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Supporting Information

Control over Multiple Nano- and Secondary Structures in Peptide Self-Assembly

Goutam Ghosh, Ranajit Barman, Anurag Mukherjee, Uttam Ghosh, Suhrit Ghosh,* and Gustavo Fernández**

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Table of Contents

1. Materials and methods

Chemicals and reagents: All the necessary chemicals and solvents were purchased from various companies such as Sigma-Aldrich (St. Louis, MO, USA), TCI Europe N.V. (Japan) or Alfa Aesar (USA). All the chemicals were used without further purification unless otherwise stated and the solvents were purified and dried following standard protocols before use.[1]

Mass spectrometry: MALDI TOF mass spectra were measured on a Bruker MicrOToF system.

CD spectroscopy: A JASCO J-1500 spectropolarimeter was used to perform CD experiments. This instrument is equipped with a Peltier module as a temperature controller. The experiments were carried out in buffer solutions at different pH values. The samples for CD were prepared by dissolving the solid peptide in an appropriate buffer solution and measured at various temperatures and concentrations in the wavelength range of 260–190 nm. Cuvettes with a path length of 1.0 cm were used. A scan speed of 500 nm/min and a response time of 2.0 s were selected. The spectra were averaged over three scans to minimize signal noise.

Fluorescence spectroscopy: Fluorescence spectra were recorded on a JASCO FP-8500 spectrofluorimeter equipped with the same water circulation unit. Sample preparation and variable temperature experiments for fluorescence spectroscopy were identical as for CD spectroscopy. The protocol for thioflavin T (ThT) assay was followed from recent reports by Ghosh et. al.[2]

FT-IR spectroscopy: FTIR spectra were collected on a PerkinElmer Spectrum 100 FTIR spectrometer. A solution of the peptide ($c = 5.0 \times 10^{-4}$ M) in D_2O (pH 7.4) was placed in a CaF_2 cell window with a 0.5 mm spacer. The spectra were recorded against the corresponding solvent background. The scans were between 1700 and 1600 cm^{-1}, with 500 accumulations at a resolution of 0.4 cm^{-1}.

Atomic Force Microscopy: AFM images were recorded on a Multimode® 8 SPM System (AXS Bruker). Silicon cantilevers with a nominal spring constant of 9 Nm^{-1} and with resonant frequency of ~150 kHz and a typical tip radius of 7 nm (OMCL-AC200TS, Olympus) were employed. To prepare the AFM samples, 10 µL of corresponding aggregated solution were drop-casted followed by spin coating at 1000 rpm onto mica surface. After that, the samples were kept for 24 h to air dry.

Angular Dependent Dynamic Light Scattering (AD-DLS): AD-DLS measurements were carried out using an ALV CGS-3 Compact Goniometer System equipped with an LSE-5004 Light Scattering Electronics (22 mW HeNe Laser (633 nm)) and Multiple Tau Digital Correlator unit. Prior to sample processing, solvents were filtered through 0.45 µm pore size nylon or Teflon filters.

Buffer solution preparation: PBS and acetate buffer were prepared by following standard protocols for pH 7.4 and 5.5 respectively. NaOH was added into Mili-Q water to make pH 13.0 solution and 0.15 M sodium chloride were used to avoid the influence of the ionic strength, and the pH values were precisely measured using a pH meter.

Hydrogel Preparation: To prepare hydrogels, desired amounts of **PEP-1** were taken into a screw-capped gel vial and 500 µL of 10.0 mM PBS buffer (pH 7.4) was added to achieve a final concentration of 5 mM. The solution was first sonicated for 2 min and then heated with a hot air gun until to yield a clear solution. The hot clear solution was allowed to cool down to room temperature. The transparent gelation was observed after 24 hrs and checked by the stable-to-inversion of a vial method. The gel was stable even after six months.

Rheometry: To investigate the mechanical strength of **PEP-1**, stress-amplitude sweep with hydrogels (C = 5 mM) in PBS buffer (pH 7.4) was measured by an advanced Rheometer AR 2000 (TA Instrument). The cone diameter, cone angle, and the plate gap were 40 mm, 4°, and 121 mm, respectively. The run took place at 25 °C and frequency of oscillation was constant at 1 Hz.

Differential Scanning Calorimetry (DSC): To examine the gel-to-sol transition temperature (T_{gel}) of the hydrogel (5 mM), a multicell differential scanning calorimeter from TA Instruments was used. The gel and solvent were placed in the corresponding sample and reference cells respectively. Cells were closed with their lids and recorded the heat change in 1 s intervals. The scans started with an isotherm at 20 °C for 5 min, followed by a 1 °C min^{-1} ramp up to 80 °C.

Bacterial Preparation. All the bacteria were cultured aerobically in Luria-Bertani broth/ agar at 37 ºC with gentle shaking at 180 rpm and bacteria of mid-logarithmic phase was used for all the antibacterial studies. Gram-positive *S. aureus* (ATCC 25923) and Gram-negative bacteria *E. coli* (ATCC 25922), were purchased from ATCC, USA.

Minimum Inhibitory Concentration (MIC) Determination. The minimum inhibitory concentration (MIC) was checked by a broth microdilution assay. A 2.0 mg/ mL concentrated peptide hydrogel solution was serially diluted to obtain different concentrations of the sample in LB broth. Then, 50 µL of bacterial suspension in LB (c = 1× 10^6 CFU/ mL) were added to each well of noncoated polypropylene 96-well plates. Next, incubation at 37 °C for 18 h took place with gentle shaking at 180 rpm. The MIC was considered as the lowest concentration of the peptide solution at which 100% reduction in growth was observed. Broth alone and broth containing only cells were considered as sterility control and growth control, respectively. All experiments were done in triplicate.

Hemolysis Assay. 1.0 mL of human blood drawn from a healthy donor was added in 9 mL of freshly prepared PBS buffer (pH 7.4) and centrifuged at 2000 rpm for 5 min to collect the RBCs. The supernatant was discarded and the pellet was resuspended in the same PBS. This washing was done for two additional times. Finally, the RBC pellet was suspended in 10 mL of volume and then four times dilution was done to prepare 2.5% (v/v) RBC solution. Then, a stock solution of peptide (c = 8 mg/ mL) was serially half-diluted in a sterile 96-well plate to obtain different concentrations of the peptide solution and 150 µL of RBC suspension 2.5 % (v/v) was added to each well and incubated at 37 °C with shaking at 180 rpm for 2 h. Triton X-100 (0.1% v/v in water) and PBS solution were used as the positive and negative lysis control, respectively. After the incubation, the supernatant (100 µL) from each well was transferred to a sterile 96-well flat-bottom polystyrene plate and the absorbance was checked at 414 nm using a Varioskan microplate reader (Thermo Fisher). The percentage of hemolysis was estimated with respect to the positive control and negative control solvents.

Cytotoxicity Assay Using MTT. HeLa cells and non-cancerous HEK-293 (ATCC) cells were cultured in DMEM medium and 10000 cells were seeded in each well of a 96-well plate and incubated for 24 h. Then, the medium was replaced by the different concentrated peptide solution and incubated for 24 h and 48 h. Then, 50 µL of freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (c = 5 mg/ mL in DMEM) was carefully added into each well and incubated for 4 h. After that, the medium with MTT solution was carefully removed and 100 µL of DMSO was added into each well, and the plate was gently shaken for 5 min at room temperature to dissolve all formed precipitates. The absorbance at 570 nm was measured by a microplate reader (VARIOSKAN, Thermo Fisher). Cell viability was calculated by the ratio of absolute absorbance of cells incubated with the peptide solution and cells incubated with only culture medium.

Scanning Electron Microscopy (SEM). 500 µL of bacterial suspensions (1×10^9 CFU/ mL) in LB broth were mixed with 500 µL of hydrogel (C = 5 x 10^{-3} M) and the solution was incubated for 6 h at 37 °C with gentle shaking at 180 rpm. After that, the solution was centrifuged at 4000 rpm for 10 min and precipitates were washed with 0.85% NaCl solution twice and then it was resuspended in 2.5% glutaraldehyde in PBS incubated at room temperature for 30 min and overnight at 4 °C. Then, the cell pellets were collected and washed with DI water twice. After that, the samples were dehydrated in different ethanol grades (30, 50, 70, 80, 90, and 100%, 15 min each step). Then, 5.0 µL of cell suspension in 100 % ethanol were spread on glass plates and 1.5 min gold spattering was done before capturing the images. The images were captured using a JEOL-JSM-7500F field-emission scanning electron microscope at 5.0 kV. The control experiments were performed with live bacteria and incubated with only media (without hydrogel) and samples were prepared following a similar procedure.

2. Details of Biocompatibility and Antimicrobial Activity

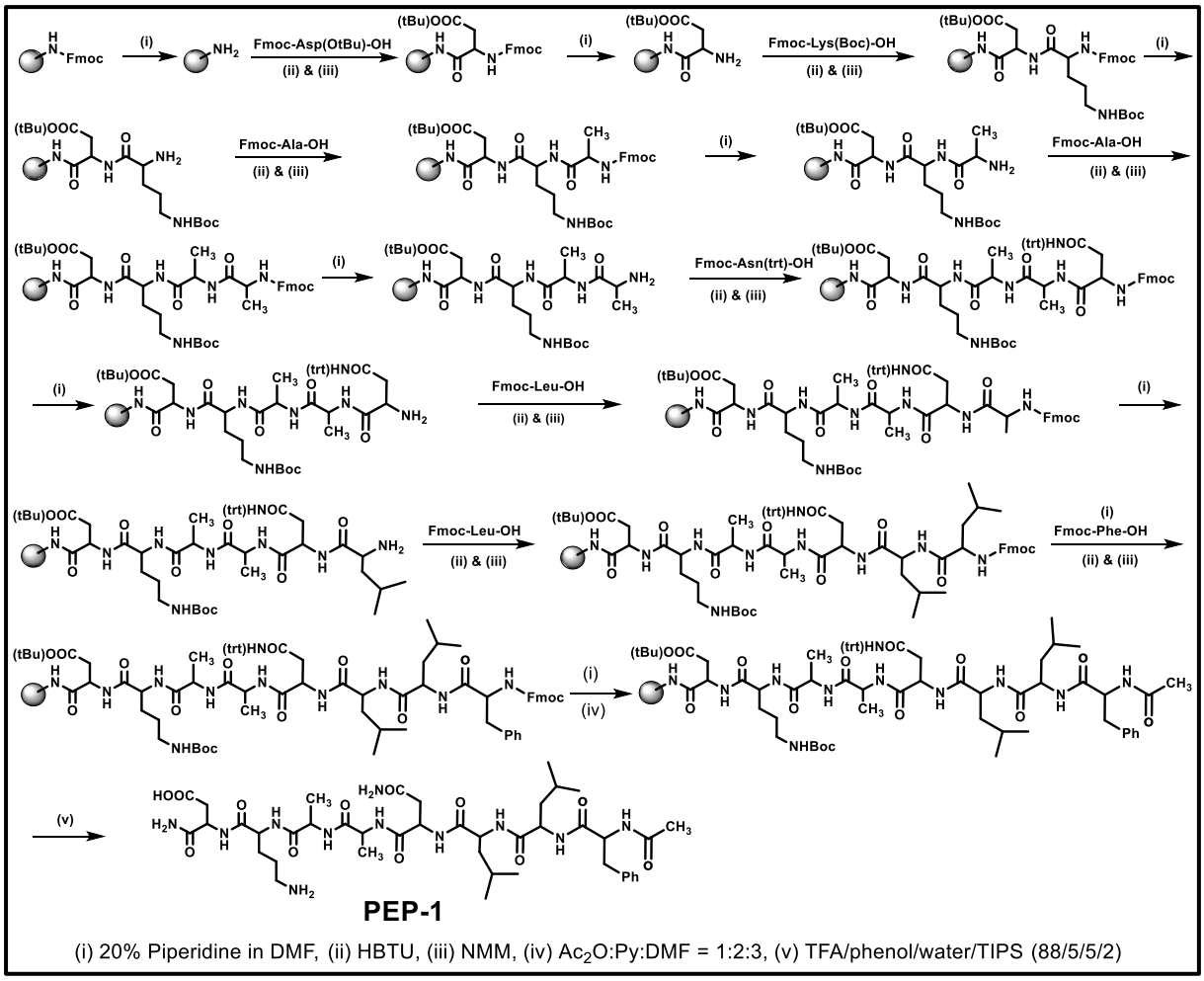
The peptide hydrogel was screened for antibacterial activity against two different clinically relevant bacterial strains, Gram-negative *E. coli* and Gram-positive *S. aureus*. The antibacterial activity was expressed in term of minimum inhibitory concentration (MIC) which was determined using different planktonic bacteria in microdilution assay following the

recommended procedure of the Clinical and Laboratory Standards Institute (CLSI) with some modification. Luria-Bertani broth and agar were used for bacteria culture and for all antibacterial experiments. The MIC data (Table S1) showed that the hydrogel is lethal for both Gram negative and Gram-positive bacteria with the MIC concentration 0.31-.62 mM. The peptide hydrogel exhibits inherent antibacterial activity due to the presence of cationic segment (lysine) and phenyl alanine or leucine as hydrophobic segments. Recently, antibacterial peptide (AMPs) hydrogel are used as a conceivable source of panacea for the treatment of antibiotic-resistant bacterial infections due to their strong antibacterial activities against a very broad spectrum of microorganisms with minimum toxicity. The toxicity profile towards mammalian cells was checked by hemolysis assay and MTT assay which showed that the hydrogel is not at all toxic. The hemolysis assay was performed with human red blood cells (RBCs) which revealed that < 10 % lysis was observed up to 2 mg/ mL peptide concentration whereas 100 % lysis was noticed for 0.1% triton x 100 which was used as positive control. Cell viability was checked by MTT assay using HeLa and HEK-293 (ATCC) cell line for 24 h and 48 h time point. The cell viability study revealed that the hydrogel is up to 85-95 % cell viable at 2 mg/ mL concentration after 48 h incubation.

The bacterial killing mechanism of the hydrogel is similar to the function of AMPs. The electrostatic interactions between the cationic heads of lysine moieties and anionic bacterial membranes facilitate the binding/ trapping of bacteria in the hydrogel resulting in the physical destruction of membrane structures and cell death. The membrane disruption can also be visualized by FESEM images of bacteria after treatment with hydrogel. Bacterial suspension was incubated with hydrogel ($C = 5 \times 10^{-3}$ M) for 6 h and after that images were captured. The images showed that the cell membrane of both the *E. coli* and *S. aureus* became wrinkled and rough and cell morphologies became irregular whereas for live bacteria (without hydrogel) smooth and regular morphologies were observed. Thus, FESEM studies reveal that the bacteria can be attached/ trapped into the hydrogel network by electrostatic interactions, followed by membrane disruption which led to cell death.

3. Synthetic procedure

Synthesis of PEP-1: The octa-peptide was synthesized (Scheme S1) manually on solid phase using a special glass apparatus under nitrogen atmosphere employing Fmoc chemistry. The detailed standard protocols were followed from a recent report by Ghosh et. al.[2] Fmoc-protected rink amide resin (0.45 g, 0.5 mmol) was used as solid support. At first, resin was soaked in 10 ml of DMF for two hours and then transferred to the special apparatus. Four different steps were followed to obtain the peptide such as: i) deprotection of Fmoc group by 20% piperidine in DMF, ii) After deprotection of Fmoc- group, coupling of Fmoc-protected amino acid (2 mM) was performed by using HBTU (2 mM, 0.76 g) as coupling agent and N-methylmorpholine (4 mM) as base. The same procedures were repeated until the desired peptide was obtained. Cleavage of the final peptide was performed by applying the cocktail (TFA/phenol/water/TIPS 88/5/5/2). The solution was drained off and concentrated to dryness in a round-bottom flask. The peptide was washed several times with cold ether. The crude peptide was purified by RP-HPLC (Figure S1) using solvent (water and acetonitrile) gradient method. After collecting the desired fraction of peptide solutions, evaporated the acetonitrile by rotary evaporator and the final water solution was lyophilized to obtain the pure peptide as white solid. The purity of **PEP-1** was confirmed by RP-HPLC (Figure S1) which revealed a single peak suggesting the pure peptide which was further checked by MALDI-TOF mass spectrometry. m/z 933.62 $[M + 2H]^+$, calculated for $C_{43}H_{71}N_{11}O_{12}$ = 933.53 (Figure S2).



Scheme S1. Synthetic procedure of **PEP-1** on solid phase.

4. Additional figures

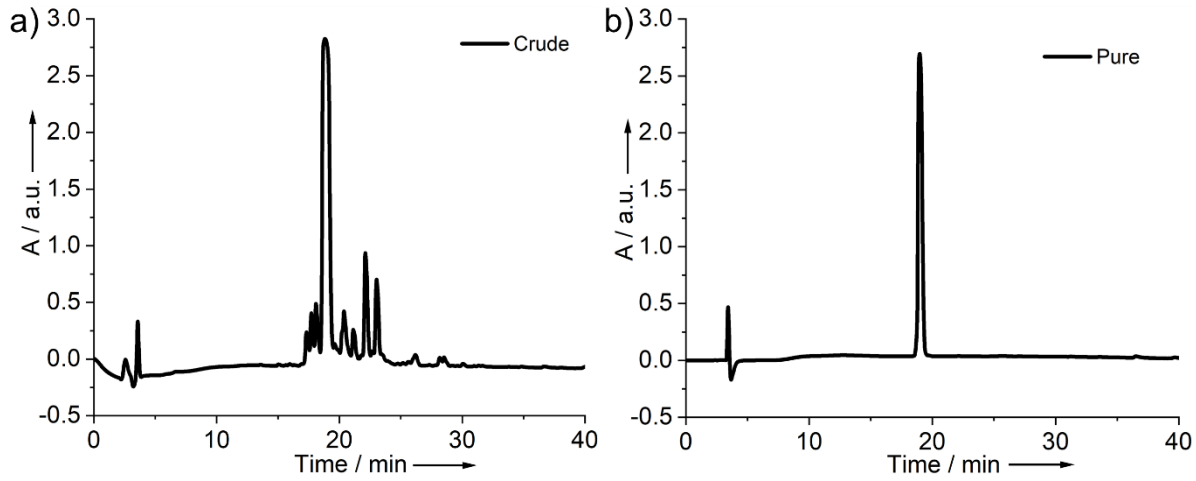


Figure S1. Reverse phase HPLC of a) crude and b) pure **PEP-1**.

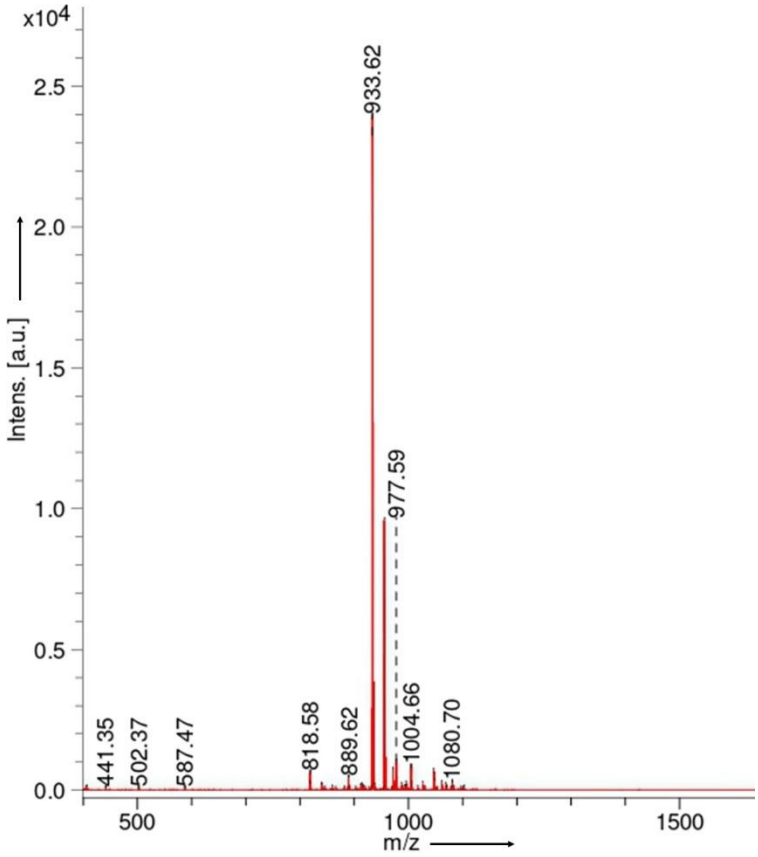


Figure S2. MALDI-TOF mass spectrum of **PEP-1**. Calculated m/z = 931.51 and observed m/z = 931.60 [M-2H$^+$].

Figure S3. Image of transparent hydrogel **PEP-1** at *C* = 5 x 10^{-3} M.

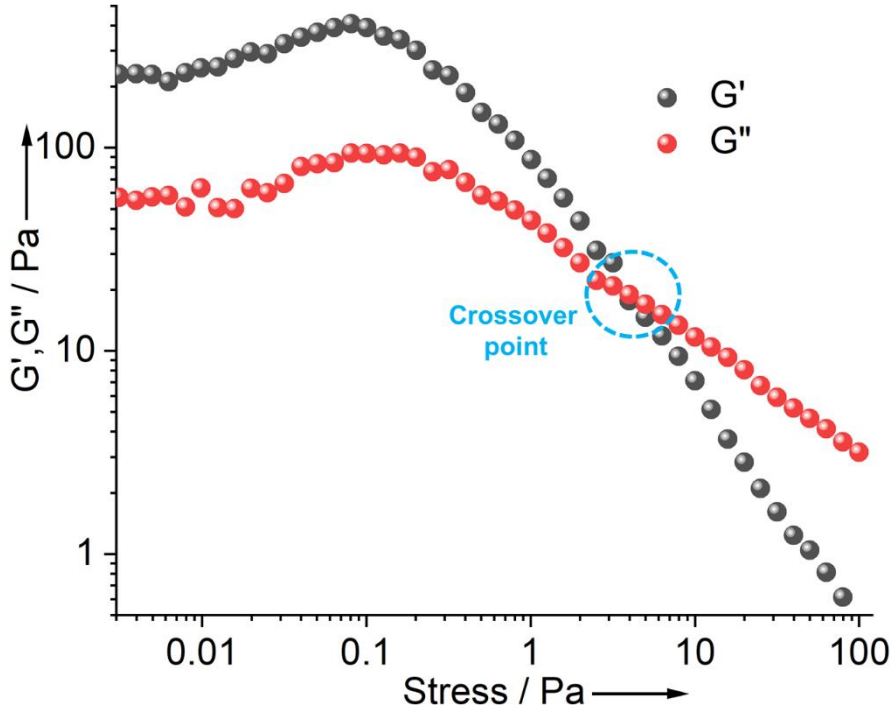


Figure S4. Rheological study of the **PEP-1** hydrogel in PBS (pH = 7.4) buffer at *C* = 5 x 10^{-3} M.

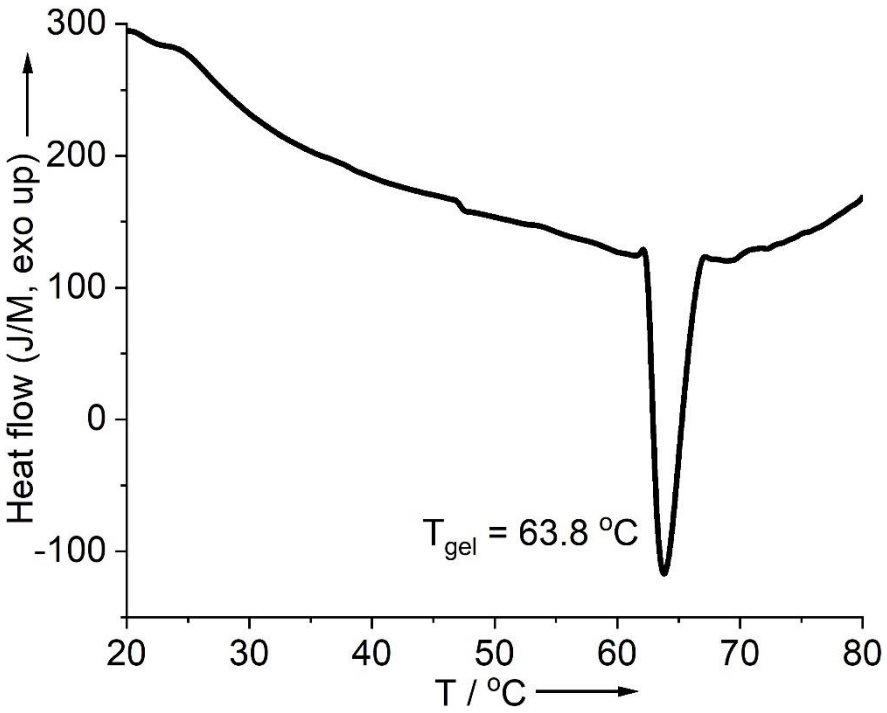


Figure S5. Differential scanning calorimetry (DSC) thermogram (heating) of the **PEP-1** hydrogel [C = 5 x 10^{-3} M]. DSC was used to investigate gel-to-sol transition (T_{gel}) of the hydrogel which revealed an endothermic peak at ~64 °C with ΔH = 1.9 J g^{-1} suggesting the disruption of the gel phase.

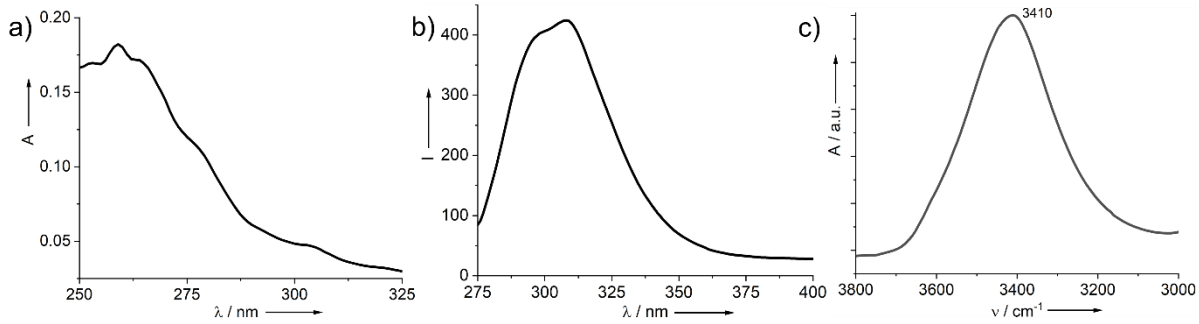


Figure S6. a) UV/Vis, b) fluorescence and c) FT-IR spectra of **PEP-1**. [C = 5 x 10^{-4} M]. To probe π-stacking interactions among the aromatic units, UV/Vis and fluorescence spectroscopy studies were conducted. The observed bands at 258 and 308 nm in UV/Vis and fluorescence spectra respectively confirmed π–π stacking among the phenyl alanine residues.[2, 3] The N-H band at 3410 cm^{-1} in the FT-IR spectrum further demonstrated the intermolecularly H-bonded state.[2, 4]

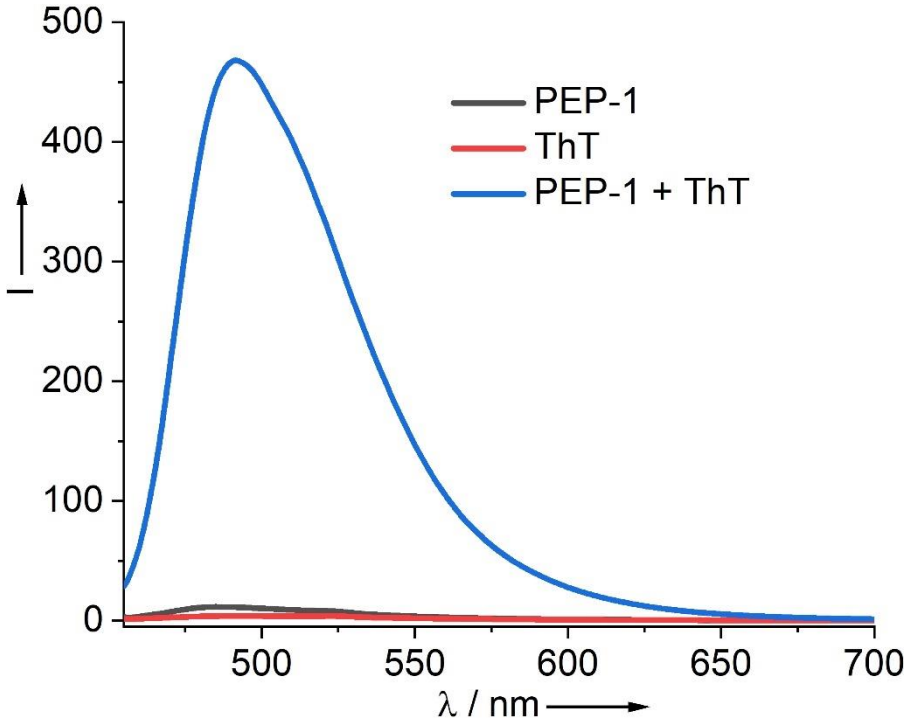


Figure S7. ThT fluorescence assay of **PEP-1** [*C* = 5 x 10^{-4} M]. Thioflavin T (ThT) fluorescence assay was performed to confirm the formation of β-sheet amyloid type fibrils as ThT is a widely used amyloid-specific fluorescent dye[5] which can bind specifically to the multistranded β-sheets. Upon excitation at 440 nm in PBS (pH = 7.4), the emission intensity of β-sheet bounded ThT increases severals orders of magnitude in comparison to free ThT, further confirming the formation of amyloid β-sheet structure as also indicated by the CD and FT-IR results.

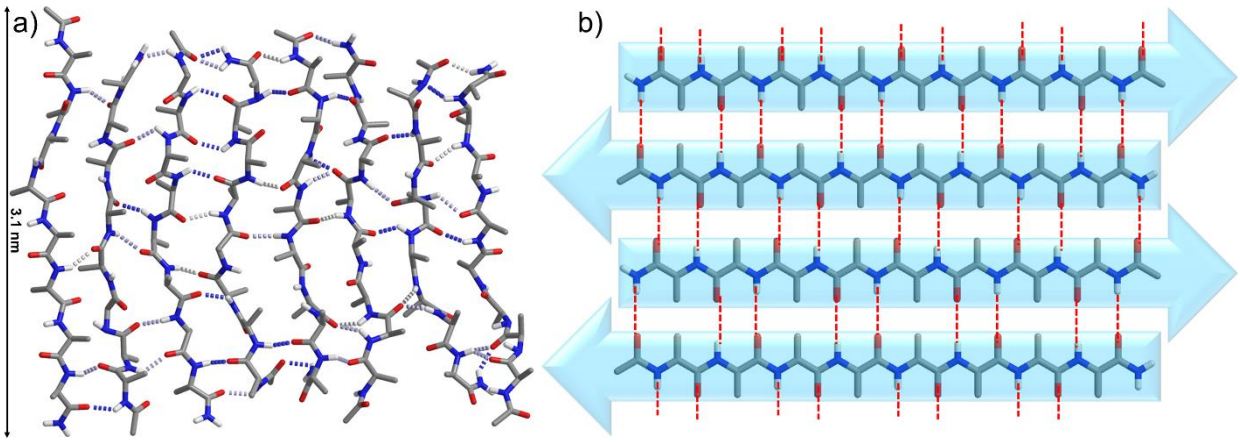


Figure S8. a) Proposed packing mode formation of nanofibers as well as β-sheet structures by intermolecular H-bonding (MM2 calculations using Chem3D 20.1); b) Schematic representation of the formation of antiparallel β-sheets.

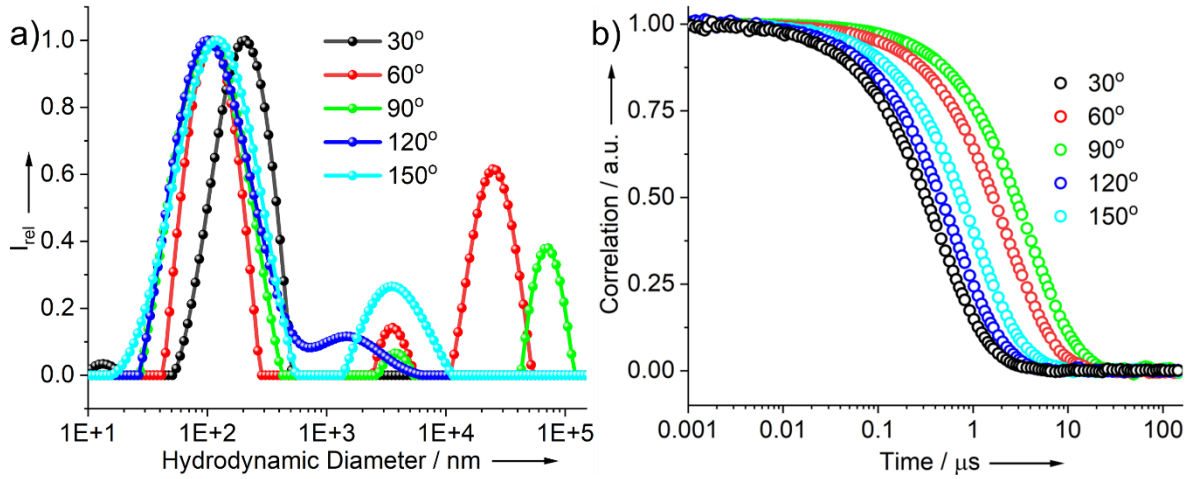


Figure S9. Angular-dependent DLS studies. A) Size distribution; b) corresponding correlation function for **PEP-1** at pH 7.4 at RT [*C* = 5 x 10^{-4} M].

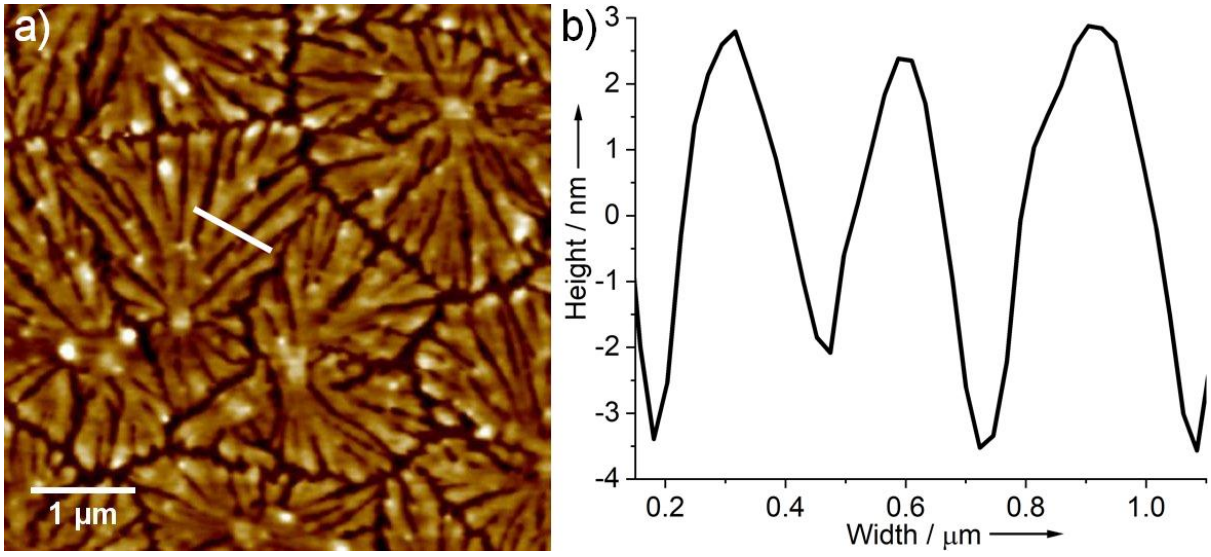


Figure S10. a) AFM image of **PEP-1** at pH 13.0 and b) height profile of the cross sectioned area in (a). [*C* = 5 x 10^{-4} M]

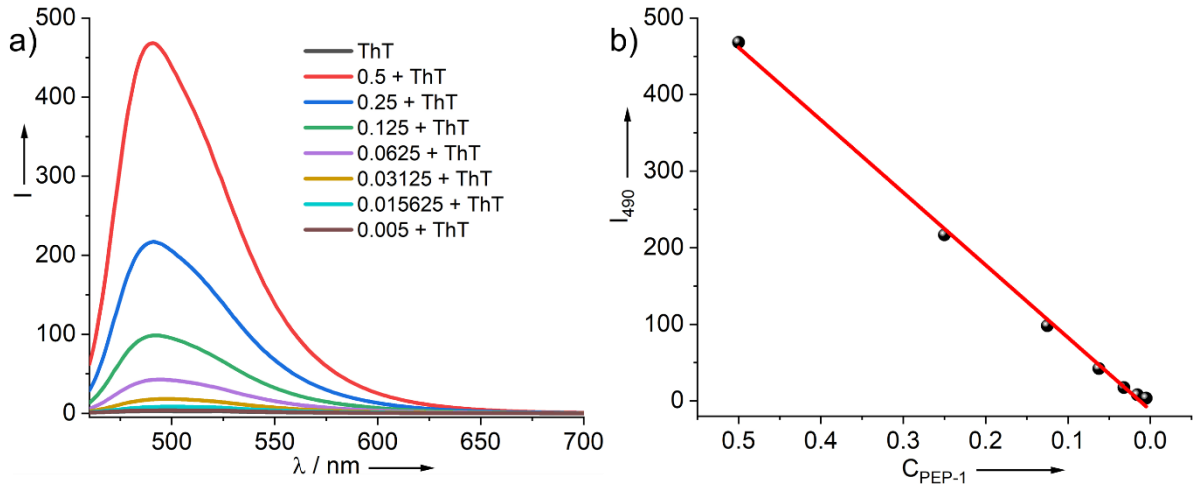


Figure S11. Concentration-dependent ThT fluorescence assay of **PEP-1**. In this experiment, the amount of ThT remained identical for all spectra. b) plot of I_{490} vs concentration of **PEP-1** indicating destruction of β-sheet structure upon lowering the concentration.

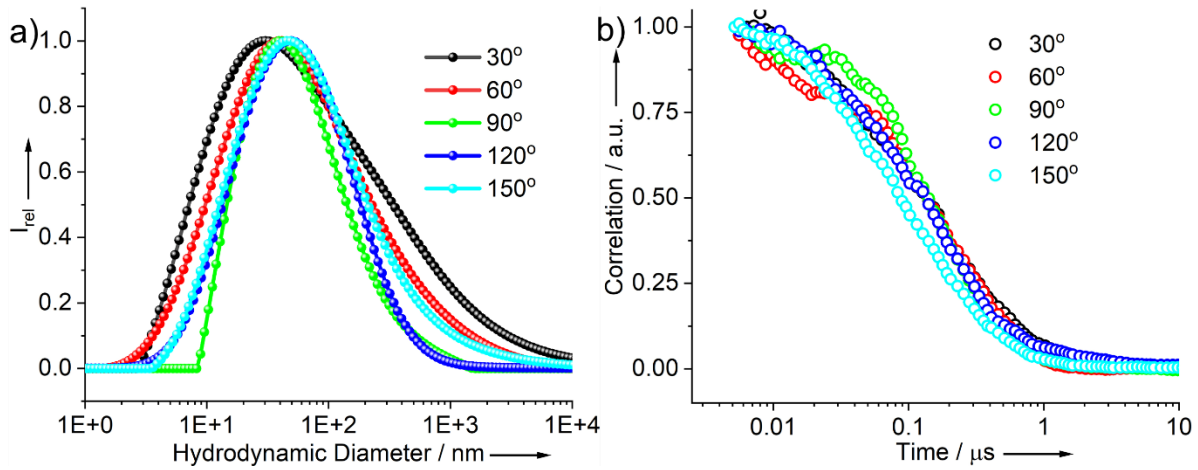


Figure S12. Angular-dependent DLS studies. A) Size distribution; b) corresponding correlation function for **PEP-1** at pH 7.4 at RT [C = 5 x 10^{-5} M]

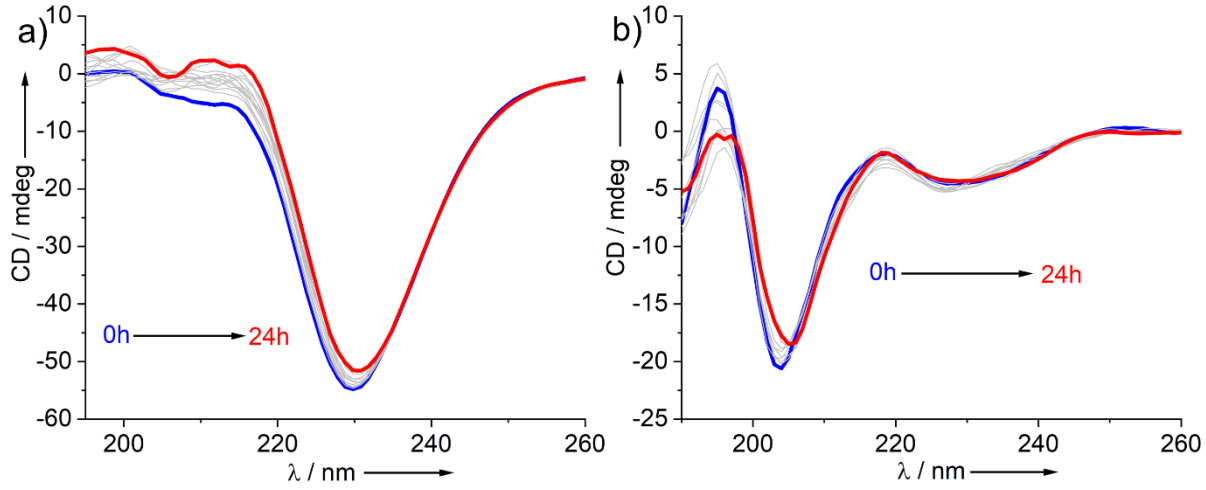


Figure S13. Time-dependent CD spectra of **PEP-1** at a) C = 5 x 10^{-4} M and b) C = 5 x 10^{-5} M at RT.

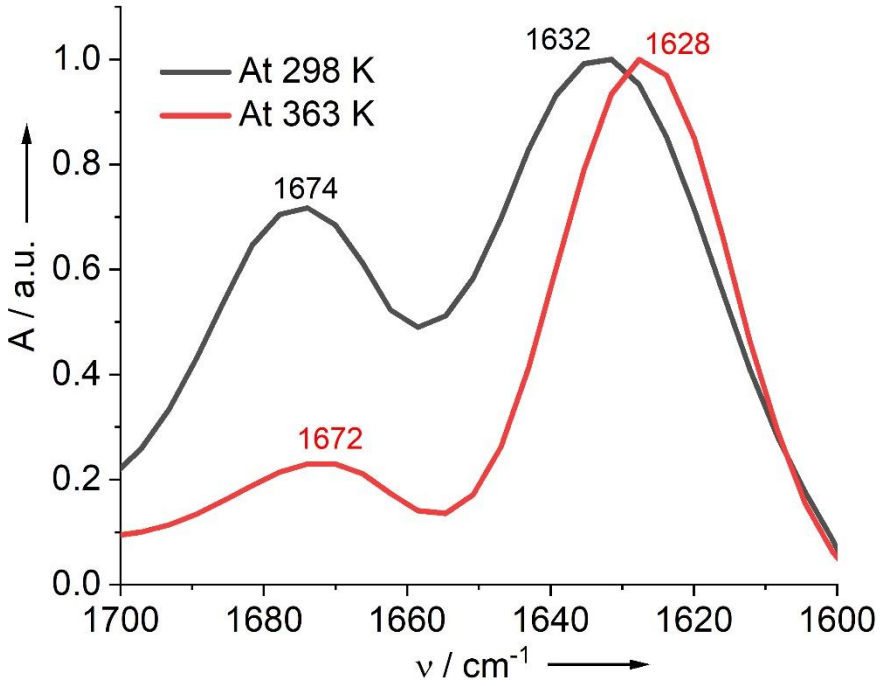


Figure S14. Temperature-dependent FT-IR spectra of **PEP-1** at pH 7.4. [C = 5 x 10^{-4} M]

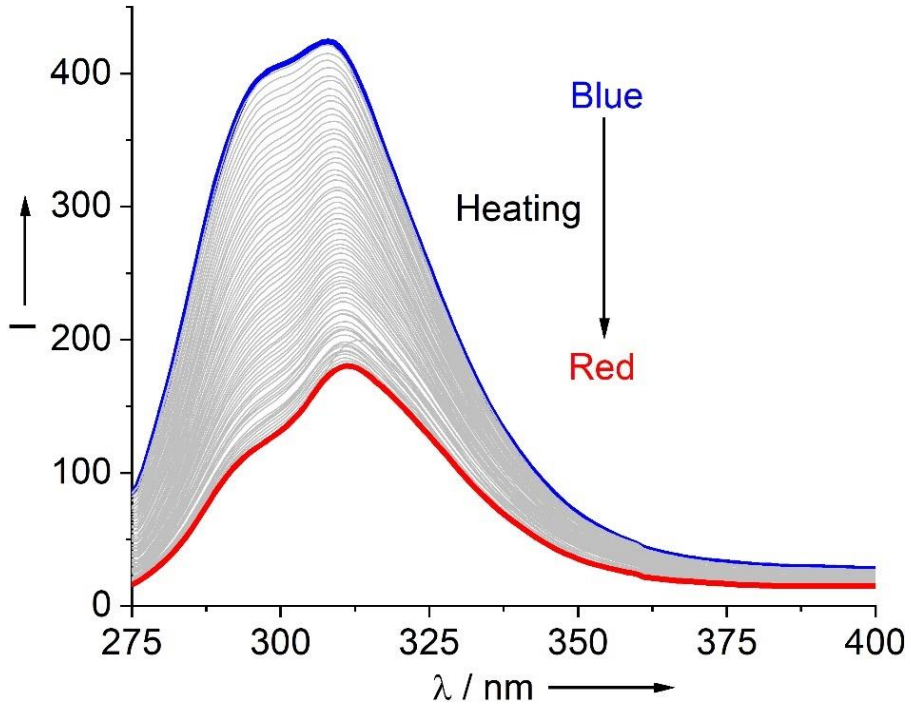


Figure S15. Temperature-dependent fluorescence spectra of **PEP-1**. [*C* = 5 x 10^{-4} M]

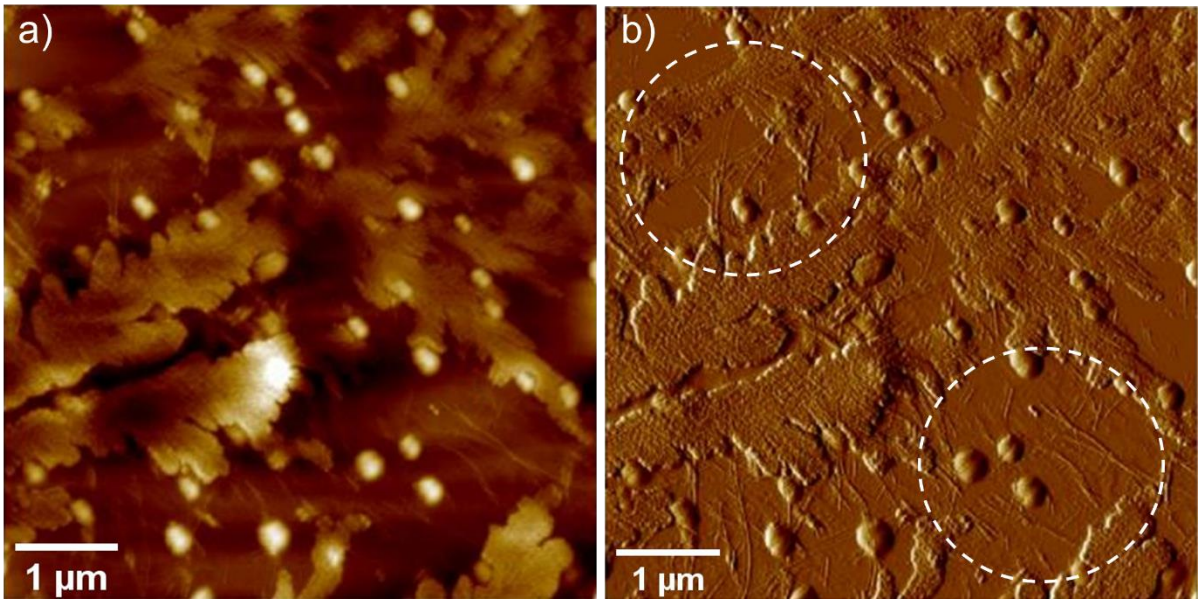


Figure S16. Zoomed AFM image of **PEP-1** at pH 7.4 at 363 K; a) height image; b) corresponding phase image. The circled areas indicate the presence of isolated fibers and the sheets are formed upon lateral interactions among fibers [*C* = 5 x 10^{-4} M].

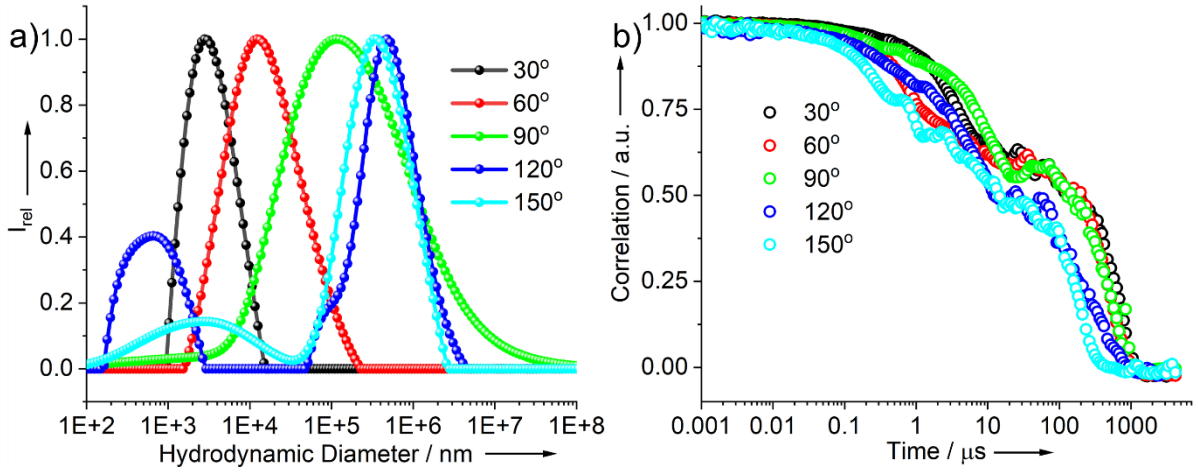


Figure S17. Angular-dependent DLS studies. a) Size distribution; b) correlation function for **PEP-1** at pH 7.4 at 363 K [*C* = 5 x 10^{-4} M].

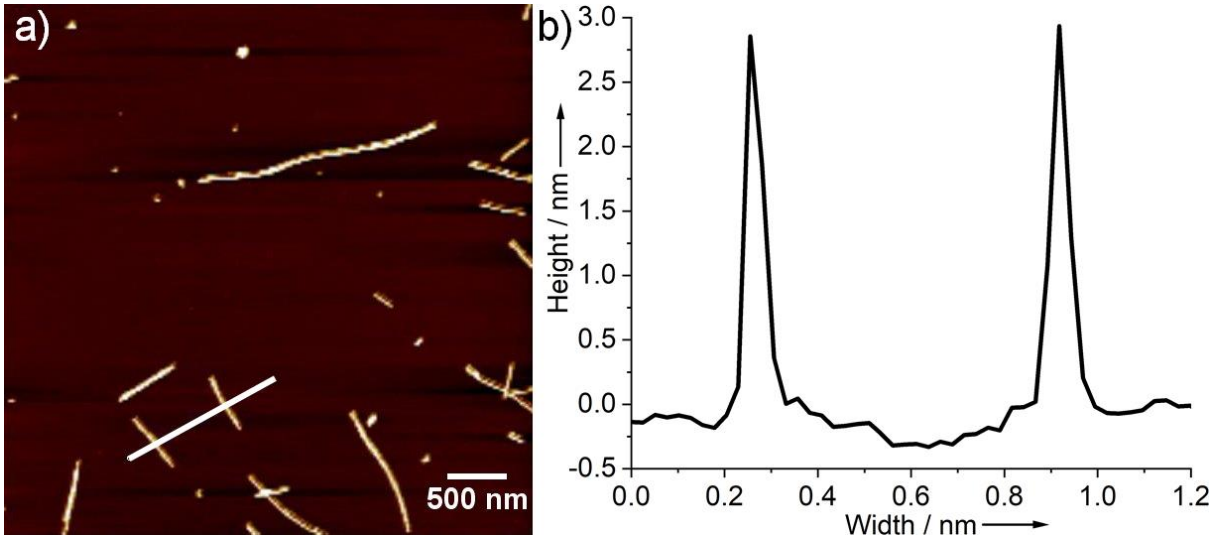


Figure S18. a) AFM image of **PEP-1** at pH 7.4 at 363 K and b) height profile of the cross sectioned area in (a). [*C* = 5 x 10^{-5} M]

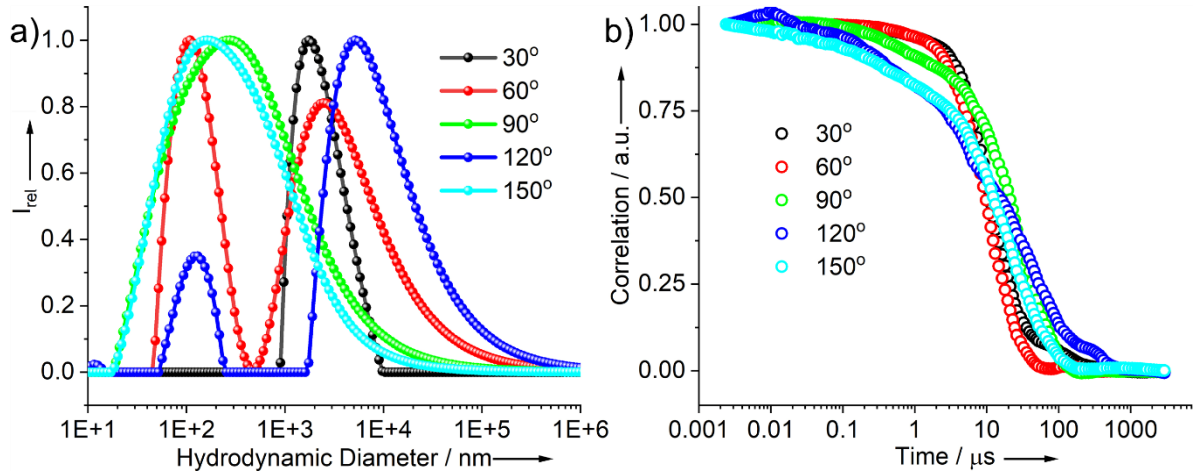


Figure S19. Angular-dependent DLS studies. A) Size distribution; b) correlation function for **PEP-1** at pH 7.4 at 363 K [*C* = 5 x 10^{-5} M].

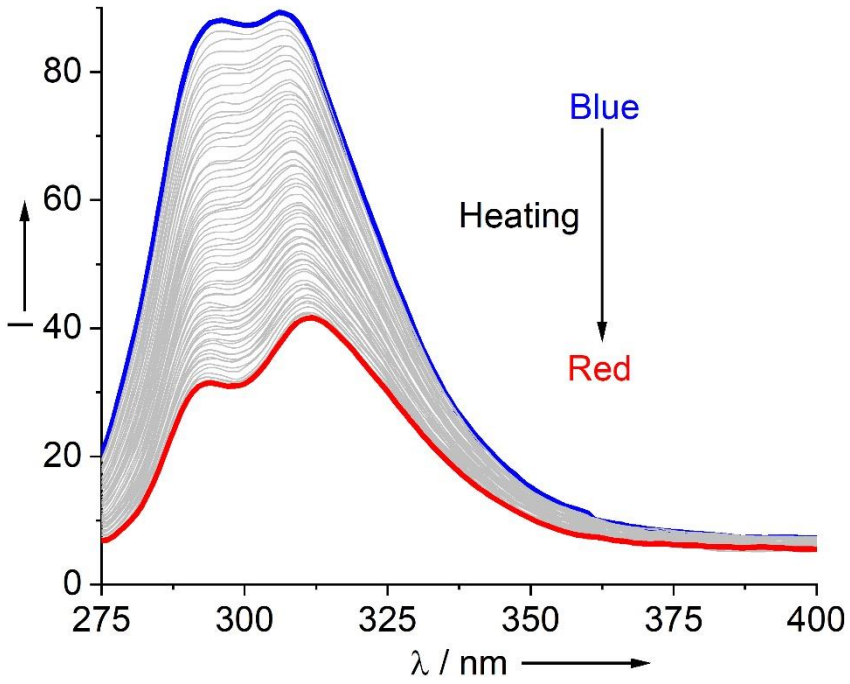


Figure S20. Temperature-dependent fluorescence spectra of **PEP-1**. [*C* = 5 x 10^{-5} M]

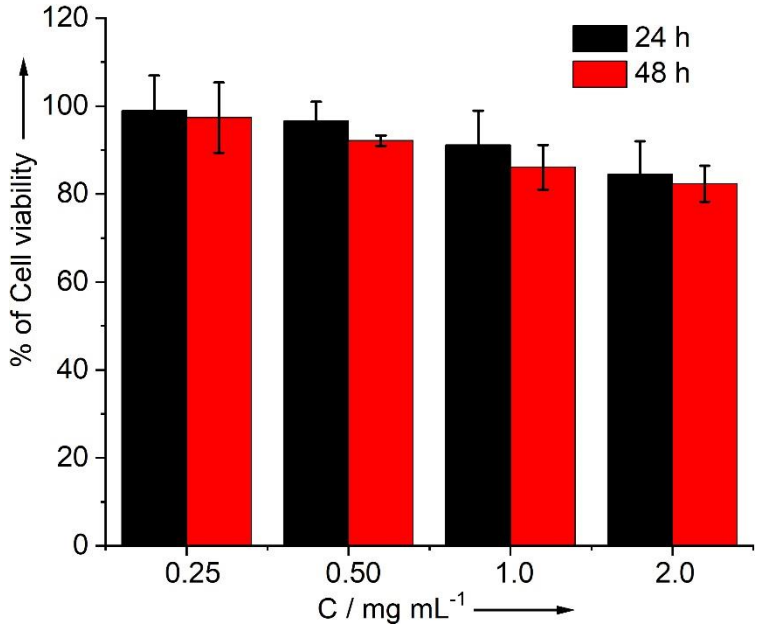


Figure S21. Cell viability of **PEP-1** studied by the MTT assay with HEK-293 (ATCC) cell line.

5. Supporting table

Table S1: Antibacterial activity and Hemolytic activity of **PEP-1** hydrogel

Compound	**Minimum inhibitory concentration (MIC)**				**HC_{10} (μg/ mL)**
	E. coli (μg/ mL)	*E. coli* (mM)	*S. aureus* (μg/ mL)	*S. aureus* (mM)	
PEP-1	291	0.31	582	0.62	> 2000

6. References

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7. Author Contributions

Dr. Goutam Ghosh made all synthetic word, studied the self-assembly behaviour and conceptualized the project jointly with Prof. Gustavo Fernández, who raised the external funding and supervised the project. Prof. Suhrit Ghosh supervised the biological studies. RB carried out the biological studies including cell viability, hemolytic assay and antibacterial activities. AM studied the mechanical properties and gel-to-sol transitions of the hydrogel and UG helped during the synthetic process and purified the peptide molecule by RP-HPLC. The manuscript was written through contributions of all authors.