Assembly of an Injectable Non-Cytotoxic Peptide-based Hydrogelator for Sustained Release of Drugs

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Experimental Section

Synthesis of peptide 1

The tripeptide was synthesized by conventional solution phase methods by using racemization free fragment condensation strategy. The Boc group was used for the N-terminal protection and the C-terminus was protected as a methyl ester. Coupling was mediated by N,N-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole monohydrate (DCC/HOBt.H_2O). C-terminal methyl group was deprotected by using aqueous sodium hydroxide. The final compound was fully characterized by mass spectrometry, ^{1}H NMR spectroscopy and ^{13}C NMR spectroscopy (Figure S1-S3).

Synthesis of Boc-AUDA-OH: 2.01g (10 mmol) of 11-amino undecanoic acid (AUDA) was taken in a round bottomed flask. Then 10 mL 1(N) NaOH, 10 mL water and 20 mL 1,4-dioxane were added to it and cooled to 0^{o}C. 2.20 g (10.1 mmol) di-tert-butyl dicarbonate (Boc anhydride) was added to the reaction mixture and stirred for 10 hours at room temperature. Then volume of the solution was reduced to one third in vacuum. The resulting mixture was acidified with saturated $KHSO_4$ solution and the aqueous layer was extracted with ethyl acetate (3 x 40 mL). The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated in vacuum to obtain the white powdered product. Yield: 2.72 g (9.04 mmol, 90.36 %).

Synthesis of Boc-AUDA-Phe-OMe: 2.72 g (9.04 mmol) of Boc-AUDA-OH was dissolved in 12mL dry N,N-dimethyl formamide (DMF) and cooled in an ice bath. H-Phe-OMe was obtained by neutralization with saturated Na_2CO_3 from its hydrochloride salt and subsequent extraction with ethyl acetate. The ethyl acetate solution was then concentrated to 10 mL and added to the DMF solution followed by 1.38 g (9.04 mmol) of HOBt.H_2O and 1.95 g (9.5 mmol) of N,N-dicylohexylcarbodiimide (DCC). The reaction mixture was allowed to come at room temperature and stirred for 24 hours. The reaction mixture was diluted with ethyl acetate and filtered to separate N,N- dicyclohexyl urea (DCU). The ethyl acetate layer was washed with 1(N) HCl (3 x 30 mL), brine (2 x 30 mL), saturated sodium carbonate solution (2 x 30 mL) and brine (2 x 30 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated to obtain the yellowish product. The product was purified through silica gel column chromatography using pet ether/ethyl acetate (5:1) as eluent to obtain the pure white product. Yield: 2.95 g (6.38 mmol, 70.63 %,).

^{1}H NMR (300 MHz, $CDCl_3$) δ: (numbering on long chain part of the peptide has been shown in Figure S1) 1.25 (12H, s, chain CH_2), 1.44 (9H, s, Boc $-CH_3$ s), 1.57 (4H, br, $^{3}CH_2$ and $^{10}CH_2$), 2.16 (2H, t, J= 7.3 Hz, $^{2}CH_2$), 3.19-3.05 (4H, m, β CH_2 of Phe and $^{11}CH_2$), 3.72 (3H, s, $-OCH_3$), 4.61-4.81 (1H, br, NH), 4.88-4.93 (1H, m, α CH of Phe), 5.92-5.94 (1H, d, J= 7.36 Hz, NH), 7.08-7.10 (2H, m, J= 7.49 Hz, aromatic CH), 7.24-7.30 (3H, m, aromatic CH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.80, 172.31, 156.10, 136.01, 129.34, 128.63, 127.18, 79.07, 53.01, 52.38, 40.71, 38.00, 36.59, 30.14, 29.54, 29.42, 29.34, 29.23, 28.52, 26.86, 25.61. Elemental analysis: Calculated (%) for $C_{26}H_{42}N_2O_5$: (462.3094): C, 67.50; H, 9.15; N, 6.06; Found C, 67.43; H, 9.11; N, 6.10. HRMS (m/z): Calculated for $C_{26}H_{42}N_2O_5$: 462.3094, Found: 485.4393 $(M+Na)^+$.

Synthesis of Boc-AUDA-Phe-OH: 2.9 g (6.28 mmol) of Boc-AUDA-Phe-OMe was taken in a round bottomed flask and dissolved in 50 mL methanol. 15 mL of 1 (N) NaOH was added to it and kept under stirring for 6 hours. The progress of hydrolysis was monitored by thin layer chromatography (TLC). After the completion of the reaction, as indicated by TLC, the methanol was removed in vacuum. The aqueous part was then taken in 50 mL water and washed with diethyl ether (2 x 30 mL). The remaining solution was acidified with 1 (N) HCl and extracted with with ethyl acetate (3 x 40 mL). The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated in vacuum to obtain a white powdered product. Yield: 2.72 g (6.07 mmol, 96.66 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ 1.09-1.20 (12H, m, chain CH_2), 1.35 (13H, m, Boc $-CH_3$s and $^{3}CH_2$ and $^{10}CH_2$), 2.01 (2H, t, J= 7.2 Hz, $^{2}CH_2$), 2.78-3.07 (4H, m, β CH_2 of Phe and $^{11}CH_2$), 4.45-4.37 (1H, m, α CH of Phe), 6.73 (1H, br, NH), 7.15-7.27 (5H, m, aromatic CH), 8.06 (1H, d, J= 8.19Hz, NH),12.58 (1H, br, −COOH). ^{13}C NMR (75 MHz, DMSO- d_6) δ 173.23, 172.13, 155.56, 137.77, 129.03, 128.06, 126.29, 77.24, 53.16, 36.73, 35.06, 29.48, 28.98, 28.77, 28.43, 28.28, 26.27, 25.17. Elemental analysis: Calculated (%) for $C_{25}H_{40}N_2O_5$: (448.2937): C, 66.94; H, 8.99; N, 6.24; Found C, 67.01; H, 8.94; N, 6.20. HRMS (m/z): Calculated for $C_{25}H_{40}N_2O_5$: 448.2937, Found: 471.2882 $(M+Na)^+$.

Synthesis of Boc-AUDA-Phe-Phe-OMe: 2.24 g (5.0 mmol) of Boc-AUDA-Phe-OH was dissolved in 8 mL dry N,N-dimethyl formamide (DMF) and cooled in an ice bath. H-Phe-OMe was obtained by neutralization with saturated Na_2CO_3 from its hydrochloride salt and subsequent extraction with ethyl acetate. The ethyl acetate solution was then concentrated to 8 mL and added to the DMF solution

followed by 0.77 g (5.03 mmol) of HOBt.H_2O and 1.09 g (5.3 mmol) of N,N-dicylohexylcarbodiimide (DCC). The reaction mixture was allowed to come at room temperature and stirred for 24 hours. The reaction mixture was diluted with ethyl acetate and filtered to separate N,N- dicyclohexyl urea (DCU). The ethyl acetate layer was washed with 1(N) HCl (3 x 30 mL), brine (2 x 30 mL), saturated sodium carbonate solution (2 x 30 mL) and brine (2 x 30 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated to obtain the yellowish product. The product was purified through silica gel column chromatography using pet ether/ethyl acetate (4:1) as eluent to obtain the pure white product. Yield: 2.05 g (3.37 mmol, 67.40 %,).

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.22-1.27 (12H, m, chain CH_2), 1.42-1.52 (13H, m, Boc $-CH_3$s and $^3CH_2$ and $^{10}CH_2$), 2.09-2.12 (2H, m, $^2CH_2$), 2.958-3.10 (6H, m, β CH_2 of Phe and $^{11}CH_2$), 3.65 (3H, s, $-OCH_3$), 4.42-4.58 (1H, br, NH), 4.63-4.67 (1H, q, α CH of Phe), 4.71-4.75 (1H, q, α CH of Phe), 6.10-6.12(1H, d, J= 7.0 Hz, NH), 6.37-6.39 (1H, d, J= 7.0 Hz, NH), 7.00-7.01 (2H, m, aromatic CH), 7.17 -7.27 (8H, m, aromatic CH). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.20, 171.41, 170.73, 155.76, 136.59, 135.76, 129.45, 129.30, 128.72, 128.67, 127.23, 127.11, 79.43, 54.18, 53.56, 52.41, 38.17, 37.99, 36.61 30.17, 29.56, 29.42, 29.35, 29.22, 28.56, 26.88, 25.62. Elemental analysis: Calculated (%) for $C_{35}H_{51}N_3O_6$: (609.3778): C, 68.94; H, 8.43; N, 6.89; Found C, 68.86; H, 8.55; N, 6.79. HRMS (m/z): Calculated for $C_{35}H_{51}N_3O_6$: 609.3778, Found: 632.3425 $(M+Na)^+$.

Synthesis of Boc-AUDA-Phe-Phe-OH (Peptide 1): 1.95g (3.2 mmol) of Boc-AUDA-Phe-OMe was taken in a round bottomed flask and dissolved in 50 mL methanol. 10 mL of 1 (N) NaOH was added to it and kept under stirring for 6 hours. The progress of hydrolysis was monitored by thin layer chromatography (TLC). After the completion of the reaction, as indicated by TLC, the methanol was removed in vacuum. The aqueous part was then taken in 50 mL water and washed with diethyl ether (2 x 30 mL). The remaining solution was acidified with 1 (N) HCl and extracted with ethyl acetate (3 x 40 mL). The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated in vacuum to obtain a white powdered product. Yield: 1.83 g (3.07 mmol, 96.11 %).

^{1}H NMR (400 MHz, DMSO- d_6) δ 1.14-1.20 (12H, m, chain CH_2), 1.25-1.35 (13H, m, Boc $-CH_3$s and $^3CH_2$ and $^{10}CH_2$), 1.90-1.95 (2H, m, $^2CH_2$), 2.63-2.69 (2H, m, $^{11}CH_2$), 2.82-3.09 (4H, m, β CH_2 of Phe), 4.42-4.46 (1H, m, α CH of Phe), 4.50-4.55 (1H, m, α CH of Phe), 6.72-6.74 (1H, br, NH), 7.074-7.27

(12H, m, aromatic CH and NH), 12.77 (1H, br, −COOH). ^{13}C NMR (100 MHz, DMSO- d_6) δ 172.66, 171.96, 171.46, 155.57, 137.97, 137.90, 137.39, 137.36, 129.24,129.12, 128.15, 128.10, 127.86, 127.81, 127.03, 126.41, 126.08, 77.25, 53.43, 53.38, 53.22, 37.78, 37.37, 37.05, 36.65, 35.17, 29.46, 28.96, 28.79, 28.76, 28.40, 28.25, 26.25, 25.13. Elemental analysis: Calculated (%) for $C_{34}H_{49}N_3O_6$: (595.3621): C, 68.54; H, 8.29; N, 7.05; Found C, 68.44; H, 8.36; N, 7.10. HRMS (m/z): Calculated for $C_{34}H_{49}N_3O_6$: 595.3621, Found: 618.3597 $(M+Na)^+$.

Instrumentation

Field emission scanning electron microscopic (FE-SEM) study: FE-SEM experiments were performed by placing a small portion of gel samples on a microscope cover glass. Then, these samples were dried first in air and then in vacuum and coated with platinum for 90 s at 10 kV voltages and 10 mA current. The average thickness of the coating layer of platinum was 3 to 4 nm. After that micrographs were taken by using a Jeol Scanning Microscope JSM-6700F.

Transmission electron microscopy (TEM) study: The morphology of the hydrogel was investigated by using a transmission electron microscope. The samples were prepared by depositing 2.34 mM of dilute gel sample onto a TEM grid (300 mesh Cu grid). After 2 min excess fluid were removed. Negative staining was obtained by covering the grid with 2% uranyl acetate in water for 2 min. Excess uranyl acetate was removed.[S1] Then, the grid was dried under vacuum at 30 °C for two days. Images were recorded on a JEOL electron microscope at an accelerating voltage of 200 kV.

Wide Angle Powder X-ray diffraction study: X-ray diffraction study of the xerogel was carried out by placing the sample on a glass plate. Experiments were carried out by using an X-ray diffractometer (Bruker AXS, Model No. D8 Advance). The instrument was operated at a 40 kV voltages and 40 mA current using Ni-filtered CuK_α radiation and the instrument was calibrated with a standard Al_2O_3 (corundum) sample before use. For scan 5°–30°, the LynxEye super speed detector was used with scan speed 0.5 s and step size 0.02°.

Small Angle X-Ray Scattering (SAXS): Measurements were performed using a Bruker Nanostar instrument using CuK_α radiation and a Vantec 2000 detector. The sample-to-detector distance was 1.07 m. The $q = 4\pi\sin\theta/\lambda$ (scattering angle 2θ) scale was calibrated using silver behenate. Samples were mounted in quartz capillaries.

FTIR spectroscopy: The FTIR spectrum of the xerogel were recorded on a Shimadzu (Japan) FTIR spectrophotometer. In the solid-state FTIR studies, the powdered samples were mixed with KBr to prepare the thin films.

UV/Vis spectroscopy: UV/Vis absorption spectra were recorded on a hewlett-packard (model 8453) UV/Vis spectrophotometer (varian carry 50.bio).

Fluorescence spectroscopy: The fluorescence spectra were recorded on a Perkin–Elmer LS55 Fluorescence Spectrometer. The gel samples were placed in a quartz cell (path length: 0.5 cm) and excited at 261 nm. The emission scans were recorded from 271–375 nm by using a slit width of 5 nm for both excitation and emission slits.

Rheology: The rheology experiment was performed by using an Anton Paar Modular Compact Rheometer MCR 302 at 25 °C.

NMR experiments: All NMR studies were carried out on a Brüker DPX 300 MHz, Brüker DPX 400 MHz and Brüker DPX 500 MHz spectrometers at 300 K. Concentrations were in the range 1–10 mM in $CDCl_3$ or DMSO-d_6.

Mass spectrometry: Mass spectra were recorded on a Q-Tof microTM (Waters Corporation) mass spectrometer by positive mode electro spray ionization process.

Circular dichroism (CD) study: Circular dichroism spectrum was recorded by using a quartz cuvette of 1 mm path length in a Jasco J-815 spectropolarimeter.

Gelation and Thermal study

The N-terminal protected long chain amino acid (11-aminoundecanoic acid) containing tripeptide Boc-AUDA-Phe-Phe-COOH (AUDA-11-aminoundecanoic acid, Phe- L-phenylalanine) (P1) was placed in a glass vial and heated on a hot plate after addition of 1 mL of 50 mM phosphate buffer (pH 7.46) to give a clear solution. The solution on standing at room temperature for a few minutes (almost 10 minutes) produced a translucent gel (Figure 1). This was confirmed by vial inversion. The gelator P1 formed a gel in the pH range 5.52 to 8.5. The minimum gelation concentration of the hydrogel at pH 7.46 was measured to be 0.29 % (w/v). Gel melting temperature (Tgel) of the hydrogel (at pH 7.46) was measured at different concentrations by using a digital water bath. The Tgel vs concentration plot suggests that the

Tgel values increase sharply in the low concentration region (Figure S4) and subsequently attains a near-plateau region as the saturation point is reached.

Circular Dichroism (CD) study

A circular dichroism study of the wet gel near its minimum gelation concentration gives a sharp negative peak at 219 nm (Figure S5) indicating the formation of a *β*-sheet like structure. Thus the CD study reinforces the knowledge obtained from the FTIR data in confirming a *β*-sheet like supramolecular network.

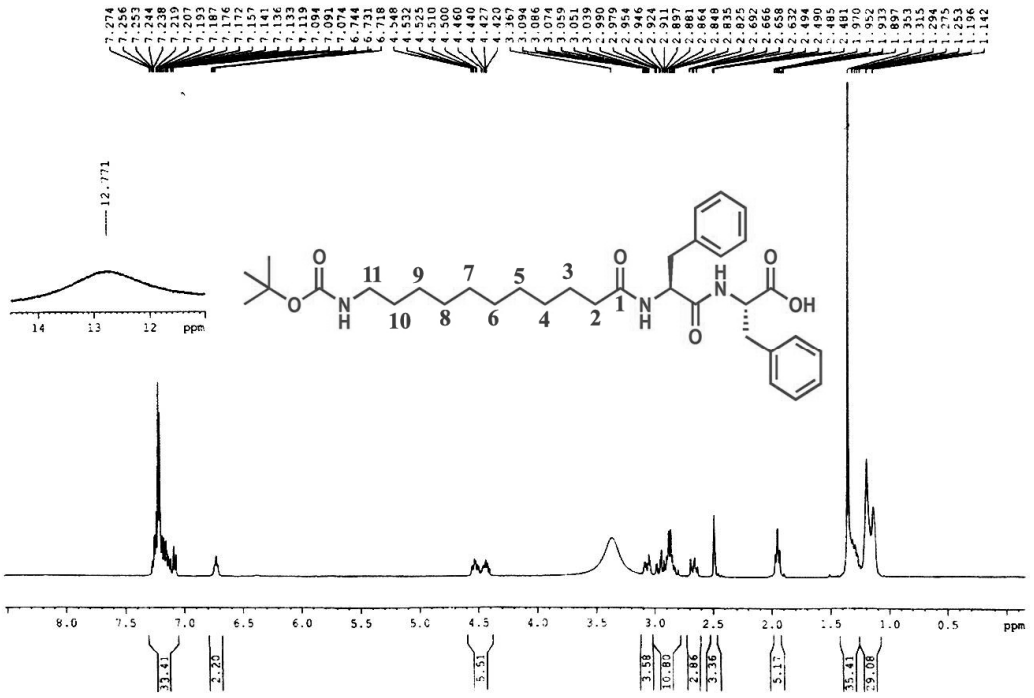

Figure S1: 400 MHz ^{1}H NMR spectra of Peptide 1 in DMSO-d_6. (Numbering have been done on the chemical structure of the gelator peptide arbritarily)

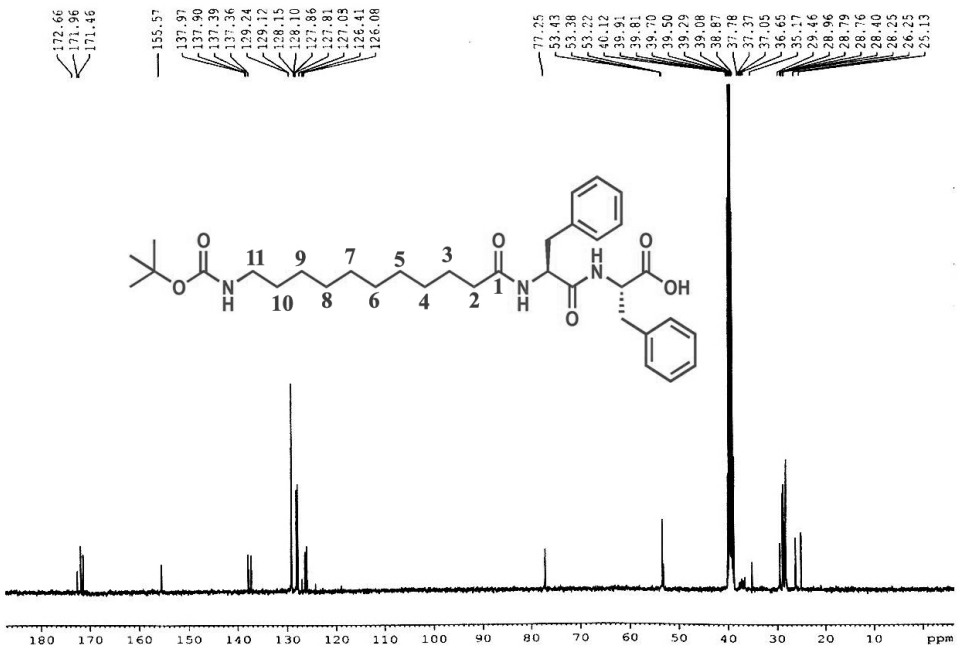

Figure S2: 100 MHz ^{13}C NMR spectra of Peptide 1 in DMSO-d_6

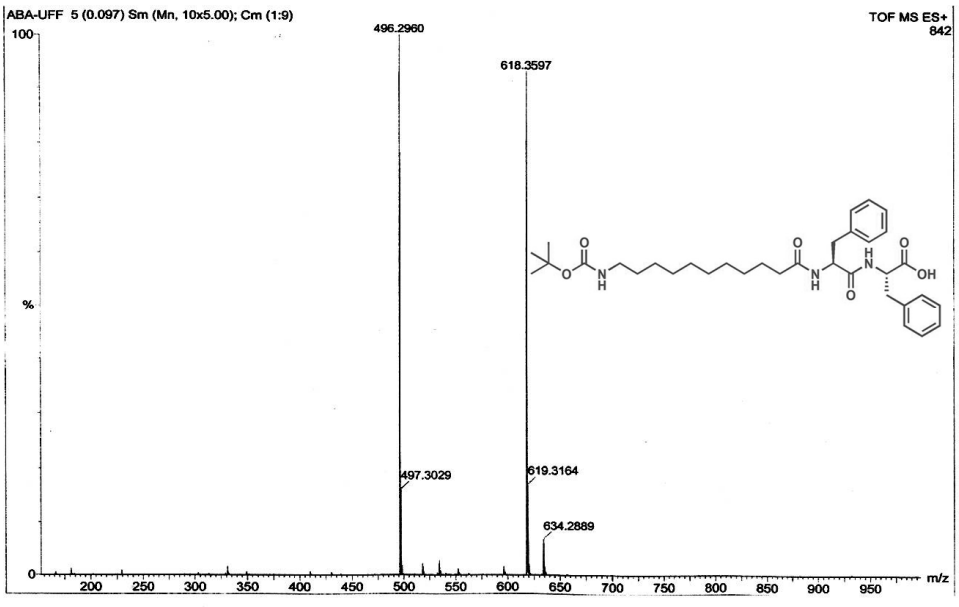

Figure S3: HRMS spectra of Peptide 1.

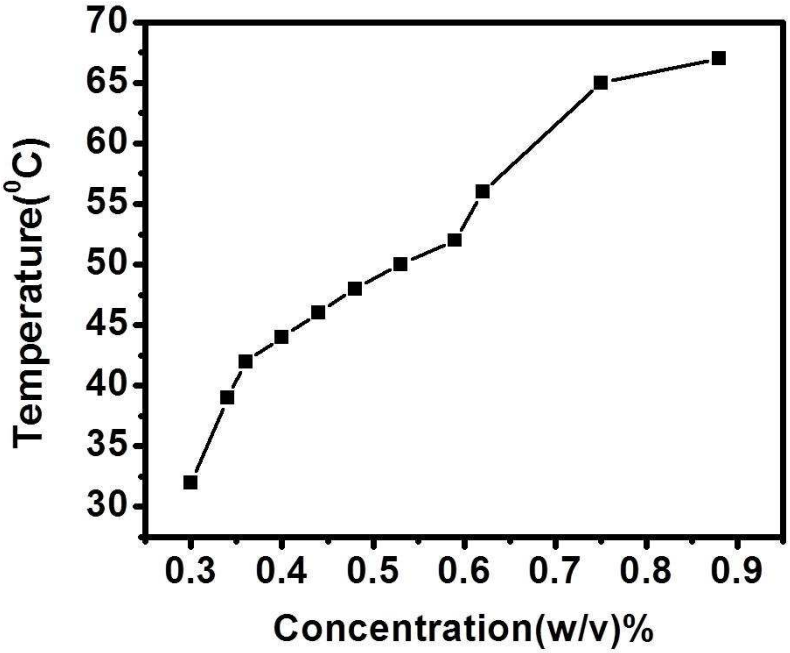

Figure S4: T_{gel} plot of Boc- AUDA-Phe-Phe-OH hydrogel.

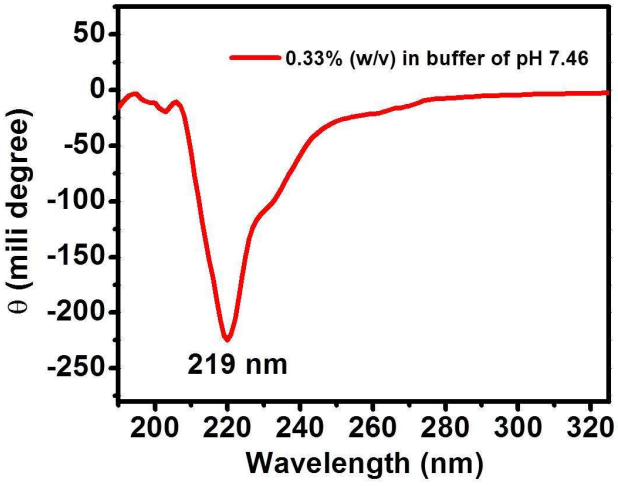

Figure S5: Circular Dichroism plot of the hydrogel near gel concentration.

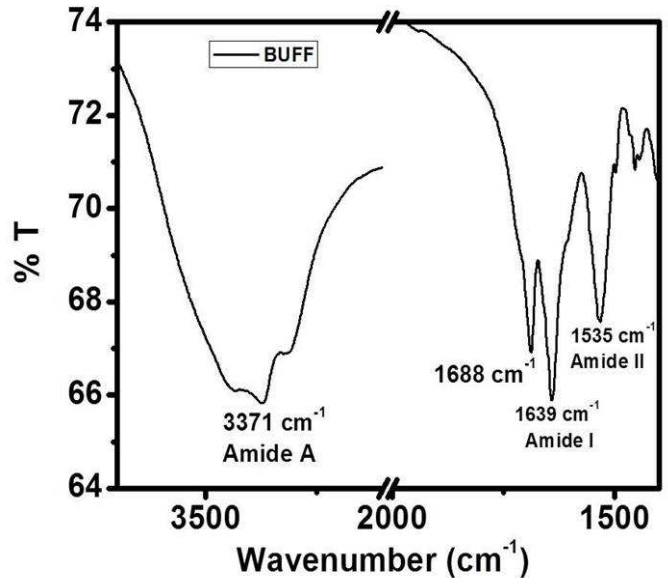

Figure S6: Fourier-transform infrared (FT-IR) spectra of the xerogel.

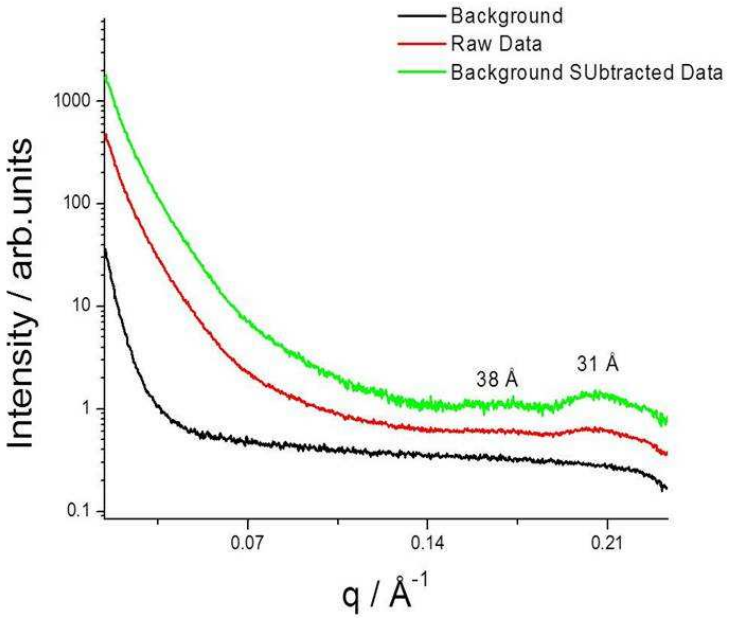

Figure S7: Small angle X-ray scattering (SAXS) pattern of the hydrogel.

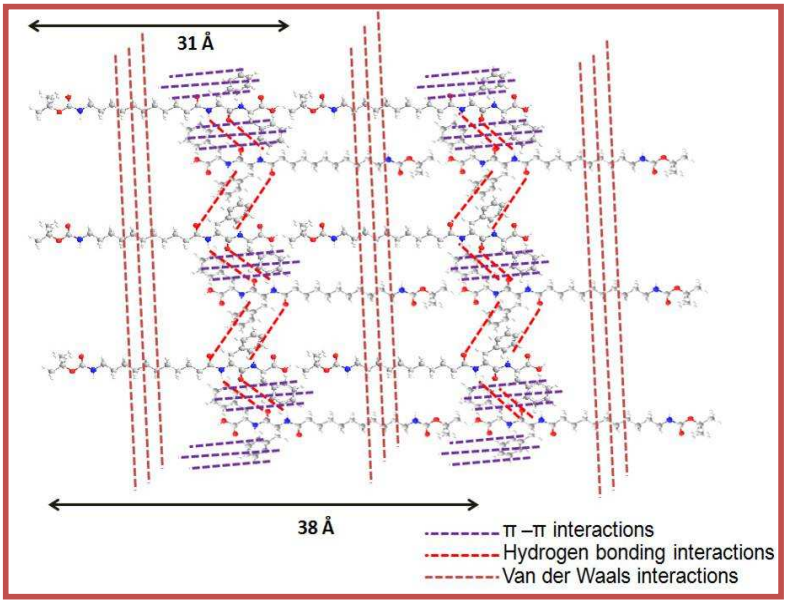

Figure S8: Probable packing pattern of the gelator molecules based on the small angle X-ray scattering (SAXS) of the hydrogel

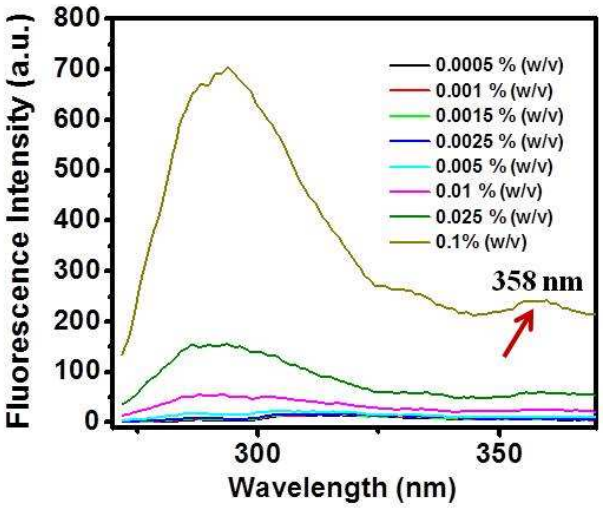

Figure S9: Fluorescence emission spectra with different concentrations of the gelator. The broad peak at 358 nm suggests π-π stacking interaction.

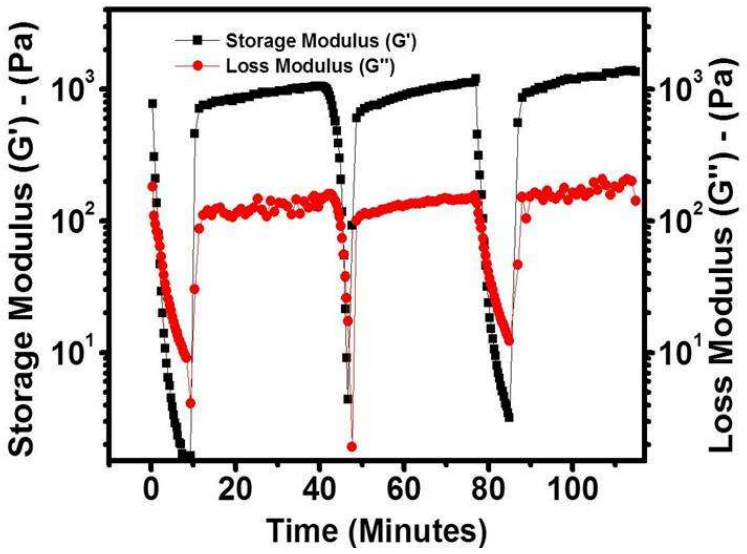

Figure S10: Continuous strain time dependent rheological analysis of the hydrogel [0.5 % (w/v)]. Strain is varied from 0.1 % to 50 % to break the gel and then restoration is observed at constant strain of 0.1 % keeping the angular frequency constant at 1 rad/sec.

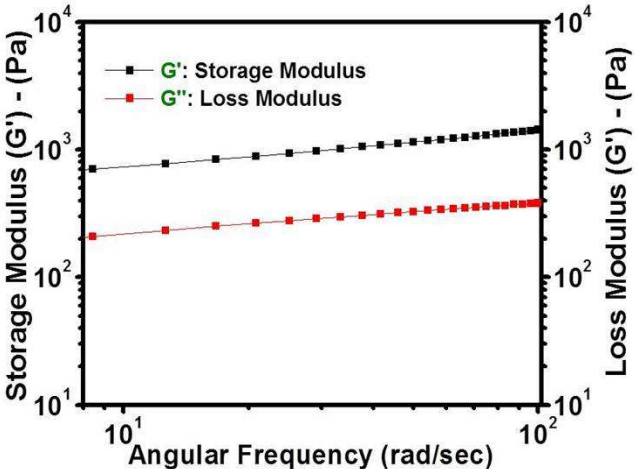

Figure S11: Frequency sweep rheological analysis of the hydrogel [(0.5 % (w/v)] at a constant strain of 0.1 %.

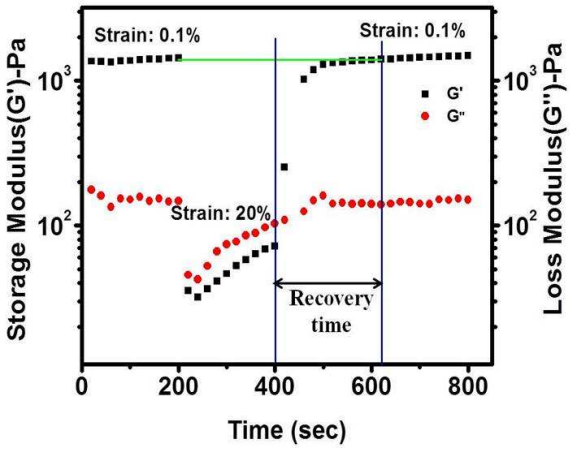

Figure S12: Step-strain time dependent rheological analysis of the hydrogel [(0.5 % (w/v)] at a fixed angular frequency of 1 rad/sec.

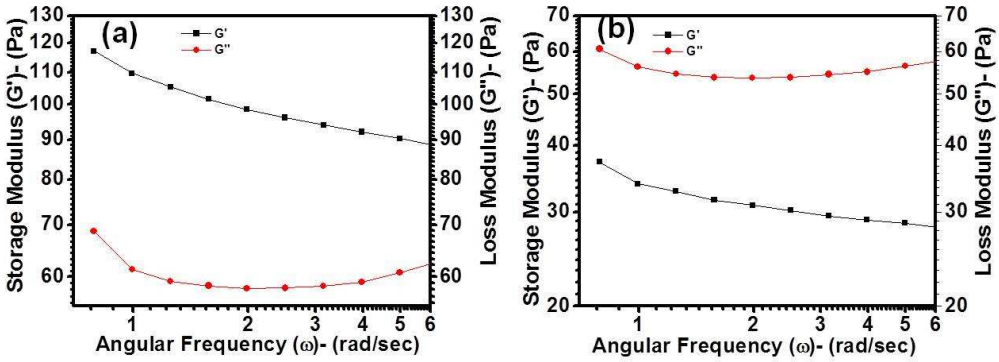

Figure S13: (a) Frequency sweep rheological analysis of the hydrogel [(0.8 % (w/v)] at a constant strain of 15 % showing the stable gel phase behaviour and (b) Frequency sweep rheological analysis of the hydrogel [(0.8 % (w/v)] after breaking the gel by using mechanical shaking at a constant strain of 15 % showing low viscous liquid/solution-like behaviour.

References:

(S1) Mahler, A.; Reches, M.; Rechter, M.; Cohen, S.; Gazit, E. Rigid, Self-Assembled Hydrogel Composed of a Modified Aromatic Dipeptide. *Adv. Mater.* **2006**, *18*, 1365–1370.