Electronic Supplementary Information

Luminescent Ultrashort Peptide Hydrogelator with Enhanced Photophysical Implications and Biocompatibility

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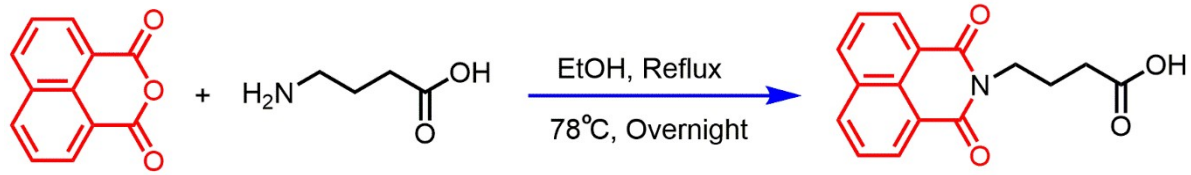
Materials and Instruments

Protected amino acids, resin, 2-(1h-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-Hydroxybenzotriazole (HOBT) and other reagents, chemicals and solvents were procured from Sigma-Aldrich, TCI, Spectrochem and SRL India. Cell culture media and supplements included RPMI 1640 (Catalog No. 1875093), MEM (Catalog No. 12492013), DMEM (Catalog No. 11965092), fetal bovine serum (FBS) (Catalog No. 10082147), and antibiotic-antimitotic (Catalog No. 15240096), all sourced from Gibco. The dyes used were 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (catalog no. M6494), live/dead™ Viability/Cytotoxicity kit (Catalog No. L3224, Invitrogen), and Hoechst 33258 (Catalog no. H21492). The other reagents included phosphate-buffered saline (PBS) (Catalog No. 10010023, Gibco), fixative (Image-IT I28900), and dimethyl sulfoxide (DMSO) (catalog no. D12345). Proteinase K (10 mg/mL, Catalog No. BUF28-5, derived from Engyodontium album) was sourced from G2P Pvt. Ltd. Unless otherwise mentioned, all the chemicals, reagents and solvents were used without further purification. The peptide is synthesized employing a standard Solid Phase Peptide Synthesis (SPPS) strategy using 9-Fluorenylmethyloxycarbonyl (Fmoc) chemistry. All the samples for the experiments were prepared using Milli-Q water with a conductivity of less than 2 $\mu S\ cm^{-1}$. The synthesized peptide was purified by Waters HPLC system equipped with UV-Vis detector using Atlantis® T3 C18 5 μm, 10 x 250 mm semi-prep HPLC column. Analytical HPLC was carried out in an Agilent 1260 Infinity II system using a YMC Triart C18 5 μm, 4.6 x 250 mm analytical column. Electrospray ionization mass spectra were acquired using a Q-TOF instrument from Waters. ^{1}H-NMR and ^{13}C-NMR were recorded using a JEOL 400 YH instrument (400 MHz). The viscoelastic property of the hydrogel was determined using an Anton Paar rheometer (MCR 102) with a 20 mm parallel plate and a 0.5 mm zero gap at a temperature of 25°C. Emission spectra were recorded on a Fluoromax Plus spectrophotometer, whereas Absorption spectra were acquired on an Agilent Cary Series spectrometer utilizing standard 10 mm and 1 mm path quartz cuvettes according to the requirements. For the FTIR experiment, a Parkin Elmer Spectrum FT-IR spectrometer was used. Circular Dichroism (CD) spectra were recorded in a Jasco J-1500 CD spectrometer using a 2 mm path quartz cuvette. Powder XRD data were acquired on a Malvern PANalytical Empyrean Series III. SEM, TEM and AFM images were captured on a ZEISS Gemini SEM 360 instrument and JEOL 2100F and Bruker DIMENSION FastScan microscopes, respectively. Fluorescence lifetimes were measured using Picosecond Time-resolved and Steady State Luminescence Spectrometer on an Edinburg Instruments Lifespec II & FSP 920 instrument pulsed diode laser with λ_{ex} = 375 nm.

Synthesis

Synthetic route of building block

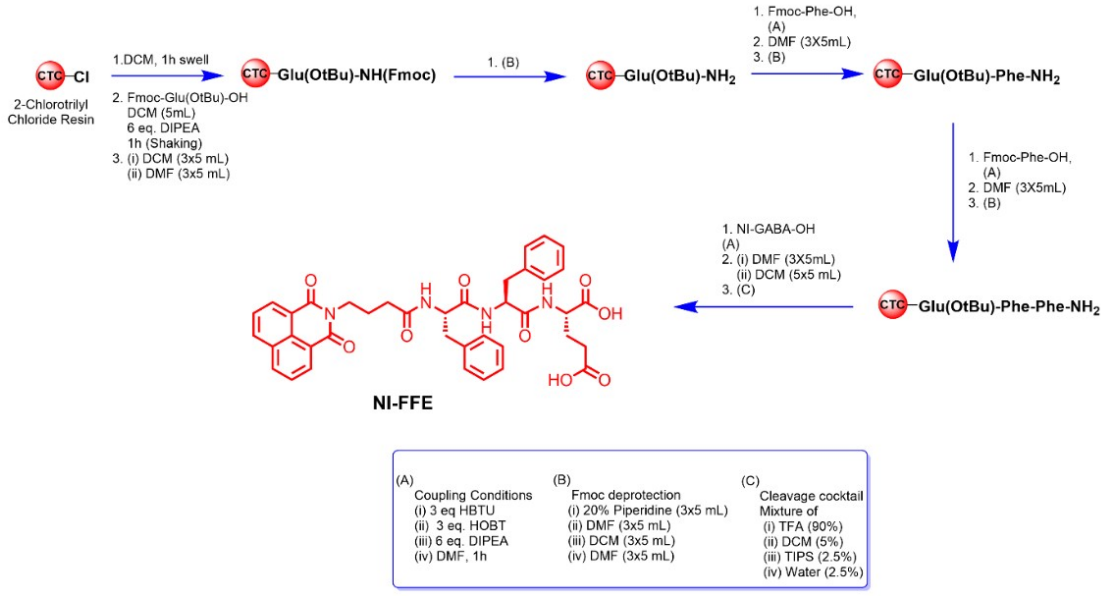


Scheme S1. Synthesis of **NI-GABA-OH**

NI-GABA-OH was synthesized using our previously published protocol.[1] For instance, 1,8-Naphthalic anhydride 4g (20.1 mmol) and 4-aminobutyric acid 2.7 (1.3 eqv. 26.2 mmol) were taken in a 100 mL round bottom flask and 30 mL ethanol was added. The reaction mixture was heated at 80 °C for 10 hours with constant stirring. After cooling to room temperature, the reaction mixture was diluted with 70 mL of cold water and vacuum filtered followed by repetitive washing with water to afford a white-colored product. Finally, the product was dried under vacuum yielding 71% (4.05g).

NMR and Mass Spectra: Compound already characterized.[1]

Synthetic route of peptide



Scheme S2. Solid Phase Peptide Synthesis

The peptide (**NI-FFE**) was synthesized by standard 'Fmoc' solid-phase peptide synthesis strategy employing 2-Chlorotrilyl Chloride Resin (2-CTC resin) as the solid support. Prior to the synthesis, the resin was appropriately dried under reduced pressure. The first coupling was carried out in DCM employing Fmoc-Glu(OtBu)-OH (3 eqv.) and DIPEA (6 eqv.), followed by washing and 'Fmoc' deprotection. The peptide chain elongation at the N-terminus was achieved by coupling the appropriate moiety (3 eqv.) under standard conditions using HBTU (3 eqv.) and DIPEA (6 eqv.) in the presence of HOBT (3 eqv.) in DMF. The final peptide was cleaved from the resin using a cleaving cocktail of TFA-DCM-TIPS-Water (90:5:2.5:2.5). The cleaved peptide was precipitated from cold dry diethyl ether. The purification was achieved using semi-prep HPLC (acetonitrile/water having 0.1% TFA system as the eluent, in a gradient of 10 to 80 % acetonitrile over 20 min) followed by lyophilization to yield the pure peptide.

Characterization of the peptides

NI-FFE: **^{1}H-NMR** (400 MHz, DMSO-d_6) δ: 8.50-8.44 (m, 4H), 8.20 (s, 1H), 8.07 (s, 1H), 7.99 (s, 1H), 7.89-7.85 (t, 2H), 7.24-7.10 (m, 10H), 4.55-4.50 (m, 1H), 4.46-4.41 (m, 1H), 4.25-4.20 (m, 3H), 3.99-3.96 (t, 2H), 3.06-3.01 (m, 1H), 2.93-2.89 (m, 1H), 2.83-2.78 (m, 1H), 2.67-2.61 (m, 1H), 2.29-2.24 (m, 2H), 2.10-2.06 (t, 2H), 2.0-1.96(m, 1H), 1.83-1.69 (m, 3H).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 173.79, 173.11, 171.51, 171.21, 171.06, 163.51, 137.99, 137.71, 134.37, 131.37, 130.80, 129.27, 129.14, 128.05, 127.96, 127.47, 127.30, 126.25, 126.12, 122.16, 53.66, 53.54, 51.18, 37.29, 33.06, 32.95, 29.97, 26.38, 23.92, 17.89.

MS-ESI: m/z: calculated 706.2639 for $C_{39}H_{38}N_4O_9$, found: 707.2714 $[M+H]^+$

Methods

Hydrogel Formation

The lyophilized peptide powder was suspended in Milli-Q water and an equimolar quantity of NaOH (10N) was added. The deprotonation of the carboxylic acid end group, facilitated by basic condition that enhanced water solubility, resulted in a clear solution. The pH of this solution was measured and found to be around 10. The solution was then sonicated and vortexed to ensure a homogeneous mixture. Instant hydrogelation was achieved by shifting the pH of the solution by adding the required amount of dilute HCl to make the final pH around 5.5. However, for uniform gelation, a small amount of GdL (10 mg/mL) was added, and the mixture was allowed to stand, forming a homogeneous, self-supporting supramolecular hydrogel. Typically, to prepare a 1 wt/v% supramolecular hydrogel, 10 mg of the lyophilized peptide was dissolved in 900 µL of Milli-Q water, followed by the addition of 10N NaOH to increase the pH and ensure complete peptide solubility and the final volume was adjusted to

1000 µL. A pinch amount of GdL (10 mg/mL) was then added to initiate the gelation process and the solution was left undisturbed until the gelation process got completed. The hydrogel formation was verified using the vial-inversion test.

Rheology

The viscoelastic property of the hydrogel was assessed using an Anton Paar rheometer (MCR 102) with a 20 mm parallel plate and a 0.5 mm zero gap at a temperature of 25°C. A strain sweep test was performed to establish the linear viscoelastic region (LVR), maintaining a constant oscillation frequency of 1 rad s^{-1} while varying the strain from 0.01% to 100%. The LVR can be defined as the region where strain has no impact upon G' and G". Following this, a frequency sweep test was conducted to evaluate the gel's mechanical strength, covering a frequency range from 0.1 to 100 rad/s at 25°C under a strain of 0.1%, determined from the LVR. Furthermore, to investigate the thixotropic and self-healing properties of the hydrogel (1 wt/v %), a cyclic dynamic strain sweep experiment was performed at a constant angular frequency of 1 rad s^{-1}. In this experiment, a higher strain (γ = 200%) and a lower strain (γ = 1%) were applied on the gel alternatively throughout 2600 s and four successive cycles.

Stability of Hydrogel in buffer of pH 7.4 and cell culture medium

The hydrogel was prepared in Tris buffer pH 7.4 or cell culture medium (DMEM from Gibco, Catalog No. 11965092). Briefly, a series of 0.5 mL 1 wt/v% hydrogels were prepared and to these 0.5 mL Tris buffer (pH 7.4) or culture medium were added and incubated. After a predetermined time periods, the solution from the hydrogel was removed and the excess surface water was gently blotted with tissue paper, and the hydrogel was weighed. The percentage degradation was calculated from the ratio of the final weight to the original weight of the hydrogels.

Stability of Hydrogels toward Proteolytic Digestion

The enzymatic degradation of the hydrogel was conducted using proteinase K. In a typical procedure, a series of hydrogels were prepared and to these 0.5 mL of proteinase K solution (0.1 mg/mL) in HEPES buffer (50 mM, pH 7.4) were added. After a predetermined time periods, the solutions were removed, excess surface water was gently soaked with tissue paper, and the hydrogels were weighed. The percentage degradation was calculated from the ratio of the final weight to the original weight of the hydrogels**.**

Swelling Property

A series of hydrogels were prepared and freeze-dried individually. The weight of the hydrogels were recorded (W_d). Then each of the dried gel was immersed in a large excess of water at room temperature for a various periods of time. The water-swollen gels were then centrifuged, removed from the bulk water, and weighed (W_s). The swelling ratio (SR) was calculated following equation

$$\mathbf{SR = [(W_s - W_d)/W_d]}$$

Scanning Electron Microscopy (SEM)

SEM analysis was conducted using a ZEISS Gemini SEM 360 Germany. SEM samples were prepared by drop casting on an aluminium-coated glass slide. Typically, a 10 μL **NI-FFE** hydrogel (1 wt/v%) or solution (at pH 10) was drop-cast onto the slide and air-dried prior to the imaging.

Transmission Electron Microscopy (SEM)

The TEM experiment was conducted by casting the sample on a carbon-coated Cu grid (300 mesh Cu grid with thick carbon film from Pacific Grid Tech, USA). In a typical method, a 10 μL hydrogel was drop-cast on the grid and allowed to absorb for 30 sec, followed by bloating the excess hydrogel with tissue paper and air drying for 2 days.

Atomic Force Microscopy (SEM)

AFM was performed on a Bruker DIMENSION FastScan with ScanAsystTM using Peak Force Mapping in Air Tapping mode. The cantilever was calibrated using the automated "no touch" calibration routine built into the software. The diluted hydrogel was dropped onto freshly cleaved mica surfaces. The sample was air-dried at room temperature for 24 h and imaged with a Peak Force set point of 500 pN with a Peak Force amplitude of 30 nm and frequency of 4 kHz.

Circular Dichroism (CD) Spectroscopy

CD Spectra of samples were recorded using a JASCO J-1500 CD Spectrometer equipped with a Peltier temperature Control. The data were collected using a 2 mm path length cuvette at a scan rate of 100 nm min^{-1} with 0.5 nm intervals and 2 nm bandwidth. For concentration-dependent CD measurement, a series of **NI-FFE** solutions (at basic pH) at different concentrations were prepared and assembly was induced by adjusting the pH around 5.5-6, followed by recording CD spectra. Additionally, to follow the CD kinetics, a 2 mM peptide solution GdL was added to initiate the assembly, and time-dependent

CD spectra were recorded from 300 to 400 nm. Prior to every CD experiment, baseline correction using comparable reference solutions was carried out.

Fourier-Transform Infrared (FT-IR) Spectroscopy

FTIR spectra were recorded on a Parkin Elmer Spectrum FT-IR Spectrometer using a Diamond ATR from Parkin Elmer. Non-assembled and assembled (hydrogel) **NI-FFE** were prepared in DMSO-d_6 and D_2O for the FT-IR measurement. In a typical experiment, 10 μL of the sample was applied to the sample holder, and transmittance was recorded.

Powder X-ray diffraction (**PXRD)**

PXRD data were collected on Malvern PANalytical Empyrean Series III, using Cu-Kα radiation (wavelength 1.540598 Å) at a 45 kV voltage and a 40 mA current. Data were interpreted using High Score Plus software. The layer thickness *d* was calculated according to the Bragg equation: $d = \lambda/2 \sin\theta$, λ = 1.540598 Å).[1] For PXRD experiments, typically, a 20 mg lyophilized hydrogel was used.

Absorption Spectroscopy

The absorption spectra of **NI-FFE** at different solvent compositions, f_w =0 % to 90 % (f_w is the volume fraction of water in DMSO), were measured using an Agilent Cary Series spectrometer utilizing standard 10 mm path quartz cuvettes at room temperature. The final concentration of **NI-FFE** was kept at 100 μM to avoid the detector's saturation. Baseline correction using comparable reference solutions was performed before every measurement.

Fluorescence Spectroscopy

Emission spectra of different **NI-FFE** samples were recorded on a Fluoromax Plus spectrophotometer utilizing standard 10 mm path quartz cuvettes by exciting at 350 nm at 25°C. For samples with different water/DMSO (f_w) compositions, the final peptide concentration was kept at 1 mM. However, for concentration concentration-dependent experiments, peptide concentration was varied from 1 μM to 3 mM. The temperature-dependent emission spectra were recorded by varying the temperature from 20^{o}C to 80^{o}C to and fro at a rate of change temperature 1^{o}C/min, keeping peptide concentration 1 mM and f_w = 90%.

For the Thioflavin T (ThT) assay, 10 μM ThT was used as the fluorescent probe for all experiments, using an excitation wavelength of 450 nm and an emission wavelength of 482 nm.

It is to be noted that we performed all the spectroscopic characterization (unless otherwise mentioned) of **NI-FFE** below its MGC value to avoid any scattering.

Computational study of NI-FFE assembly

Desmond in Schrodinger Release 2021-1 [Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2021] was utilized to perform molecular dynamics (MD) simulations to investigate molecular interactions in the **NI-FFE** tetramer. The tetramer was created with the ChimeraX tool.[2] The orthorhombic box was used for the solvation of the system using the TIP3P water model.[3] To neutralize the system, the appropriate amounts of Na^+ and Cl^- ions were added to achieve a salt concentration of 0.15 nM. Minimization and relaxation were executed using the NPT ensemble method.[4] The MD simulations of the **NI-FFE** tetramer lasted for 500 ns, with trajectories recorded every 100 ps. During the simulation, the temperature and pressure were maintained at 300 K and 1.01325 bar, respectively. For equilibration, a standard relaxation procedure utilizing the RESPA integrator was applied.[5] Each system underwent an initial relaxation of 1 ps using the thermostat principle through the Nose-Hoover chain method[6] followed by applying the barostat principle with the Martyna-Tobias-Klein approach[7], allowing for an additional 2 ps of relaxation. Energy minimization for all complexes was conducted using the OPLS4 force field.[8]

Cell Culture

The human keratinocyte (HaCaT), mouse fibroblasts (McCoy), mouse embryonic fibroblast (MEF) and Rat bone marrow mesenchymal stem cells (BMSCs) and human immortalized monocyte cell line (THP-1) were obtained from the National Centre for Cell Science (NCCS), Pune, India. The HaCaT, MEF and Rat BMSC cells with passage numbers 4, 13 and 11 were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) and the McCoy cells with passage number 18 were cultured in MEM media supplemented with 8% FBS and THP-1 cells with passage number 13 cultured in RPMI media 1640 supplemented with 10% FBS and 1% antibiotic-antimycotic at 37°C in a 5% CO_2/95% air humidified incubator (Thermo HERA cell, USA).

MTT Assay

Cell viability study was performed in HaCaT, McCoy and THP-1 cell lines cell lines using MTT assay. Overnight grown cells (1 x 10^5 cells) in 96 well plates were treated with different concentrations of **NI-FFE** hydrogelator for 24h. The cells were then washed twice with 1X PBS, followed by a 3h treatment with 10µL of MTT (5 mg/mL). Finally, the MTT solution was decanted cautiously, and 100 µL of DMSO was added into each well and incubated for 15 min for proper dissolution of the formazan aggregates. The cell viability was calculated by recording the optical density (OD) at 570 nm using a Plate reader

(MultiskanTM Spectrophotometer, A51119600C). The experiment was performed in triplicate, and the IC50 values were determined.

Live/Dead Analysis

The cytocompatibility of the **NI-FFE** hydrogelator was evaluated in four cell lines, HaCaT, McCoy, MEF, and Rat BMSC, by live/dead assay. The assay employs a Double Staining Kit that utilizes Calcein-AM and Ethidium bromide (EtBr) to distinguish viable from dead cells, respectively. Briefly, cells were cultured in a 6-well plate with coverslips, with a seeding density of 1×10^5cells/well, and placed in an incubator with 5% CO_2 at 37°C (Thermo HERA cell, USA) for 24h. Then, the confluent cells were treated with IC50 of **NI-FFE** hydrogelator for 24h. After rinsing the cells twice with 1 × PBS, calcein and ethidium homodimers in phosphate buffer were added, and the plate was incubated at 37°C for 45 min. The solution was then removed, and the cells were washed thrice with 1 × PBS. The cells were then fixed with fixative (Image-IT I28900) for 15 minutes, rinsed twice with 1 × PBS, and incubated with Hoechst solution (H21492) for 10 minutes. Further, the cells were extensively washed with 1 x PBS, and the coverslips were monitored under a confocal microscope (Leica DMi8, STELLARIS 5).

Cellular uptake

A comparative cellular uptake study was performed to evaluate the selective uptake of **NI-FFE** hydrogelator under a controlled in vitro milieu. Briefly, cells were cultured in a 6-well plate with coverslips, a seeding density of 1×10^5 cells/well, and placed in an incubator with 5% CO_2 at 37°C (Thermo HERA cell, USA). After that, the cells were treated with ½ IC_{50}, IC_{50}, and 2 IC_{50} of **NI-FFE** hydrogelator for 24h. The media was discarded, and cells were washed twice with 1X PBS. Then, the adhered cells on the coverslips were directly visualized under a confocal microscope (Leica DMi8, STELLARIS 5) with an excitation wavelength of 422 nm and an emission wavelength of 450 nm.

Biocompatibility and Immunogenicity assessment

The biocompatibility of the hydrogelator was assessed using a live/dead dual staining in the human immortalized monocyte cell line (THP-1). THP-1 cells were cultured in 6-well plate, with a seeding density of 1×10^5cells/well. After proper growth, the cells were treated with IC_{50} of **NI-FFE** hydrogelator for 24 h. Later, calcein and ethidium homodimer stains were used to differentiate viable and non-viable cells. The slides were visualized under a confocal microscope (Leica DMi8, STELLARIS 5) with an excitation wavelength of 422 nm and an emission wavelength of 450 nm.

Further, to confirm the immune response, the expression of CD14 was evaluated by immunofluorescence after 24 h treatment with hydrogelator. The undifferentiated monocytes were

fixed for 20 min with 4% paraformaldehyde, washed with PBS, and blocked with BSA. After washing, the monocytes were incubated overnight with CD14 at 4°C. Then, the cells were washed twice with PBS and incubated with Alexa-Fluor secondary antibody for 3 h. Subsequently, the cells were washed with PBS, followed by actin staining for 20 min. The cells were then washed with PBS and stained with Hoechst solution for 10 min. After extensive washing with PBS, the coverslips were mounted onto slides using gold anti-fade and imaged using a confocal microscope (Leica DMi8, STELLARIS 5).

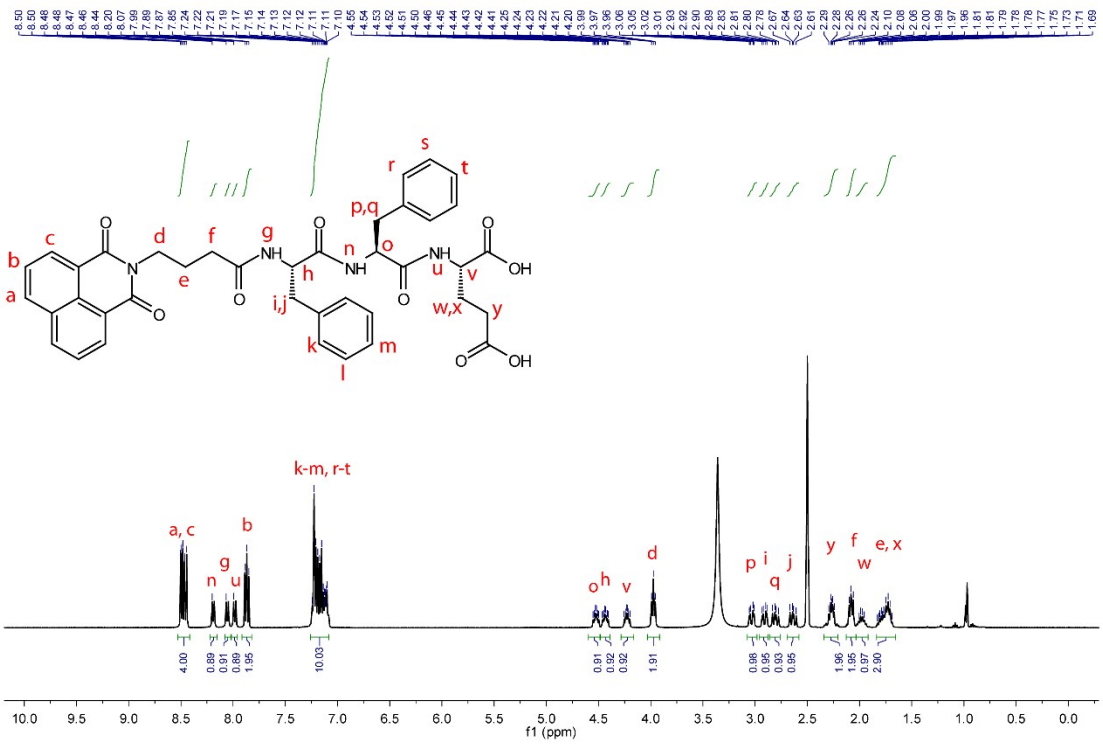


Fig. S1 ^{1}H-NMR spectrum of **NI-FFE** in DMSO-d_6.

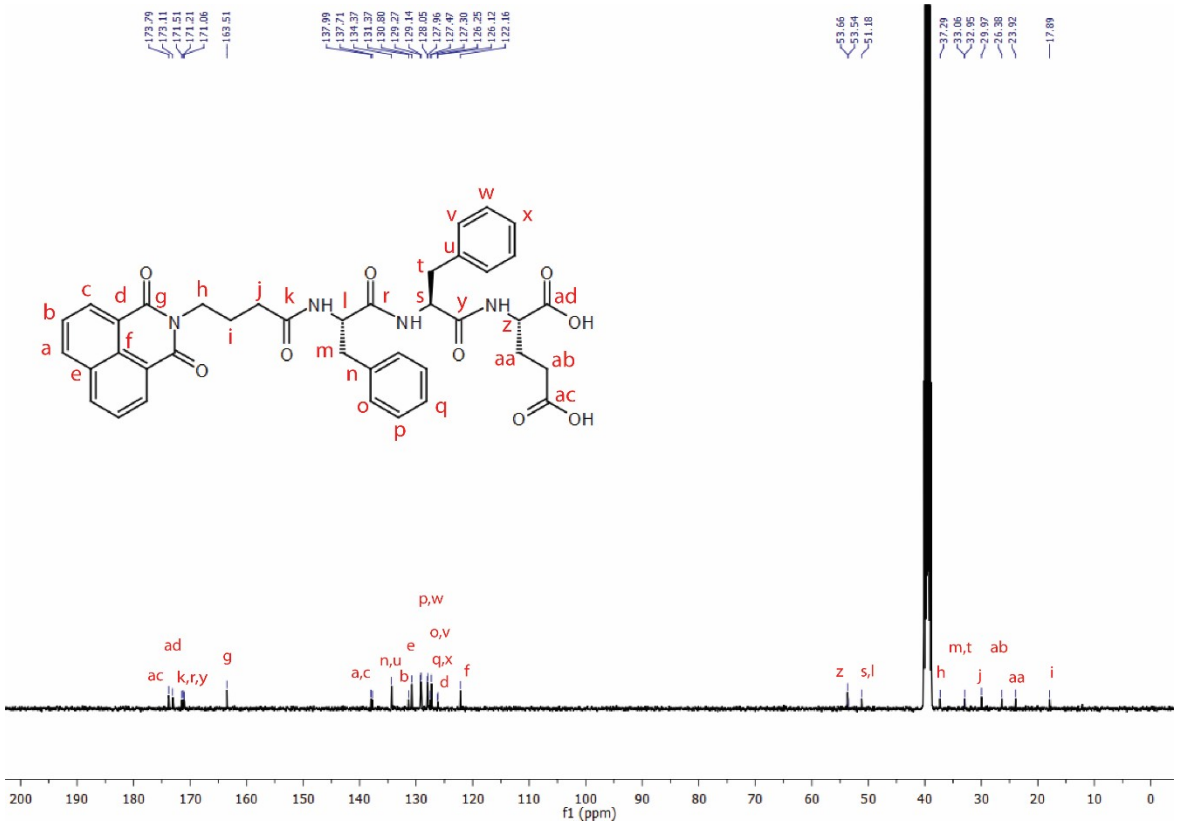


Fig. S2 ^{13}C-NMR spectrum of **NI-FFE** in DMSO-d_6.

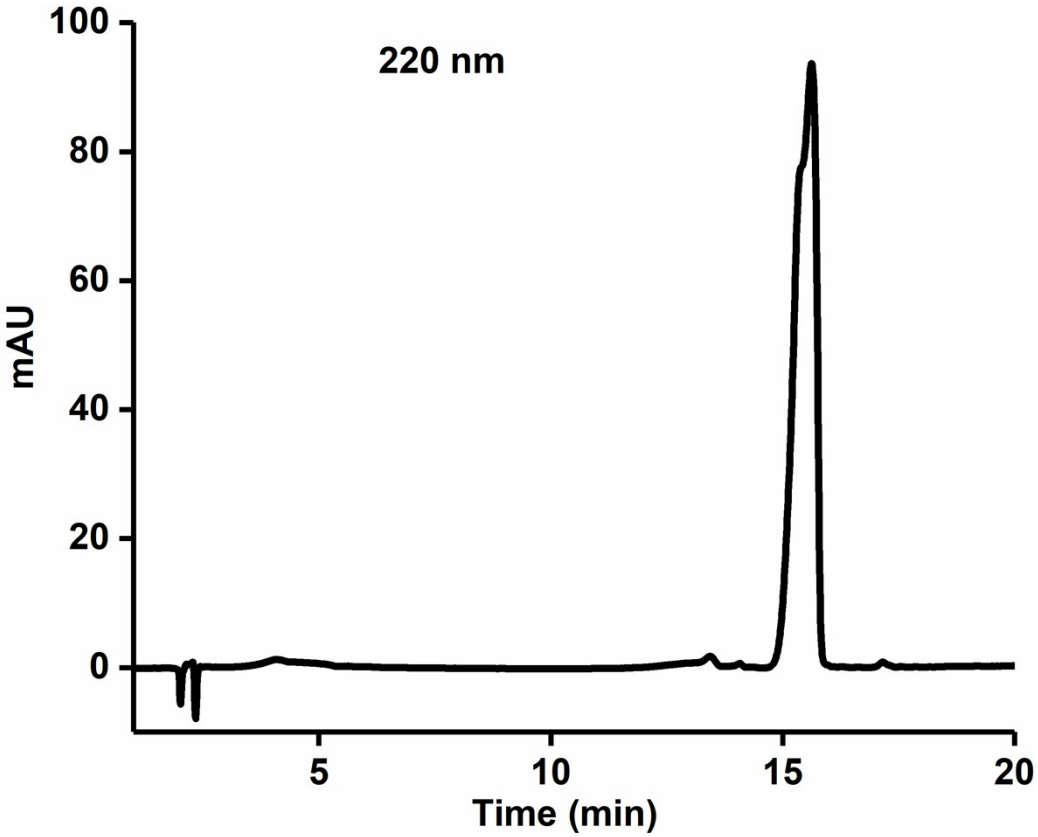


Fig. S3 HPLC Chromatogram of **NI-FFE** recorded at 220 nm wavelength

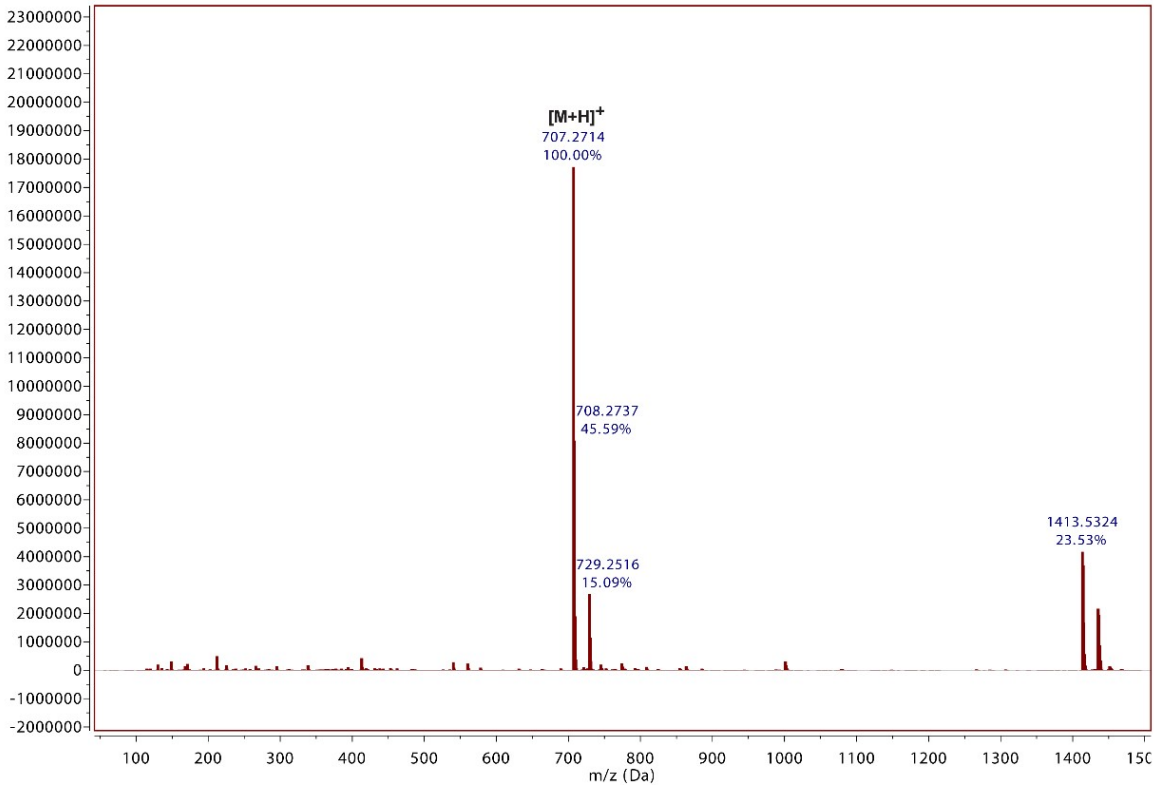


Fig. S4 ESI-MS of **NI-VLD**.

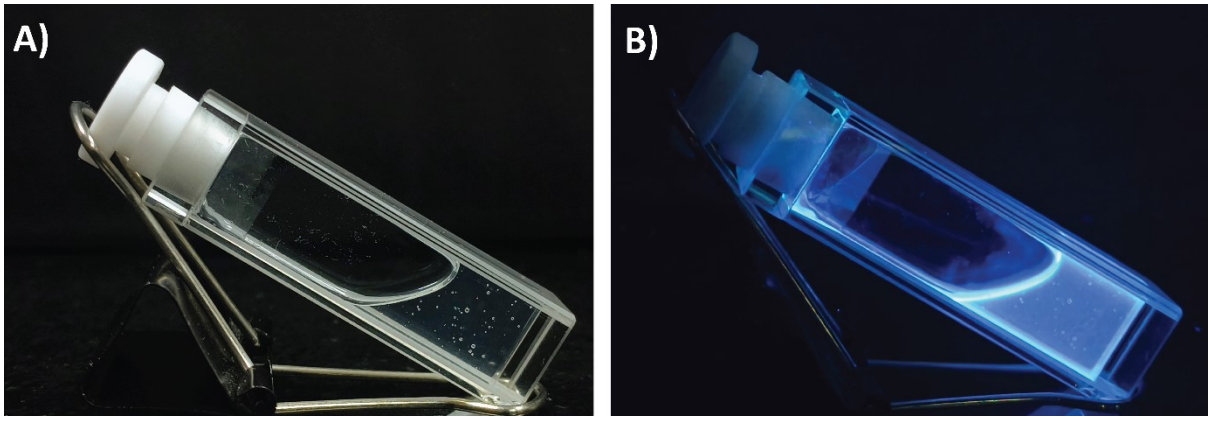


Fig. S5 A) 1 wt/v% **NI-FFE** at pH 7 as a viscous solution but not hydrogel, visualized under ambient light. **B)** The same viscous solution exhibiting strong blue emission when visualized under uv lamp of 365 nm.

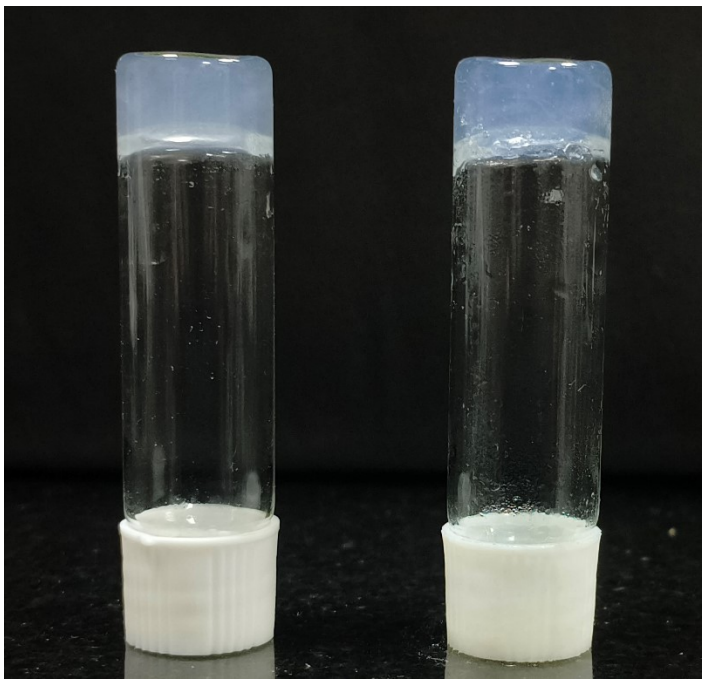


Fig. S6 Left**: Freshly prepared NI-FFE** Hydrogel. Right: **NI-FFE** hydrogel after slowly heating from room temperature up to 80 ^{o}C, demonstrating high thermal stability. [**NI-FFE**] = 1 wt/v% = 14.14 mM).

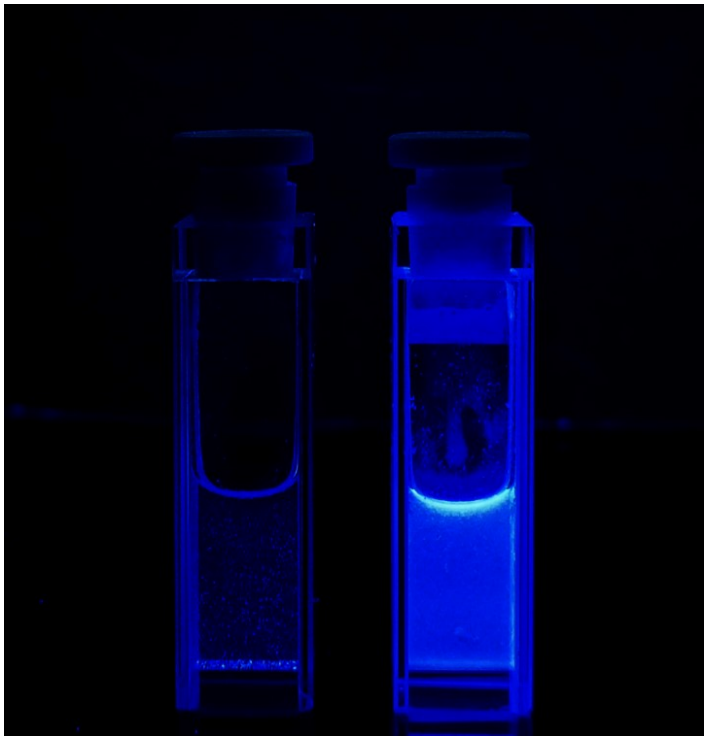


Fig. S7 Left**: NI-FFE** solution at low concentration (100 **×** 10^{-3} mM) showing faint blue emission. Right: **NI-FFE** at gelation concentration (1 wt/v% = 14.14 mM) showing blue solid emission. Both systems are under UV lamps of 365 nm, solution pH 5.5.

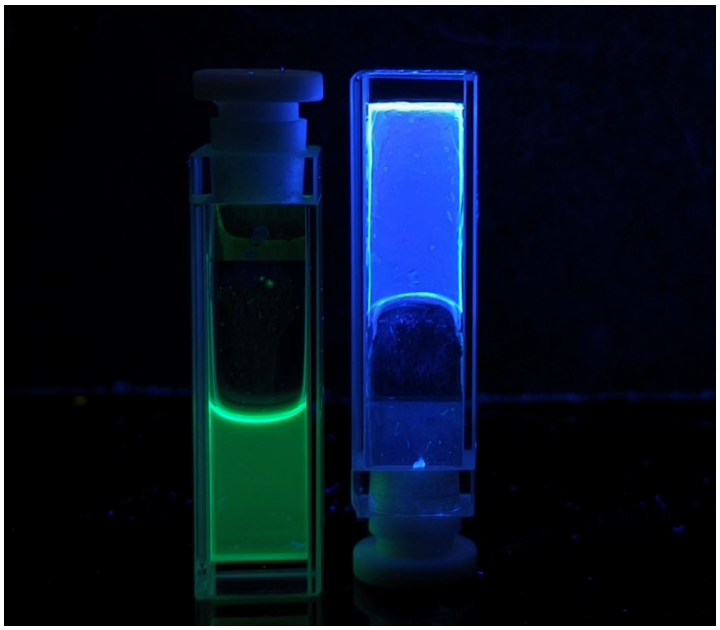


Fig. S8 Left**: NI-FFE** solution at basic condition (pH 10) showing strong green emission. Right: **NI-FFE** hydrogel at acidic condition (pH 5.5) showing strong blue emission. Both systems are under UV lamp of 365 nm. [**NI-FFE**] = 1 wt/v%.

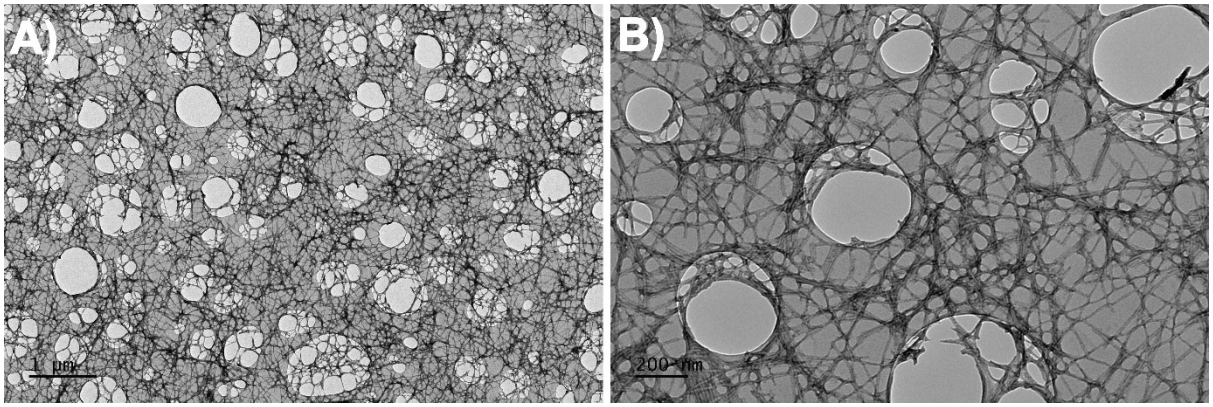


Fig. S9 TEM images of **NI-FFE** hydrogel at different magnifications. [**NI-FFE**] = 1 wt/v%.

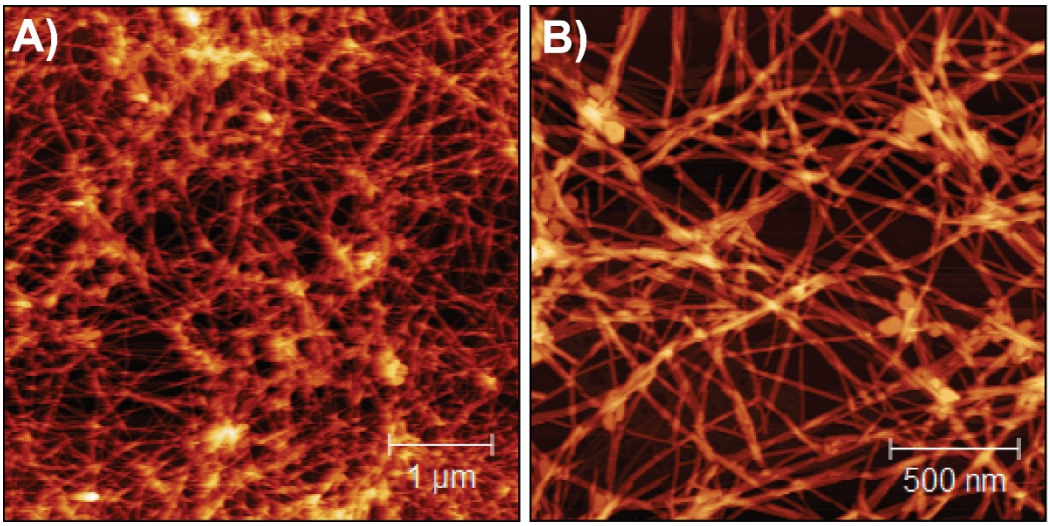


Fig. S10 AFM images of **NI-FFE** hydrogel at different magnifications. [**NI-FFE**] = 1 wt/v%.

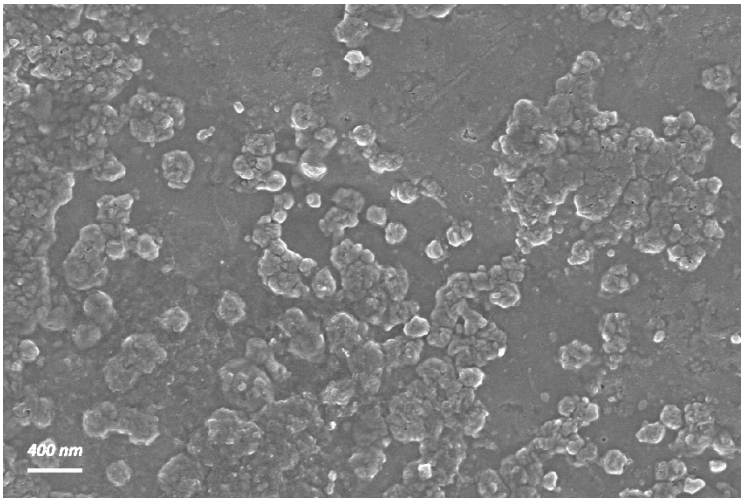


Fig. S11 SEM Image of **NI-FFE** at pH 10. [**NI-FFE**] = 1 wt/v%.

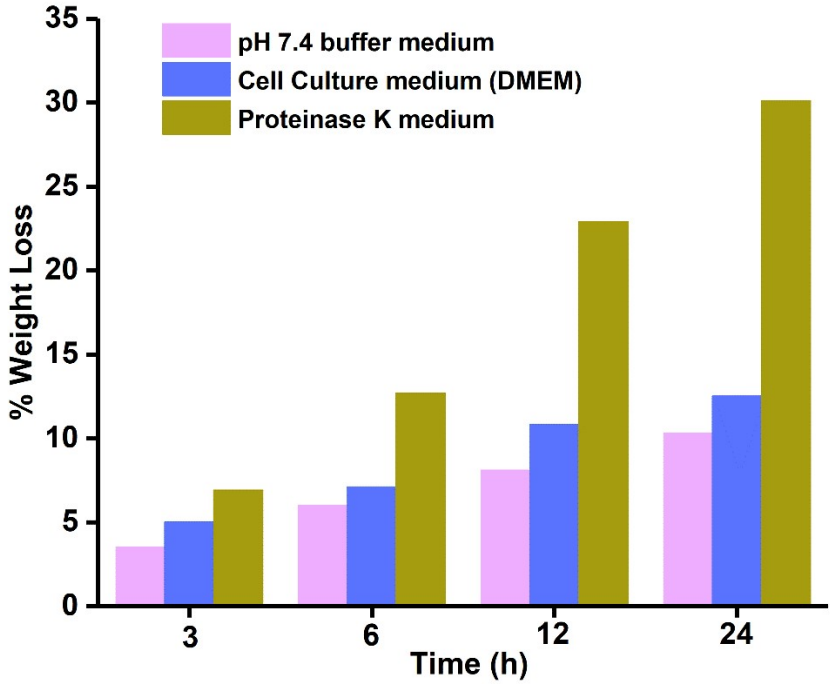


Fig. S12 Stability profile of **NI-FFE** hydrogel in different medium for different time periods.

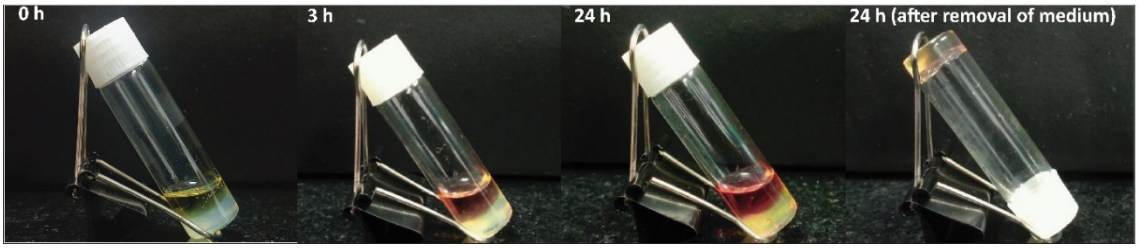


Fig. S13 Photographs of **NI-FFE** hydrogel incubated in DMEM for different time periods demonstrating high stability of the hydrogel in the culture medium.

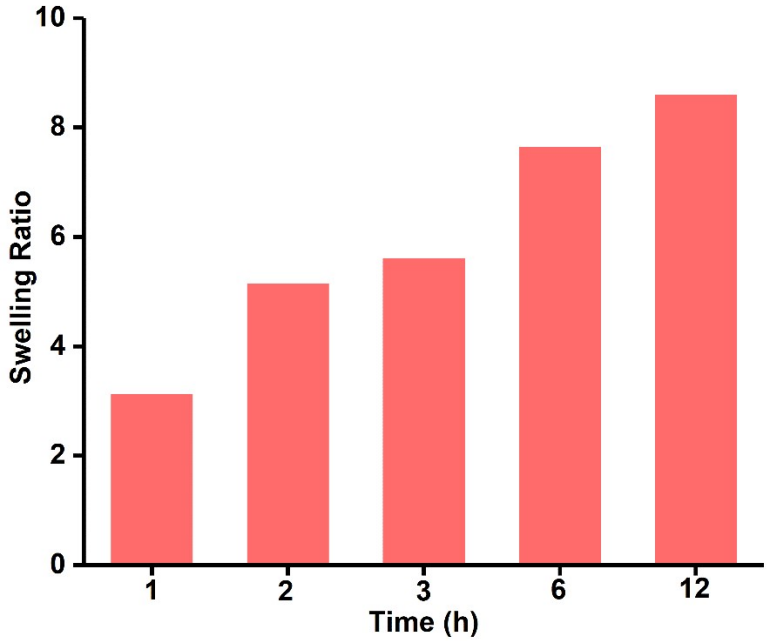


Fig. S14 Swelling ratio of dried **NI-FFE** gel at different time intervals.

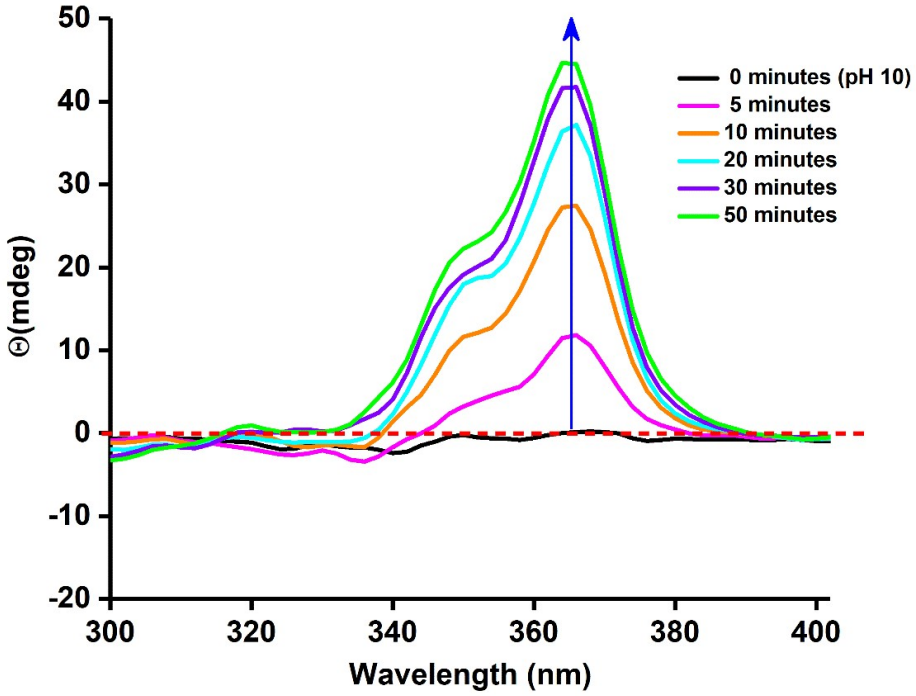


Fig. S15 CD kinetics, *i.e*. gradual appearance of signature signal of NI chromophore after addition of GdL, indicating generation of induced chirality. [**NI-FFE**] = 2 mM

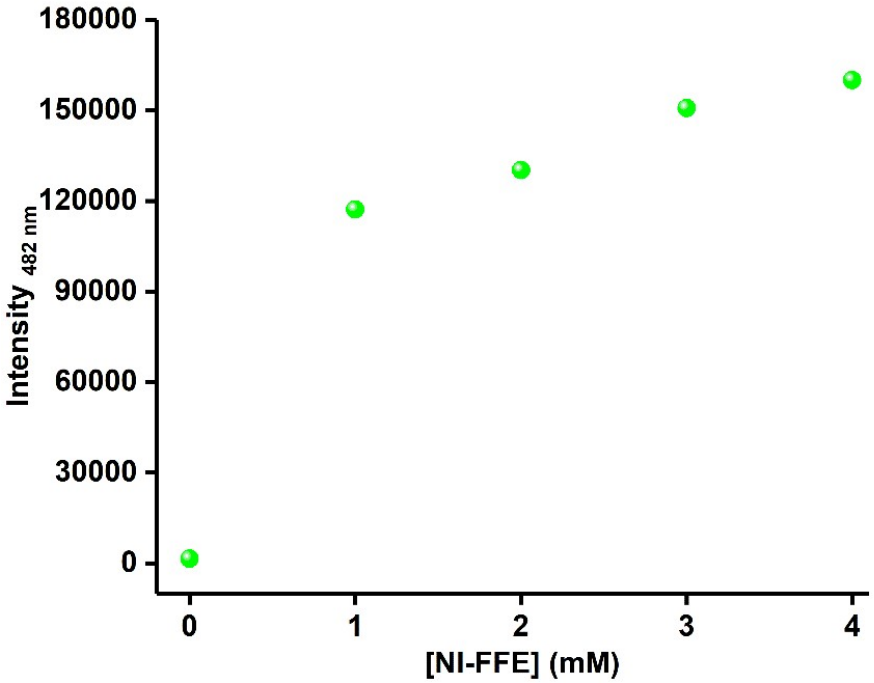


Fig. S16 ThT emission intensity at 482 nm as a function of peptide concentration. [ThT] = 10 μM and λ_{ex} = 450 nm.

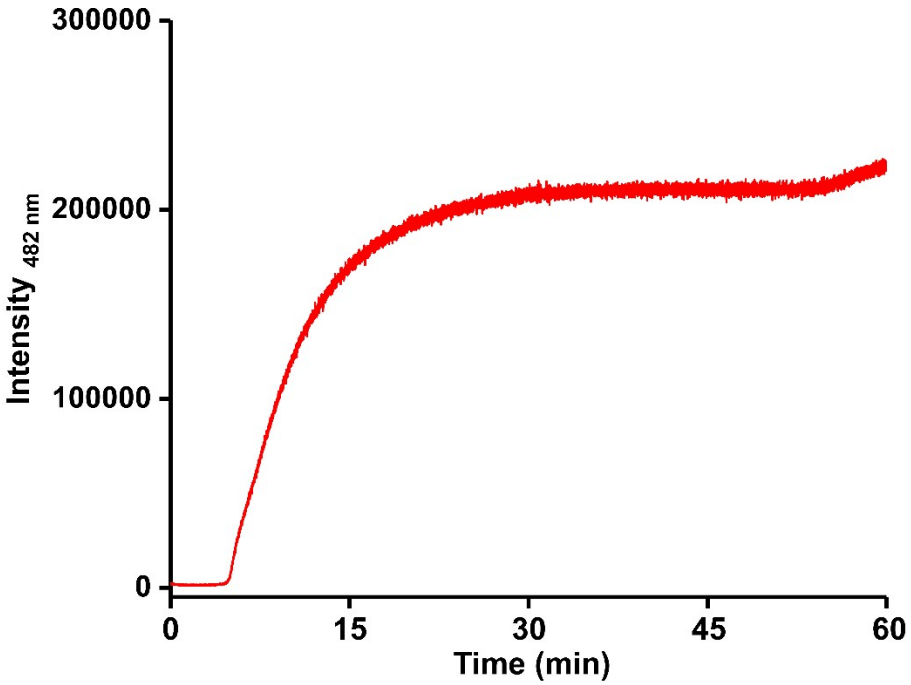


Fig. S17 Change of ThT emission intensity at 482 nm on the addition of GdL to 1 mM peptide solution demonstrating assembly kinetics.

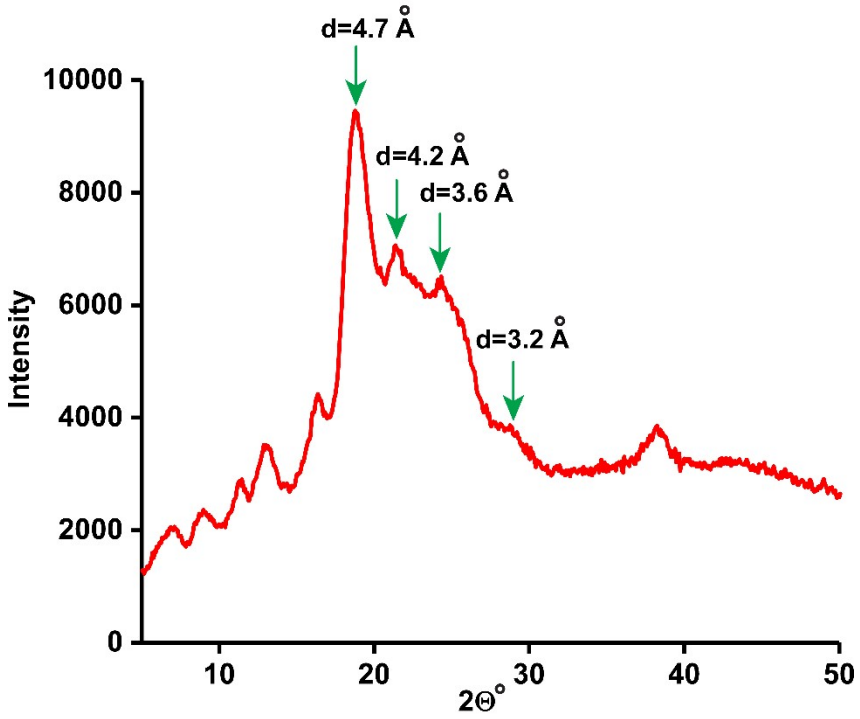


Fig. S18 PXRD data of **NI-FFE** xerogel. The green arrows indicate *d* spacing.

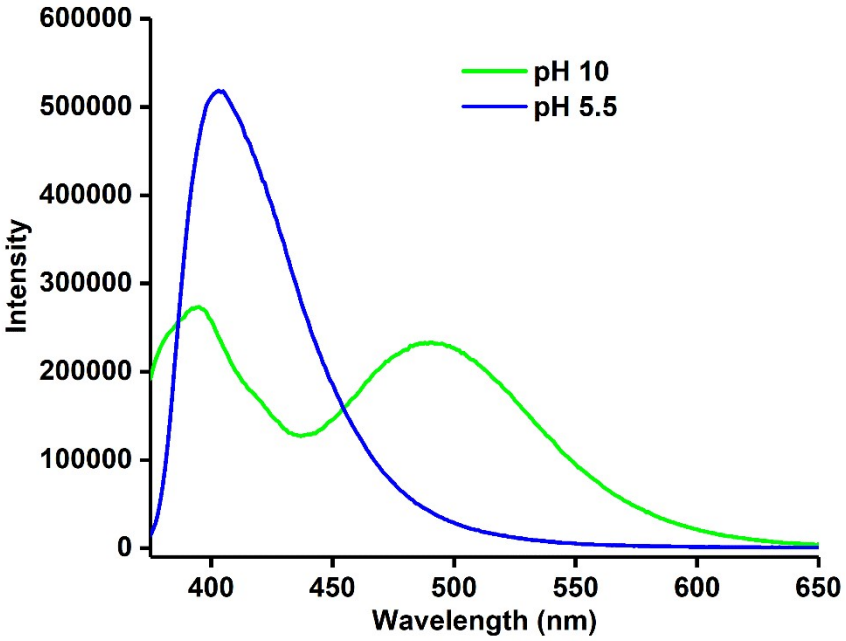


Fig. S19 Emission spectra of **NI-FFE** at pH 10 and pH 5.5. [**NI-FFE**]= 1 mM, λ_{ex} = 350 nm.

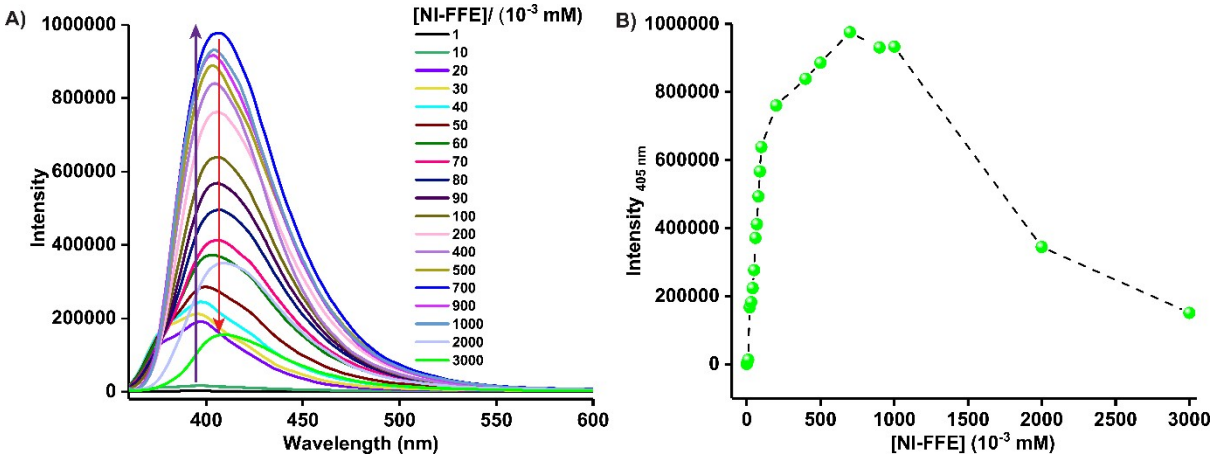


Fig. S20 A) Concentration-dependent emission spectra of **NI-FFE**. **B)** Emission intensity at 405 nm as a function of **NI-FFE** concentration. λ_{ex} = 350 nm.

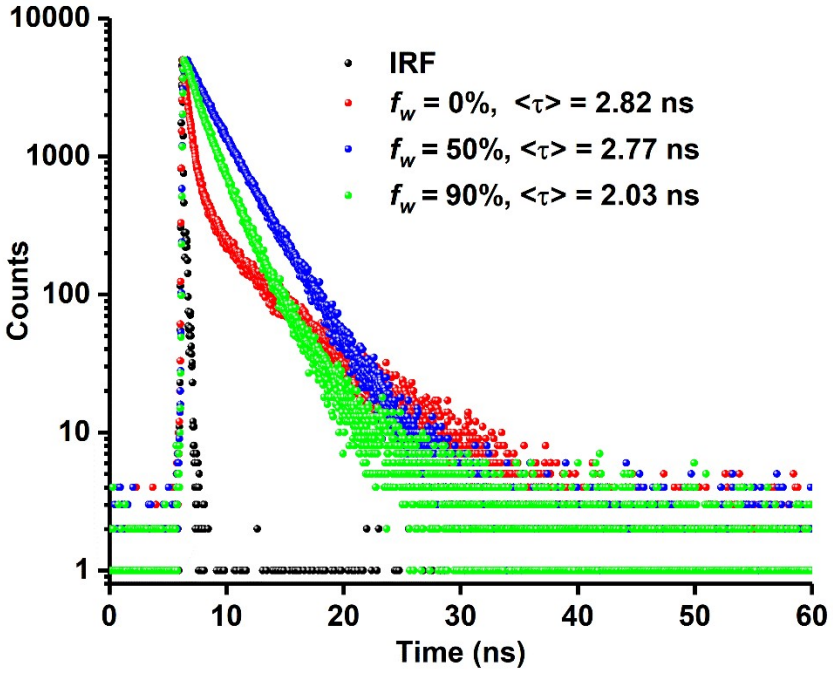


Fig. S21 Fluorescence decay profile of **NI-FFE** at different states acquired at λ_{em} = 405 nm, when λ_{ex} = 375 nm**.**

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