Supporting information

Synthesis, Characterization and *In Vitro* Biocompatibility Assessment of Novel Tripeptide Hydrogelator as a Promising Scaffold for Tissue Engineering Applications

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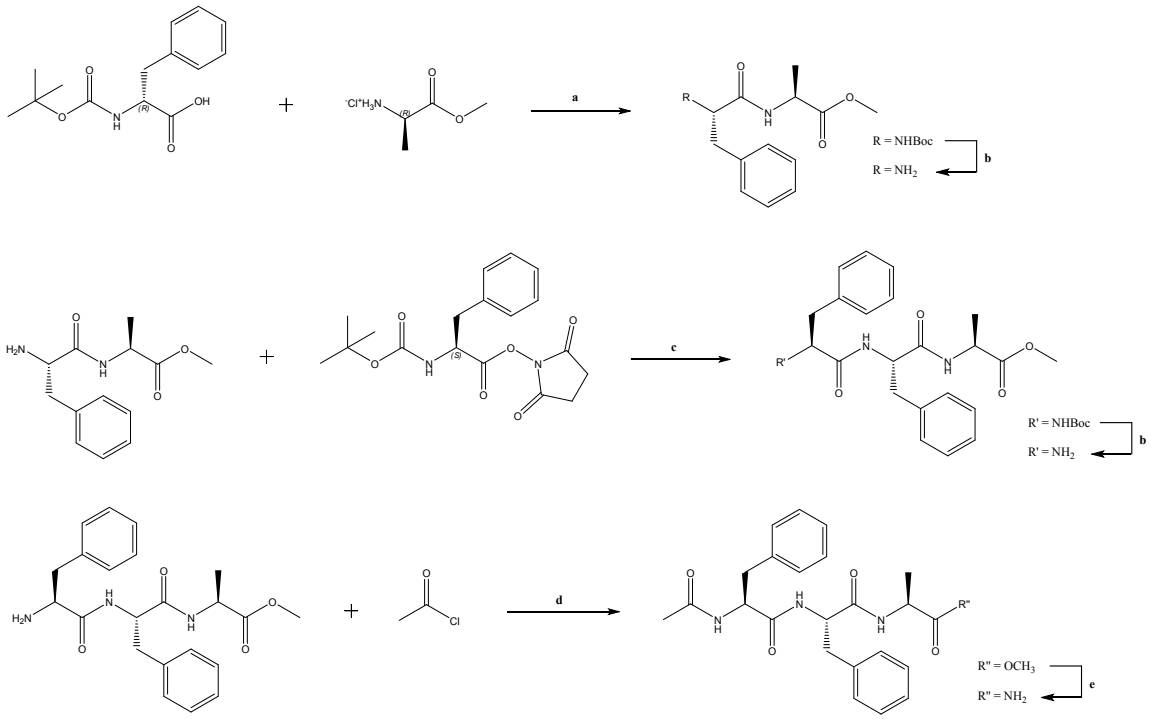
Synthesis and characterization of Ac-L-Phe-L-Phe-L-Ala-NH_2

Ac-L-Phe-L-Phe-L-Ala-NH_2 was synthesized according to Scheme S-1.



Scheme S-1 a) DCC, DMAP, Et_3N; b) TFA/CH_2Cl_2; c) CH_2Cl_2; d)Et_3N; e) NH_3/MeOH

Experimental procedures: syntheses of compounds 1–6 and the corresponding analytical data

General

Melting points were determined on Kofler stage and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on the Bruker Advance 300 MHz spectrometer, ^{1}H operating at 300.13 MHz and ^{13}C at 75.475 MHz. Proton chemical shifts were referenced to tetramethylslane (TMS) as an internal standard, whereas ^{13}C chemical shifts were referenced to the solvent signal $\delta^{13}C(DMSO\text{-}d_6)$ = 39.5 ppm. Sample concentration was approx. 20 mg mL^{-1}. FTIR spectra were recorded on an ABB Bomen MB 102 FTIR-spectrometer. Optical rotations were measured on an Optical Activity AA-10 Automatic Polarimeter in a 1 dm cell at 589 nm. Thin-layer chromatography (t.l.c.) was performed on Merck Kieselgel HF254 plastic sheets and spots were made visible using a UV lamp (254 nm) or I_2 vapours. Preparative T. L. C. was performed on silica gel (type 60 F254, Merck), the plates were activated at 110 °C for 1 h. HRMS analyses were carried out on a Bruker Ultraflex MALDI TOF/TOF (Bruker, Bremen) mass spectrometer, in reflectron positive mode using α-Cyano-4-hydroxycinnamic acid (FLUKA) as a matrix. High-resolution mass spectra were obtained by internal calibration using Verapamil and Leu-enkephalin as internal standards. Transmission electron micrographs were taken on an EM10A Zeiss transmission electron microscope (TEM). A small amount of sample was placed on carbon-coated grid (copper, 100 mesh). The sample was negatively stained with PWK (dipotassium phosphotungstate) and Pd shadowing. Reagents were purchased from Aldrich or Fluka and were used without further purification. All solvents were purified and dried according to standard procedures. The physiological saline for parenteral (intravenous) application was purchased from Croatian Institute for Transfusion Medicine (Petrova 3, 10000 Zagreb) with pH range from 4.5 to 7.0 (measured pH was 6.12). The plastic used for cell culturing were from Sarstedt (Germany). Dulbecco's modified free medium was purchased from Lonza (Verviers, Belgium). Quant-iT™ PicoGreen® dsDNA Kit was obtained from Promega (Madison, WI USA). LIVE/DEAD Cell Imaging Kit 488/570, Molecular Probes was from Thermo Fisher Scientific (Waltham, MA USA).

1. Boc-L-Phe-L-Ala-OMe (**1**)[s1]

Compound **1** is synthesized according to the literature.[s1]

^{1}H-NMR ($CDCl_3$): 7.32-7.19 (m, 5H, Ph), 6.41 (d, 1H, *J* = 6.90, N**H**C*H), 4.98 (bs, 1H, N**H**C*H), 4.52 (dt as quintet, 1H, *J* = 7.20, C^*H_{Phe}), 4.35 (d, 1H, *J* = 6.90, C^*H_{Ala}), 3.71 (s, 3H, OCH_3), 3.07 (dd, 2H, *J* = 6.6, 2.4, CH_2Ph), 1.41 (s, 9H, $C(CH_3)_3$), 1.35 (d, 3H, *J* = 7.1, CH_{3Ala}).

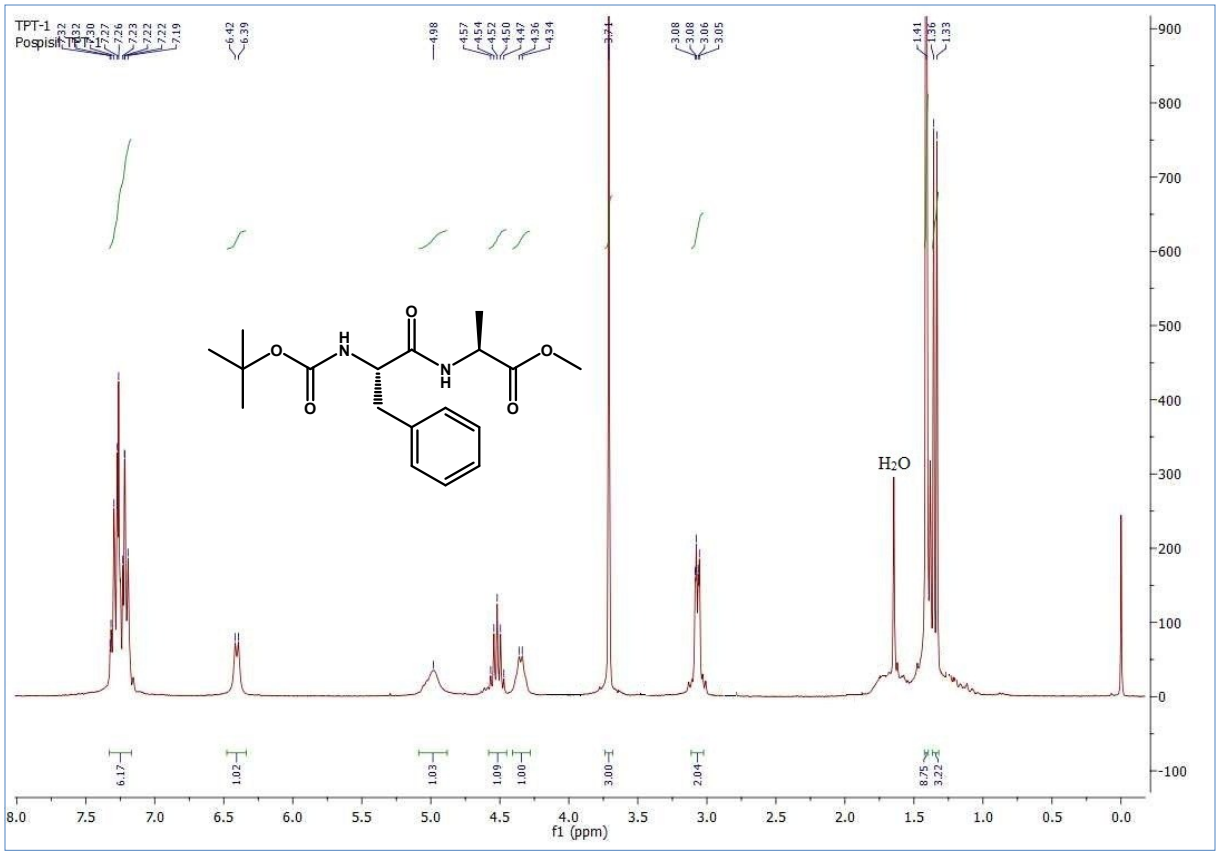


Fig. S-1 ^{1}H-NMR of Boc-L-Phe-L-Ala-OMe

2. L-Phe-L-Ala-OMe **(2)**[s2]

Compound **2** is synthesized according to the literature.[s2]

^{1}H-NMR ($CDCl_3$): 7.77 (d, 1H, J = 7.10, N**H**C*H), 7.34-7.21 (m, 5H, Ph), 4.60 (dt as quintet, 1H, J = 7.30, C*H_{Ala}), 3.74 (s, 3H, OCH_3), 3.64 (dd, 1H, J = 4.0, 9.2, C*H_{Phe}), 3.25 (dd, 1H, J = 3.90, 13.80, CH_2Ph), 2.73 (dd, 1H, J = 9.30, 13.70, CH_2Ph), 1.44 (s, 2H, NH_2), 1.39 (d, 3H, J = 7.20, CH_{3Ala}).

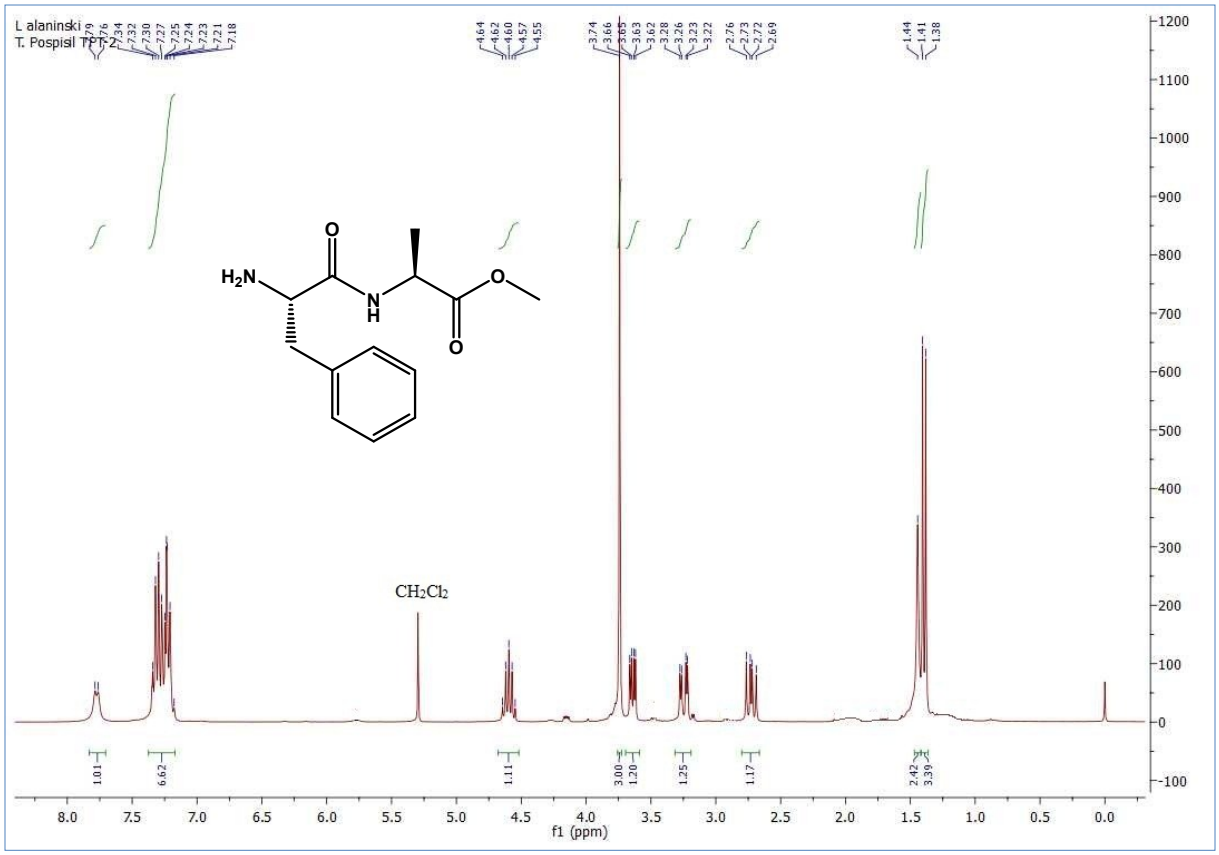


Fig. S-2 ^{1}H-NMR of L-Phe-L-Ala-OMe

3. Boc-L-Phe-L-Phe-L-Ala-OMe **(3)**[s3]

To a solution of Boc-Phe-OSu (1.00 g, 2.76 mmol) in a mixture of dry dioxane and CH_2Cl_2 (35 ml), Phe-Ala-OMe (0.69 g, 2.76 mmol) was added and stirred at room temperature overnight. The reaction mixture was evaporated and water (25 mL) and EtOAc (25 mL) added. The layers were separated, aqueous one extracted with 25 mL EtOac, the combined extracts were washed with water, dried (Na_2SO_4) and evaporated to the white solid residue that had to be chromatographically purified (CH_2Cl_2 : MeOH = 50:1) to give additional pure Boc-Phe-Phe-Ala-OMe (0.85 g, 62 %). ^{1}H-NMR ($CDCl_3$): 7.31-7.07 (m, 10H, 2 x Ph), 6.35 (bs, 2H, 2 x N**H**C*H), 4.79 (bs, 1H, N**H**C*H), 4.61 (dd, 1H, J = 7.1, 13.8, C*H_{Phe}), 4.45 (dt as quintet, 1H, J = 7.20, C*H_{Phe}), 4.30 (d, 1H, J = 6.30, C*H_{Ala}), 3.71 (s, 3H, OCH_3), 3.16-2.89 (m, 4H, 2 x CH_2Ph), 1.34 (s, 9H, $C(CH_3)_3$), 1.31 (d, 3H, J = 7.20, CH_{3Ala}). Compound **3** was obtained previously by the DCC method,[s3] the characterization data are in accordance with the earlier described in the literature.[s3]

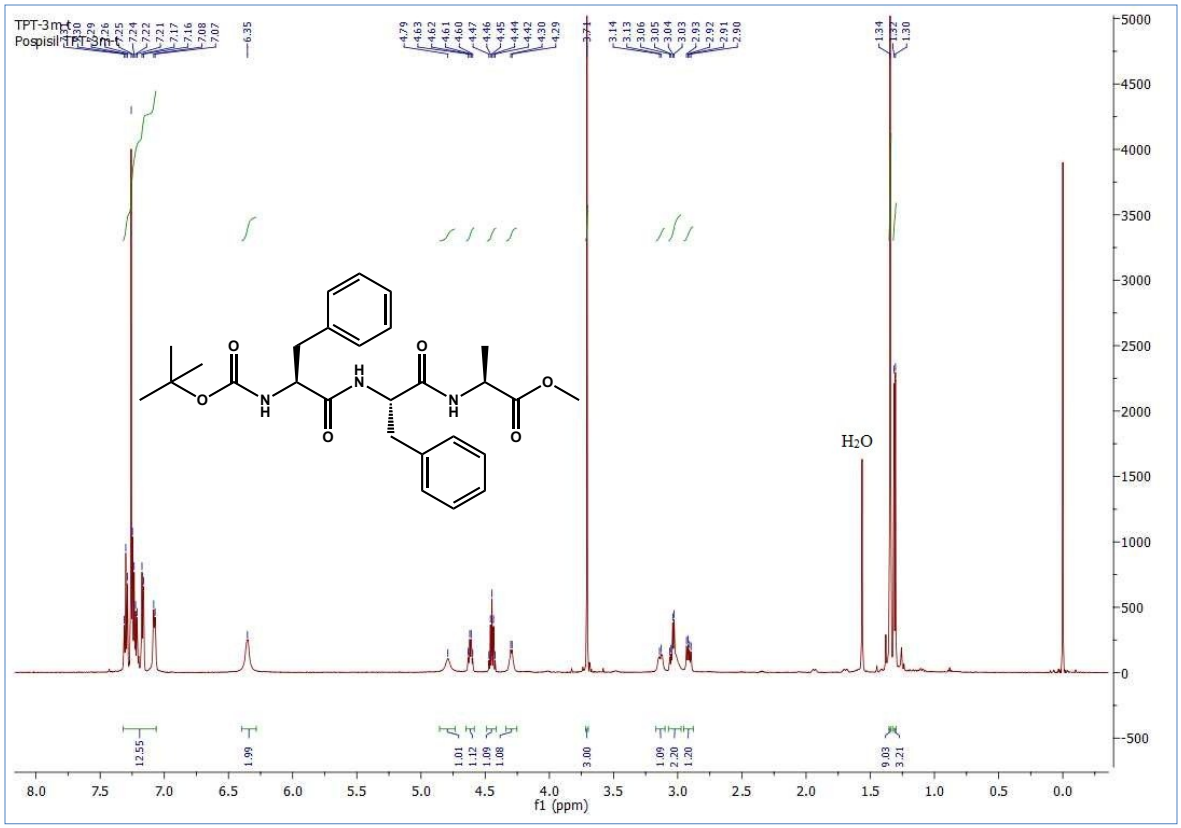


Fig. S-3 ^{1}H-NMR of Boc- L-Phe - L-Phe-L-Ala-OMe

4. L-Phe-L-Phe-L-Ala-OMe **(4)**[4]

To a solution of Boc-Phe-Ala-OMe (0.793 g, 1.59 mmol) in dry CH_2Cl_2 (5 mL) cooled down in an ice-bath, TFA (1.59 mL) was added, and the reaction mixture stirred for 0.5 h at 0 °C and at room temperature overnight. The reaction mixture was evaporated, some CH_2Cl_2 (15 mL) were added and the mixture neutralized with 10 % $KHCO_3$ under cooling in an ice-bath. The layers were separated, aqueous one extracted with 2 x 15 mL CH_2Cl_2, the combined extracts were washed with water, dried (Na_2SO_4) and evaporated to give Phe-Phe-Ala-OMe (0.63 g, 92 %). ^{1}H-NMR (DMSO-d_6): 8.48 (d, 1H, *J* = 7.1, N**H**C*H), 8.01 (d, 1H, *J* = 8.5, N**H**C*H), 7.27-7.13 (m, 10H, 2xPh), 4.61 (dd, 1H, J = 8.4, 13.2, C*H_{Phe}), 4.29 (dt as quintet, 1H, *J* = 7.2, C*H_{Ala}), 3.02-2.77 (m, 4H, 2x CH_2Ph), 1.63 (bs, 2H, NH_2), 1.29 (d, 3H, *J* = 7.3, CH_{3Ala}), Compound **4** was obtained previously by deprotection of Boc-Phe-Ala-OMe (**3)** with formic acid,[s4] the characterization data are in accordance with the earlier described in the literature.[s4]

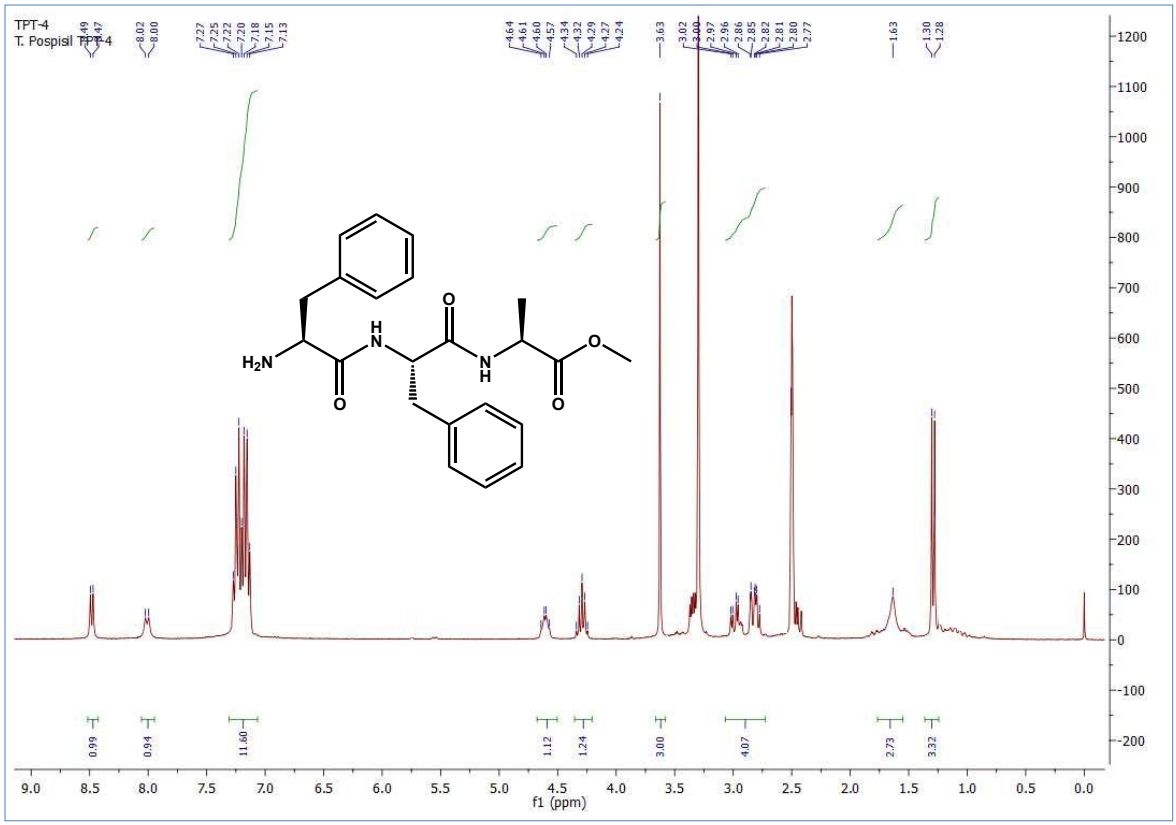


Fig. S-4 ^{1}H-NMR of L-Phe-L-Phe-L-Ala-OMe

5. Ac- L-Phe-L-Phe-L-Ala-OMe **(5)**

To a suspension of L-Phe-L-Phe-L-Ala-OMe (0.578 g, 1.45 mmol) in dry CH_2Cl_2 (10 mL) cooled down in ice-bath, Et_3N (0.203 mL, 0.146 g, 1.45 mmol) and acetyl chloride (0.103 mL, 0.114 g, 1.45 mmol) was added dropwise. The reaction mixture was stirred 1 h at 0 °C and at room temperature overnight. The reaction mixture was cooled down in an ice-bath, water (25 mL) added and the layers separated. Organic layer was washed with 5 % $NaHCO_3$, water, 1 M HCl and water, dried (Na_2SO_4) and evaporated to give Ac-L-Phe-L-Phe-L-Ala-OMe (0.579 g, 92 %).^{1}H-NMR (DMSO-d_6): 8.34 (d, 1H, *J* = 7.00, N**H**C*H), 7.97(dd, 2H, *J* = 8.30, 14,30, 2xN**H**C*H), 7.27-7.14 (m, 10H, 2 x Ph), 4.54 (dt, 1H, *J* = 4.70, 8.90, C***H**$_{Phe}$), 4.47-4.41(m, 1H, C***H**$_{Phe}$), 4.29 (dt as quintet, 1H, *J* = 7.20, C***H**$_{Ala}$), 3.62 (s, 3H, OC**H**$_3$), 3.05 (dd, 1H, *J* =4.6, 14.0, 2 x C**H**$_2$Ph), 2.92 (dd, 1H, *J* = 4.4, 13.9, 2 x C**H**$_2$Ph), 2.81 (dd, 1H, *J* = 9.3, 14.0, 2 x C**H**$_2$Ph), 2.65 (dd, 1H, *J* = 10.0, 13.9, 2 x C**H**$_2$Ph), 1.72 (s, 3H, COC**H**$_3$), 1.29 (d, 3H, *J* = 7.30, C**H**$_3$). ^{13}C-NMR (DMSO-d_6): 172.78, 171.13, 170.76, 169.07 (4 x CO), 137.95, 137.56, 129.22, 129.03, 127.98, 127.93, 126.23, 126.11 (2 x Ph), 53.80, 53.41 (2 x C*H$_{Phe}$), 51.83 (O**C**H$_3$), 47.59 (C*H$_{Ala}$), 37.42, 37.28 (2 x **C**H$_2$Ph), 22.38 (CO**C**H$_3$), 16.86 (**C**H$_3$); IR(KBr): ν = 3278 (NH), 1740 (COOMe), 1638 (amide I), 1537 (amide II) cm^{-1}; m.p. 214-216 ^{0}C; $[\alpha]\frac{RT}{D}=$ +29° (c = 0.02 in MeOH). HRMS: m/z [M+Na]+ exact mass, $C_{24}H_{29}N_3O_5Na$: 462.2005, found: 462.1999.

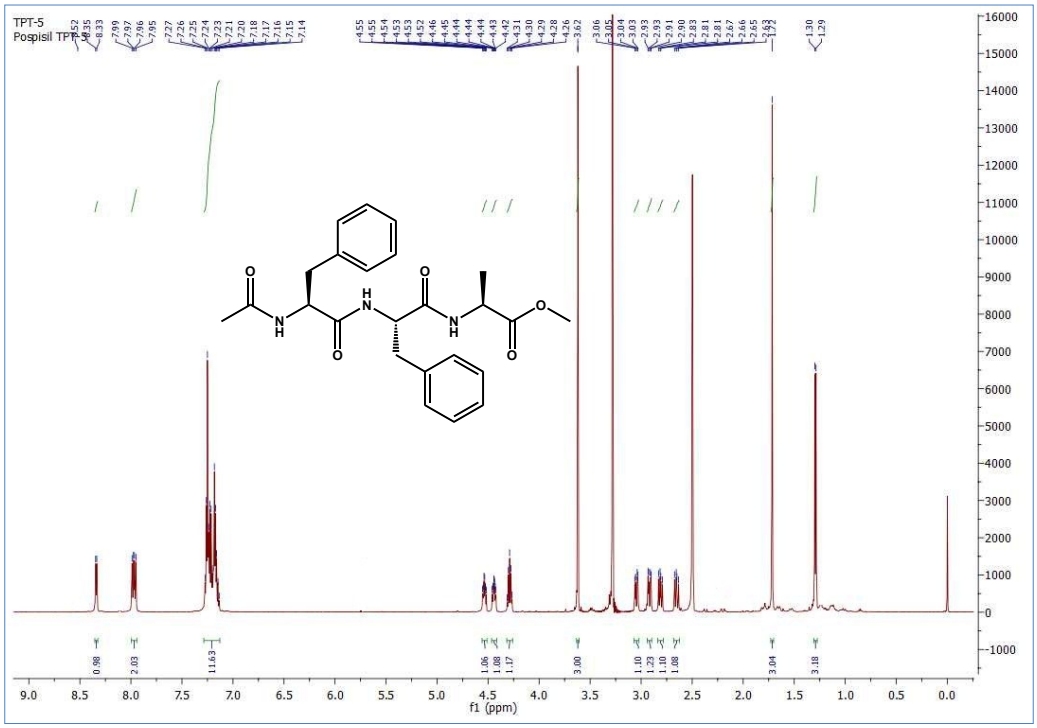


Fig. S-5 ^{1}H-NMR of Ac-L-Phe-L-Phe-L-Ala-OMe

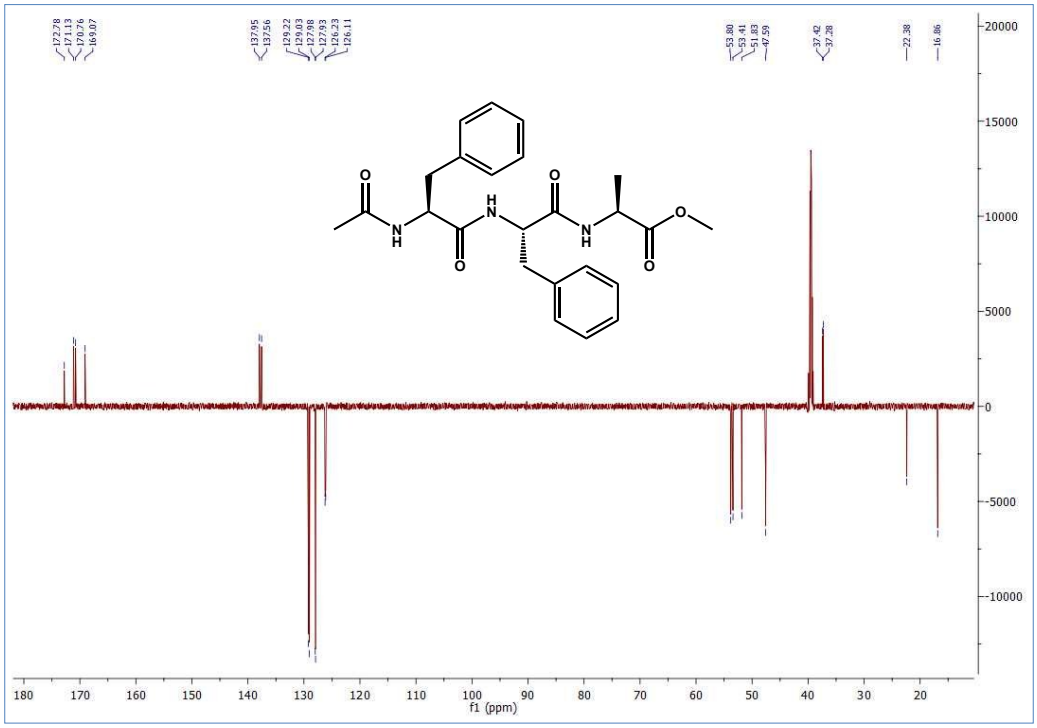


Fig. S-6 ^{13}C-NMR of Ac-L-Phe-L-Phe-L-Ala-OMe

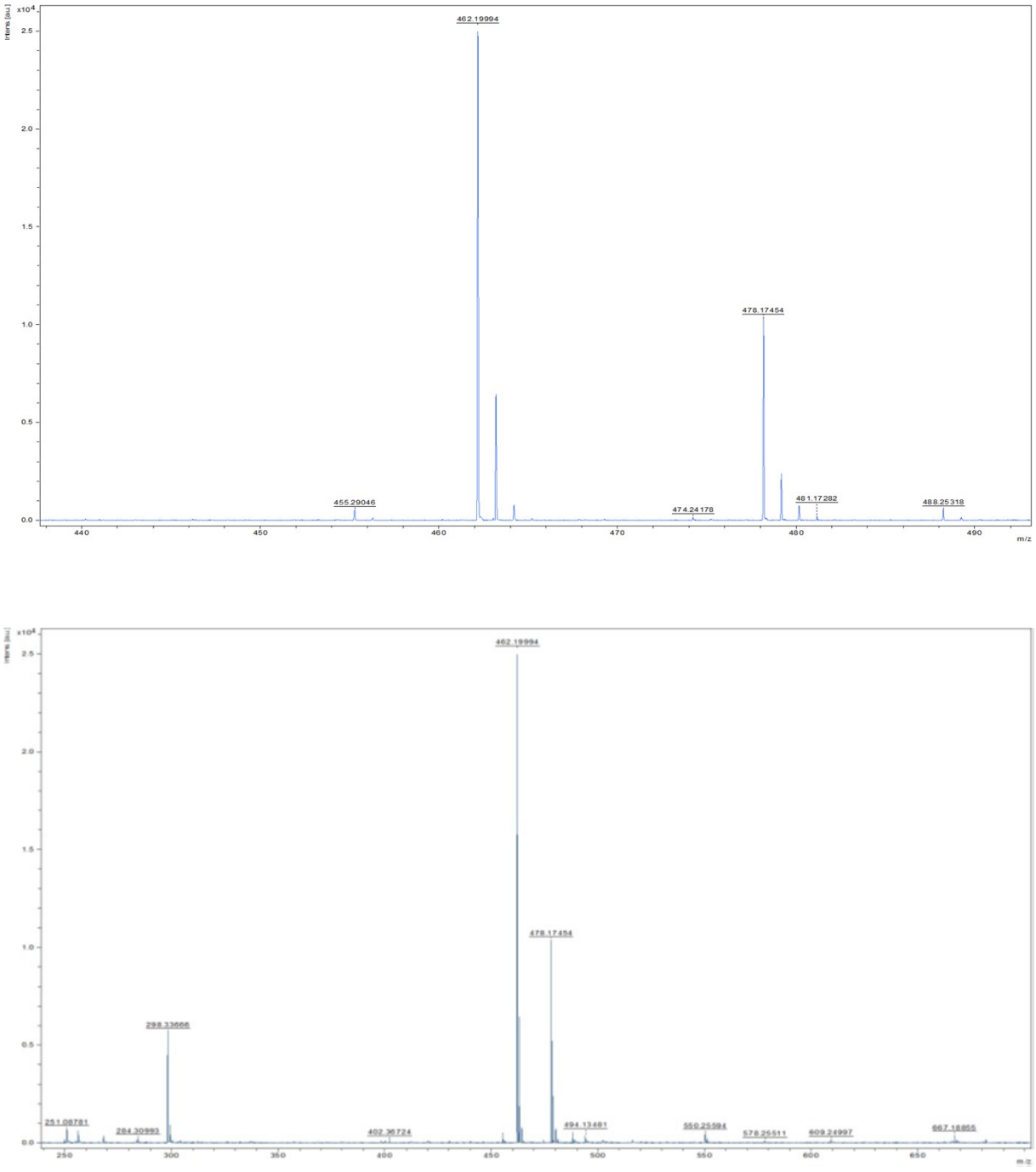


Fig. S-7 HRMS spectra of Ac-L-Phe-L-Phe-L-Ala-OMe

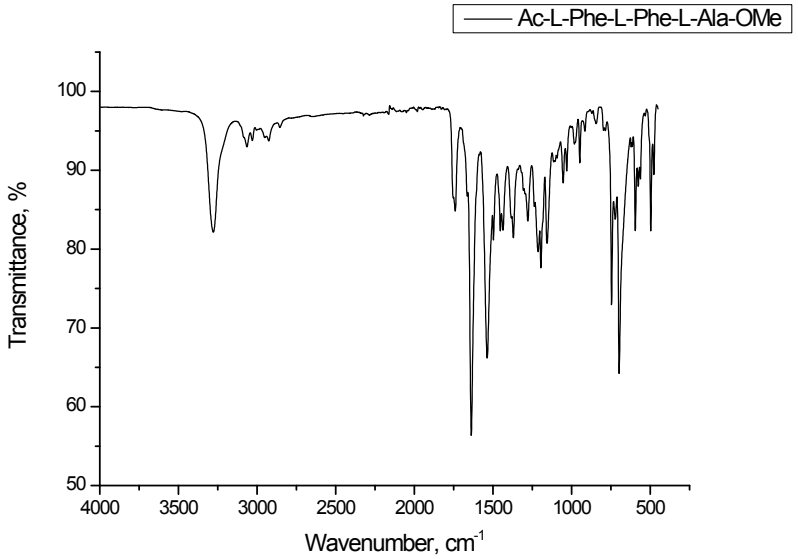


Fig. S-8 IR spectrum of Ac-L-Phe-L-Phe-L-Ala-OMe

6. Ac- L-Phe-L-Phe-L-Ala-NH_2 **(6)**

L-Phe-L-Phe-L-Ala-OMe (0.573 g, 1.30 mmol) was dissolved in saturated methanol solution of ammonia and held for seven days at 4 °C. Final product Ac-L-Phe-L-Phe-L-Ala-NH_2 precipitated from the solution (0.540 g, 98%). ^{1}H-NMR (DMSO-d_6): 8.08 (d, 1H, J = 8.00, N**H**C*H), 8.00 (d, 1H, J = 8.03, N**H**C*H), 7.93 (d, 1H, J = 7.50, N**H**C*H), 7.29-7.10 (m, 10H, 2 x Ph), 6.99 (bs, 1H, NH_{NH2}), 4.56-4.39 (m, 2H, 2 x C*H_{Phe}), 4.19 (dt as quintet, 1H, J = 7.1, C*H_{Ala}), 3.11-2.60 (m, 4H, 2 x C**H**$_2$Ph), 1.72 (s, 3H, COC**H**$_3$), 1.21 (d, 3H, J = 7.10, C**H**$_{3Ala}$). ^{13}C-NMR (DMSO-d_6): 173.87, 171.30, 170.28, 169.08 (4 x CO), 137.94, 137.66, 129.20, 129.04, 128.01, 127.93, 126.22, 126.12 (2 x Ph), 53.84, 53.83 (2 x **C***H_{Phe}), 48.00 (**C***H_{Ala}), 37.25, 37.18 (2 x **C**H_2Ph), 22.37 (CO**C**H_3), 18.31 (**C**H_3).); IR(KBr): ν = 3277 (NH), 1679 ($CONH_2$), 1626 (amide I), 1544 (amide II) cm^{-1}; Elem. Anal. Calcd. (%) for $C_{23}H_{28}N_4O_4$ (424.49): C 65.08 H 6.65 N 13.2; found C 65.09 H 6.48 N 13.0; m.p. 243-245 °C, $[\alpha]\frac{RT}{D} =$ -18° (c = 0.02 in EtOH)

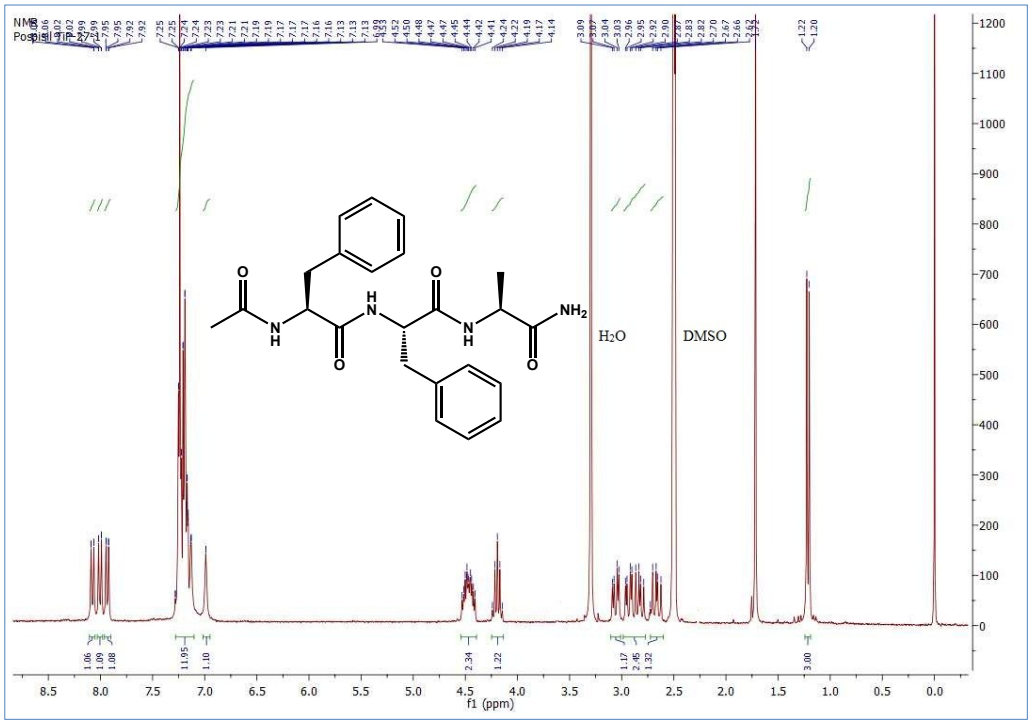


Fig. S-9 ^{1}H-NMR of Ac- L-Phe-L-Phe-L-Ala-NH_2

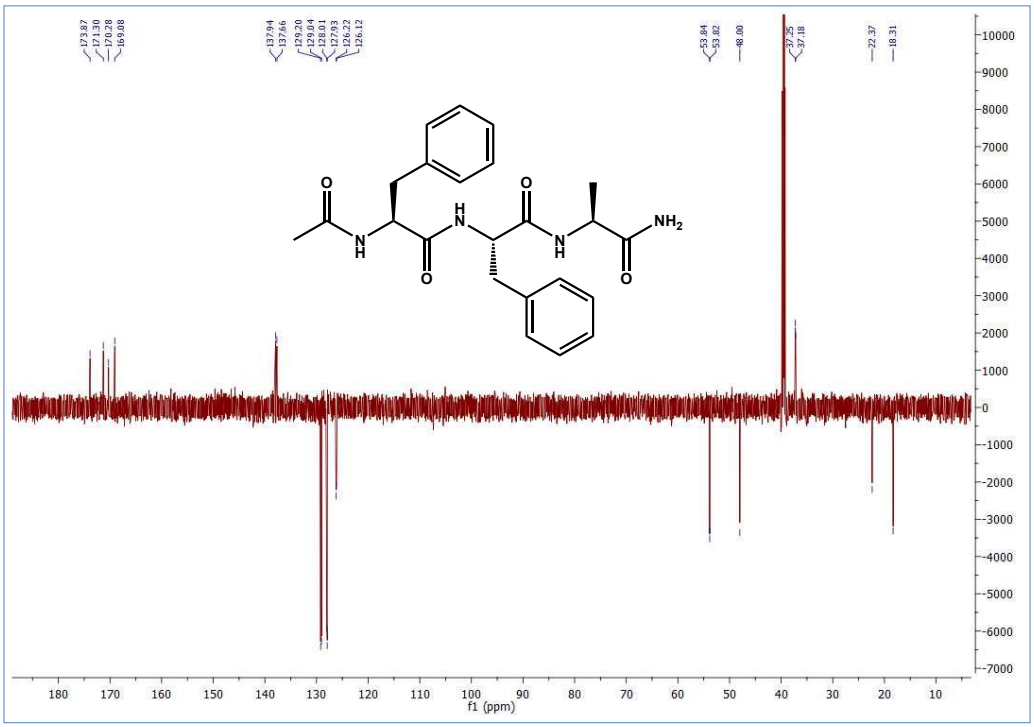


Fig. S-10 ^{13}C-NMR of Ac- L-Phe-L-Phe-L-Ala-NH_2

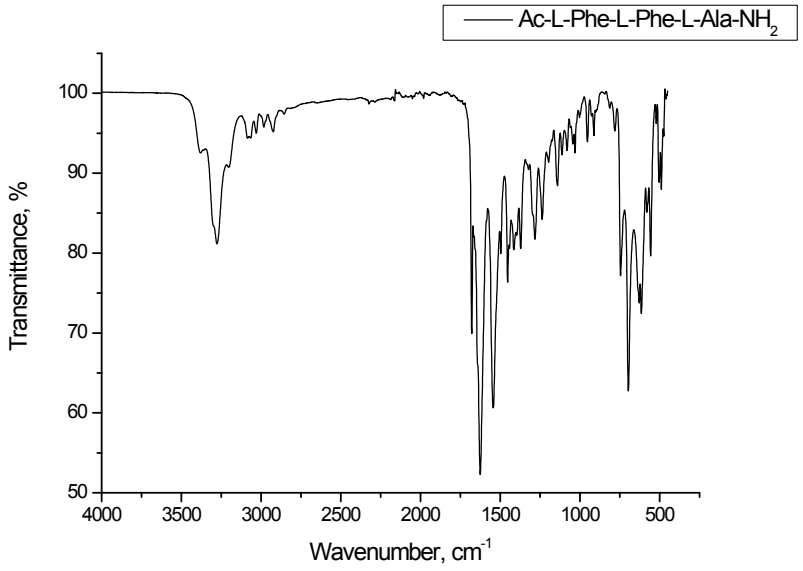


Fig. S-11 IR spectrum of Ac-L-Phe-L-Phe-L-Ala-NH_2

Preparation of the Ac-L-Phe-L-Phe-L-Ala-NH_2 tripeptide hydrogel

Tripeptide sample was slowly heated to 80 ºC to dissolve in saline and then allowed to cool down to room temperature and form an opaque hydrogel at the concentration of 2.64 mg ml^{-1} within 20 minutes. The "tube inversion method in which the glass vial was turned upside down, provides strong visual evidence of the formation of stabile hydrogel which is able to support its own weight.

Rheometry

Dynamic viscoelastic and time evolution measurements were carried out with an Anton Paar rheometer (MCR 301), by using a parallel-plate geometry (the plate diameter was 25 mm and the gap width was 300 μm). All viscoelastic measurements were performed at (25 °C). Tripeptide sample was slowly heated to dissolve in saline and then allowed to cool down and form a hydrogel at the concentration of 2.64 mg ml^{-1}. A gap of 300 μm was set, a constant strain of 2% applied, and a frequency of 10 rad/s was used. A portion of gel was set on a sensor plate. Over a period of an hour, storage (elastic, G') and loss (viscous G") moduli were measured and reported as a function of time.

The Figure S-12. shows the storage modulus (G′) and loss modulus (G″) of the hydrogel prepared from the tripeptide Ac-Phe-Phe-Ala-NH_2 as a function of time.

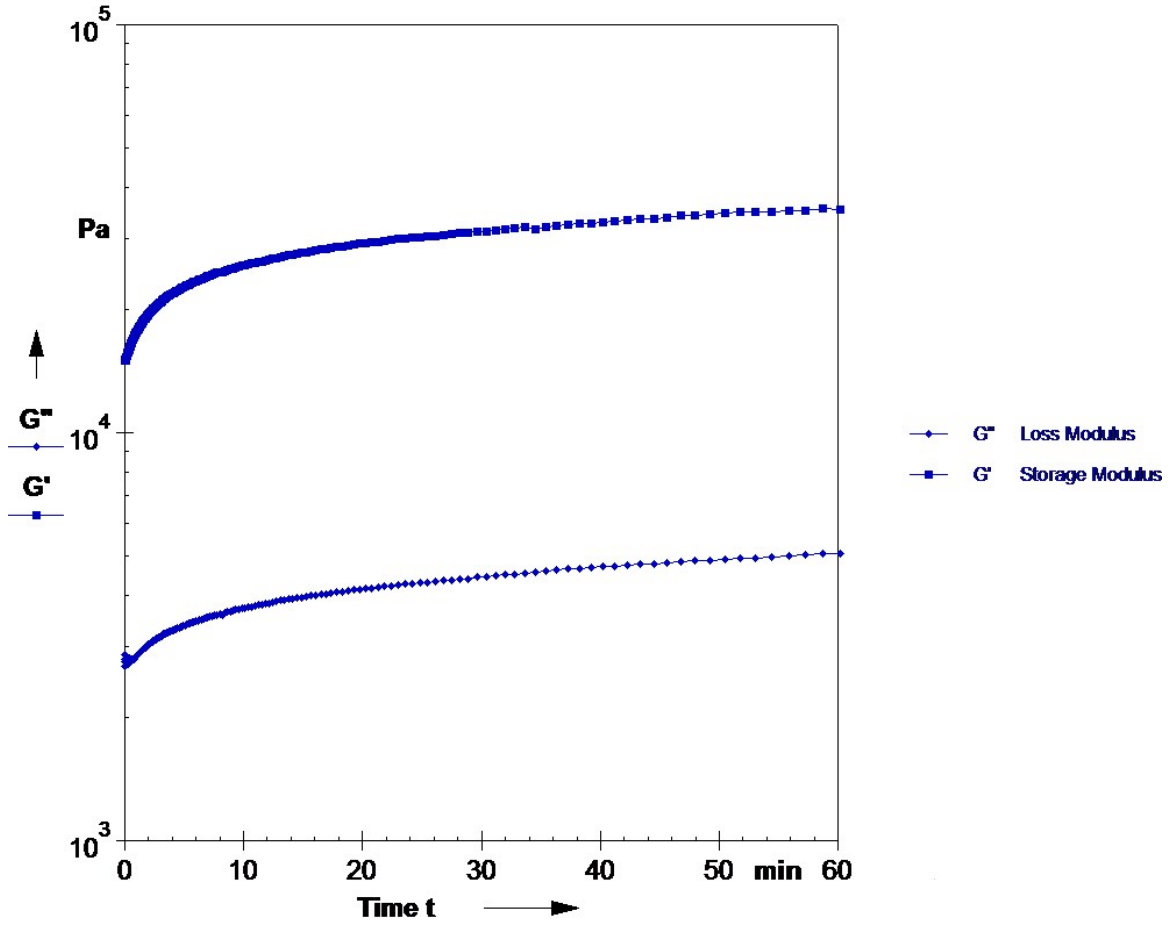


Fig S-12 Dynamic time sweep rheological data of Ac-Phe-Phe-Ala-NH_2 hydrogel (c = 2.64 mg ml-1).

***In vitro* screening of cell viability and proliferation of the tripeptide based hydrogel**

Freshly prepared Ac-L-Phe-L-Phe-L-Ala-NH_2 tripeptide hydrogel in minimal gelation concentration was left 20 min to cool down to 37 °C in the water bath. HEK293T cells were grown in DMEM medium supplemented with 1% Pen/Strep, 1% L-glutamine and 10% Fetal Bovine Serum. After reaching 80% confluency, cells were trypsinized and centrifugated at 300 x g for 5 minutes. After centrifugation, the medium was aspirated and the cell pellet was resuspended in Ac-L-Phe-L-Phe-L-Ala-NH_2 tripeptide hydrogel at 37 °C in concentration

$1x10^6$cells/ml. Cells encapsulated in the hydrogel were seeded to 24-well plates, with each well containing 150 µl of the heterogeneous cell/hydrogel mixture, and then 600 µl of total medium was added carefully to each well to avoid disturbation of the mixture. Cells were also seeded at the same concentration in Matrigel (Croning) hydrogel, which was used as positive control, together with pure total medium. Latex rubber was used as negative control. Obtained results are expressed as mean of four independent experiments each performed in a triplicate. Cells were kept for 5 days in humidified atmosphere at 37 °C with 5% of CO_2. Samples were taken on day 0 and day 5 of the experiment.To determine the total cell number in samples, we used Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Cells were washed with PBS, cell/hydrogel mixtures were broken by adding the 1 ml PBS to each well and vigorously titurating the mixture for 20 times. After that, the obtained solution was centrifuged at 300 x g for 5 min and PBS containing disrupted gel was aspirated. Cells were then resuspended in 250 µL of MiliQ water and left for 1h at 37 °C in the water bath. Cells were then stored at -80 °C until analysis with spectrofluorometer. Prepared samples were sonicated in the ultrasound waterbath, mixed with TE-buffer and PicoGreen colour, incubated in dark for 10 min and the fluorescence was read in a fluorescent microplate reader (Fluoroskan Ascent FL, Thermo Electron Corporation) at 485 nm excitation and 538 nm emission. The exact cell number was determined using HEK293T cell number standards.

To determine cell viability we used LIVE/DEAD Viability/Cytotoxicity Assay. Cells were incubated with a mixture of calcein-AM (live) and ethidium homodimer-1 (dead) for 15 minutes and then observed under Axiovert 200 fluorescent microscope with the 10x objective. Five micrographs of green fluorescence (live cells) and red fluorescence (dead cells) were taken per each well. Micrographs were analysed with Fiji software and percentage of live cells was calculated per each field of view.

Transfection

To observe cell morphology, cells were transfected transiently with tubulin-mCherry plasmid, by electroporation using Nucleofector Kit R (Lonza, Basel, Switzerland) with the Nucleofector 2b Device (Lonza, Basel, Switzerland), using the high-viability O-005 programme. Transfection protocol provided by the manufacturer was followed. In all, $1x10^6$ cells and 2 μg of plasmid DNA were used. Transfection was performed 72 h before imaging.

Confocal microscopy

Cells encapsulated in SAP hydrogel and cells grown in pure medium were imaged by using a Leica TCS SP8 X laser 15 scanning confocal microscope with 100x oil immersion objective (Leica, Wetzlar, Germany) heated with an objective integrated heater system (Okolab, Burlingame, CA, USA). Excitation and emission lights were separated with AOBS (Acousto-Optical Beam Splitter, Leica, Wetzlar, Germany). Cells were maintained at 37 °C in Okolab stage top heating chamber (Okolab, Burlingame, CA, USA). For excitation, gated STED supercontinuum visible white light laser (WLL) 575 nm was used. Emission of mCherry was detected with HyD (hybrid) detector in range 585–665 nm. Pinhole diameter was set to 0.8 μm. Images were acquired at 20 focal planes with 1 μm spacing and 400 Hz unidirectional xyz scan mode. In addition, both groups of cells were imaged under bright light using 20x dry objective.

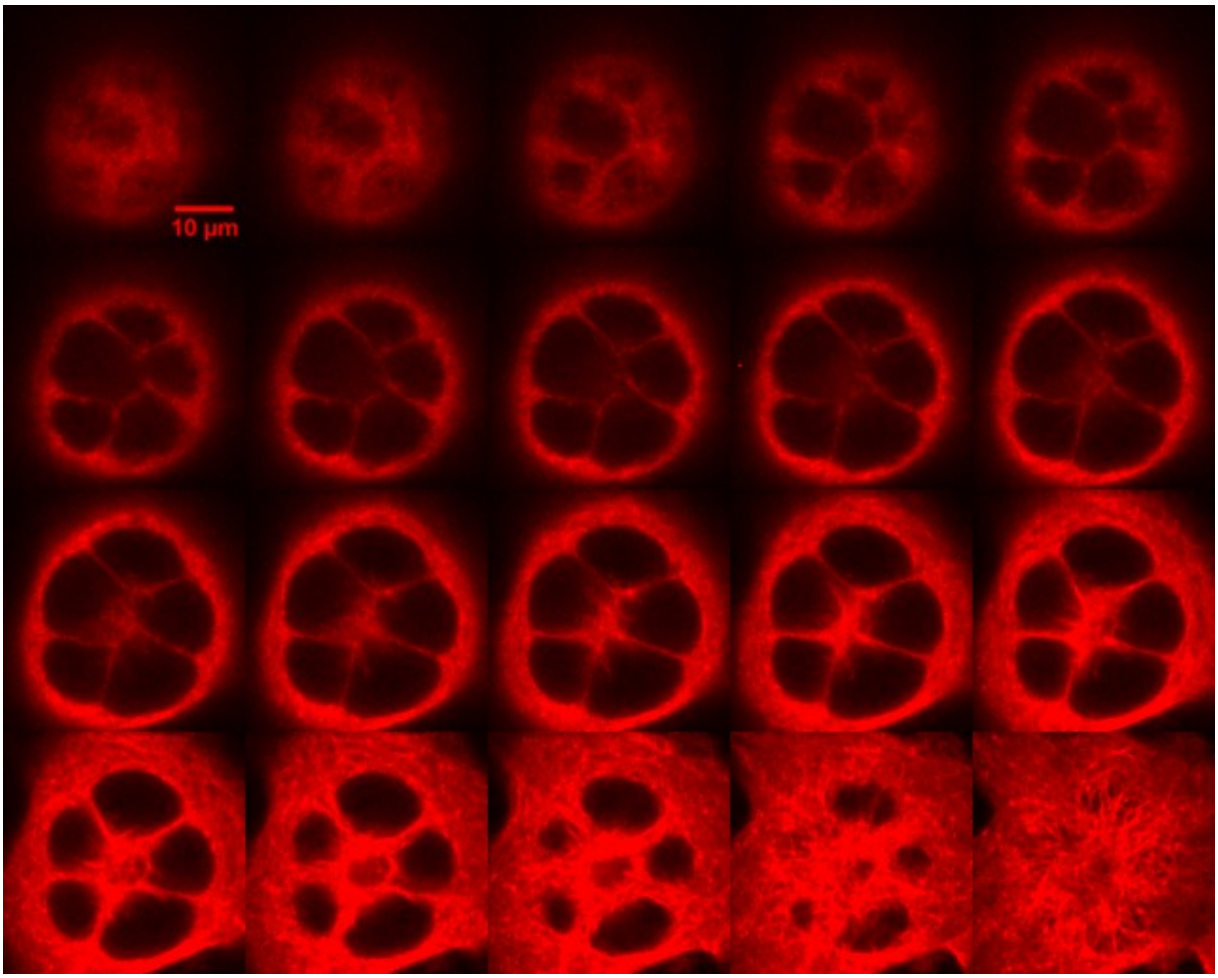


Fig. S-13 HEK293Tcells transfected with tubulin-mCherry, grown in medium and observed under confocal microscope 72h after. Cells are adhered to the bottom of the well and are all visible in same *z* planes. Magnification 100x, scale bar applies to all.

Statistical analysis

Statistical analysis (GraphPad Prism; GraphPad Software, San Diego, CA, USA) was performed using one-way analysis of variance followed by unpaired T-test, and significance was set at $p < 0.05$.

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