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Supporting information

Stacking interactions by two Phe side chains stabilize and orient assemblies of even the minimal amphiphilic β-sheet motif

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Methods

Lyophilized peptide powders were custom synthesized and purified to >95%, by Caslo (Lyngby, Denmark). Unless otherwise specified, all reagents were purchased from Sigma–Aldrich (Rehovot, Israel) and were of the highest available purity. All solutions were prepared with deionized water (18.2 MΩ x cm, Direct Q-5 Merck Millipore, Billerica, MA).

Formation of peptide hydrogels

Phe-Glu-Phe lyophilized peptide powders were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at concentrations of 2% or 1% w/v (45mM and 22.5mM, respectively). Samples were then vortexed and placed in a sonication bath (Elmasonic S10, Elma, Singen, Germany) until full dissolution was observed. Next, the solutions were diluted with deionized water to 1% and 0.1% w/v (22.5 mM and 2.25 mM), respectively. The 1% w/v sample formed the hydrogel instantly while the 0.1% w/v sample gelated within ~1h. The gelation of Phe-Thr-Phe, Phe-Cys-Phe and Ac-Phe-Glu-Phe-NH_2 was initiated by dissolution of the peptides' powders in HFIP at concentrations of 8% w/v (the samples were vortexed and sonicated). Following the addition of deionized water to reach a peptide concentration of 4% w/v, hydrogels were obtained within several minutes. Phe-Lys-Phe at 5% w/v only partially dissolved in 0.1M KCl solution, yet on heating the sample for 10 min to 70°C in a water bath, a clear solution was obtained that gelated during cooling to room temperature. The pH of all

of the formed hydrogels was in the range 3.5–5, probably due to residual trifluoroacetic acid (TFA) used in the peptide synthesis and purification.

Peptide Titration

Peptide titration measurements were performed using a pH meter (CyberScan pH 510; Eutech Instruments, Thermo Scientific, Waltham, MA) equipped with a temperature probe. Freshly prepared Phe-Glu-Phe solutions (3 mL) at concentrations of 1, 5, 10 and 20 mM were obtained by first suspending the peptide powders in 2 ml deionized water followed by the addition of 10 µl aliquots of NaOH 1M until a pH~10.5 was reached. The volume of all samples was then adjusted to 3 ml by the addition of deionized water. Titration experiments were performed by titrating 0.1 M HCl aliquots to the basified peptide solution. During titration, the solutions were continuously stirred using a magnetic stirrer at room temperature. 10 µl of the 10 mM Phe-Glu-Phe solution were sampled during the titration onto carbon-coated copper grids (300 mesh, TED PELLA, CA, USA) that were previously glow discharged for 1 min. After 5 min incubation, the grids were blotted with Whatman 42.5 mm paper and the samples were then allowed to dry overnight. TEM micrographs were obtained on a 120KV Tecnai G12 TWIN microscope (FEI, Oregon, USA).

Secondary structure

The structure of the peptide assemblies were analysed by circular dichroism (CD), thioflavin T (ThT) assay and by Fourier transforms infrared (FTIR) and fluorescence spectroscopy. CD spectra in the 190–260 nm range were recorded at room temperature on a Jasco J-715 spectropolarimeter (Tokyo, Japan), using 1 mm or 0.1 mm quartz cuvettes (Starna, Essex, England). Spectrum points with HT exceeding 800 V were emitted. The influence of pH on the secondary structure was tested with the 5 mM Phe-Glu-Phe and the 1mM Phe-(Glu-Phe)$_2$ solutions prepared similarly as samples used in the titration experiments (see above).

ThT was used to evaluate the formation of β-sheet fibrillar structures as a function of concentrations of the Phe-Glu-Phe and Val-Glu-Val peptides. Upon binding to β-sheet assemblies ThT increases its fluorescent emission at 490 nm when excited at 440nm. Peptides samples, dissolved in 1:9 v/v HFIP:deionized water (200 µl) at varying concentrations of 0.22 - 2.2 mM , were supplemented with ThT solution (50 µl) at a final concentration of 0.1 mM (0.032 mg ml^{-1}). After 2 h of incubation at room temperature, ThT fluorescent emission spectra were recorded using a black 96-well plate on a microplate-reader set at excitation = 440 and emission = 490 nm (BioTek instruments, Winoosky, VT).

FTIR spectra were collected on a Nicolet iN10 FTIR microscope (Thermo Scientific) fitted with a liquid nitrogen cooled MCT detector. Droplets (10 µl) of Phe-Glu-Phe and Phe-(Glu-

Phe)$_2$ 2.2mM samples in 1:9 v/v HFIP:deionized water were placed on a ZnSe prism that was cooled quickly on a -80°C shelf and further lyophilized (LABCONCO-Triad, Labotal , Israel). Dried peptide samples were scanned in reflection mode with an incident infrared beam focused at the surface of the samples. Before each spectrum collection a background for the gold-coated disk was obtained. The spectra were recorded in the 1350–1850 cm^{-1} range. After data collection, automatic atmospheric suppression and baseline correction functions were applied.

Atomic Force Microscopy

Peptide solutions 0.1% w/v prepared as described above, were sampled 30 min after the addition of the deionized water to the peptide in HFIP solution. A 10 µl aliquot was placed on a freshly cleaved mica surface, and the sample allowed to dry overnight under ambient conditions. Tapping mode scans with a scan size of up to 10µm at a scan rate of 1 Hz were obtained at room temperature on a Digital Instrument Dimension 3100 AFM (Veeco, NY, USA) with a 100 µm scanner mounted on an active anti-vibration table. High-resolution images were acquired with micro-fabricated silicon oxide DLCS (Diamond-Like Carbon Spike) tip (Bruker, CA, USA).

Visualizing assemblies by Cryo-TEM

Direct imaging of Phe-Glu-Phe solutions at 0.1% w/v (1:9 v/v HFIP: deionized water) were obtained by cryo-TEM. Thissolution which forms a hydrogel in ~1h, was sampled every 15 min from the addition of the deionized water to the peptide in HFIP solution. The samples, 4 µl, were placed on a copper grid coated with a perforated lacy carbon 300 mesh (Ted Pella Inc., CA, USA) and blotted with a filter paper to form a thin liquid film of solution. These grids were then plunged into liquid ethane in an automatic plunger (Lieca EM GP). The vitrified specimens were transferred into liquid nitrogen for storage. The grids were scanned using a Tecnai 12 G2 TEM at 120kV (FEI, Oregon, USA) equipped with a cryo-holder maintained at -180 °C. Images were recorded on a slow-scan, cooled, charge-coupled device (CCD) camera (Gatan manufacturer). In order to minimize electron beam radiation damage, images were recorded with the Digital Micrograph software package at low-dose conditions. Fibril width (n = 11 images) and angle analysis was performed with ImageJ freeware (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/) with the following number of angles measured for each sample: Phe-Glu-Phe at 0 and 15 min, 157 and 387, respectively. Phe-(Glu-Phe)$_2$ at 0 and 15 min, 165 and 377, respectively.

Silver nanorod formation

Hydrogel of Phe-Glu-Phe 0.1% (1:9 v/v HFIP:deionized water) was disassembled by vortexing the sample until an apparently uniform solution was observed. To 250 µl of the peptide solution, 250 µl of 40 nm gold colloids (BBI Solutions, Cardiff, UK) were added. The gold-nanoparticles peptide solution was incubated for ~7h then supplemented with 10 µl, 1M AgNO3 and again incubated for 7h. Next, the reduction of silver was triggered by the addition of 10 µl, 1M ascorbic acid. Samples of 5 µl of the peptide-gold reduced silver solution were placed on a 300 mesh carbon coated copper grid (Ted Pella Inc., CA, USA) for 2 min and blotted with filter paper. TEM scans were performed with a Tecnai G2 TEM operating at 120 kV and for high resolution images JEOL JEM 2100F TEM operating at 200 keV.

Titration:

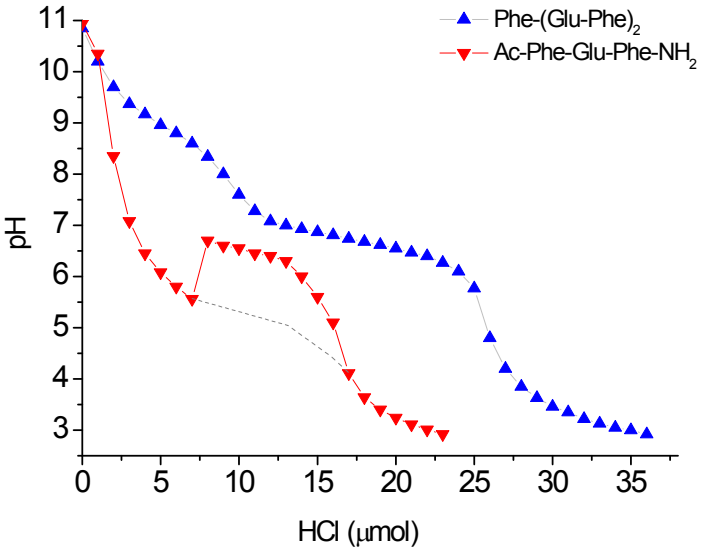


Figure S1: Titration curves of basified 3.3 mM Phe-(Glu-Phe)$_2$ and 5 mM Ac-Phe-Glu-Phe-NH$_2$ showing a pKa at ~8.5 only for the first peptide in accordance with the expectation that the acetylated analogue would lack this region. With the further addition of the acid another pKa region appeared at pH of 6-6.5 and at 6.8 for peptides Ac-Phe-Glu-Phe-NH$_2$ and Phe-(Glu-Phe)$_2$ respectively. Both these pKa values are positively offset relative to the free Glu side chain pKa value as a consequence of a cooperative effect between neighbouring Glu residues within the fibrillar assemblies. Peptide Ac-Phe-Glu-Phe-NH$_2$ shows a large positive increase in pH starting at ~ 5.5 similarly to the behavior of Phe-Glu-Phe at this pKa region, discussed in the main text.

Secondary Structure Analysis:

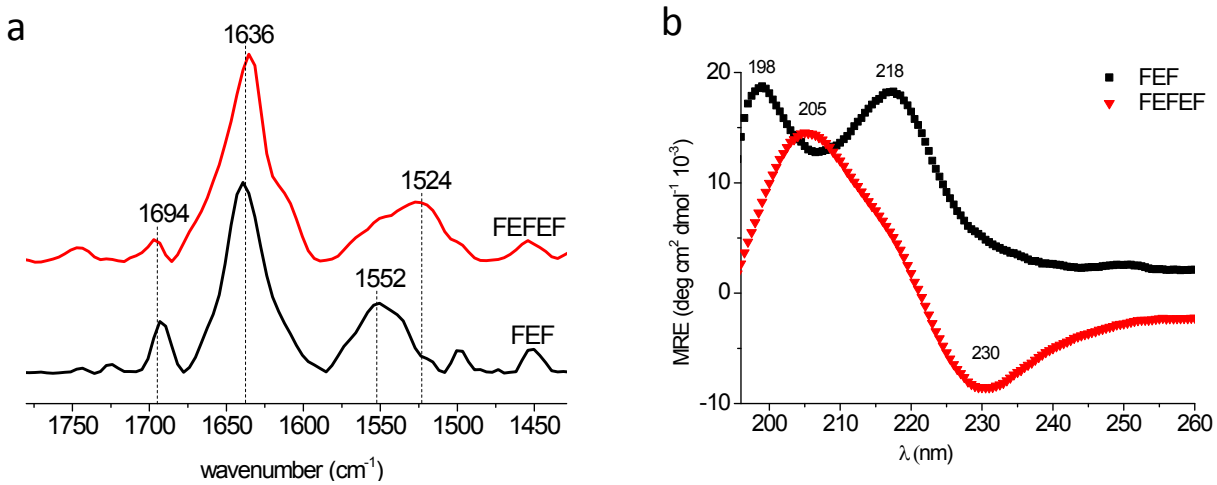


Figure S2: (a) FTIR spectra of lyophilized Phe-Glu-Phe and Phe-(Glu-Phe)$_2$ samples (2.2 mM in 1:9 v/v HFIP:deionized water) laid on ZnSe prism and (b) CD spectra of 1 mM Phe-Glu-Phe and Phe-(Glu-Phe)$_2$ solutions at 1:9 (v/v) HFIP:deionized water. These CD spectra are intended to demonstrate that the two peptides in the HFIP:deionized water solution used in the FTIR measurements, show CD spectra, hence similar conformation in solution, as those reported in the main text that were obtained by acidification.

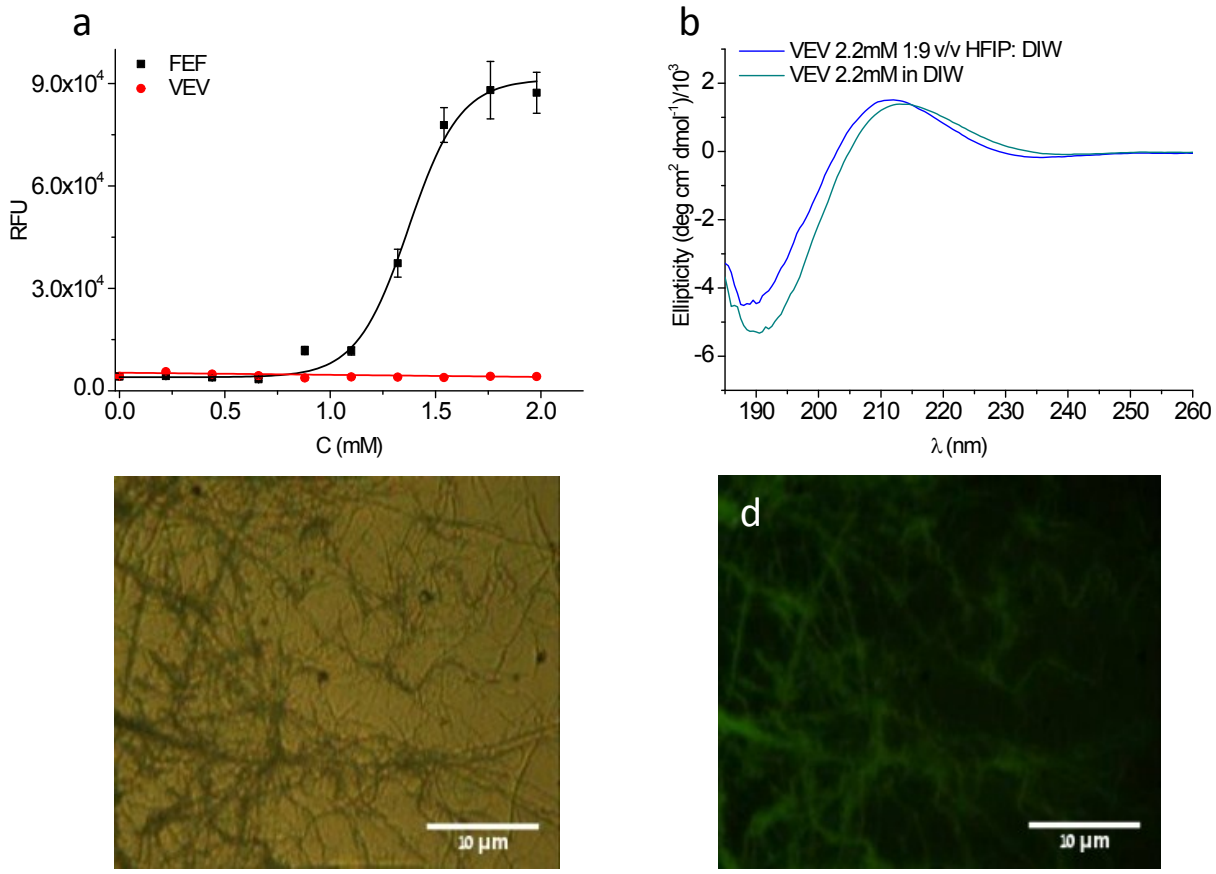
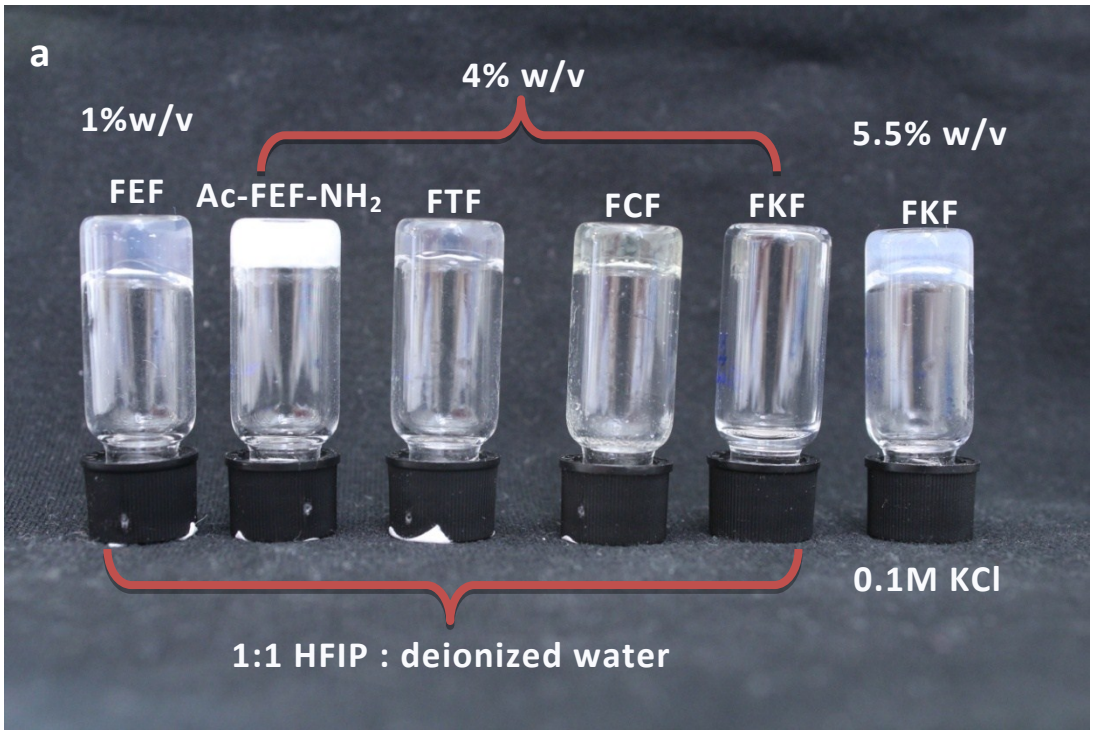


Figure S3: Characterization of fibrillar formation by (a) ThT fluorescence assay with increasing concentration of Phe-Glu-Phe and Val-Glu-Val peptides (at 1:9 v/v HFIP:deionized water (DIW)) and (b) CD spectra of Val-Glu-Val confirming no β-sheet formation at 2.2 mM concentration. Aliquot of Phe-Glu-Phe 2.2 mM solution stained with ThT was placed on a glass slide and viewed with a light microscope operating in bright field (c) and in fluorescence mode (d) showing the micrometre-long fibrils.

Phe-Glu-Phe was first dissolved in HFIP and further diluted with deionized water to overall ratio of 1:9 (v/v) HFIP:deionized water to a final concentration of 2.2 mM. Immediately after the addition of deionized water ThT was added at invariant concentration. In the same manner Val-Glu-Val was dissolved and supplemented with the ThT. This probe binds to β-sheet assemblies and as a result the fluorescent emission at λ=480 nm increases (λ_{exc} = 440 nm). The curve shown in figure S3a illustrates a sigmoidal behaviour of ThT fluorescence emission as a function of the Phe-Glu-Phe concentration with a critical value of β-sheet formation at 1.1 mM. The plateau observed for higher peptide concentrations is probably due to saturation of interactions between ThT and peptides assembled in β-sheets. Sample of the 2.2 mM Phe-Glu-Phe ThT bound was dried on a glass slide and viewed under a light

microscope. These results emphasize the crucial role of the aromatic diphenylalanine frame in stabilizing the fibril of the pleated β-sheet tripeptide.

Hydrogel formation:



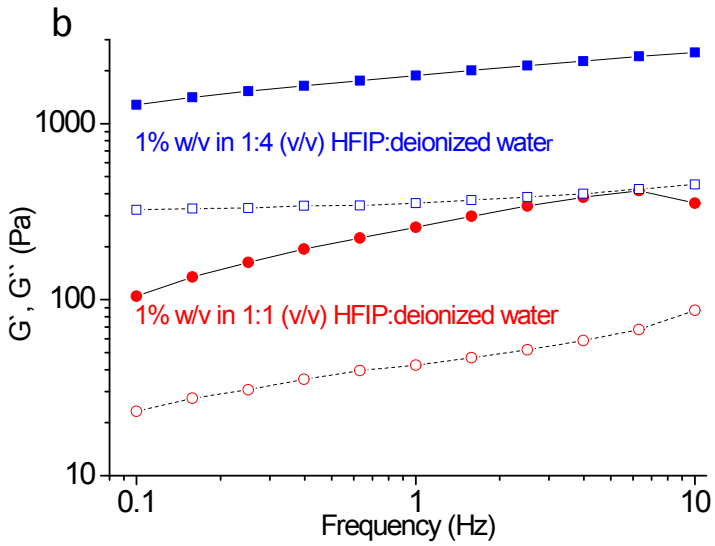


Figure S4: Hydrogel formation as determined by visual cessation of flow in inverted vials. Hydrogels were prepared as described in Experimental.(b) Rheology analysis of Phe-Glu-Phe hydrogels at different compositions of HFIP:deionized water illustrating both the storage G`, and loss, G``, moduli (solid and dashed lines, respectively).

Phenylalanine Fluorescence:

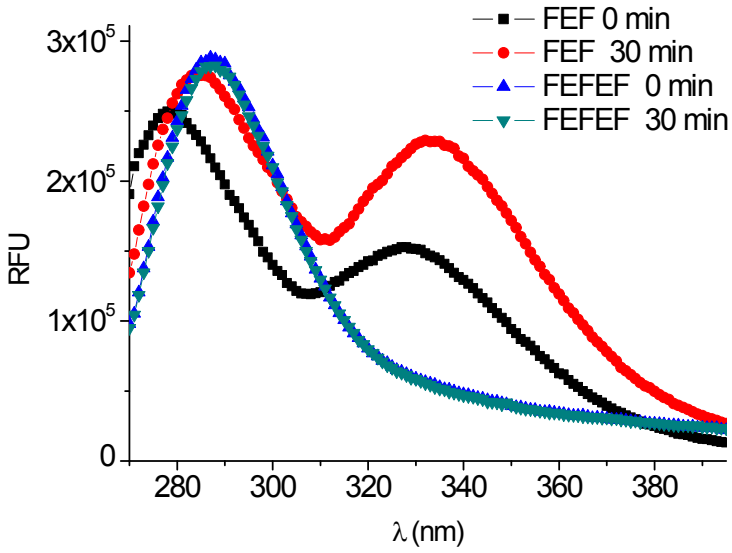


Figure S5: Fluorescence emission spectra (Exc=255 nm) of Phe-Glu-Phe (FEF) and Phe-(Glu-Phe)$_2$ (FEFEF) 5 and 3.33 mM (1:9 v/v HFIP:deionized water (DIW)), respectively, measured immediately after the subjection of peptides HFIP solutions to DIW and after 30 min.

The intrinsic fluorescence of phenylalanine benzyl moiety is highly dependent on the environment surrounding the residue and can reveal π-stacking interactions. Phe-(Glu-Phe)$_2$ at 3.33 mM shows a time-consistent peak at 288 nm. Phenylalanine equimolar concentration of Phe-Glu-Phe at 5 mM immediately after the addition of deionized water shows two peaks at 279 and 327 nm. Both peaks increased in intensity and underwent a red shift, over a course of 30 min, to 284 and 332 nm, respectively. The reason for the appearance of 332 nm peak is not clear as noted in several previously reported fluorescence measurements of short peptides containing more than one phenylalanine. Phenylalanine monomers have an emission peak at 280-282 nm and as suggested by Ahand and Mukherjee a red shift of several nanometres can indicate the formation of π−π stacking between adjacent residues (*Langmuir* **29**, 2713-2721 (2013)).

AFM Scans and analysis:

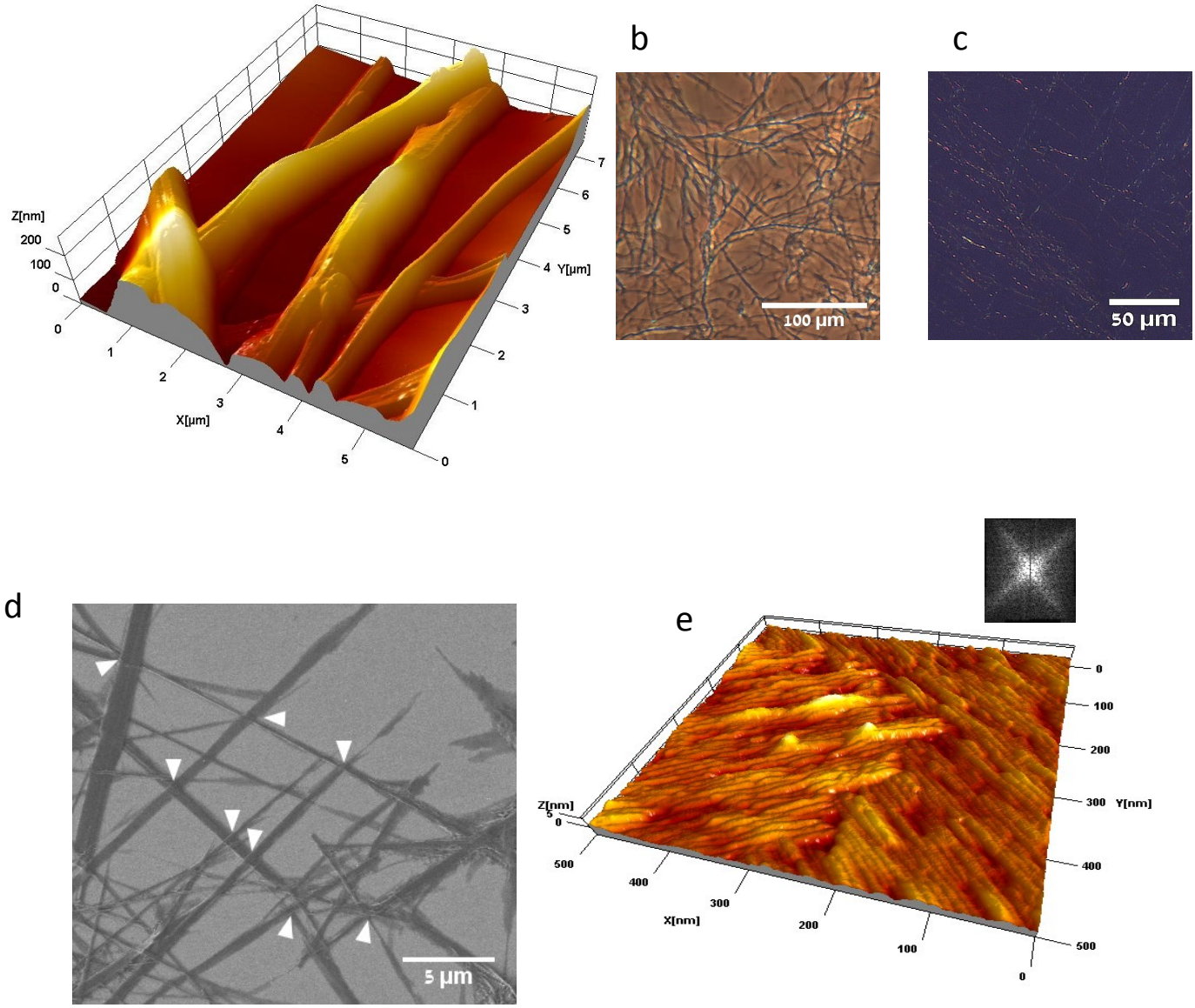


Figure S6: AFM scans of (a) Phe-Glu-Phe dried film (2.2 mM in 1:9 (v/v) HFIP:deionized water) on mica and light microscopy image of the sample obtained in (b) bright field and (c) between crossed polarizers demonstrating crystallinity along the fibrils' long axis.(d) The same sample as for (a) applied on Si wafer and imaged by SEM showing preferred angular alignment of several fibers (white arrows). (e) AFM scan of dried Phe-Thr-Phe (2.2 mM in 1:9 v/v HFIP:deionized water) on mica with the corresponding Fourier transform of the image showing neighbouring fibril domains aligned at ~85° angle similar to the angle observed between Phe-Glu-Phe domains in Fig. 2.

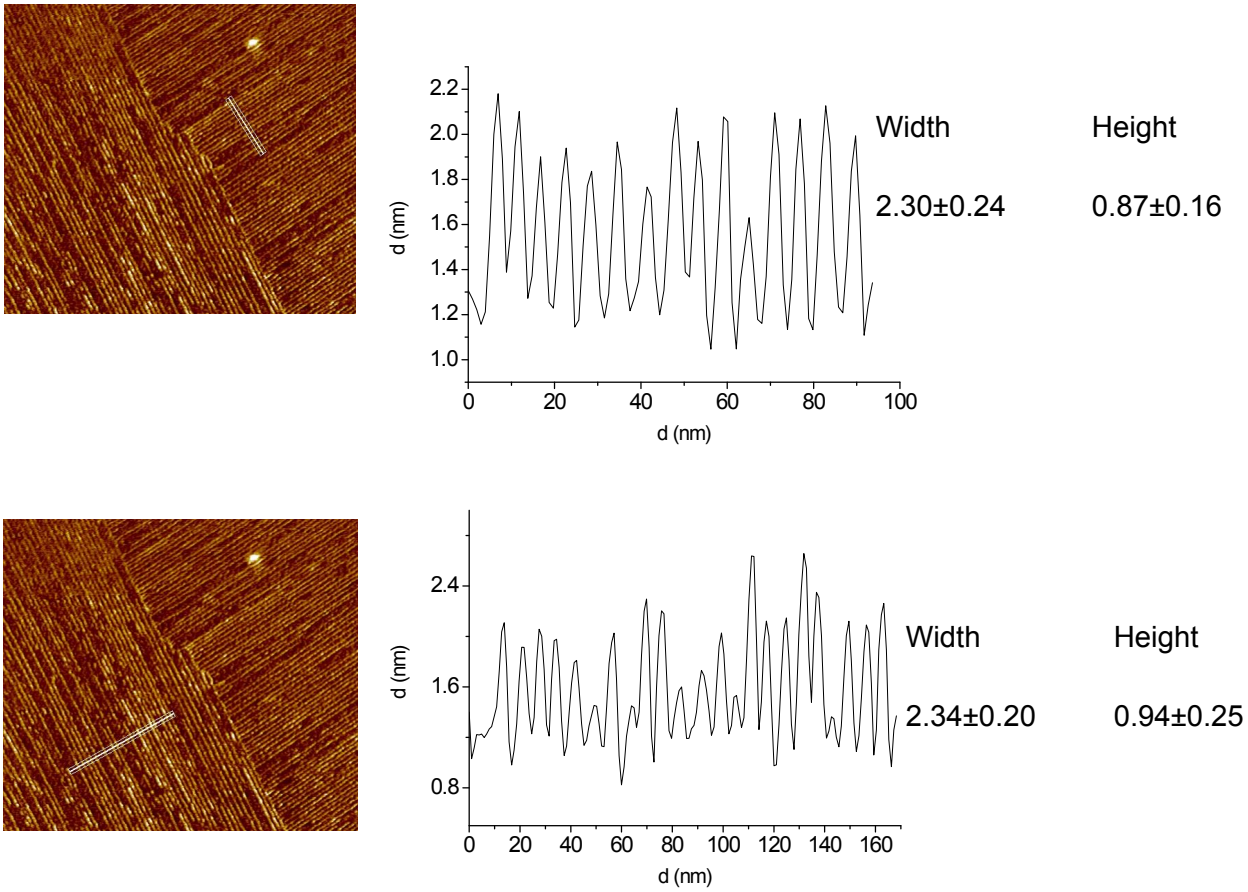


Figure S7: Topography AFM pattern of sample described in Fig. 2 showing peaks average FWHM in two regions corresponding to the fibrillar width. Standard deviation was calculated based on 15 peaks in each direction.

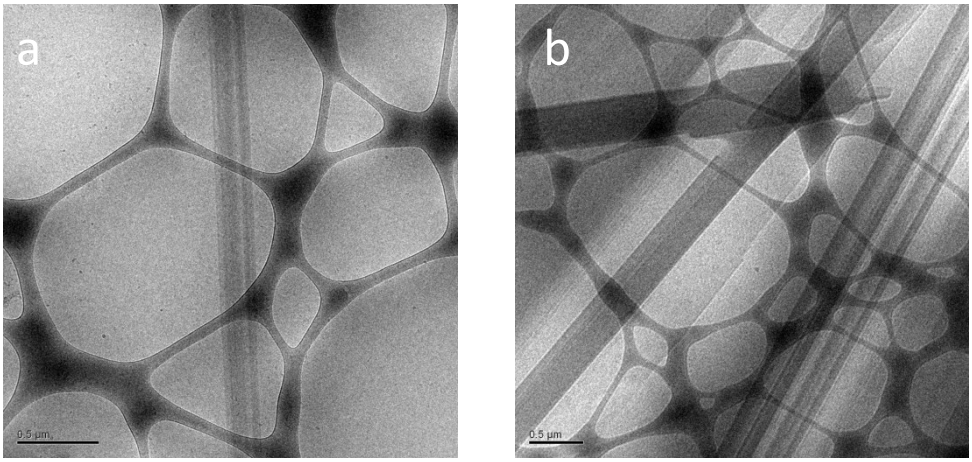


Figure S8: Cryogenic TEM images of Ac-Phe-Glu-Phe-NH2 peptide (2.2 mM in 1:9 v/v HFIP: deionized water) sampled at (a) 0 and (b) 15 min after the addition of deionized water demonstrating a lack of crossing and stemming fibrils. As oppose to the free termini analogue, this peptide shows excessive formation of elongated nanosheets. These differences in fibril morphologies point to the effect of peptide termini as well as Phe---Phe interactions over neighbouring peptide fibrils.

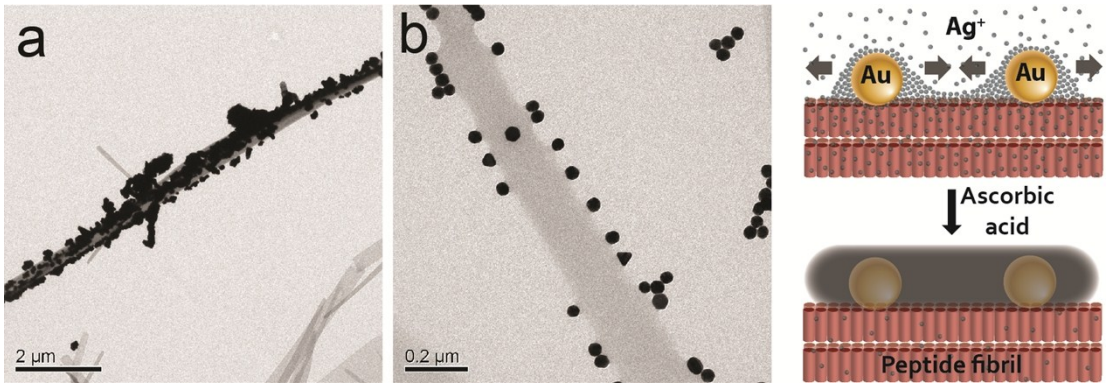


Figure S9: TEM images of peptide fibrils formed in 2.2 mM Phe-Glu-Phe decorated by (a) reduced silver submicron clusters and by (b) gold nanoparticles (dAu=40 nm).

Scheme: Hypothetical model for elongated silver nanorod single crystal formed by the co-nucleating effect of the anchored gold nanoparticles and the peptide fibrils.