Supporting Information

Co-assembly generates peptide hydrogel with wound dressing material properties

Chaitanya Kumar Thota†, Allison A. Berger†, Laura Elomaa†, Chaunxiong Xie†, Christoph Böttcher‡, Beate Koksch†*

†Freie Universität Berlin, Department of Chemistry and Biochemistry, Takustrasse 3, 14195, Berlin, Germany

‡Freie Universität Berlin, Research Center for Electron Microscopy, Fabeckstrasse 36a, 14195 Berlin, Germany

Corresponding Author

Phone: +49-30-838-55344. E-Mail: beate.koksch@fu-berlin.de

Synthesis, Purification, and Characterization of L∆F and fMLF, and Co-Assembly Sample Preparation

*Solution Phase Peptide Synthesis of Leucine-α,β-Dehydrophenylalanine (**L∆F**)*

A solution of sodium hydroxide (0.022 g, 0.55 mM) and D,L-threo-β-phenylserine (0.1 g, 0.55 mM) was prepared and stored at 4°C. Boc-Leu-OH (0.116 g, 0.55 mM) was dissolved in dry tetrahydrofuran and then stirred in an ice-salt bath at -20°C. *N*-Methylmorpholine (0.05 mL, 0.55 mM) followed by isobutyl chloroformate (0.07 mL, 0.5 mM) were added to the above solution and stirred for 10 min. Later, the pre-cooled solution of sodium hydroxide and D,L-threo-β-phenylserine was transferred to the above solution and stirred overnight at room temperature. Later the resultant azalactone was hydrolysed to obtain Boc-LeuΔPhe-OH. Boc deprotection from Boc-LeuΔPhe-OH was achieved by dissolving it in anhydrous TFA and DCM (1:1 v/v) and stirred for 30 min on ice. The solvent was evaporated and the compound was precipitated and washed thrice with anhydrous diethyl ether. The final compound **L∆F** was lyophilized to obtain the final products as white powder. Yield obtained was: 0.124 g, 90%, *Rf* was 0.36 ($CHCl_3$-MeOH, 9:1).

*Solid Phase Peptide Synthesis of **fMLF***

Wang resin with 0.5 mmol substitution factor was loaded with Fmoc-Phe-OH using standard amino acid loading protocol. Wang resin was swelled in DMF. 10-fold excess of Fmoc-Phe-OH (1.93 g, 5 mM) was dissolved in DCM and then activated with DIC (0.63 mL, 5 mM) on ice for 20 min. The solvent was evaporated, the product dissolved in DMF and transferred to the swelled resin. Immediately, 4-dimethylaminopyridine (6.1 mg, 0.05 mM, 0.1 *equiv.* with respect to resin) was added and the mixture gently stirred overnight at room temperature. Subsequently, the resin was washed thrice with DMF/DCM/Methanol and dried in a desiccator for 24 hours under vacuum. Using a standard SPPS protocol fMLF peptide was synthesized on a 0.05 mmol scale. Fmoc removal at each step was achieved by using 20% piperidine in DMF (3×10 min, 5 mL of deprotecting solution), washed thrice with DMF. 10-fold excess amino acid (2×60 min, 5 mL), HOAt (0.68 g, 5 mM) and DIC activation (0.63 mL, 5 mM) was used for coupling Fmoc-Leu-OH (1.76 g, 5 mM) while 3-fold excess amino acid (2×60 min, 5 mL), DIPEA (0.072 mL, 3.75 mM) and HATU (0.57 g, 1.5 mM) activation was used for coupling *N*-Formyl-L-methionine (0.27 g, 1.5 mM). The resin was washed with

DMF/DCM/Methanol thrice and then subjected to acid cleavage with cocktail TFA (95.6%)/Phenol(2%)/Water(2%)/TIS(0.4%) for 3 hours, followed by solvent evaporation, washing with diethyl ether (three times) and lyophilization to obtain the final product **fMLF** as a white powder.

Characterization of Peptides using RP-HPLC and ESI–ToF–MS

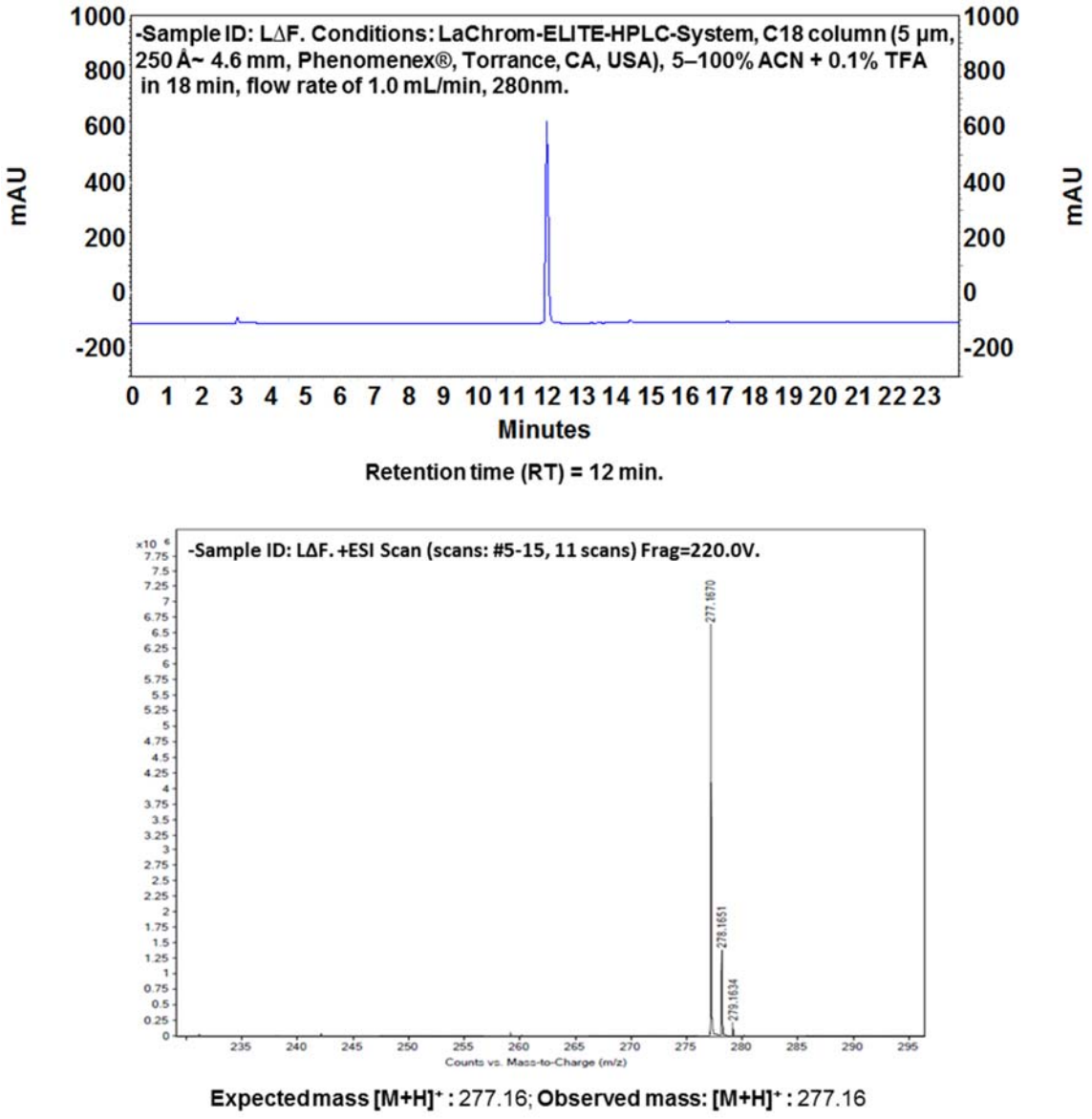


Figure S1. Characterization of purified **LΔF** using RP-HPLC and ESI–ToF–MS.

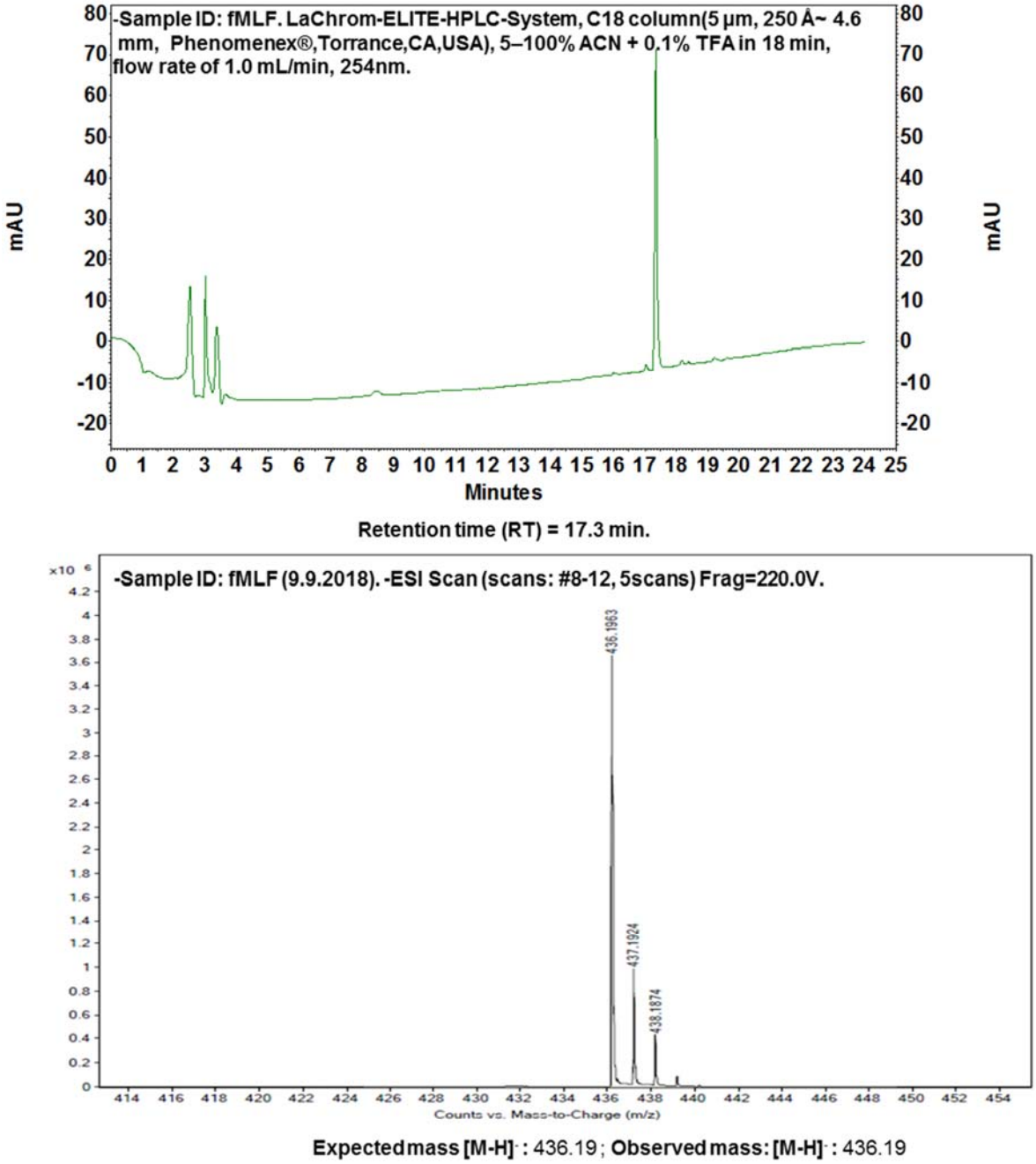


Figure S2. Characterization of purified **fMLF** by RP-HPLC and ESI–ToF–MS.

Preparation of Dipeptide Hydrogel and Ligand Co-Assembled Hydrogels

LΔF and **fMLF** peptides were dissolved in methanol to 50 mg/mL. When the peptide stocks were diluted with an appropriate volume of 0.8 M sodium acetate buffer pH 7.4 to obtain 0.5 to 1% w/v hydrogels at room temperature. Gels form instantaneously. Methanol was removed by exposure to air overnight.

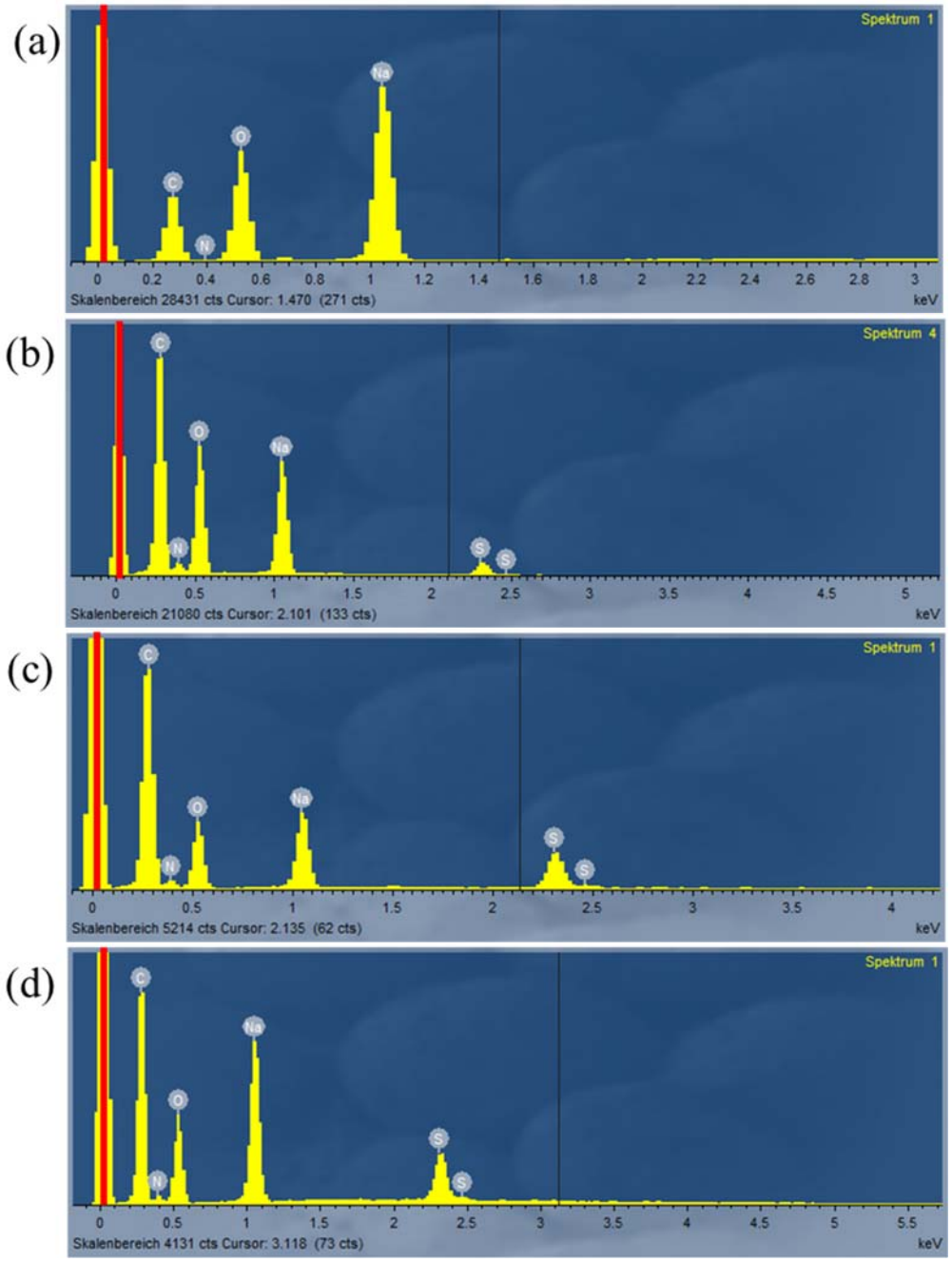


Figure S3. EDX analysis of the nanofiber of the hydrogels. Signals for carbon (C), nitrogen (N), oxygen and sodium (Na) in the spectra originate from peptide and sodium acetate buffer. Presence of sulfur (from methionine of **fMLF** peptide) show that the nanofibers are composed of both the peptides **LΔF** and **fMLF**. (a) Control 1% w/v **LΔF** hydrogel shows the absence of sulfur content, (b) 1% w/v **LΔF** + **fMLF** (1:0.25), (c) 1% w/v **LΔF** + **fMLF** (1:0.5), and (d) 1% w/v **LΔF** + **fMLF** (1:1) hydrogel all show the presence of sulfur content.

Transmission Electron Microscopy (TEM)

Aliquots (5 µL) of the aqueous samples of, **LΔF**, **LΔF** + **fMLF** peptides at **1:0.2, 1:0.5** and **1:1** molar ratios in 0.8 M sodium acetate buffer at pH 7.4 were applied to a Formvar/carbon covered copper grid (400 mesh, PLANO GmbH, Wetzlar) which was hydrophilized by 60 s glow discharging at 8 W in a BALTEC MED 020 device directly before use. After 45 s of sedimentation, excess liquid was removed with blotting paper. A droplet of contrasting material (1% phosphotungstic acid, pH 7.0) was added and incubated for 45 s, blotted again and air-dried. The grid was then transferred to a Talos L120C transmission electron microscope (FEI Company, Oregon) equipped with a LaB6 electrode operating at an acceleration voltage of 120 kV. Image data were recorded using a 4k × 4k Ceta CMOS camera.

Samples	Length (in nm)	Standard Deviation (SD)
LΔF	13.461	2.464
LΔF + fMLF (1:0.25)	13.760	0.971
LΔF + fMLF (1:0.5)	15.939	2.140
LΔF + fMLF (1:1)	15.603	1.679

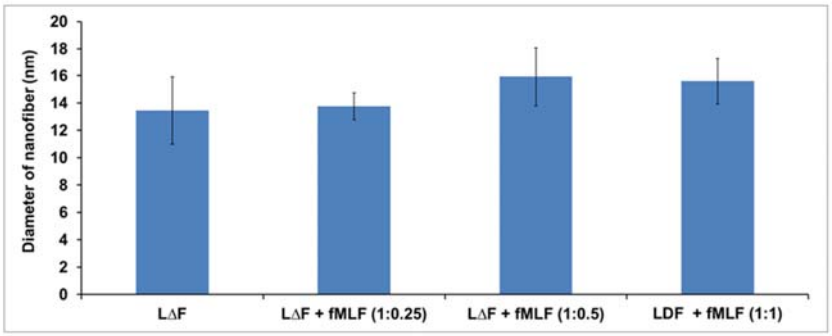
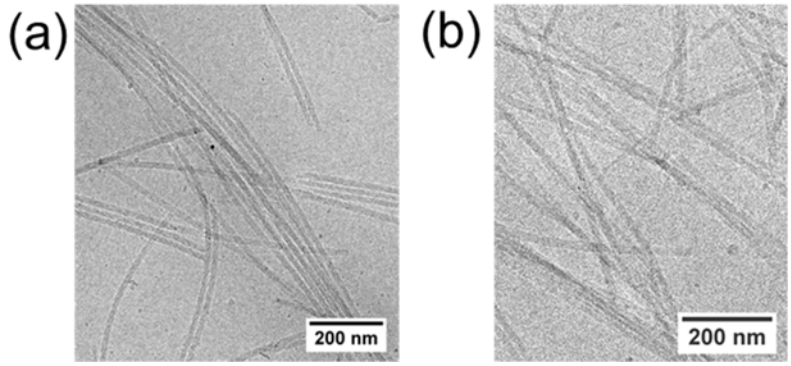


Figure S4. Analysis of nanofiber dimensions in hydrogels of **LΔF** and **LΔF**:**fMLF** at molar ratios 1:0.25, 1:0.5 and 1:1 using cryo-TEM.



(a) LΔF+fMLF (1:0.25) (b) LΔF+fMLF (1:0.5)

Figure S5. Analysis of nanofiber dimensions in hydrogels of **LΔF**:**fMLF** at molar ratios 1:0.25 and 1:0.5 using cryo-TEM.

Scanning Electron Microscopy (SEM)

Double sided tape was placed on the SEM holder and onto it **1 % w/v LΔF** and **1 % w/v LΔF + fMLF** peptide hydrogels were spread. These samples were gold coated by ion sputtering (Safematic, CCU-010) for 30 s at an operating vacuum of 6.6 mbar. A Hitachi Scanning Electron Microscope (SU8030) equipped with an Oxford energy-dispersive X-ray (EDX) detector was used to acquire SEM images with 2.0 nm resolution at an acceleration voltage of 15 kV and EDX spectra, respectively.

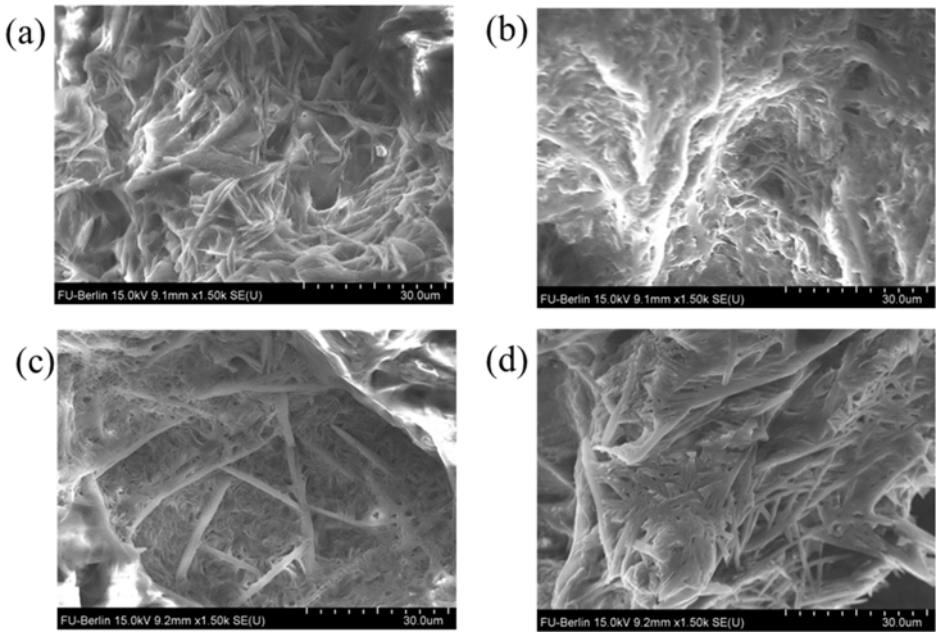


Figure S6. SEM analysis of hydrogels showing a network of nanofiber bundles. (a) SEM image of 1%w/v LΔF hydrogel, (b) SEM image of 1%w/v LΔF + fMLF (1:0.25)

hydrogel, (c) SEM image of 1%w/v LΔF + fMLF (1:0.5) hydrogel, (d) SEM image of 1%w/v LΔF + fMLF (1:0.25) hydrogel.

CD Spectroscopy

CD studies of, **LΔF**, **LΔF + fMLF** peptides at **1:0.2, 1:0.5** and **1:1** molar ratios were carried out at 0.2 mg/mL concentration in 0.8 M sodium acetate buffer at pH 7.4. A 0.1 cm path length quartz cuvette was used. JASCO-8-10 polarimeter with a temperature controller set at 20°C, over a wavelength range of 190 nm to 300 nm, with an average of 5 scans was used to record the spectrum. A constant flush of N_2 at a flow rate of 3.0 L/min was maintained.

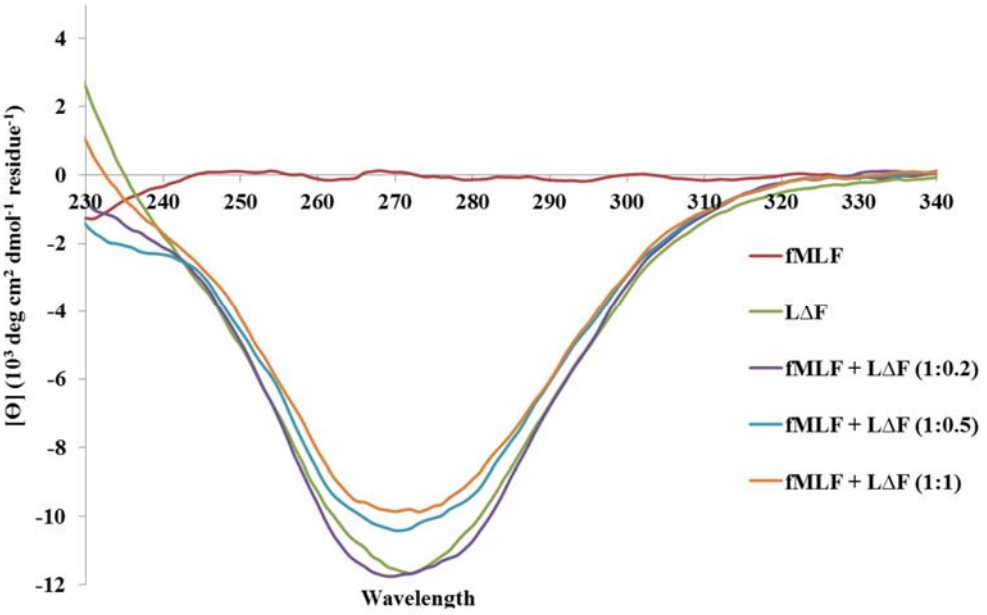


Figure S7. CD studies on **LΔF** and **LΔF** + **fMLF** hydrogels at 1:0.2, 1:0.5 and 1:1 concentrations in 0.8 M sodium acetate buffer of pH 7.4 at 20°C.

Rheology

Rheology studies were conducted to investigate the strength, stability and thixotropic nature of co-assembled gels. *Malvern Kinexus Rheometer was used with* an 8 mm plate to plate geometry. The gel strength and linear viscoelastic regime (LVR) of the **LΔF** and the co-assembled hydrogels **LΔF+fMLF (1:0.25)**, **LΔF+fMLF (1:0.5)**, **LΔF+fMLF (1:1)** hydrogels at 1 % w/v were determined by measuring their storage

modulus (G') and loss modulus (G") while performing an amplitude sweep (0.01% - 10% strain) at a constant oscillatory frequency of 1Hz. In frequency sweep measurements, G' and G" values were also recorded as a function of angular frequency (0.1-100rad/s) maintaining a constant strain of 0.1%. Time dependent step-strain rheological measurements were carried out to study the impact co-assembly on the thixotropic behaviour of the **LΔF** gel. For this, **1 % w/v LΔF+fMLF** was first subjected to low constant strain of 0.1% and then was quickly followed by application of a higher strain of 50% that disrupts the gel structure completely. Subsequently, the high strain was reduced to 0.1%, to study the thixotropic behaviour or a time dependent recovery of gel strength.

Cytotoxicity

All cell experiments were conducted according to German genetic engineering laws and German biosafety guidelines in the laboratory (biosafety level 1). Human embryonic kidney cells (HEK293 cells) and U937cellines (monocyte/macrophage) were obtained from DSMZ and fibroblasts were isolated from human foreskins kindly gifted by Institute of Pharmacy, Freie Universität Berlin. HEK293 cells and fibroblasts were cultured in Dulbecco Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10 % (v/v) FBS and 1 % penicillin by incubating with 5 % CO_2 at 37 °C under a humidified atmosphere. U937cellines (monocyte/macrophage) were cultured in Roswell Park Memorial Institute (RPMI) 164 media with 10 % (v/v) FBS and 1 % penicillin by incubating with 5% CO_2 at 37 °C under a humidified atmosphere. 24 h before the tests, all the cell lines were seeded separately in 96-well plates with 100 µL DMEM/RPMI with a density of 1×10^4 cells per well and incubated overnight at 37 °C. **LΔF**, **LΔF+fMLF (1:0.25)**, **LΔF+fMLF (1:0.5)**, **LΔF+fMLF (1:1)** hydrogels at 0.5 wt %, 1 wt % and 1.5 wt % were prepared and incubated in a cell culture hood overnight to allow for methanol evaporation. Subsequently, 50 µL of the gels each in triplicate were transferred to respective wells and then incubated for 24 hr. The control group consisted of cells treated with culture medium alone. Cell viability was determined using a CCK8 assay.

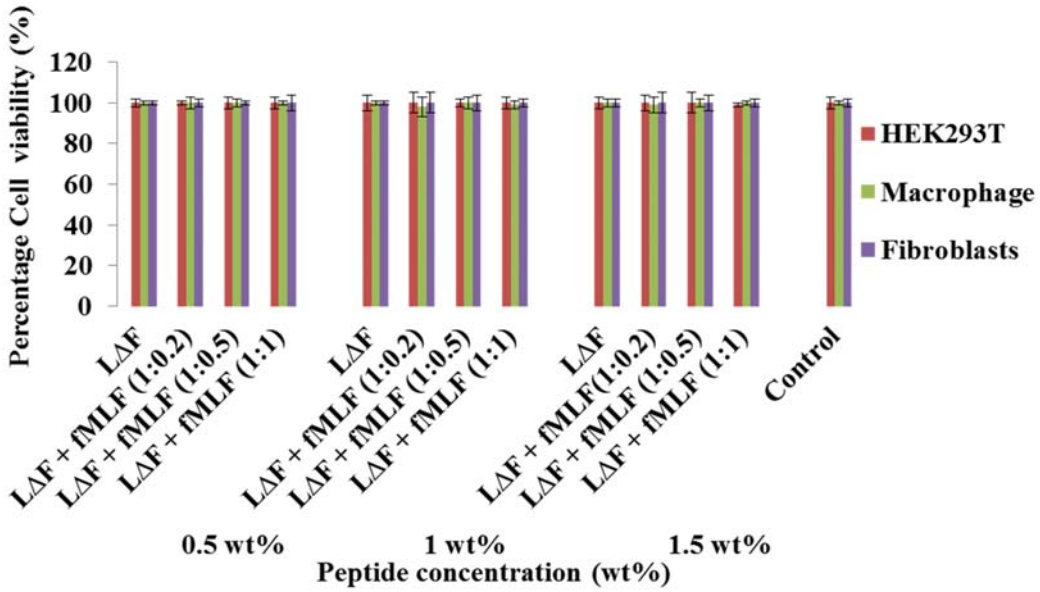


Figure S8. Cytotoxicity studies of LΔF, LΔF + fMLF (1:0.25), LΔF + fMLF (1:0.5), LΔF + fMLF (1:1) hydrogels at 0.5 wt%, 1 wt% and 1.5 wt% on HEK293T, macrophage and fibroblast cell lines using CCK8 assay.

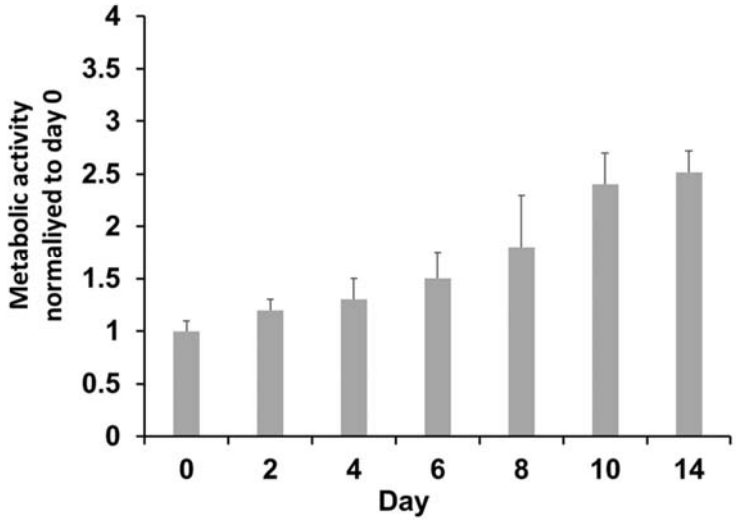


Figure S9. Quantification of cellular metabolism of fibroblast 3D cultures in 1 % w/v LΔF + fMLF (1:0.25) hydrogel using PrestoBlue assay as a function of cell culture time up to a duration of 14 days. The UV absorbance from each well was normalized to the initial day 0 level.

Drug Loading and Release

Drug loaded **1 % w/v LΔF+fMLF (1:0.25)** gels were prepared by separately incorporating the drugs (Isoniazid, Ciprofloxacin and Amphotericin-B) to a final concentration of 4 mM during gel formation. For the next 30 mins, the gels were kept undisturbed and then overlayed with PBS. At regular time intervals 2 hr, 4 hr, 8 hr, 16 hr, 24 hr, 48 hr, 72 hr, 96 hr and 120 hr, 1 mL of the overlaid PBS was withdrawn and replaced with fresh PBS. UV-Vis spectroscopy was used to monitor the amount of drug released into the overlaid PBS. The following equation was used to calculate the percentage release: percentage release (%) = (absorbance of drug released into overlaying PBS/absorbance of total drug amount encapsulated in gel) X 100.

Drug	Net Charge (pH 7)	Molecular mass (Da)	Clog P
Isoniazid	0	137.05	- 0.7
Ciprofloxacin	0	331.346	-0.57
Amphotericin B	0	923.48	0.8

The ClogP values of the drugs were obtained from "drug bank" and "pubchem".

Figure **S10**. Physicochemical features of the drugs studied here.

The percentage release of Interleukin-4 (IL-4) from **1 % w/v LΔF+fMLF (1:0.25)** gels loaded with 100ng IL-4 was monitored using the protocol described above (Figure-**S11**).

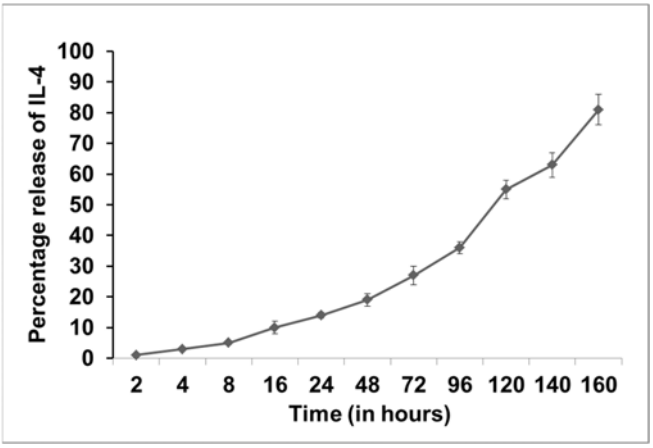


Figure **S11**. Percentage release of Interleukin-4 (IL-4) from 1 % w/v LΔF + fMLF (1:0.25) hydrogel for a duration of 160 hours.