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A Heterochiral Diphenylalanine Auxin Derivative empowers Remarkable Mechanical Integrity with promising Antiinflammatory and Antimicrobial Performances

Naureen Khan,[a] Arindam Gupta, [b] Vaibhav Shivhare, [a] Rishabh Ahuja, [a] Mayank Varshney, [c] Anindya Basu,,[d,e],and Anita Dutt Konar [a,d,e]*

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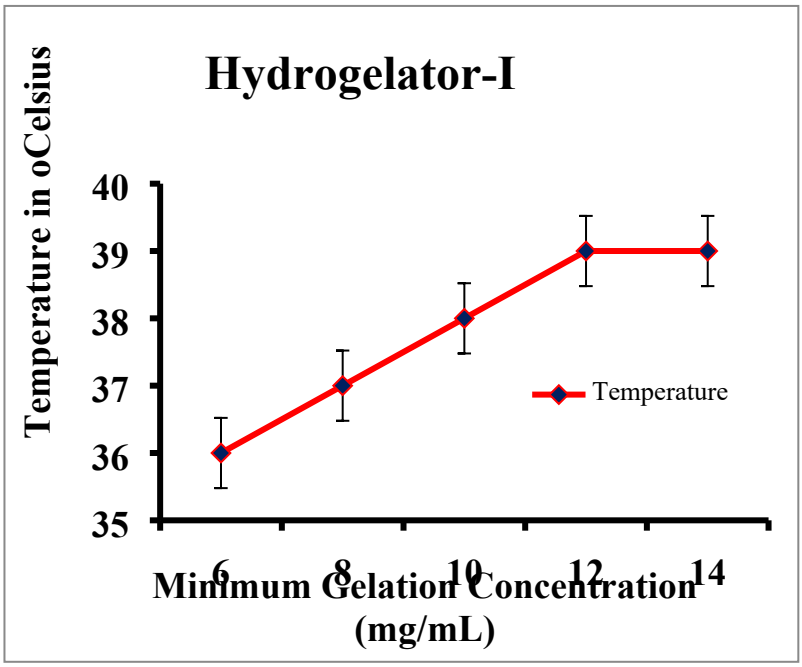


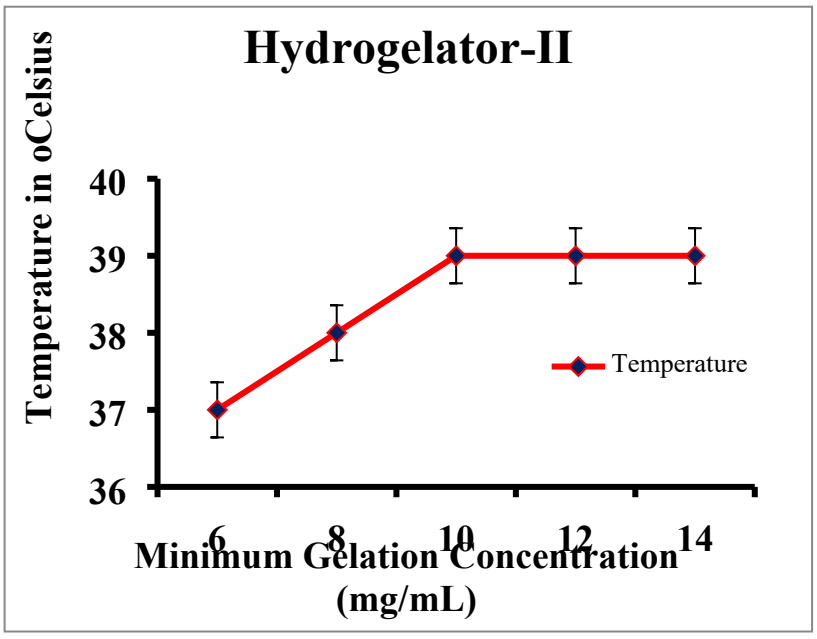
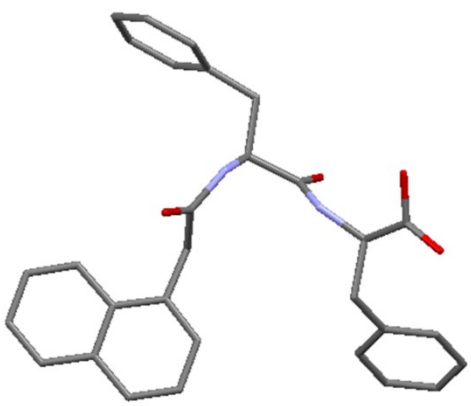
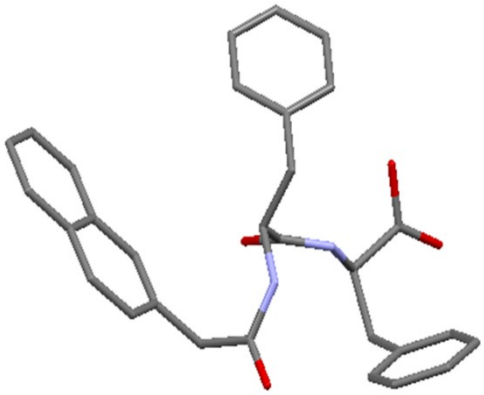


Figure S1: - T_{gel} Graphs of Hydrogelator **I & II**



Hydrogelator I



Hydrogelator II

Figure S2: - Energy Minimized Structures of Hydrogelator-**I & II**

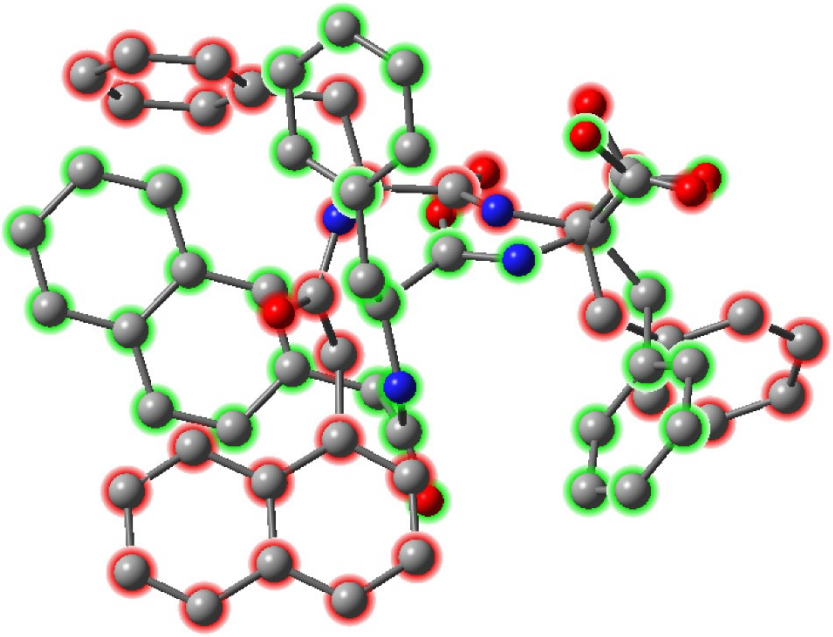


Figure S3: - Overlayed Structures of Hydrogelator-**I & II**

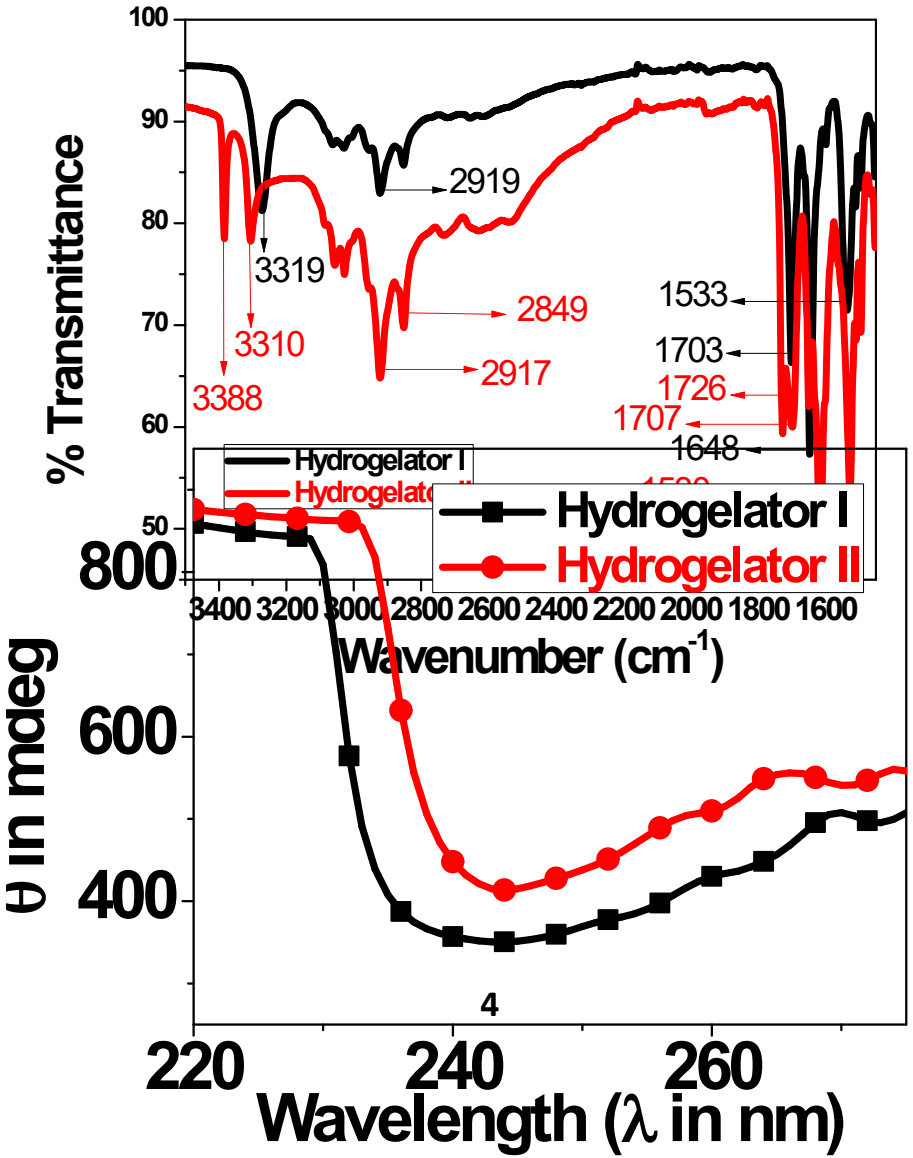


Figure S4: - Enlarged view of the FT-IR Spectra of Hydrogelator **I – II** Xerogels for

Figure S5: - Enlarged CD data of the Hydrogelator **I - II.**

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Table S1:- Enlarged view of the MTT assay of Hydrogelator **I – II** with data in tabular format.

	200	**100**	**50**	**25**	**12.5**	**6.25**	**NC**
Hydrogelator-I	**68.77**	**81.29**	**84.98**	**88.01**	**94.33**	**99.86**	**100**
Hydrogelator-II	**67.85**	**75.76**	**79.84**	**82.21**	**93.81**	**96.57**	**100**

Table S2: - Data of Antibacterial assay determined by turbidity experiments

S. No.	**Samples**	**100 µg/ml**	**50 µg/ml**	**25 µg/ml**	**12.5 µg/ml**	**6.25 µg/ml**	**3.12 µg/ml**	**1.6 µg/ml**	**0.8 µg/ml**	**0.4 µg/ml**	**0.2 µg/ml**
E.coli											

01	**Hydrogelator-I**	R	R	R	R	R	R	R	R	R	R
02	**Hydrogelator-II**	R	R	R	R	R	R	R	R	R	R
Klebsiella											
01	**Hydrogelator-I**	R	R	R	R	R	R	R	R	R	R
02	**Hydrogelator-II**	R	R	R	R	R	R	R	R	R	R
S.aureus											
01	**Hydrogelator-I**	S	S	S	R	R	R	R	R	R	R
02	**Hydrogelator-II**	S	S	S	S	R	R	R	R	R	R
S.mutans											
01	**Hydrogelator-I**	S	S	S	S	**S**	R	R	R	R	R
02	**Hydrogelator-II**	S	S	S	S	R	R	R	R	R	R
Candida											
01	**Hydrogelator-I**	S	S	S	S	S	S	S	S	R	R
02	**Hydrogelator-II**	S	S	S	S	S	R	R	R	R	R
A.niger											
01	**Hydrogelator-I**	S	S	S	S	S	S	R	R	R	R
02	**Hydrogelator-II**	S	S	S	S	S	R	R	R	R	R

S.No.	Samples	100 mg / mL	50 mg / mL	25 mg / mL	12.5 mg / mL	6.25 mg / mL	3.12 mg / mL	1.6 mg / mL	0.8 mg / mL	0.4 mg / mL	0.2 mg / mL
E.coli											
01	**Hydrogelator I**	1.099	1.506	1.711	1.799	1.836	1.862	1.876	1.906	1.908	1.922
02	**Hydrogelator II**	1.256	1.501	1.719	1.757	1.829	2.001	2.013	2.055	2.061	2.069
Klebsiella											
01	**Hydrogelator I**	1.612	1.855	1.877	1.881	1.886	1.934	1.961	1.973	1.988	2.205
02	**Hydrogelator II**	1.450	1.885	1.886	1.900	1.905	1.955	1.922	1.932	2.012	2.020
S.aurues											
01	**Hydrogelator I**	0.438	0.500	0.657	0.714	1.073	1.227	1.411	1.443	1.500	1.715
02	**Hydrogelator II**	0.355	0.550	0.781	0.809	0.938	0.952	1.361	1.405	1.535	1.695
S.mutans											
01	**Hydrogelator I**	0.517	0.588	0.695	0.938	0.695	0.942	1.029	1.037	1.042	1.047
02	**Hydrogelator II**	0.428	0.928	0.940	0.955	0.968	1.021	1.076	1.087	1.268	1.510
Candida											
01	**Hydrogelator I**	0.685	0.775	1.083	1.149	1.267	1.294	1.312	1.345	1.365	1.382
02	**Hydrogelator II**	0.669	0.830	1.246	1.316	1.374	1.382	1.540	1.545	1.552	1.748
A.niger											
01	**Hydrogelator I**	0.355	0.368	0.377	0.382	0.384	0.407	0.412	0.417	0.848	1.004
02	**Hydrogelator II**	0.377	0.378	0.383	0.385	0.390	0.423	0.429	0.430	0.430	0.458

Table S3: - Zymographic study of the Anti-Inflammatory activities of Hydrogelator **I – II.**

S.NO	NAME OF THE COMPOUND	% BANDS OF MMP		% INHIBITION OF MMP	
		MMP 2	MMP 9	MMP 2	MMP 9
1	Hydrogelator-I	20	22	80	78
2	Hydrogelator-II	31	38	69	62

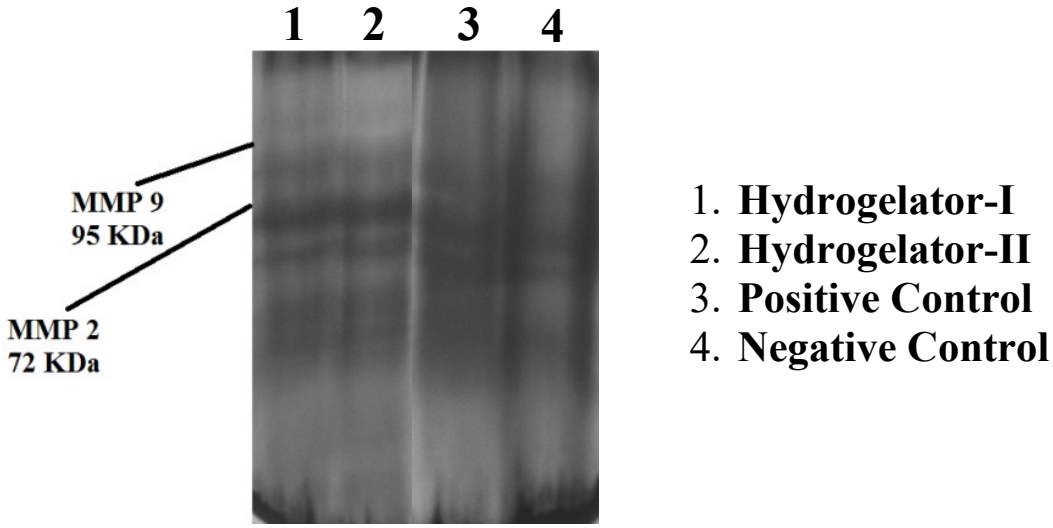

Figure S6: - Diagram of Zymographic study of the anti-inflammatory activities of Hydrogelator **I – II.**

DILUTIONS OF Hydrogelator I

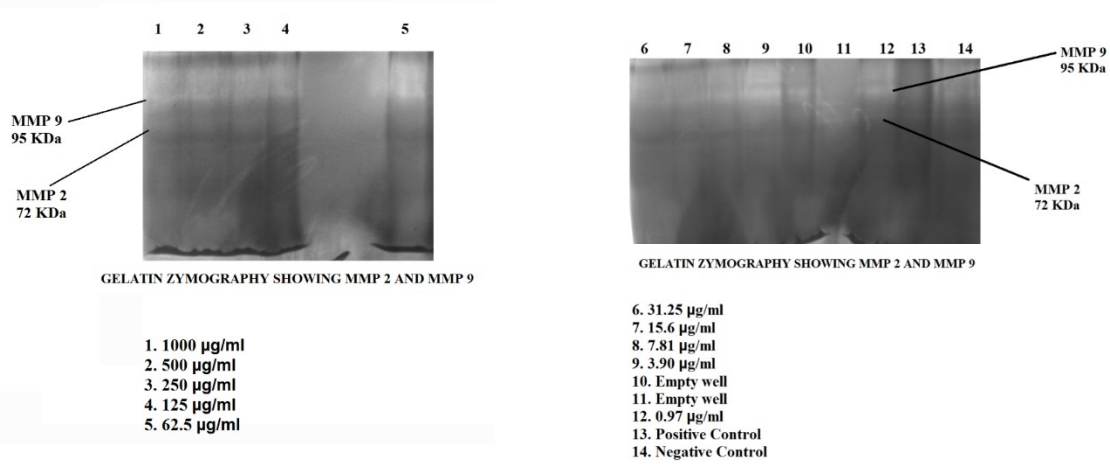

Figure S7- Concentration dependent zymographs of Hydrogelator **I**

Table S4:- The Diversified concentrations of Hydrogelator **I** used for the experiment

S.NO	SKNK 1 DILUTIONS	% BANDS OF MMP		% INHIBITION OF MMP	
		MMP 2	MMP 9	MMP 2	MMP 9
1	1000 µg/ml	20	26	80	74
2	500 µg/ml	19	22	81	78
3	250 µg/ml	17	18	83	82
4	125 µg/ml	14	22	86	78
5	62.5 µg/ml	13	35	87	65
6	31.25 µg/ml	05	10	95	90
7	15.6 µg/ml	16	12	84	88
8	7.81 µg/ml	17	32	83	68
9	3.90 µg/ml	32	50	68	50
10	Empty well	-	-	-	-
11	Empty well	-	-	-	-
12	0.97 µg/ml	27	39	73	61
13	POSITIVE CONTROL	00	08	92	100
14	NEGATIVE CONTROL	100	100	00	00

Experimental Procedure

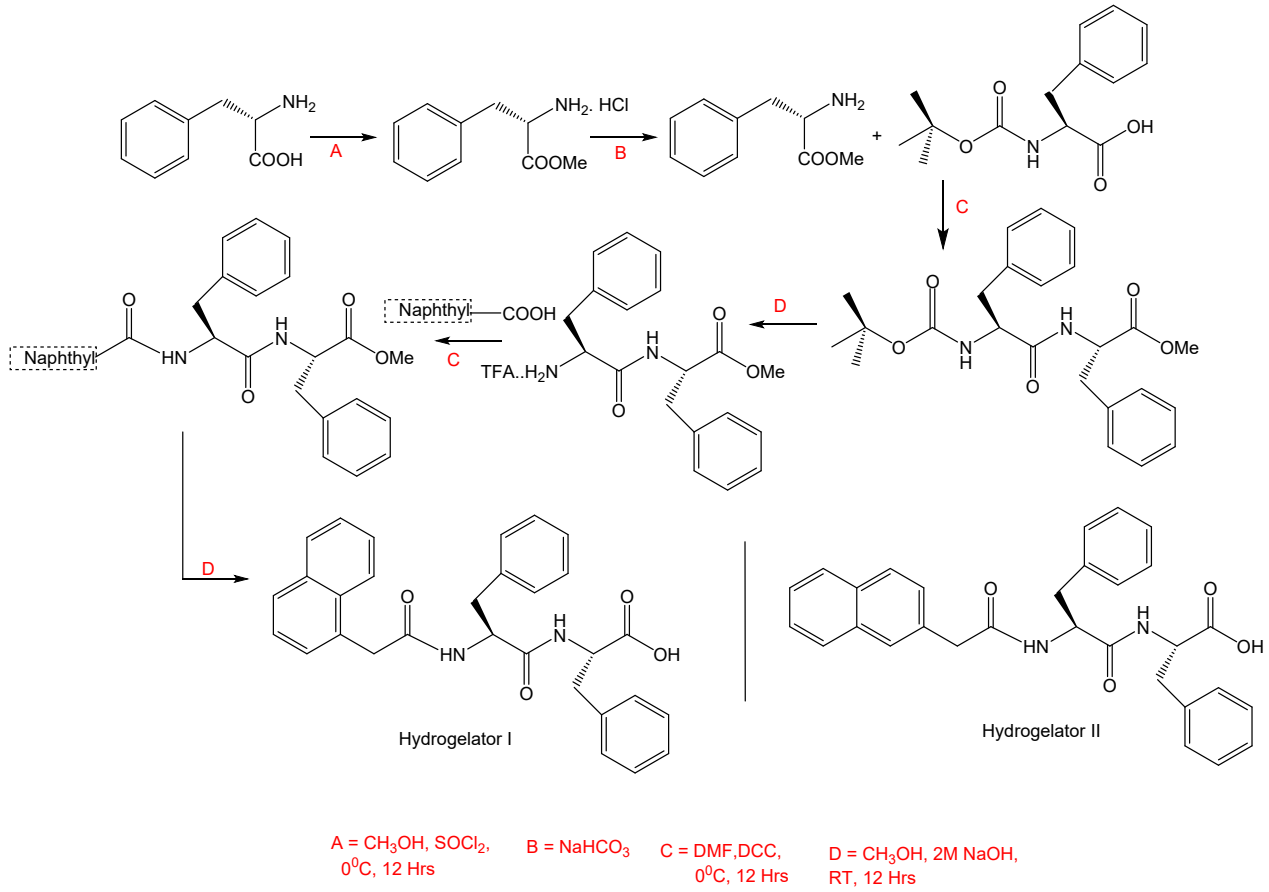

Figure S7: Schematic Representation of the Hydrogelators

Synthesis of the Compounds I - III
D-Phe.HCl (1)[1]:
Yield: 7.05 gm (90%)
Boc-L-Phe-D-Phe- OMe (2)[1]:
Yield: 5.05 gm (90%)

1-Naph-LF-DF-OMe (3)/2-Naph-LF-DF-OMe (4): To obtain Compound **3/4** initially the tertiary butyloxycarbonyl was removed from **compound 2** (2.5 g, 5.88 mmol), using trifluoroacetic acid at 0°C and stirring the reaction at room temperature, until complete deprotection was noted (probed by TLC). Thereafter the trifluoroacetate salt was dissolved in water, washed with diethyl ether, followed by extraction with ethyl acetate and addition of sodium bicarbonate solution. The organic layer was dried using anhydrous sodium sulphate and cooled and then added to Naphthyl Acetic Acids (900 mg, 4.90 mmol) dissolved in DMF (1-Naphthyl Acetic Acid: Hydrogelator **I**; 2-Naphthyl Acetic Acid: Hydrogelator **II**;) at 0°C, followed by DCC (1.51g, 7.35 mmol) and stirring at room temperature.

The progress of the reaction was monitored by TLC. After completion of the reaction, the DCU was filtered off; the organic layer was worked up with washings of dilute HCl/sodium carbonate solution, followed by its drying using sodium sulphate and dried to obtain a white solid.
Yield: 1.94 gm (80%)

Hydrogelator I/II: To obtain Hydrogelator **I/II**, Compound **3/4** (2.32 g) was dissolved in calculated amount of methanol (15 ml) and NaOH (2M NaOH: 18 ml) was added dropwise to the solution. The progress of the reaction was monitored by TLC. After completion of the reaction, as determined by TLC, the methanol was evaporated. The residue containing the sodium salt was dissolved in water and extracted with diethyl ether to remove the unreacted stuff. The aqueous layer obtained was cooled, acidified with 2N HCl and extracted with ethyl acetate. The solvent was evaporated *in vacuo* to obtain a white solid.
Hydrogelator **I** :Yield: 1.74 gm, (90%); LC-MS: $C_{30}H_{28}N_2O_4$: 503.2 $[M + Na]^+$; MS (calculated) m/z: 480.20 $[M]^+$; FT-IR: 3319, 2919, 1703, 1648, 1533 cm^{-1}; ^{1}1H-NMR (500 MHz; d_6-DMSO; δ in ppm); 12.46-12.77 (Phe(2) -COOH, 1H, *br*); 8.5 (Phe(2) -NH, 1H, *d*; *J*=10 Hz); 8.27-8.29 (Phe(1) -NH, 1H, *d*; *J*=10 Hz); 7.76-7.99 (3H, *m*, 1-naphthyl ring aromatic Hs); 7.35-7.58 (4H, *m*, 1-naphthyl ring aromatic Hs); 7.18-7.28 (8H, *m*, Phe(1) aromatic Hs); 7.02-7.07 (2H, *m*, Phe(2) aromatic Hs); 4.56-4.60 (1H, *m*, Phe(1) $C^{\alpha}H$); 4.47-4.51 (1H, *m*, Phe(2) $C^{\alpha}H$); 3.80-3.91 (2H, *m*, 1-naphthyl ring $C^{\alpha}H$); 2.65-3.13 (4H, *m*, $C^{\alpha}H$ of Phe(1) & Phe(2)).
^{13}C NMR (125 MHz; d_6-DMSO): 173.20, 171.48, 133.68, 132.36, 132.14, 129.74, 128.84, 128.45, 128.34, 128.17, 127.38, 126.95, 125.98, 125.86, 124.68, 124.48, 54.05, 53.84, 38.96, 38.46, 37.72.

Hydrogelator **II**: Yield: 1.54 gm, (80%); LC-MS: $C_{30}H_{28}N_2O_4$: 503.2 $[M + Na]^+$; MS (calculated) m/z: 480.20 $[M]^+$; FT-IR: 3388, 3310, 2917, 2849, 1726, 1707, 1612, 1530 cm^{-1};^{1}H-NMR (500 MHz; d_6-DMSO; δ in ppm); 12.59 (Phe(2) -COOH, 1H, *br*); 8.49 (Phe(2) -NH, 1H, *d*; *J*=10 Hz); 8.22 (Phe(1) -NH, 1H, *d*; *J*=10 Hz); 7.78-7.91 (3H, *m*, 2-naphthyl ring aromatic Hs); 7.39-7.60 (4H, *m*, 2-naphthyl ring aromatic Hs); 7.02-7.29 (10H, *m*, Phe(1) aromatic Hs); 4.56-4.61 (1H, *m*, Phe(1) $C^{\alpha}H$); 4.44-4.51 (1H, *m*, Phe(2) $C^{\alpha}H$); 3.49-3.61 (2H, *m*, 2-naphthyl ring $C^{\beta}H$); 2.65-3.12 (4H, *m*, $C^{\beta}H$ of Phe(1) & Phe(2))

DFT Calculations
The molecules (reactants and products) were modelled using Spartan08 software and energy minimization was done within the software. Calculations were performed on a single molecule as described in the reference 1.[1]

Preparation of the Hydrogels
The requisite amounts of hydrogelators were separately dissolved in 7.5 pH phosphate buffer by slightly warming until a transparent solution was produced. It was kept undisturbed for some time when hydrogel formation took place, confirmed by inverted vial method.

Conformational analysis of the Hydrogels.
The temperature dependent ^{1}H, ^{13}C NMR, COSY and ROESY experiments were performed using Bruker Advance instrument operating at 500MHz NMR, with d_6-DMSO as solvent.

Fourier-Transform Infrared (FTIR) Spectroscopy
FTIR spectra for both the xerogels were recorded using a KBr pellet on an Agilent CARY 620 FTIR spectrophotometer. The background was collected using a blank KBr pellet.

Circular Dichroism
Far-UV CD measurements of the Hydrogelators were recorded in methanol at 25^{o}C with a 0.5 s averaging time, a scan speed of 50nm/min, using a JASCO spectropolarimeter (J 720 model) equipped with a 0.1 cm path length cuvette. The measurements were taken at 0.2 nm wavelength intervals, 2.0 nm spectral bandwidth, and five sequential scans were recorded for each sample.

Morphological Study of the Hydrogels.
Field-emission scanning electron microscopy (FE-SEM) experiment was performed on a JEOL scanning electron microscope (model no. JSM-7600F) with xerogels, obtained from the hydrogels of same concentration 8 mg/ml.

Rheological Properties of the Hydrogels. Rheological experiments were performed at 25 °C on an Anton Paar Physica MCR 301 rheometer. The viscoelastic properties of hydrogels were measured by measuring the storage modulus (G′) and loss modulus (G″). Hydrogel (1 mL) was transferred on a rheometer plate by using a microspatula and kept hydrated by using a solvent trap. A stainless steel parallel plate (diameter: 25 mm) was used to sandwich the hydrogels with TruGap (0.5 mm). The dynamic strain sweep experiment was performed to determine the region of deformation of hydrogels in which linear viscoelasticity is valid. The exact strains for hydrogel materials were determined by linear viscoelastic regime at a constant frequency of 10 rad s^{-1}. The mechanical strengths of the hydrogels were determined by frequency sweep experiment. In the frequency sweep measurement, the graph was plotted as a function of frequency in the range of 0.05−100 rad s^{-1}. The thixotropic properties were investigated by step-strain experiments at the constant frequency of 10 rad s^{-1}, and applied strains were varied from 0.1 to 40%. The concentrations of hydrogelators used were 8 mg/mL.

The PROTEOLYTIC STABILITY: The hydrogelators were incubated with the proteolytic enzyme proteinase K and recording the degradation rate by Mass Spectrometry at regular intervals of time as described in References 2 and 3. [2, 3]

MTT Assay: This is a colorimetric assay that quantifies the reduction of *yellow* 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinated dehydrogenase. Since the reduction of MTT occurs in metabolically active cells, the level of activity is a measure of the viability of the cells. Eventually the IC_{50} values were determined which would give a measure of the biocompatibility $IC_{50} > 100\mu M$).

Antibacterial Experiment.
Bacterial Culture. *S. Aureus* ATCC – 25923, *S. Mutans* ATCC – 25175, *E. Coli* ATCC – 25922, *Klebsiella Pneumonia* ATCC - 1705 were obtained as a lyophilized powder. Before beginning the experiments, fresh inoculums of the organisms were prepared.

Antimicrobial Properties: *The* antimicrobial activities were done through MIC (minimum inhibitory concentration) approach using Turbidity Experiment. Before beginning the experiments, fresh inoculums of the organisms were prepared from their lyophilized powder.
For this study various dilutions of the hydrogelators were used and the experiments were done in triplicates. In each experiment, tentatively ten microliter of peptide hydrogels of a particular concentration were added to each well which were further diluted for rest of the experiments. The bacterial solution devoid of hydrogels in nutrient broth was considered as the control and only nutrient broth as blank. These plates containing test organisms and hydrogels were incubated at 37 °C for 24 h. Finally the antibacterial properties of peptide hydrogels were confirmed with a microplate reader using 96-well microplates at 25°C by comparing the absorbance of the test solution with the control experiment.[1]

The Antifungal activity was done using the same protocol.

In vitro Anti-inflammatory Activity
The Matrix Metalloproteinase enzymes are mainly known to regulate this activity and they were extracted in tris buffer as described in Reference 1.[1] Again in the same Tris buffer an equal amount of hydrogel was incubated for an hour. Only the ENZYME Solution was used as negative control (NC) and ENZYME Solution with an equal amount of standard anti-inflammatory drug were used as positive control (PC). The final solutions were mixed with a non reducing buffer in equal volume and from this a certain volume of sample was loaded in each well, connected with the electrodes and POWER was started until the bromophenol blue reached the bottom of the plates. After electrophoresis, the apparatus were dissembled; the gel was removed and washed with zymogram renaturing buffer i.e. 2.5%Triton x-100 for one hour to remove SDS completely allowing the gels to renature. It was further incubated at 37ºC overnight, followed by staining with Coomassie blue R-250 for one hour and then destained with appropriate solution. After staining, the background was stained blue with Coomassie stain where the gelatin was degraded, while white bands appeared indicating the presence of gelatinases.

The percentage inhibition of protein denaturation was calculated by using the following formula % Inhibition = 100 x (Abs of control -Abs of sample) /Abs of control.[1]

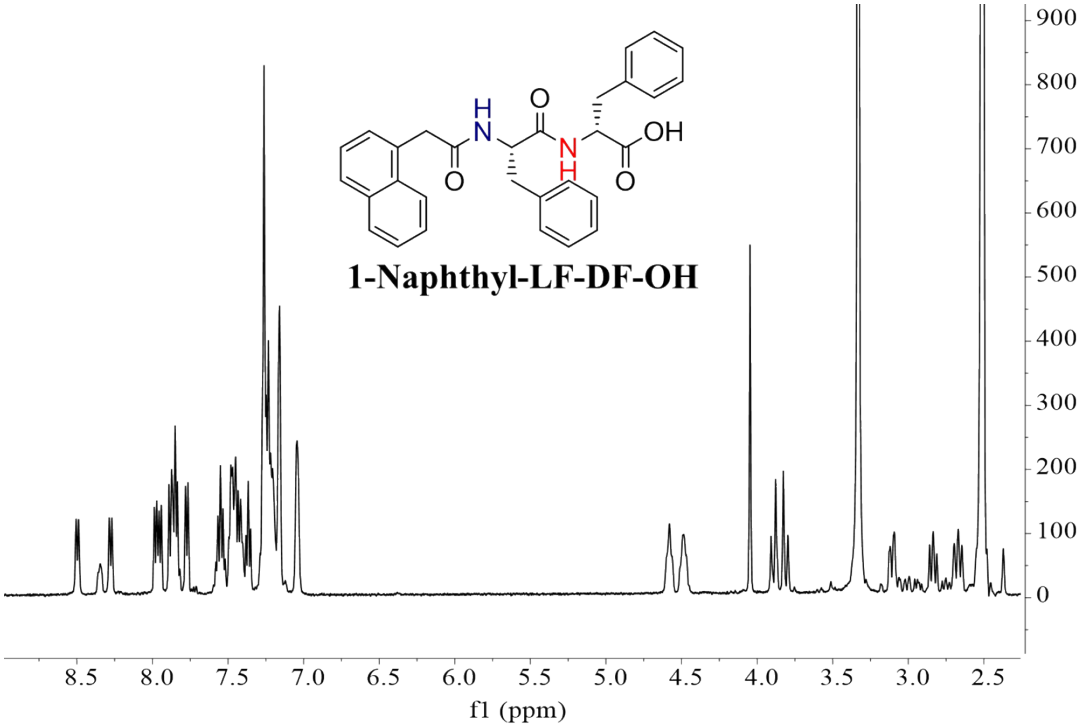

Figure S8: - ^{1}H NMR of Hydrogelator- **I**

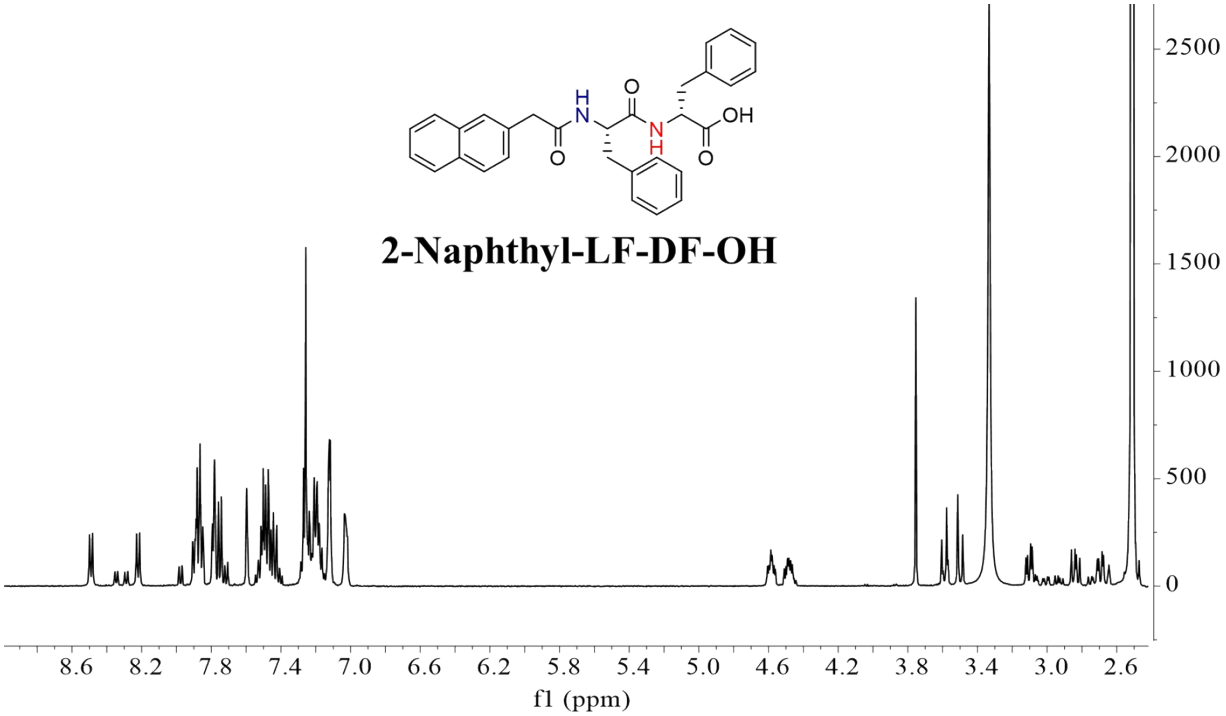

Figure S9: - ^{1}H NMR of Hydrogelator**- II**

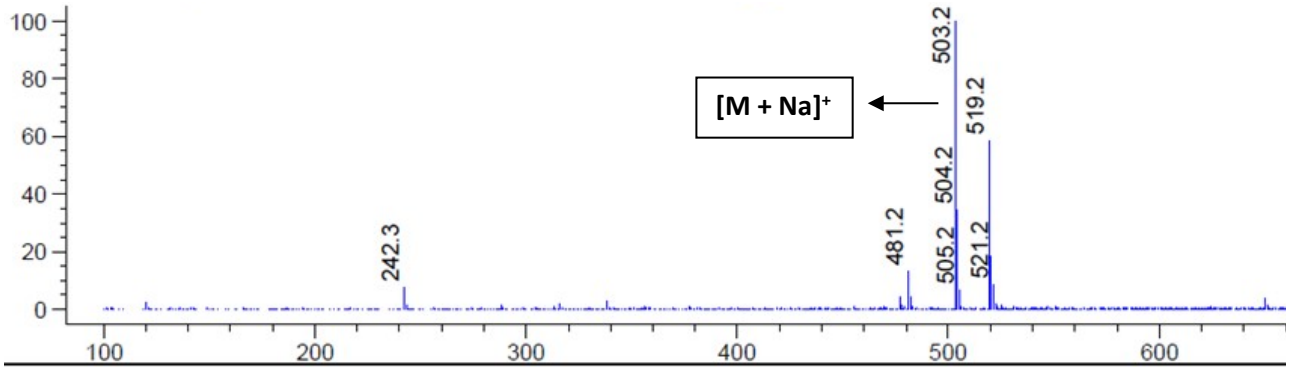

Figure S10: - Mass Spectra of Hydrogelator**- I**

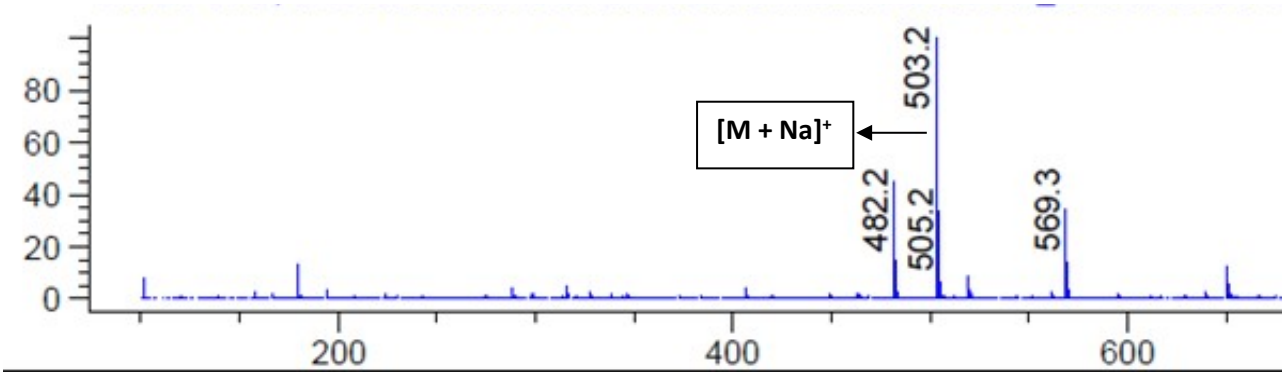

Figure S11: - Mass Spectra of Hydrogelator**- II**

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