Electronic Supplementary Material (ESI) for Faraday Discussions.

Supplementary Information:

UV-Responsive Cyclic Peptide Progelator Bioinks

Carlini, et. al.

1. Abbreviations

AcOH: acetic acid; **ANP**: (S)-3-amino-3-(2-nitrophenyl)propionic acid; **CTC**: 2-chlorotrityl chloride; **DCM**: dichloromethane; **DIPEA**: N,N-diisopropylethylamine; **DMF**: N,N-Dimethylformamide; ECD: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; **Fmoc**: 9-Fluorenylmethoxycarbonyl; **2-nF**: 2-nitrophenylalanine; **HATU**: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; **HBTU**: 3-[Bis(dimethylamino)methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate; **HPLC**: high-pressure liquid chromatography; **SPPS**: solid phase peptide synthesis; **TFA**: trifluoroacetic acid; **TIPS**: triisopropylsilane.

2. Materials and methods

All reagents were purchased from Commercial Sources and used without further purification. Solvents for organic synthesis were of reagent grade. HPLC grade solvents were used for all LC analysis techniques. Purified water was obtained from a Thermo Scientific Barnstead GenPure xCad Plus system. Peptides were synthesized on an AAPPTec Focus XC automated peptide synthesizer. Analytical HPLC analysis of peptides was performed on a Jupiter 4u Proteo 90Å Phenomenex column (150 x 4.60 mm) using a Hitachi-Elite LaChrom L-2130 pump equipped with UV-Vis detector (Hitachi- Elite LaChrom L2420). Thermo Scientific™ HyPURITY™ C18 HPLC column (3µm particle size). LCMS analyses were performed with a Bruker AmaZon X, which couples an Agilent 1200 Series LC system with a quadrupole ion trap mass analyzer configured with an electrospray ionization (ESI) source and using a Thermo ScientificTM HyPURITYTM C18 column (3µm particle size). Preparative purification was performed on a Jupiter® 4 µm Proteo 90 Å LC Column 250 × 21.2 mm (Phenomenex®) using an Armen Glider CPC preparatory HPLC. General mass spectra were analyzed on Bruker AmaZon SL quadrupole ion trap mass analyzer configured with an electrospray ionization (ESI) source.

Peptide Synthesis. All peptides were synthesized according to classic manual and automated Fmoc SPPS. Peptides **linGelator-I, cyc(2-nF), lin(ANP),** and **cyc(ANP)** were synthesized on 2-chlorotrityl chloride (CTC) resin (0.81 mmol/g). Briefly, CTC was swollen in dry DMF for 20 min. The first amino acid (3 eq) was coupled with DIPEA (9 eq) overnight, followed by capping unreacted trityl groups with MeOH for 1 hr. The resin was swollen in DMF again and transferred to a peptide synthesizer for standard SPPS. Peptide **linGelator-II** which was synthesized on Wang resin (0.73 mmol/g). Briefly, resin was swollen in DCM/DMF **(**90:10 %v/v) at 15 mL/g of resin in a three-way valve peptide synthesis vessel. Fmoc-Ser(tBu)OH (2.5 eq) was dissolved in DMF

(3 mL) along with HOBt (2.5 eq) and added to the resin. EDC (2.5 equivalents) was added to resin mixture followed by a solution of DMAP (0.1 eq) in a minimum of DMF. N_2 was bubbled through the mixture overnight. The mixture was drained, and the resin resuspended in DCM. To cap excess unreacted hydroxyl groups on the resin, acetic anhydride (2 eq) and pyridine (2eq) were added to the suspension. The mixture was bubbled with N_2 for 30 min then the solvent was removed, and the resin was washed with DMF, DCM, and methanol (× 3 each). The resin was dried under vacuum. The remaining sequence was synthesized using standard SPPS on the peptide synthesizer. For UV cleavable amino acids, either Fmoc-L-2-nitrophenylalanine or Fmoc-(S)-3-amino-3-(2-nitrophneyl)propionic acid (2 eq) were coupled with HATU (1.99 eq) and DIPEA (6 eq) in DMF overnight. For cyclic peptides, Fmoc-Lys(Mtt)-OH was used for mild acid deprotection. Acetylation of the N-terminal amine was performed by incubating peptide-bound resin with acetic anhydride (50 eq) and DIPEA (50 eq) in DMF for 1 hr. Fully deprotected peptides were obtained through deprotection in a cleavage cocktail of TFA/TIPs/H_2O (95:2.5:2.5 %v/v) for 2 hr. Following cleavage, the mixture was filtered, precipitated in diethyl ether, and the pellet was dried under vacuum to yield a white crude solid. Semi-protected peptides **open(2-nF)** and **open(ANP)** were obtained through selective removal of the 4-methyltrityl (Mtt) group and simultaneous cleavage of peptide from resin by swelling the resin in DCM for 30 min, then treating with TFA/TIPs/DCM (1:5:94% v/v) for 1 hr. The mixture was filtered, and solvent removed by rotary evaporation to yield a viscous oil residue.

Cyclization of cyc(2-nF) and cyc(ANP). Cyclic peptides were obtained by treating dilute solutions of **open(2-nF)** and **open(ANP)** in DMF (500 μM) with a mixture of HBTU (6 eq), HOBt (6 eq), and DIPEA (15 eq) for 24 hr with vigorous stirring. The solvent was removed by rotary evaporation and the resulting residue was washed with anhydrous diethyl ether. Removal of the remaining protecting groups was performed by treatment of the peptides with a cleavage cocktail of TFA/H_2O/TIPS (95:2.5:2.5 %v/v) for 2 hrs. Peptides were precipitated in cold diethyl ether with sonication the pellet was dried under vacuum to give a white crude solid.

Peptide Analysis and Purification. All purification and reaction analyses were conducted via LCMS, HPLC, and preparatory phase HPLC at 214 nm using gradients over 7 min, 30 min, and 50 min, respectively. The solvent system for HPLC, LC-MS, and preparatory phase. Running solvents consisted of Buffer A (H_2O with 0.1% TFA) and Buffer B (ACN with 0.1% TFA), and all peptides were run using a gradient of 20-60% Buffer B.

Photocleavage of UV-Responsive Peptides for Cleavage Analyses. UV-responsive peptides **cyc(2-nF), lin(ANP),** and **cyc(ANP)** were placed in a Uspicy USND-3601 Professional UV GEL Lamp, with 36W (4 x 9W bulbs) source installed 2 cm over the sample and irradiation conducted at 365 nm. **cyc(2-nF)** was dissolved in H_2O/ACN/MeOH (60:35:5 %v/v) ACN-H_2O (4:6) to a concentration of 500 μM, heated to 60 ºC, and sonicated for 2 hours. Peptide was subjected to photoirradiation and aliquots were taken at 0, 5, 20, 60, and 180 min. **lin(ANP)** was dissolved in H_2O/ACN/MeOH (60:35:5 %v/v) ACN-H_2O (4:6) to a concentration of 500 μM, heated to 60 ºC, and sonicated for 2 hours. Peptide was subjected to photoirradiation and aliquots were taken at 0, 1, and 3 hr. **cyc(ANP)** was dissolved in H_2O/ACN/MeOH (50:45:5 %v/v) at 500 μM, heated to 60 ºC, and sonicated for 2 hours. Peptide was subjected to photoirradiation and aliquots were taken at 0, 1, 2, 3, and 4 hr.

Formulations of cyc(ANP). cyc(ANP) was formulated in aqueous mixtures using two methods, Formulation 1 and Formulation 2. Formulation 1: Peptide was dissolved in H_2O/ACN (66:33 %v/v) with heating (60 ℃), then heated at 80 ℃ until acetonitrile was evaporated from solution, yielding a sample of peptide in H_2O. Cooling induced slow peptide aggregation in solution. Formulation 2: Peptide was dissolved in H_2O/ACN/AcOH (85:10:5 %v/v) with heating (60 ℃) and slowly cooled to produce a clear, free-flowing solution. The pH was adjusted with NH_4OH to pH 6.4.

Dissolution of linGelator-II. Peptide was prepared by dissolution into an aqueous solution of NH_4OH at (20 mM), followed by dilution to 1 mM with H_2O, and neutralization to pH 6.4 with a small amount of AcOH. This peptide solution was used for TEM and MALDI analyses without modification.

Rheology. Peptide gels were prepared at 15 mg/mL in H_2O/acetonitrile or PBS/acetonitrile (90:10 %v/v). Viscous and viscoelastic properties were assessed using a stress-controlled rheometer (TA Instruments AR-G2) equipped with a Peltier plate to control temperature and a 20 mm diameter parallel plate geometry. All measurements were taken at a strain of 0.5%, gap height of 1000 μm, and temperature of 25 °C. To prevent solvent evaporation, mineral oil was wrapped around the edge of the geometry at the air-sample interface. For viscoelastic measurements the apparatus was used in oscillatory mode. To ensure the measurements were made in the linear viscoelastic regime (LVR), strain sweeps were performed between 0.05-50 % strain and showed no variation in storage (G') and loss (G'') moduli up to a strain of 0.5%. The dynamic moduli of the hydrogels were measured as a function of frequency in the range 0.25–100 rad s^{-1} (n=3 repeats). For viscosity measurements the

apparatus was used in steady state flow mode. The viscosity of the samples was measured as a function of shear rate in the range 0.1–10 s^{-1} (5% tolerance).

Standard Dry-State Transmission Electron Microscopy (TEM). Dry-state samples were prepared by applying 4 μL aliquots of sample onto TEM grids (Formvar stabilized with carbon on 400 copper mesh, Ted Pella Inc.) that had been glow discharged in a PELCO easiGlow glow discharge unit for 30 s. The sample grid was blotted with filter paper, stained with a 1% uranyl acetate solution, then rinsed with water. Excess solution was removed by blotting with filter paper, then the grid was dried. TEM imaging was performed on a JEM-ARM300F (JEOL, Ltd., Tokyo, Japan) operated at 300 keV. Micrographs were recorded on a 2k x 2k Gatan OneView-IS CCD camera (Gatan Inc., Pleasanton, CA, USA) using Gatan Digital Micrograph image acquisition software (Roper Technologies, Sarasota, FL).

Preparation of Dry-State TEM Samples on Silicon Nitride LCTEM Chips. Samples were prepared by applying a 5 μL aliquot of sample onto LCTEM chips with 50 nm thick silicon nitride (SiN_x) membranes (Hummingbird Scientific, Lacey, WA, USA) that had been glow discharged in a PELCO easiGlow glow discharge unit for 30 s. The sample solution was allowed to dry.

Liquid Cell TEM (LCTEM). LCTEM chips with 50 nm thick silicon nitride (SiN_x) membranes (Hummingbird Scientific, Lacey, WA, USA) were glow discharged in a PELCO easiGlow glow discharge unit for 30 s. Next, 0.8 μL of sample was pipetted manually onto the bottom chip, then the liquid cell was assembled. LCTEM imaging was performed in a JEM-ARM300F (JEOL, Ltd., Tokyo, Japan) operated at 300 keV. Micrographs were recorded on a 2k x 2k Gatan OneView-IS CCD camera (Gatan Inc., Pleasanton, CA, USA) using Gatan Digital Micrograph image acquisition software (Roper Technologies, Sarasota, FL). Electron flux values were calculated using the beam current for each aperture selection, as measured by a Faraday Holder through vacuum, and the beam diameter incident upon the sample. After the LCTEM experiments, the SiN_x chips were separated and dried.

MALDI-Imaging Mass Spectrometry (MALDI-IMS). LCTEM chips were prepared for and analyzed by MALDI-IMS as described previously.[1] Raw mass spectra within a giving region of interest (ROI) were averaged and the baseline subtracted. For each 50 μm diameter pixel, integrated total signal from the mass spectra within

a mass range filter was calculated. Visual 2D maps were generated from these pixels and colorized according to 0-100% maximal signal on a logarithmic scale.

3. Supplementary Figures

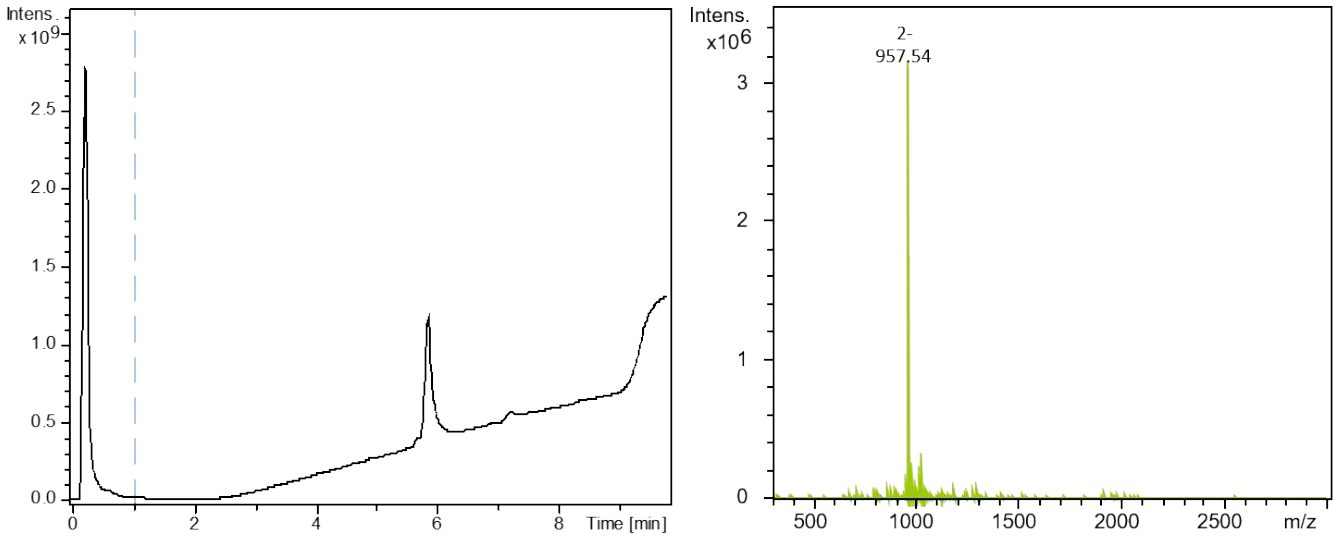


Figure S1. LCMS and ESI spectrum of **linGelator-I.** Expected mass 959.05 g/mol.

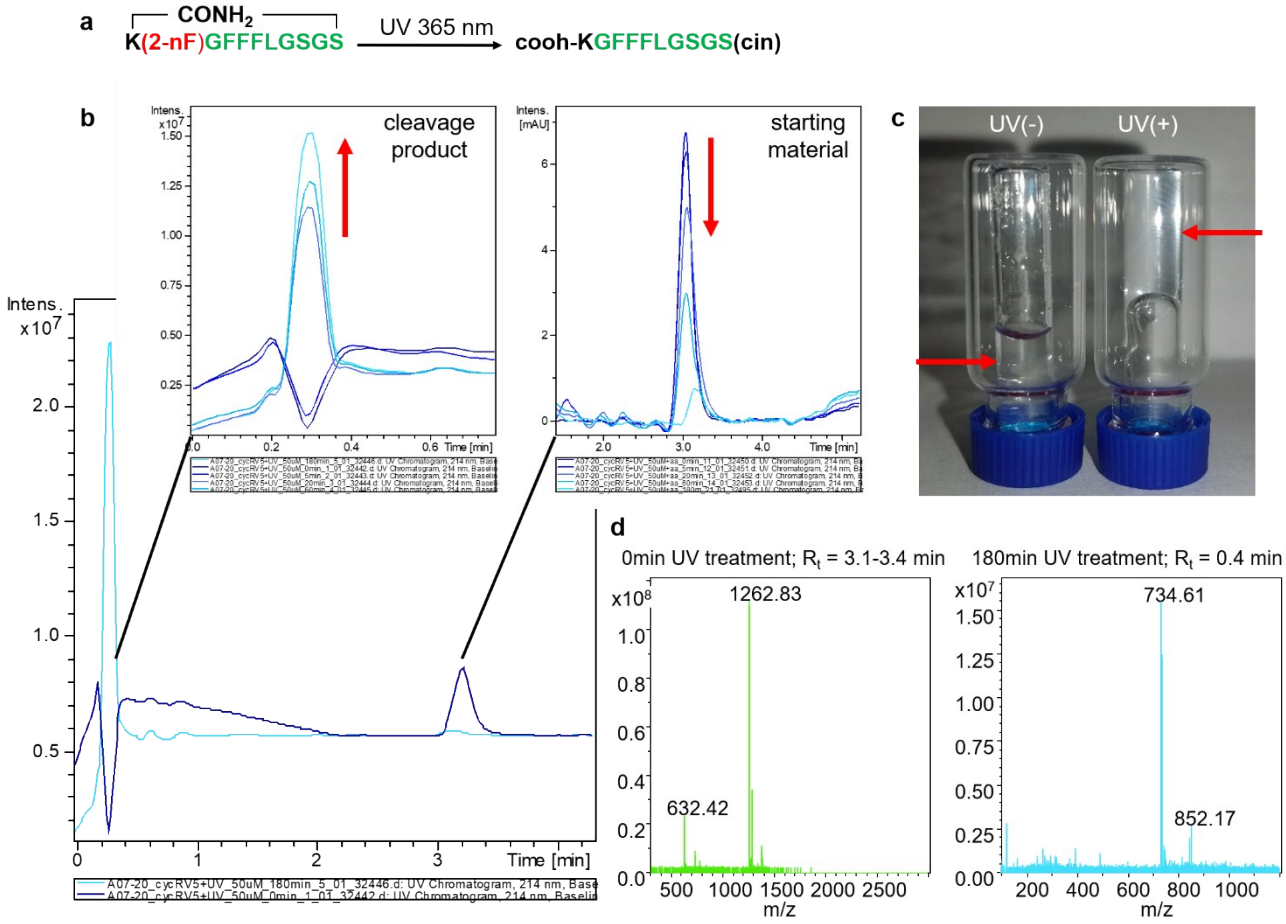


Figure S2. Photocleavage of **cyc(2-nF)** ink. **a**) Proposed synthetic UV cleavage scheme based on Peters, *et. al*.[2] **b**) LCMS spectra at 0, 5, 20, 60, and 180 min of UV (365 nm) treatment. **c**) Images of vial flips depicting (left) untreated peptide as a free-flowing solution and (right) treated peptide as a gel. **d**) Corresponding ESI-MS spectra for peaks at 0 min (R_t = 3.1-3.4 min) and 180 min (R_t = 0.4 min) of UV treatment.

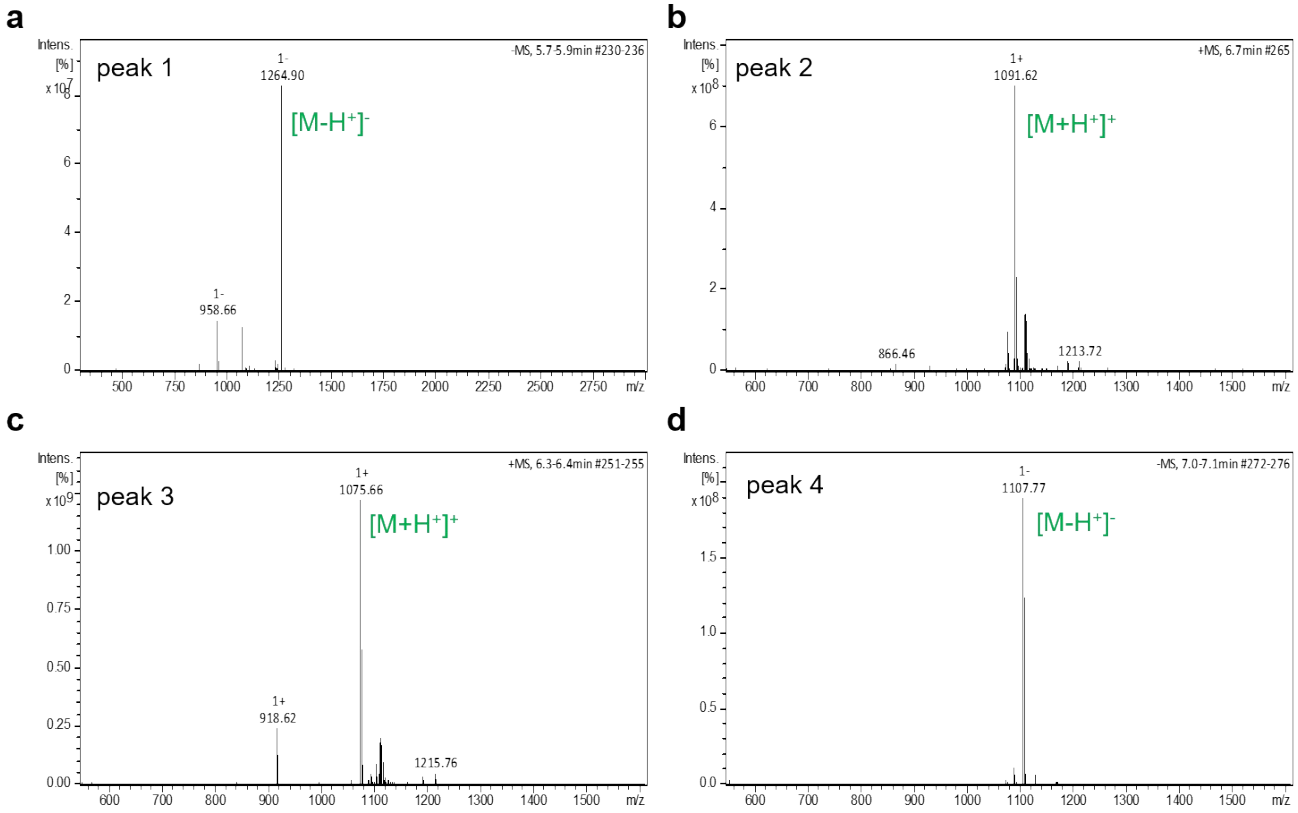


Figure S3. Representative mass spectra of photocleavage products. **a-d**) Corresponding ESI-MS spectra of labeled peaks collected at t = 1 hr of UV treatment (365 nm) in **Figure 3c**. (**a**) peak 1: 1264.90 m/z $[M\text{-}H^+]^-$, (**b**) peak 2: 1091.62 m/z $[M+H^+]^+$, (**c**) peak 3: 1075.66 m/z $[M+H^+]^+$, and (**d**) peak 4: 1107.77 m/z $[M\text{-}H^+]^-$.

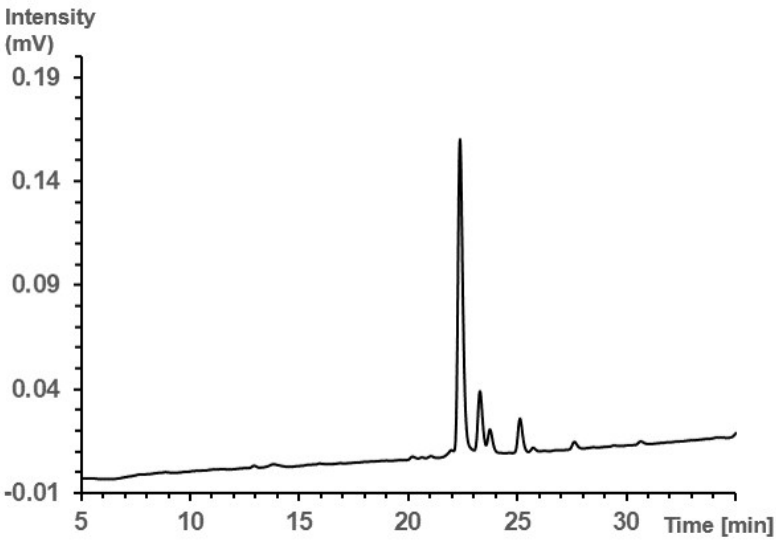
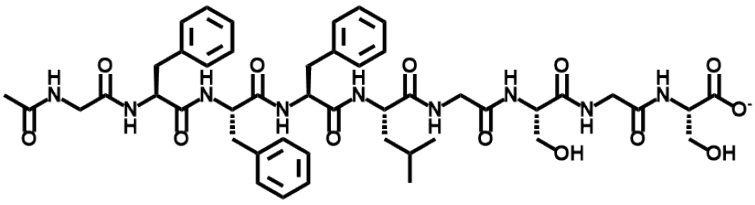


Figure S4. HPLC spectrum for crude **linGelator-II.** Corresponding MALDI-MS provided in main text **Figure 7.**

4. Supporting Chemical Structures

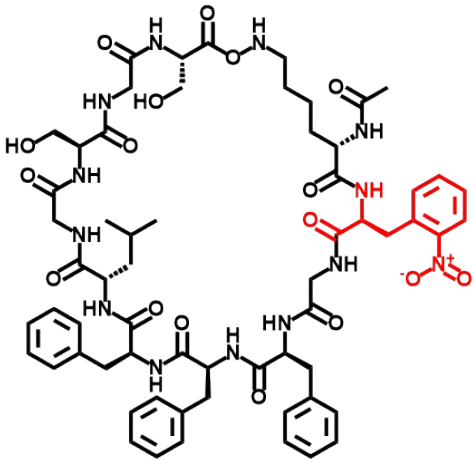
A) **linGelator:** Ac-GFFFLGSGS



Ac-Gly-Phe-Phe-Phe-Leu-Gly-Ser-Gly-Ser

Exact Mass: 958.43

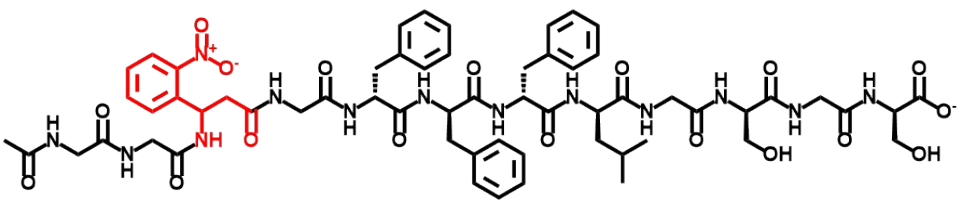
B) cyc(2-nF): Ac-*K(2-nF)GFFFLGSGS*



Ac-*Lys-(2NO_2-Phe)-Gly-Phe-Phe-Phe-Leu-Gly-Ser-Gly-Ser

Exact Mass: 1277.57

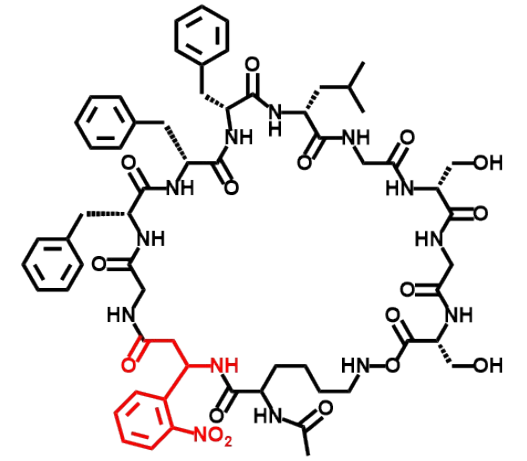
C) lin(ANP): Ac-GG(ANP)GFFFLGSGS



Ac-Gly-Gly-(ANP)-Gly-Phe-Phe-Phe-Leu-Gly-Ser-Gly-Ser

Exact Mass: 1264.53

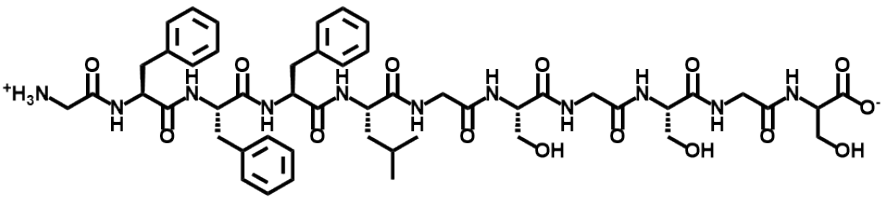
D) cyc(ANP): Ac-*K(ANP)GFFFLGSGS*



Ac-*Lys-(ANP)-Gly-Phe-Phe-Phe-Leu-Gly-Ser-Gly-Ser

Exact Mass: 1277.57

E) linGelator-II: GFFFLGSGSGS



Gly-Phe-Phe-Phe-Leu-Gly-Ser-Gly-Ser-Gly-Ser

Exact Mass: 1061.48

5. Supplementary References

1. Touve MA, Carlini AS, Gianneschi NC. *submitted*.
2. Peters FB, Brock A, Wang J, Schultz PG. Chem Biol. 2009;16(2):148-52.