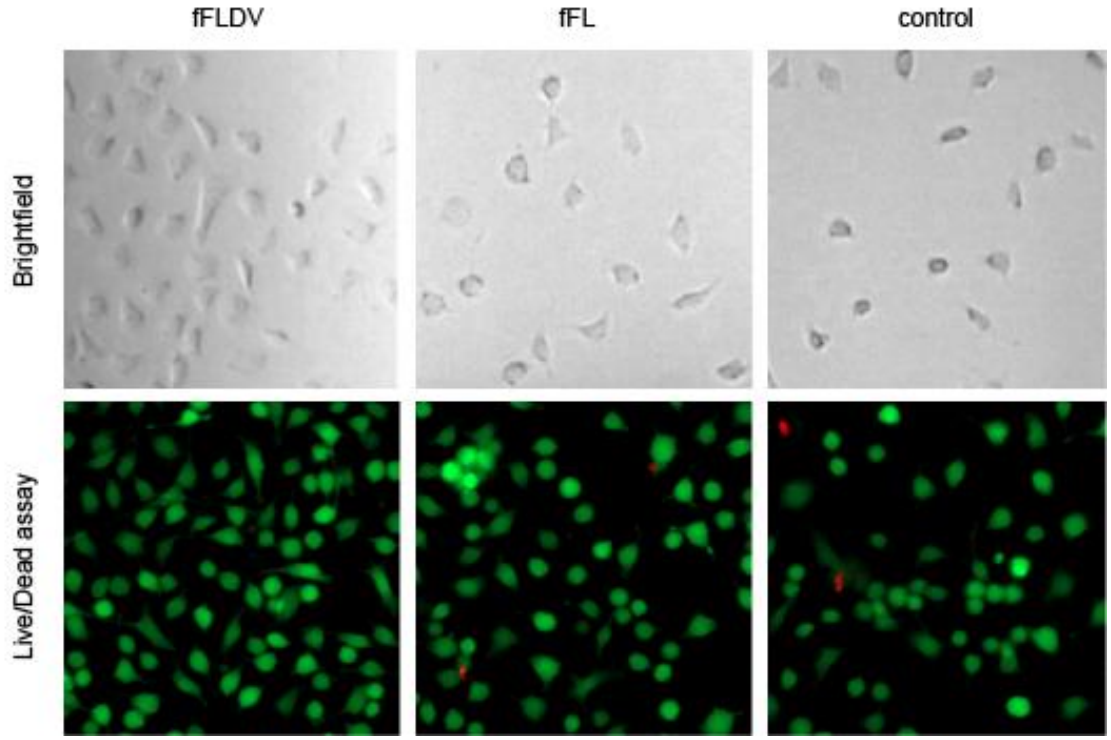


Fig. S23. Brightfield images of L929 cells allowed to adhere on fFLDV, fFL or BSA-coated wells for 48 h. Live/dead assay of L929 cells show living cells are green and dead cells as red.

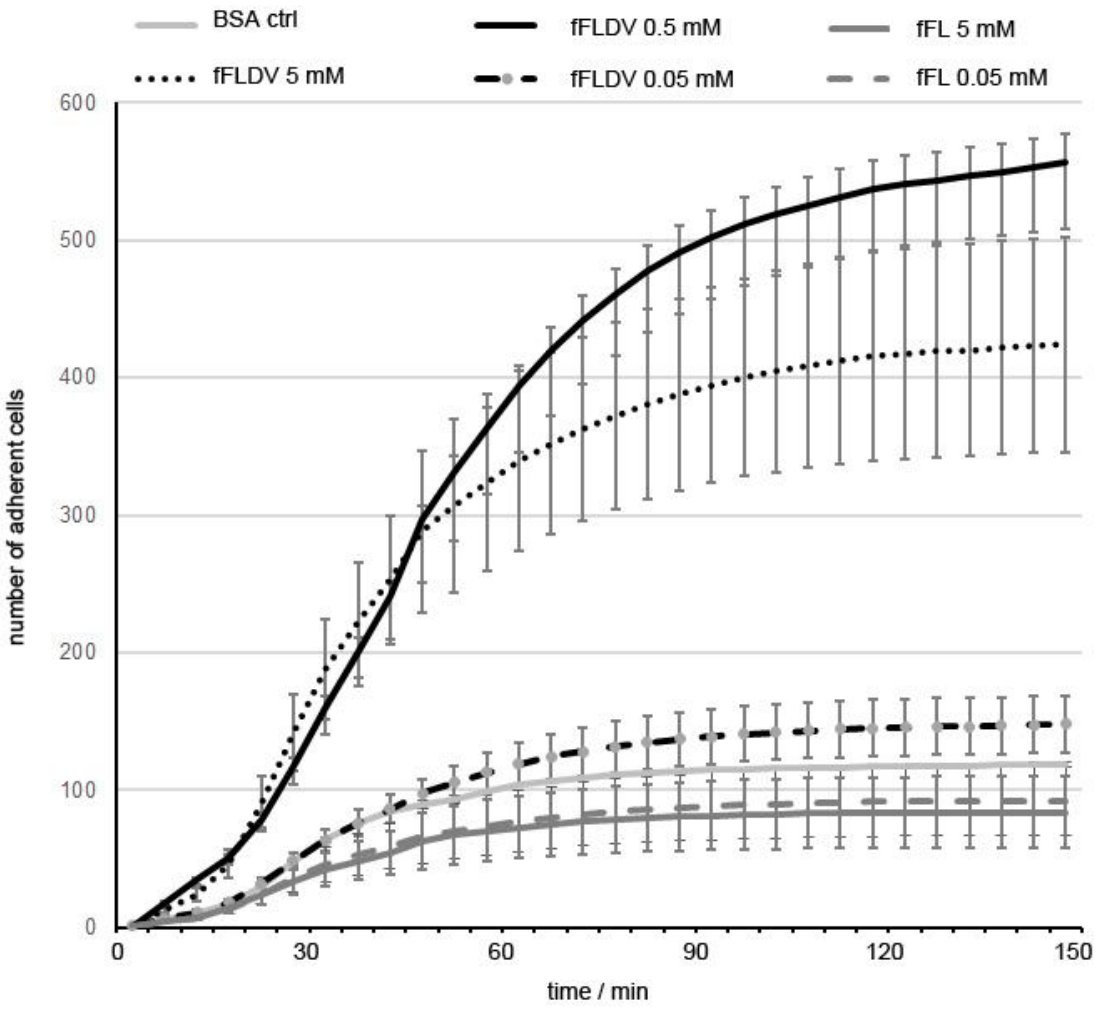


Fig. S24. iCELLigence assay of L929 cells spreading on coated surfaces.

12. Single crystal XRD data

Crystals of the peptide ^{D}Phe-Phe-Leu-Asp-Val were grown using the sitting-drop vapour diffusion method. 1.5 mL of pentapeptide solution in MeOH (< 1 mg/mL) was placed in a small vial and then introduced into a bigger vial used as reservoir containing 3.0 mL of MilliQ water and MeOH (1:1) solution. Single crystals were grown upon a one week - ten days period. A needle-shaped single crystal of the ^{D}Phe-Phe-Leu-Asp-Val peptide was collected with a loop, cryoprotected by dipping it in glycerol and stored frozen in liquid nitrogen. The crystal was mounted on the diffractometer at the synchrotron Elettra, Trieste (Italy), beamline XRD1, using the robot present at the facility. Temperature was

kept at 100 K by a stream of nitrogen on the crystal. Diffraction data were collected by the rotating crystal method using synchrotron radiation, wavelength 0.70 Å, rotation interval 1°/image, crystal-to-detector distance of 85 mm. A total of 360 images were collected. Reflections were indexed and integrated using the XDS package [1], space group $P2_1$ was determined using POINTLESS [2] and the resulting data set was scaled using SCALA [3] of the CCP4 package [4]. Phase information were obtained by direct methods using the software SHELXT [5]. Refinements cycles were conducted with SHELXL-14 [5], operating through the WinGX GUI [6], by full-matrix least-squares methods on F^2. Unit cell parameters and scaling statistics are reported in Table S1.

The asymmetric unit of crystals of ^{D}Phe-Phe-Leu-Asp-Val contains two molecules of the peptide. In addition, electron density attributed to a 2,2,2-trifluoroacetic acid, 4 MeOH molecules, and 2 water molecules has been identified in the asymmetric unit. Trifluoroacetic acid and 3 methanol molecules are statistically present in 70%, 70%, 60% and 60% of the unit cells, respectively. Considering the presence of trifluoroacetic acid, the N-terminal moieties of the peptide are probably protonated, as are the carboxylic groups of the aspartate residues, while only one of the C-terminal carboxylic moieties is protonated. Therefore, one of the crystallographically independent peptides is in its zwitterionic form, with a neutral net charge. The other crystallographically independent peptide has a net positive charge of +1. The trifluoroacetic acid is deprotonated, with a net negative charge of -1. A strong hydrogen bond, with a short distance of 2.45 Å, is present between the C-terminal carboxylic moieties of crystallographically independent peptides, confirming the conclusions regarding their protonation state. It was not possible to locate the hydrogen atom present on the C-terminal groups. A total of 4 peptide molecules, related by symmetry operators of the $P2_1$ space group, are present in the unit cell.

All atoms, except the hydrogen atoms, within the asymmetric unit have been refined with anisotropic thermal parameters. Hydrogen atoms of the peptide and of the methyl groups of the alcohol molecules were added at geometrically calculated positions and refined isotropically, with thermal parameters dependent on those of the attached atom, except

hydrogen atoms present on the carboxylic groups of aspartic residues and C-termini of the peptides. The side chain of the valine residue of one peptide shows a rotational disorder in 3 positions at occupancy of 80%, 10% and 10%, respectively. Refinement statistics are reported in Table S1.

Table S1: Crystallographic data.

	^{D}Phe-Phe-Leu-Asp-Val
Formula	$C_{33}H_{46}N_5O_8$,$C_{33}H_{45}N_5O_8$,$C_2F_3O_2$, 4(CH_4O), 2(H_2O)
Temperature (K)	100
Wavelength (Å)	0.7
Crystal system	Monoclinic
Space group	*P 2₁*
a (Å)	9.529(2)
b (Å)	23.743(5)
c (Å)	18.276(4)
α (°)	90
β (°)	96.35(3)
γ (°)	90
V (Å^3)	4109(1)
Z, ρcalc (g/cm^3)	2, 1.194
μ (mm^{-1})	0.088
F (000)	1640
Data collection θ range	1.104 - 28.253
Refl. Collected / unique	63291 / 20107
Rint	0.057
Completeness (%)	95.5
Data/Restraints/Parameters	20107 / 1 / 1001
GooF	0.971
R1, wR2 [I>2σ(I)]	0.0939 / 0.256
R1, wR2 all data	0.1155 / 0.2819
CCDC code	1916887

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[5] Sheldrick, G. M. Acta Cryst., Sect. C. 2015, 71, 3–8.

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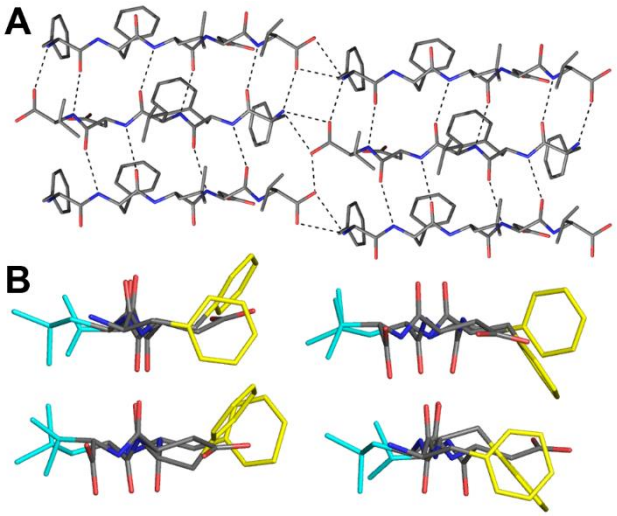

Fig. S25. Single-crystal XRD data for ^{D}Phe-Phe-Leu-Asp-Val (**2**). **A)** Hydrogen bonds (dashed lines) in peptide stacks (distances range from 2.4 to 3.1 Å). **B)** Amino acid side chains of Phe (yellow) and Val, Leu (cyan) lie on opposite sides of the stack. CCDC, 1916887.