Electronic Supplementary Information

Chemically-Fueled Transient Peptide Hydrogel enabling Programmable Time-Gated Functions

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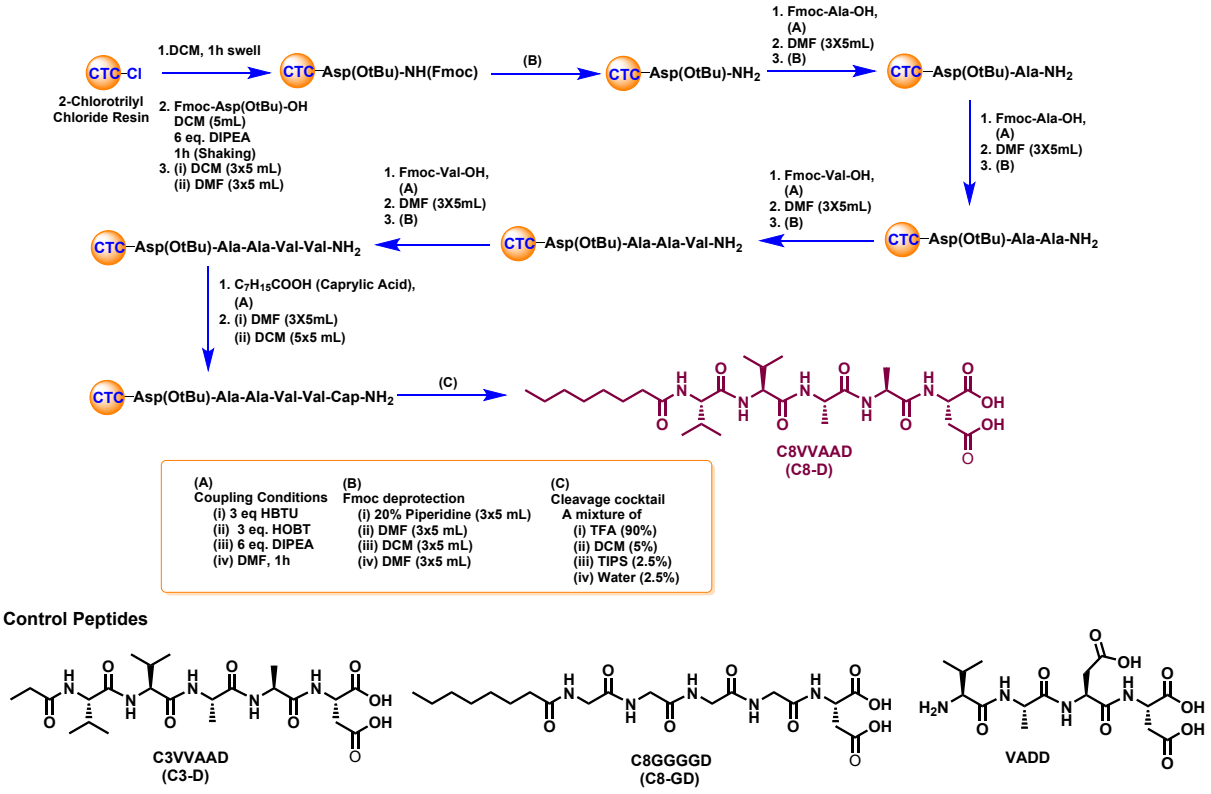
Materials and Instruments

Resin (2-Chlorotrilyl Chloride), all the protected amino acids, caprylic acid, 2-(1h-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole were purchased from Merk, India. All other reagents, chemicals and solvents were procured from TCI, Sigma-Aldrich, Spectrochem and SRL India and used without further purification unless otherwise mentioned. The peptide, C8VVAAD, was synthesized following the standard Solid Phase Peptide Synthesis (SPPS) strategy using 9-Fluorenylmethyloxycarbonyl (Fmoc) chemistry. All the samples for the experiments were prepared in Milli-Q water with a conductivity of less than 2 $\mu S\ cm^{-1}$. The synthesized peptide was purified by a Waters HPLC system equipped with a UV-Vis detector using a YMC-Triart C18 5 μm, 10 x 250 mm semi-prep HPLC column. Analytical HPLC was conducted on an Agilent 1260 Infinity II system using a YMC Triart C18 5 μm, 4.6 x 250 mm analytical column. Acetonitrile and water mixture having 0.1 % TFA was used as eluent in a gradient manner. High-resolution mass spectra (HRMS) were recorded in a 1290 Infinity II UPLC System with Agilent 6545 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) and UHPLC-QTOF-HRMS from Agilent, Model: G6546A. ^{1}H-NMR and ^{13}C-NMR were acquired using a JEOL 400 YH instrument (400 MHz). Absorption spectra were acquired on an Agilent Cary Series spectrometer and Emission spectra were recorded on a Fluoromax Plus spectrophotometer utilizing standard 10 mm path quartz cuvettes. A Parkin Elmer Spectrum FT-IR spectrometer was employed to record FTIR spectra. Circular Dichroism (CD) spectra were acquired in a Jasco J-1500 CD spectrometer using a 2 mm path quartz cuvette. The viscoelastic property of the temporal hydrogel was assessed using an Anton Paar rheometer (MCR 102) with a 20 mm parallel plate and a 0.5 mm zero gap at 25°C. TEM images were captured in a JEOL 2100F transmission electron microscope, operated at an acceleration voltage of 200kV and equipped with a Gatan K3-IS imaging system. Further, SEM and AFM images were taken in a ZEISS Gemini SEM 360 instrument and Bruker DIMENSION FastScan microscope respectively.

Synthetic route of peptide C8VVAAD (C8-D) and others



Control Peptides

Scheme S1. Solid Phase Peptide Synthesis

The peptide **C8VVAAD** (**C8-D**) was synthesized using standard 'Fmoc' solid-phase peptide synthesis strategy employing 2-Chlorotrilyl Chloride Resin (2-CTC resin) as the solid support. The loading capacity of 2-CTC resin was 1.082 mmol/g. Prior to the synthesis; the resin was adequately dried under vacuum. The first coupling was carried out in DCM employing Fmoc-Asp(OtBu)-OH (3 eqv.) and DIPEA (6 eqv.) followed by washing and 'Fmoc' deprotection. The peptide chain elongation at the N-terminus was carried out by coupling the appropriate moiety (3 eqv.) under standard conditions employing HBTU (3 eqv.) and DIPEA (6 eqv.) in the presence of HOBT (3 eqv.) in DMF. The final peptide was cleaved from the resin support using a standard cleaving cocktail of TFA-DCM-TIPS-Water (90:5:2.5:2.5). Next, the cleaved peptide was precipitated from cold and dry diethyl ether. The crude peptide was purified using semi-prep HPLC (acetonitrile/water having 0.1 % TFA system as the eluent, in a gradient of 10 to 90 % acetonitrile over 20 min) followed by lyophilization to afford the pure peptide. Using a similar strategy, the control peptides **C3-D**, **C8-GD** and **VADD** were synthesized and purified.

Characterization of the peptides

C8-D

^{1}HNMR (400 MHz, DMSO-d_6) δ: 8.14-8.12 (d, 1H), 8.0-7.94 (q, 2H), 7.87-7.84(d, 1H), 7.73-7.71 (d, 1H), 4.53-4.48 (m, 1H), 4.30-4.23 (m, 2H), 4.20-4.13 (m, 2H), 2.69-2.55 (m, 2H), 2.18-2.08 (m, 2H), 1.97-1.92 (m, 2H), 1.48-1.17 (m, 16H), 0.86-0.80 (m, 15H).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 172.34, 172.22, 171.90, 171.64, 171.53, 171.19, 170.43, 57.77, 57.36, 48.43, 47.93, 47.77, 35.94, 35.16, 31.23, 30.58, 29.95, 28.54, 28.45, 25.45, 22.05, 19.19, 18.18, 17.97, 17. 85, 13.95.

MS-ESI (HRMS): m/z: calculated 599.3530 for C28H49N5O9, found: 600.3576 $[M+H]^+$.

C3-D

MS-ESI (HRMS): m/z: calculated 529.2748 for C23H39N5O9, found: 530.2827 $[M+H]^+$.

C3-GD

MS-ESI (HRMS): m/z: calculated 487.2278 for C20H33N5O9, found: 488.2368 $[M+H]^+$.

VADD

MS-ESI (HRMS): m/z: calculated 418.1700 for C16H26N4O9, found: 419.1769 $[M+H]^+$.

Methods

Sample preparation

A stock solution of peptide was prepared by dissolving lyophilized **C8-D** in 50 mM MES buffer of pH 6. Stock solutions of EDC were prepared by dissolving the EDC powder in MQ water. Importantly, each time freshly prepared EDC was used for experiment. The transient hydrogelation cycle was initiated by adding the required volume of 5 M freshly prepared EDC stock to the peptide solution. The final EDC concentrations were kept 20 mM, and 50 mM and peptide concentration was kept 2 mM for different experiments.

Turbidity measurement

The transient evolution of turbidity was recorded by measuring the change in absorbance at 450 nm (where no absorbance peak appeared from the sample) as a function of time after adding EDC to

peptide solution at room temperature. The final EDC concentrations were kept 20 mM and 50 mM, and peptide concentration was kept 2 mM. For multiple assembly cycle, the system was refueled with 20 mM EDC (final conc.).

Rheology

The viscoelastic property of the transient hydrogel was characterized 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. At first, to determine the linear viscoelastic region which is defined as the region where strain has no significant impact on G' (storage modulus) and G" (loss modulus) of the hydrogel, a rapid strain sweep experiment was performed with varying the strain from 0.01 % to 100 % at a fixed oscillatory frequency of 1 rad s^{-1}. The frequency-sweep experiment was conducted at a fixed strain of 1 % and varying angular frequency from 0.1 rad s^{-1} to 100 rad s^{-1}. For validating the transient feature of the hydrogel, the variation of storage modulus (G') and loss modulus (G") were recorded as a function of time in time-sweep experiment with at a fixed strain of 1 % and fixed angular frequency 1 rad s^{-1}.

Circular Dichroism

CD Spectra of the peptide 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 quartz cuvette at a scan rate of 100 nm min^{-1} with 0.5 nm intervals and 2 nm bandwidth. In a typical experiment, 2 mM **C8-D** peptide solution was prepared and to it EDC (20 mM final conc.) was added and the CD spectra from 190 nm to 350 nm were recorded at different time intervals. Prior to CD experiment, baseline correction using comparable reference solution was carried out.

Fourier-Transform Infrared Spectroscopy (FT-IR)

FTIR spectra were recorded on a Parkin Elmer Spectrum FT-IR Spectrometer using a Diamond ATR from Parkin Elmer. For the FT-IR measurement **C8-D** peptide, MES buffer and EDC were all prepared in deuterated solvents described in the sample preparation section. After triggering the reaction on addition of EDC to peptide solution, samples at different time intervals were aliquoted and FT-IR spectra were recorded.

Analysis of the reaction kinetics by HPLC

The kinetics of the EDC fueled conversion of **C8VVAAD-An (C8-D-An)** from **C8VVAAD (C8-D)**, was monitored over time employing analytical HPLC (Agilent 1260 Infinity II system using a YMC Triart C18 5 μm, 4.6 X 250 mm analytical column). In a typical method, 1 mL of 2 mM **C8-D**, sample was prepared

as mentioned in the sample preparation section. The reaction cycle was initiated by adding a solution of EDC (20 mM as the final concentration). At different time intervals, 125 μL reaction mixtures were aliquoted and added to 250 μL of 20 mM benzylamine solution which acts as a quencher and irreversibly converts the cyclic anhydride into corresponding monoamides.[1] Next, these quenched samples were diluted by adding 750 μL acetonitrile making the total volume as 1125 μL and mixed thoroughly. After that, the diluted samples were filtered through 0.22 μM syringe filter and injected into HPLC through an automated injector. The elution was made using a solvent system of 0.1% TFA in acetonitrile and 0.1% TFA in water with a linear gradient of acetonitrile: water from 2: 98 to 98:2 where all the compounds in the reaction cycle were well resolved. The concentrations of the unreacted **C8-D** at different time points were calculated by using a calibration curve of pure **C8-D**. From the unreacted concentration of **C8-D** present at a particular time, we determined the **C8-D** consumed at that time which we considered as the indirect measure of anhydride (**C_8-D-An**) formation in the reaction cycle over time.[1] Measurements were carried out at 25 °C, with a flow rate of 1 mL/min at a data collection rate 2.0 Hz, and the UV detector's wave lengths were kept as 220.

Importantly, for the calibration curve, a series of pure **C8-D** peptide solutions were prepared having a concentration range 0 mM to 0.5 mM and injected in the analytical HPLC. The chromatogram was recorded at 220 nm wavelength for each of the samples. Then area under the curves was calculated and plotted against the whole concentration range. Linear fitting gives a straight line having a slope and intercept. Exploiting this calibration curve, unknown concentrations of **C8-D** at different time points during the reaction were determined. Briefly, the area of the diluted aliquots of the reaction time points was obtained from the chromatograms which were put in the calibration curve (straight line equation) and corresponding concentrations were calculated. After multiplying with the dilution factor, the variation in **C8-D** concentration over the time was estimated.

Emission Spectra

Emission spectra for ThT and Nile Red assays were recorded on a Fluoromax Plus spectrophotometer from Horiba at 25 °C. For ThT assay, 2 μM ThT was mixed to the 2 mM peptide and the emission spectra at different time points were recorded on adding EDC to the solution, exciting at 450 nm (which is the excitation wavelength if ThT). For, Nile Red assay dye concentration was kept 2 μM and excitation wavelength was 550 nm.

Transmission electron microscopy (TEM)

TEM images were acquired in a JEOL 2100F transmission electron microscope, operated at an acceleration voltage of 200kV and equipped with a Gatan K3-IS imaging system. For transmission

electron microscopy, a solution of **C8-D** peptide, **C8-D-An** gel and raptured gel was diluted in MES buffer (pH 6). 10 μL of each sample solution was placed on the surface of a holey carbon-coated copper grid, which had been previously treated with plasma, and left to adhere for 1 minute. Excess solution was then removed using filter paper. Following this, 5 μL of 2 % uranyl acetate was added on top of the grids and allowed to sit for 30 seconds. The excess uranyl acetate solution was also removed with filter paper. The grids were washed with ultrapure water for 30 s and air-dried for 24 h at room temperature (RT), prior to imaging.

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy imaging of the samples was acquired in a ZEISS Gemini SEM 360, Germany. SEM samples were prepared by drop casting method on silicon wafer. In a standard procedure, 10 μL **C8-D** solution was drop-cast on silicon wafer and air dried prior to imaging. However, for **C8-D-An** hydrogel, the sample was dehydrated through a series of ethanol water gradients (20 % to 100 % ethanol). After dehydration, the sample was dried at the critical point of CO_2 using a Critical Point Dryer. Samples were coated with 10 nm of Iridium before imaging.

Atomic Force Microscopy (AFM)

AFM imaging was carried out 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 built into the software. Typically the sample was drop cast onto freshly cleaved mica surface. The sample was left for 2 minutes. Then the excess solution was tap off onto a paper towel and then washed with 2 x 200 μL ddH_2O before drying. The images were captured with a Peak Force set point of 500 pN having a peak Force amplitude of 30 nm and frequency of 4 kHz.

Photograph of the reaction cycle in a cuvette under ambient light and UV Light

2 mM **C8-D** peptide solution was taken into a 1 mL quartz cuvette of path length 10 mm and EDC was added (20 mM final concentration). The reaction cycle was observed under the ambient light. Under the ambient light, the addition of EDC to the **C8-D** solution turned it into a hydrogel and which slowly dissolved over time of 35-40 mins. The time course photographs were captured documenting the dynamic physical transformation. Refuelling experiments were carried adding 20 mM EDC for each cycle after the braking of the hydrogel and photographed accordingly.

Time-programmed release experiments

Hydrogels were prepared in a total volume of 1 mL incorporating Doxorubicin (Dox), maintaining final **C8-D** concentration as 2 mM, Dox as 25 μM, buffer as 50 mM (MES, pH 6) and EDC as 20 or 50 mM. Once the gel formed, 500 μL buffer, as release medium, was added on the top of the gel very gently. At each time point, 200 μL solution was aliquoted from the liquid layer above the gel. Release was monitored by measuring the absorbance at 480 nm (λ_{max} for Dox). After each measurement, the solution was carefully returned to the release medium. The samples were tested in triplicates. The release percentage was determined as the absorbance (at 480 nm) for each point relative to the final i.e. absorbance when the gel broke completely (ie. 40 min and 50 min when 20 nm and 50 mM EDC were used respectively). To be specific, we marked the release at 40 min and 50 min when EDC concentrations were 20 mM and 50 mM respectively, as 100% release and accordingly we calculated the relative percentage at different time points during the course for the sol-gel-sol transformation.
Please note that, in this work the programmability refers to the ability to systematically regulate the hydrogel lifetime, other properties and corresponding functional outputs by varying the concentration of chemical fuel.

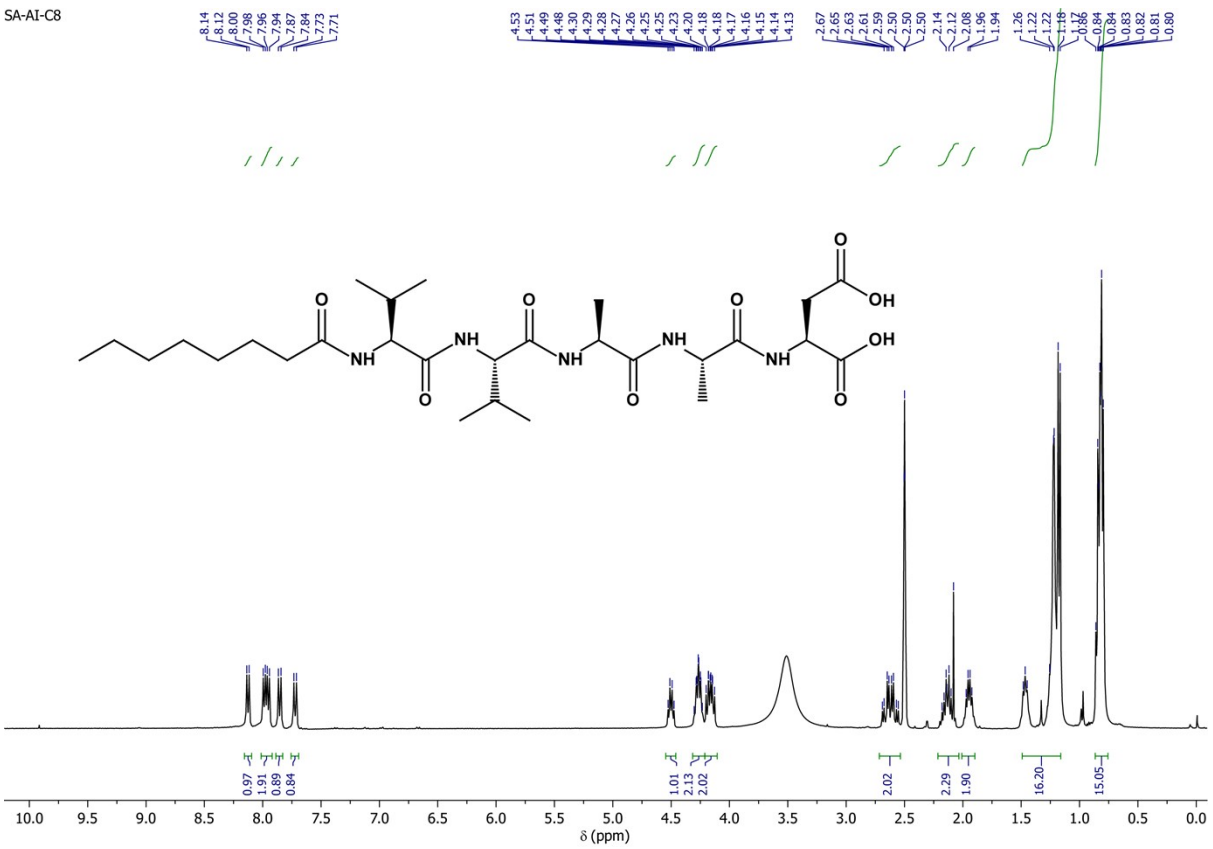


Figure S1. ^{1}H spectrum of **C8-D**.

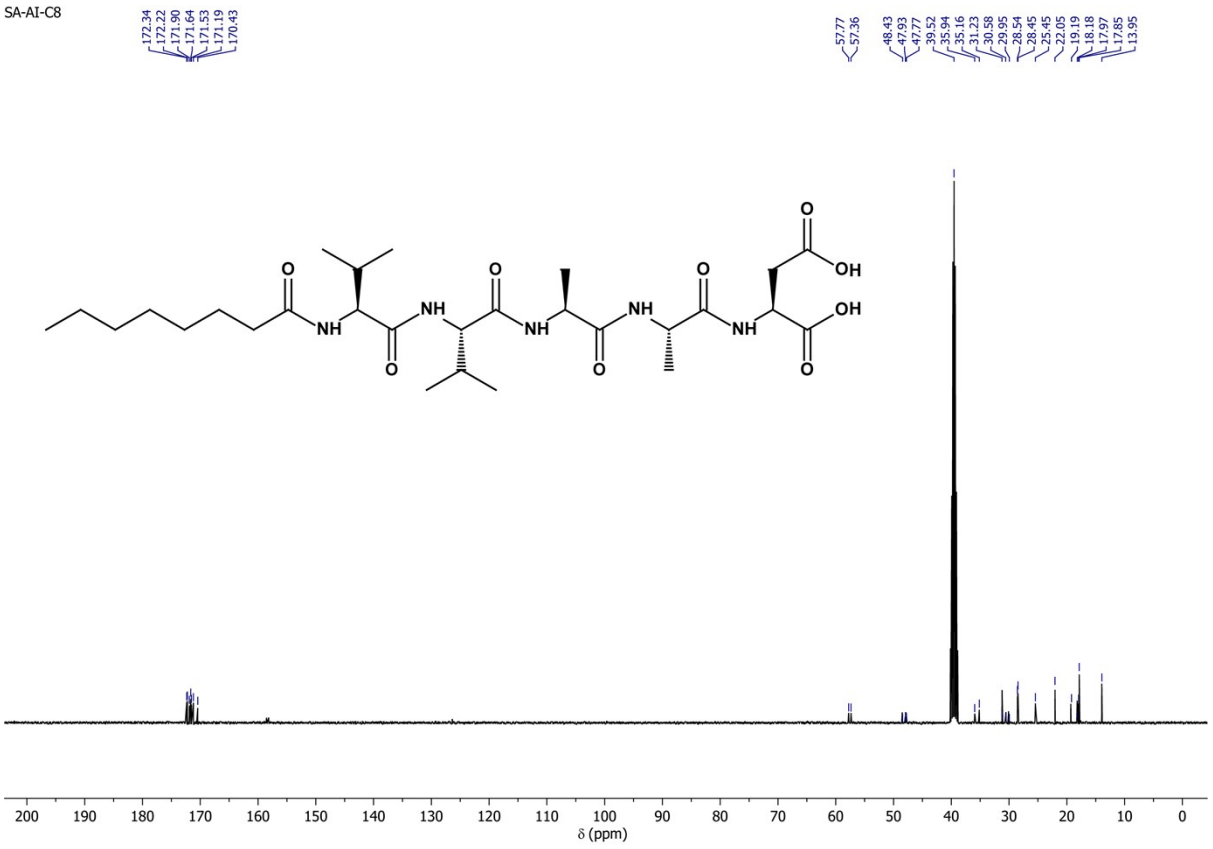


Figure S2. ^{13}C spectrum of **C8-D**.

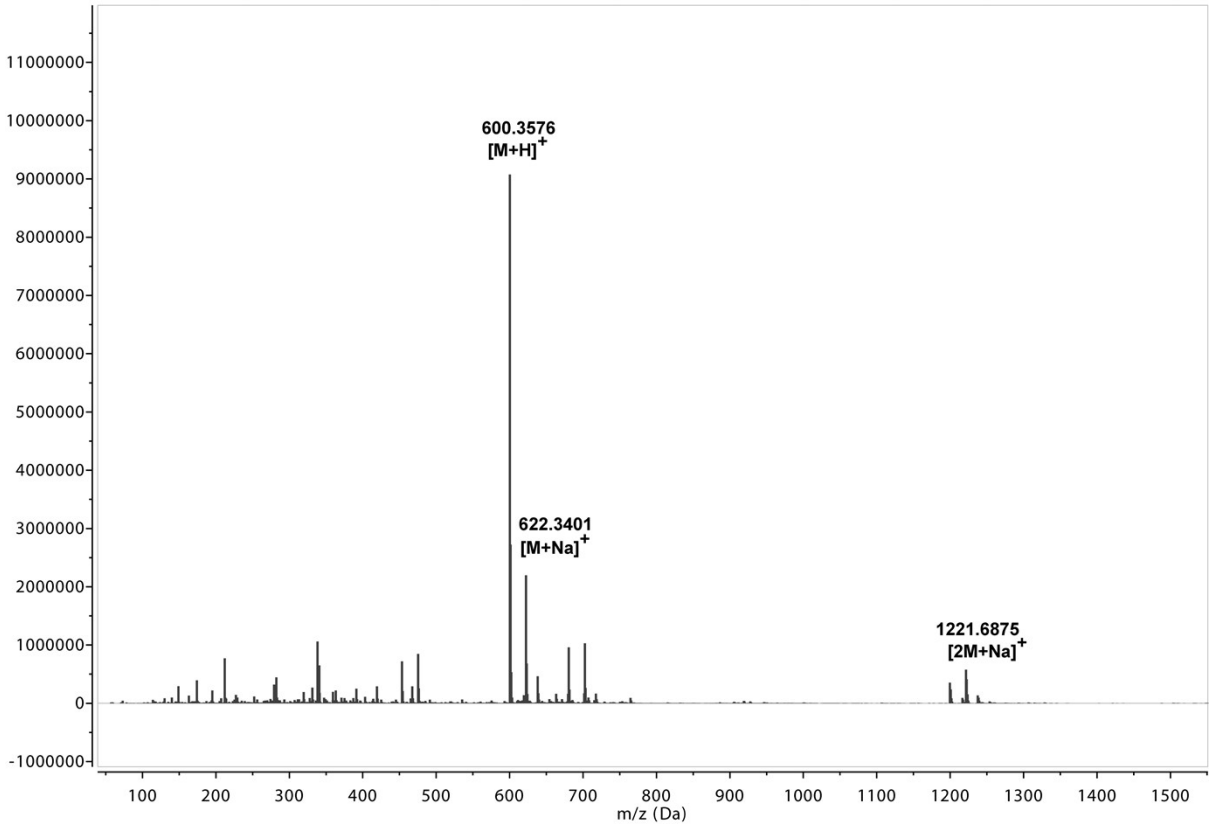


Figure S3. HRMS of **C8-D**.

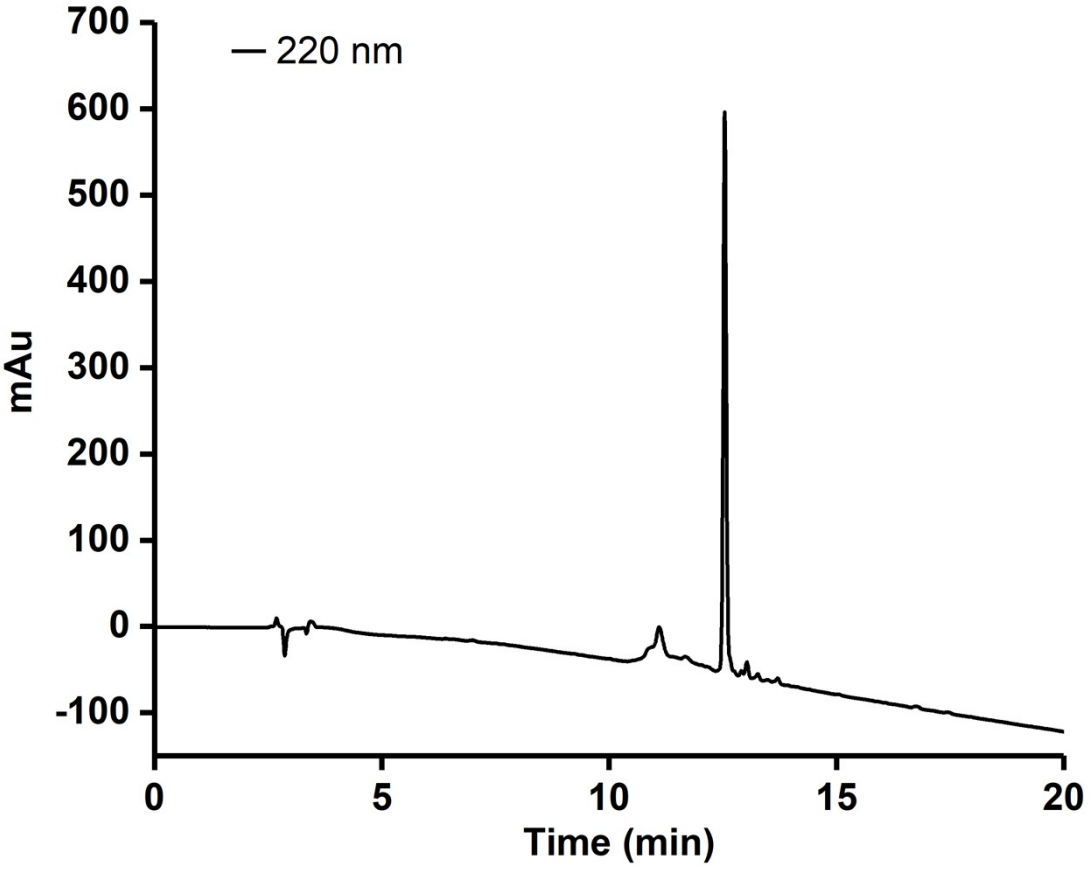


Figure S4. HPLC Chromatogram of **C8-D**.

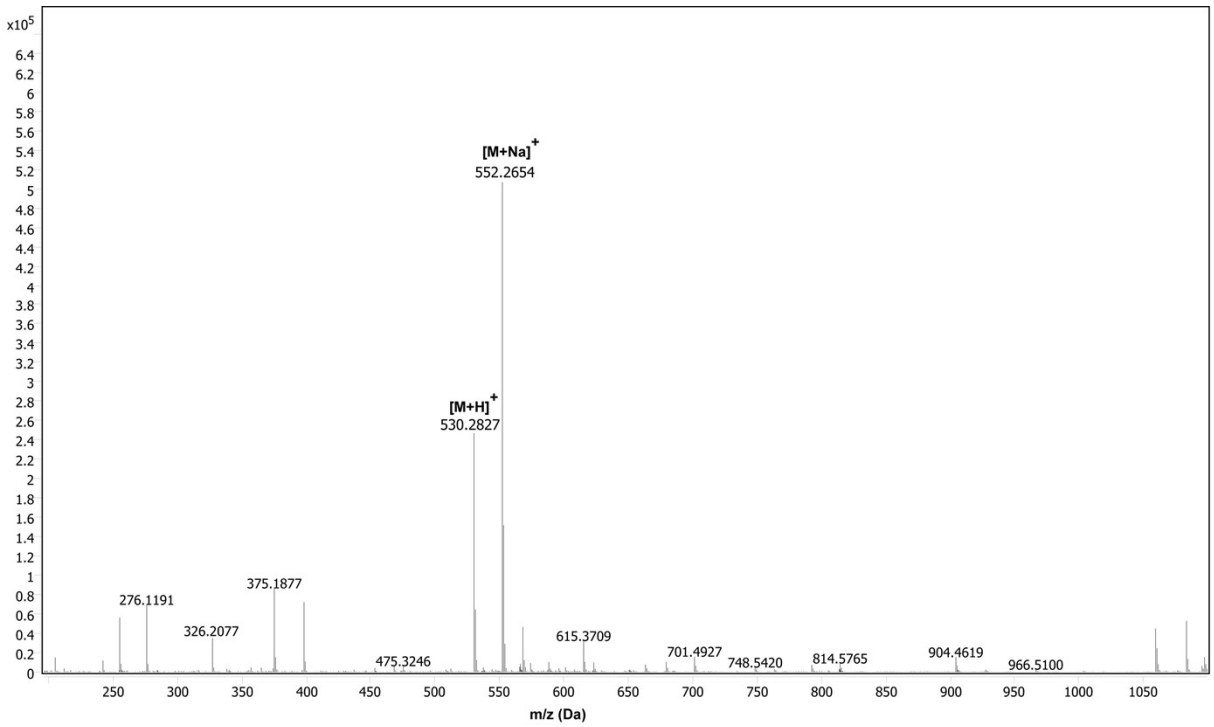


Figure S5. HRMS of **C3-D**.

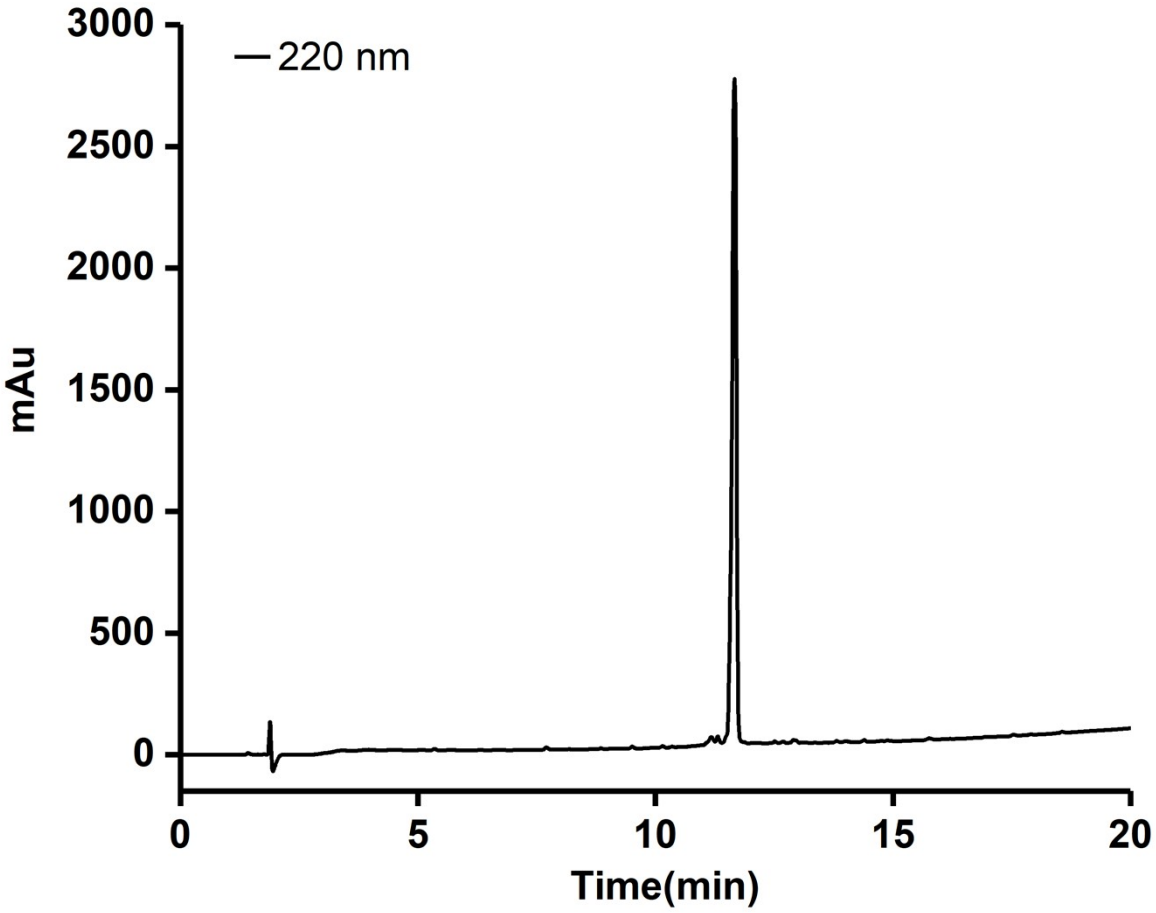


Figure S6. HPLC Chromatogram of **C3-D**.

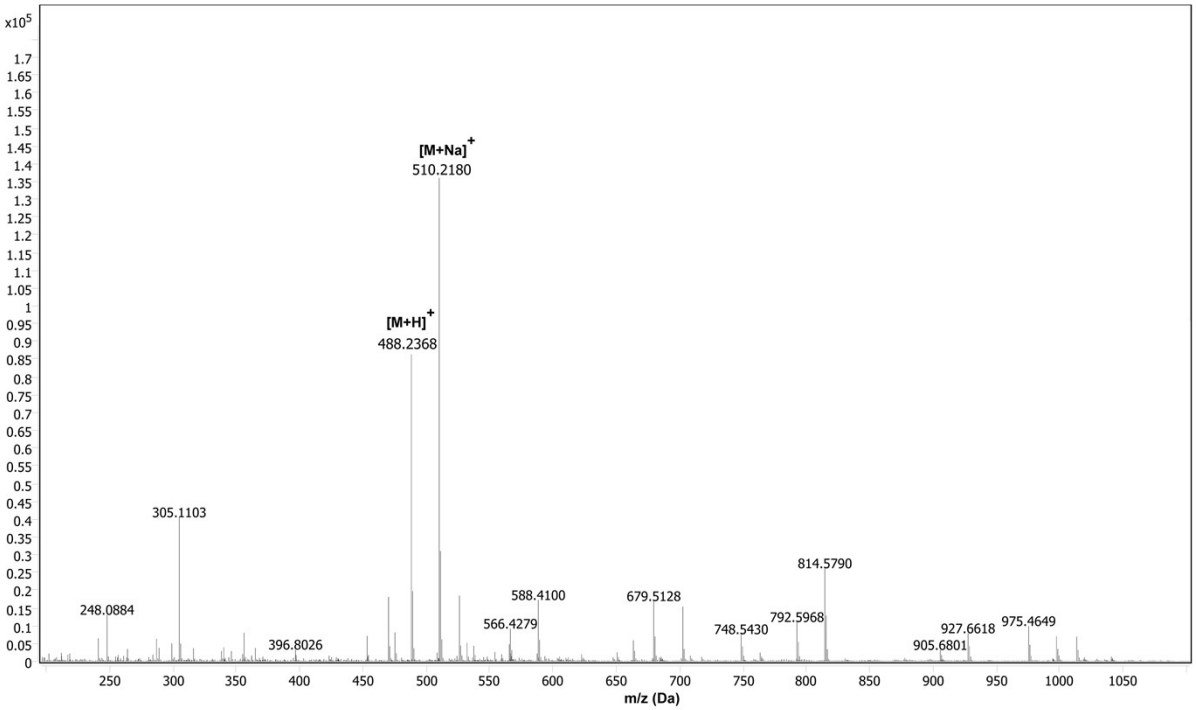


Figure S7. HRMS of **C8-GD**.

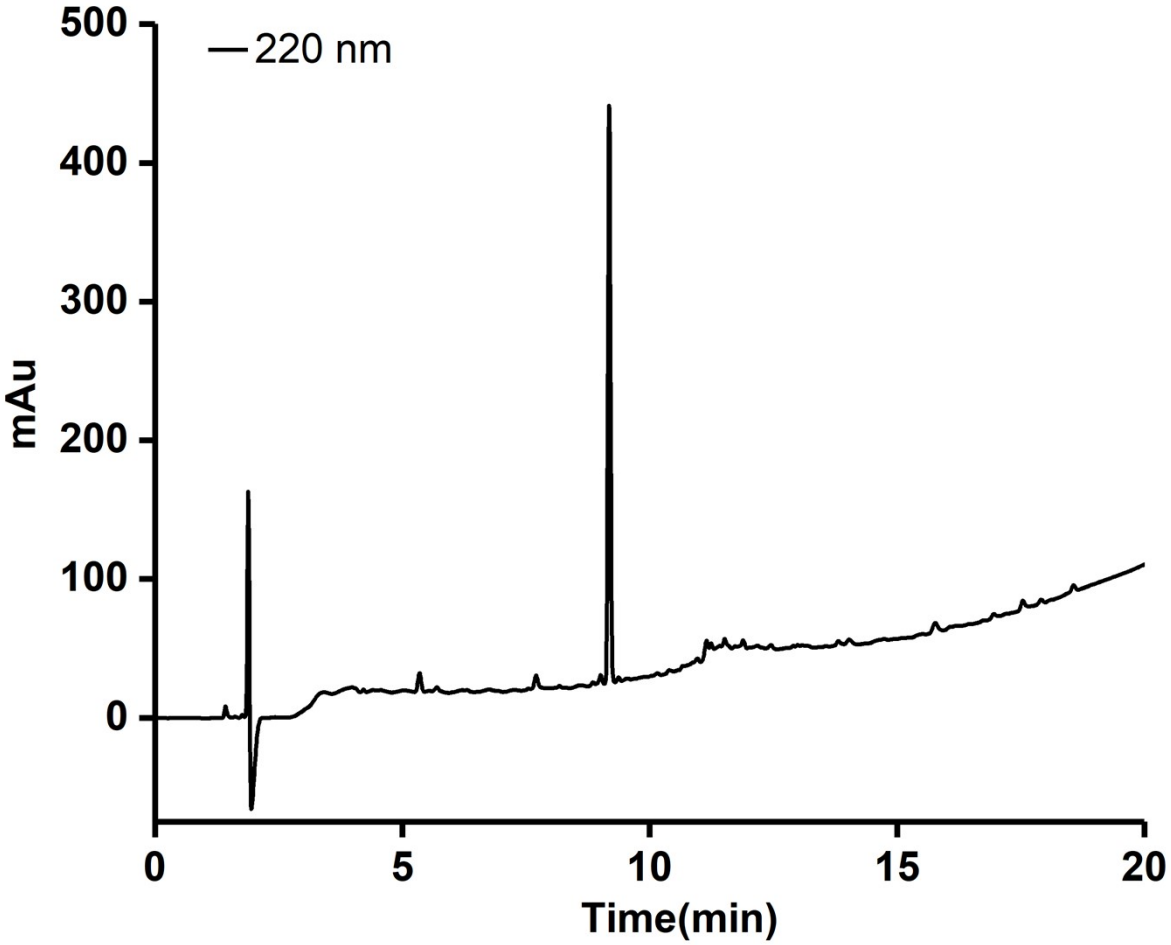


Figure S8. HPLC Chromatogram of **C8-GD**.

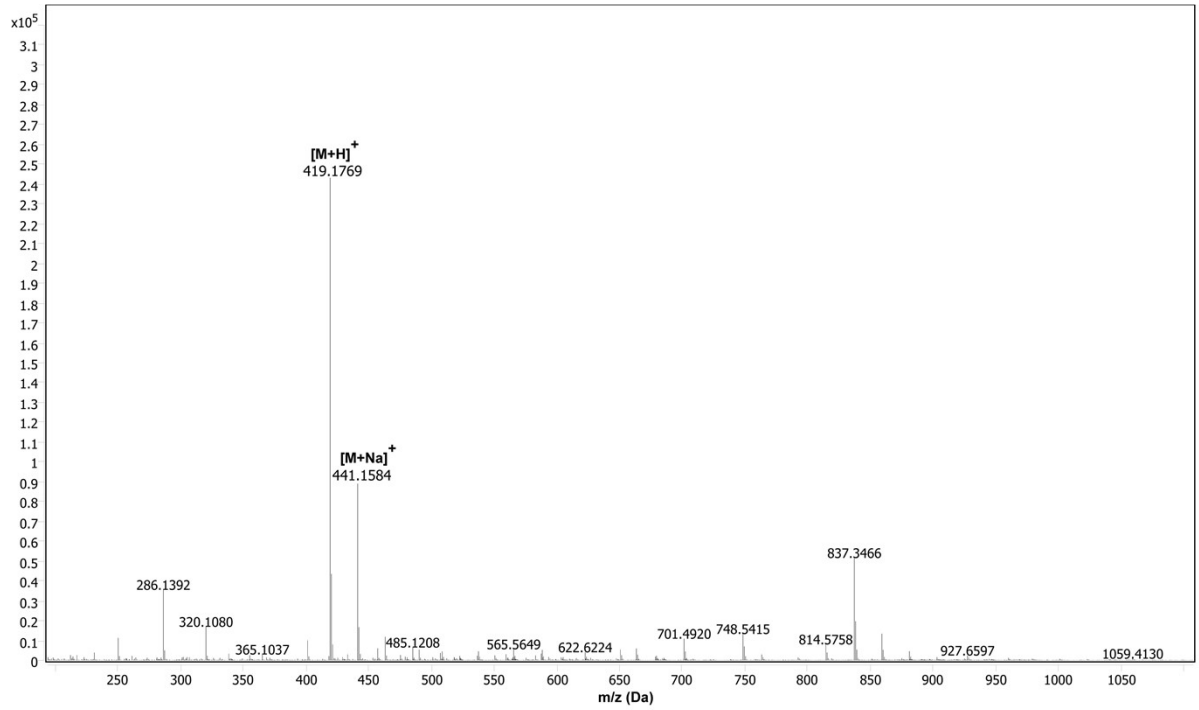


Figure S9. HRMS of **VADD**

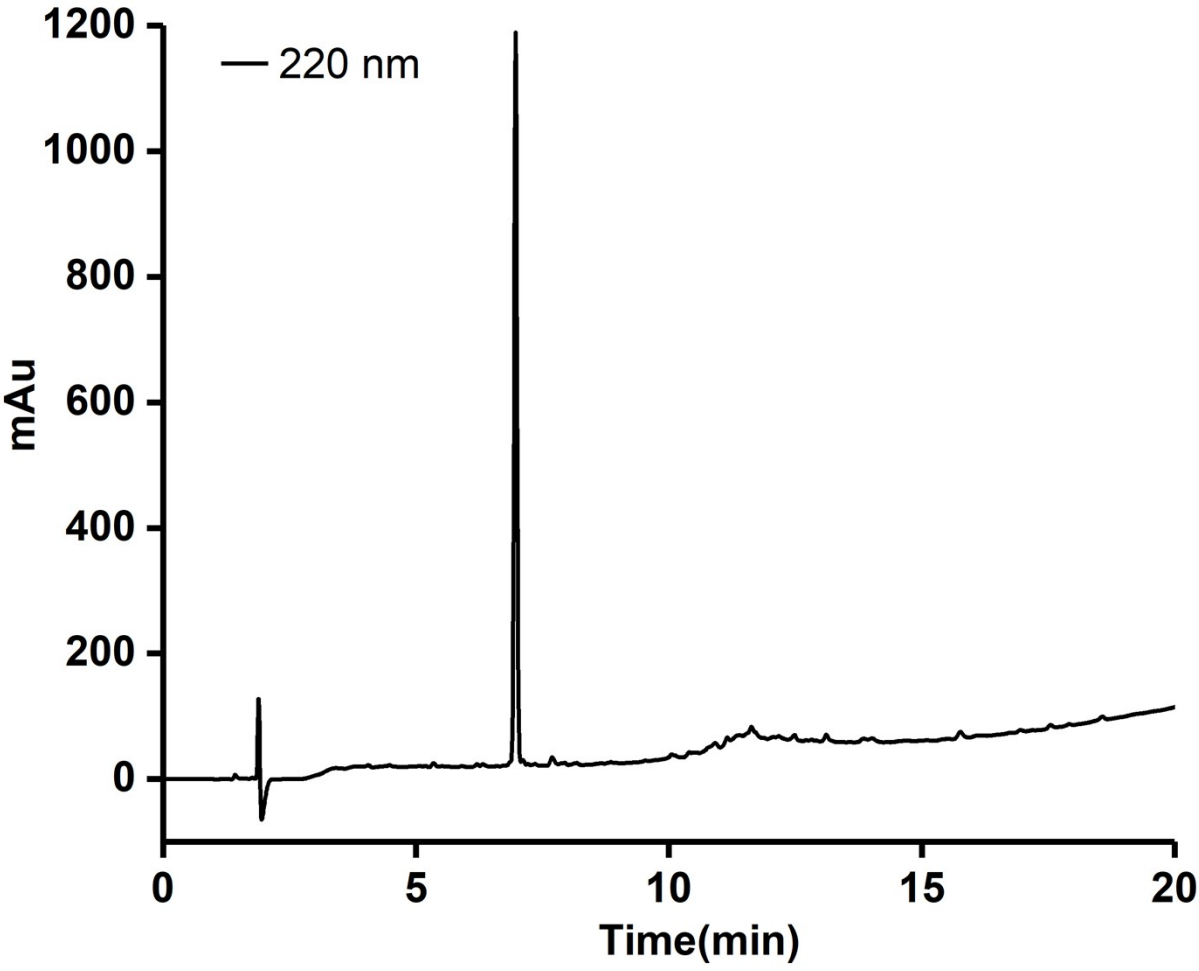


Figure S10. HPLC Chromatogram of **VADD**.

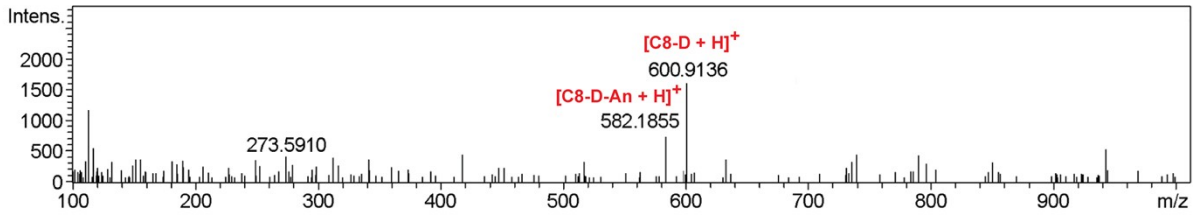
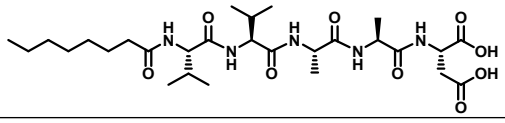
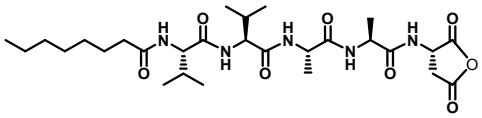
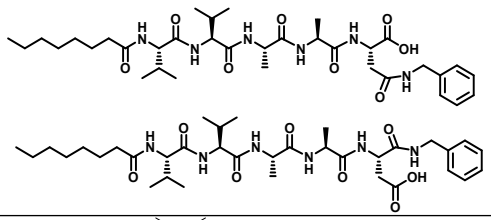
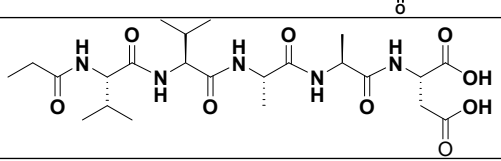
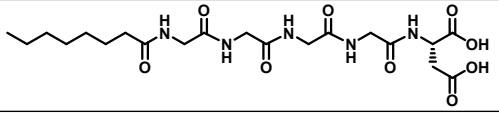
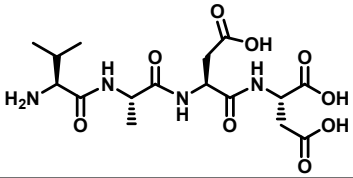
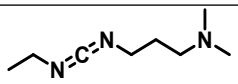
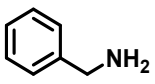


Figure S11. ESI-MS spectrum during the course of reaction showing the presence of both **C8-D** peptide and **C8-D-An** anhydride.

Table S1: The synthesized peptides and other components of the reaction cycle.

Sl. No.	Name	Structure	Exact Mass [gmol^{-1}]	Mass found [gmol^{-1}]
1	C8-D		599.3530 $C_{28}H_{49}N_5O_9$	600.3576 $[M+H]^+$
2	C8-D-An		581.3425 $C_{28}H_{47}N_5O_8$	582.1855 $[M+H]^+$
3	Amide 1 and Amide 2		688.4160 $C_{35}H_{56}N_6O_8$	689.4238 $[M+H]^+$
4	C3-D		529.2748 $C_{23}H_{39}N_5O_9$	530.2827 $[M+H]^+$
5	C3-GD		487.2278 $C_{20}H_{33}N_5O_9$	488.2368 $[M+H]^+$
6	VADD		418.1700 $C_{16}H_{26}N_4O_9$	419.1769 $[M+H]^+$
7	EDC		155.14 $C_8H_{17}N_3$	156.15 $[M+H]^+$
8	Benzylamine		107.07 C_7H_9N	-

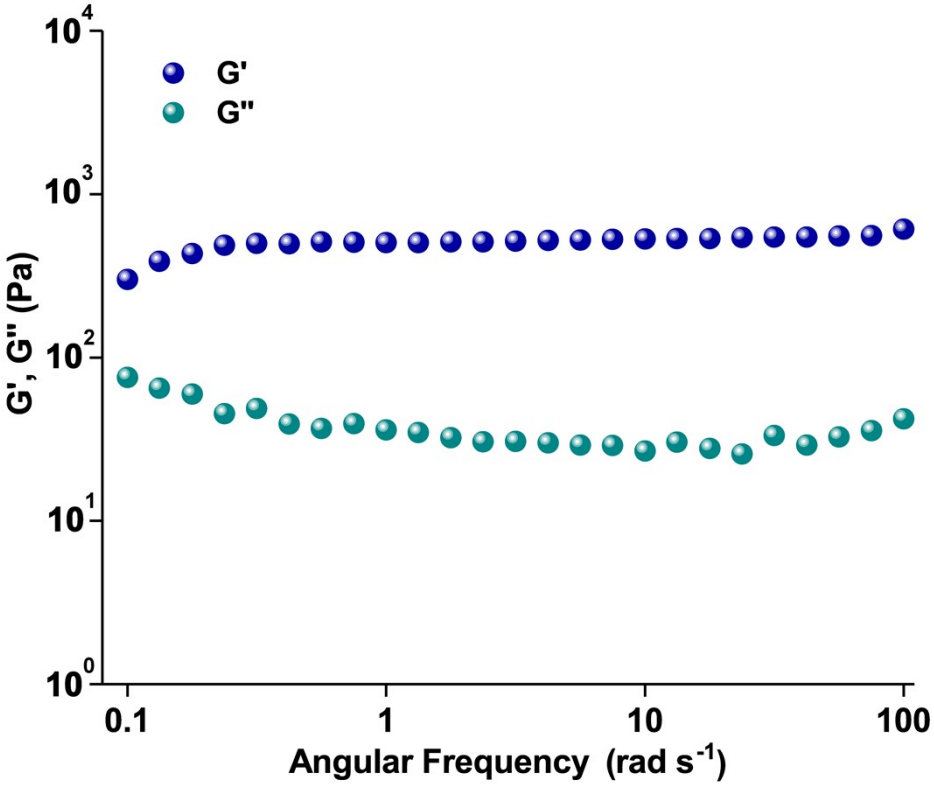


Figure S12. Frequency-sweep test of the EDC fueled hydrogel, at a fixed strain of 1 %, [**C8-D**] = 2 mM and [EDC] = 20 mM.

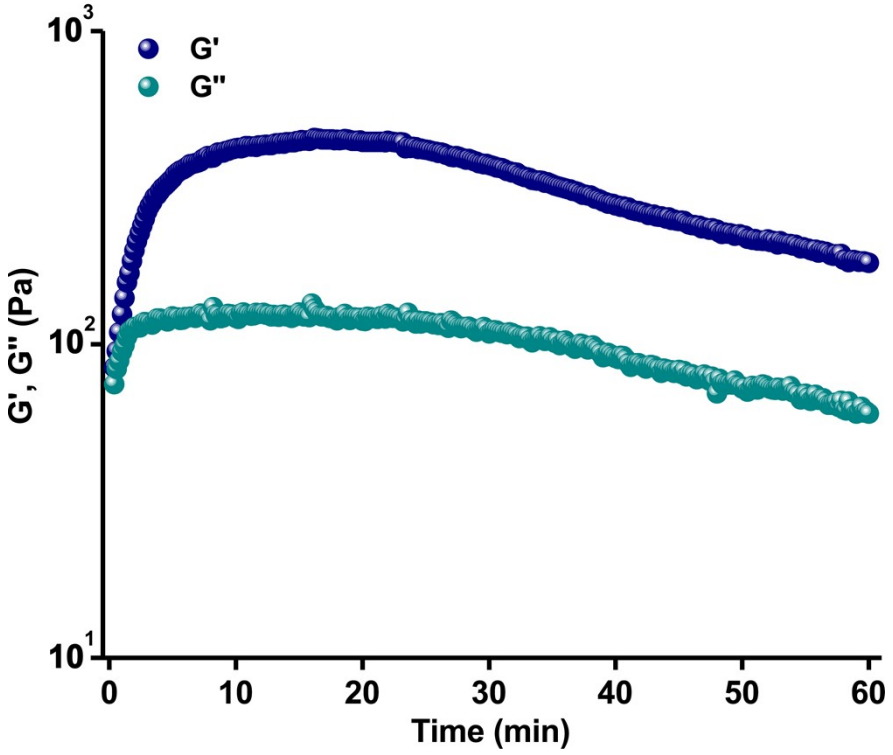


Figure S13. Time-sweep experiment with 50 mM EDC concentration. [**C8-D**] = 2 mM. It is important to note that, though the G' value decreased over time, but crossover point was not achieved.

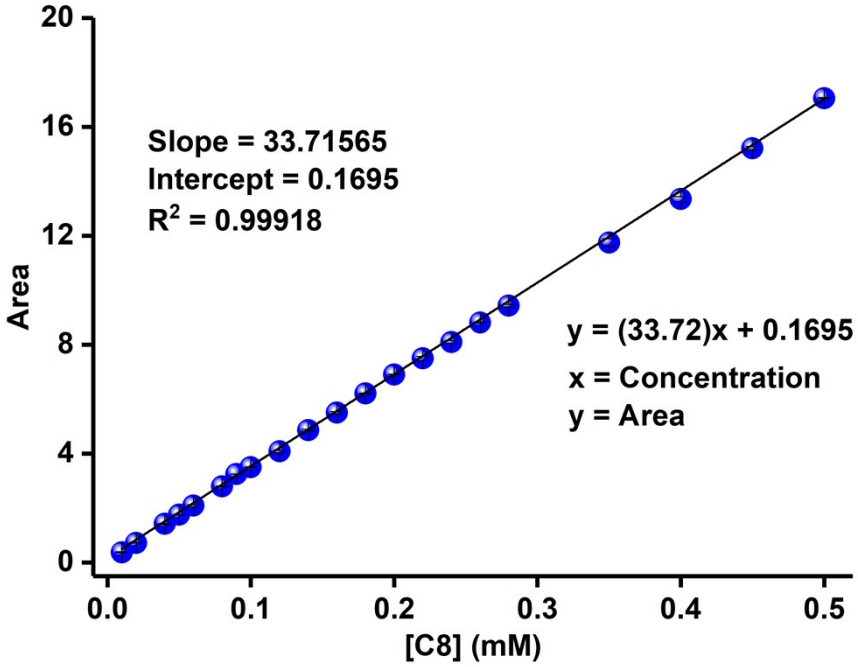
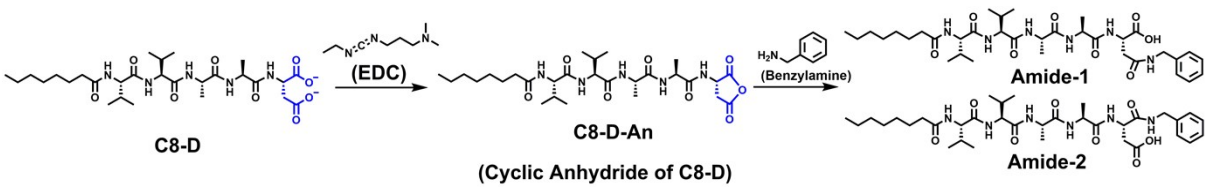


Figure S14. HPLC Calibration Curve of **C8-D** Peptide. n = 3 (mean ± SD).



Scheme S2. Reaction scheme for the formation of cyclic anhydride (**C8-D-An**), and quenching of this cyclic anhydride by benzylamine to form quenched product: Amide-1 and Amide-2.

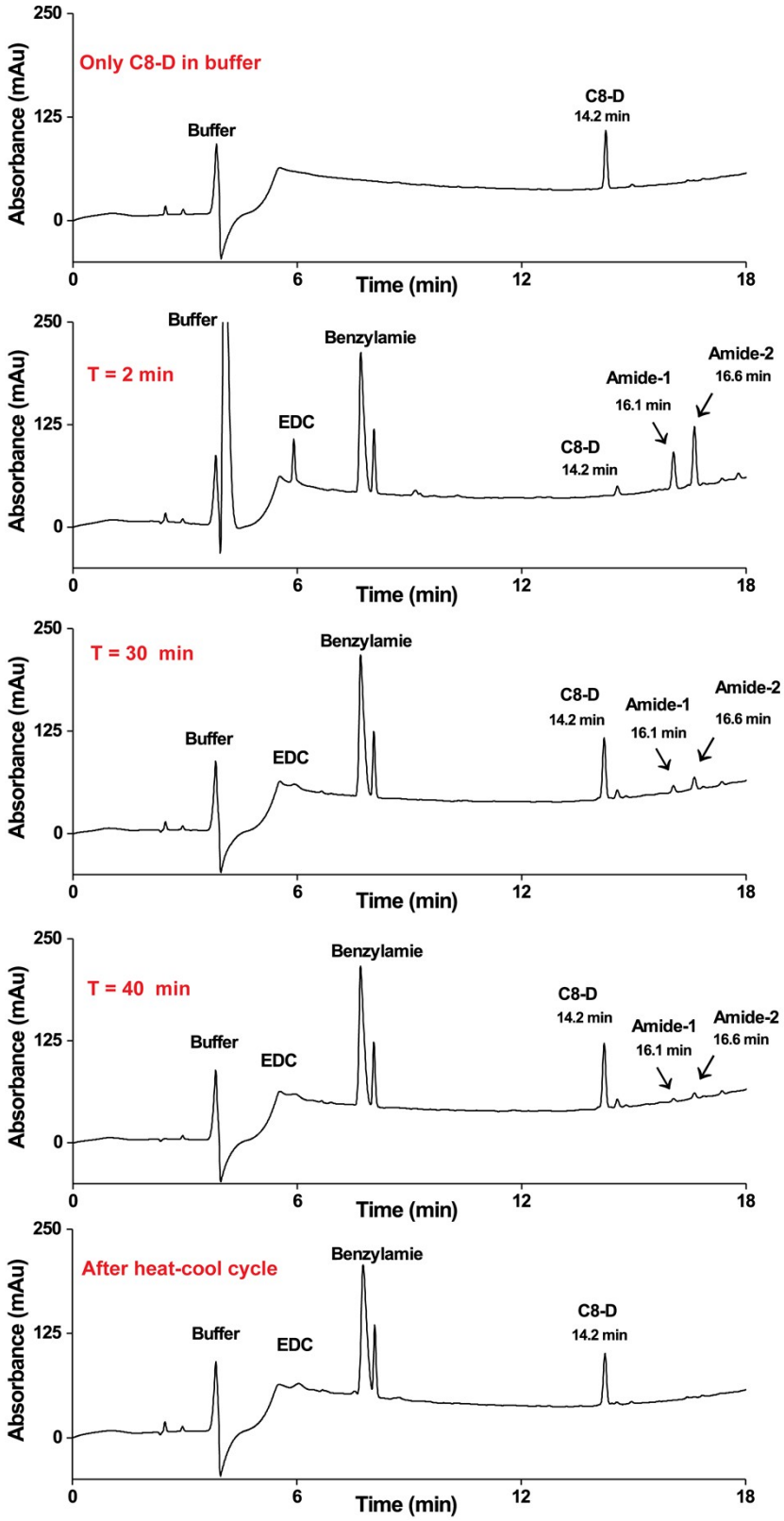


Figure S15. HPLC chromatographic stacks of pure **C8-D**, EDC-fueled reaction products after quenching with benzylamine, after different time intervals (T = 2 min corresponds to gel, T = 30 and 40 min correspond to sol and finally, the heat-cool treated sample). It is important to note that there is a negligible amount of Amide-1 and Amide-2 remaining after 40 min of reaction, which after heat-cool treatment completely disappeared. HPLC data were recorded at 220 nm wavelength.

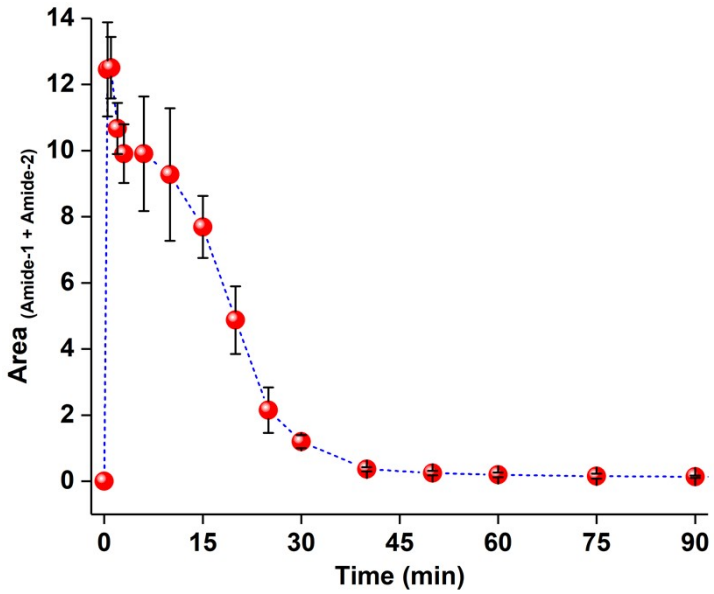


Figure S16. Total area obtained from the time-course HPLC chromatogram of quenched **C8-D-An** (Amide-1 and Amide-2, formed during the reaction cycle) vs Time. It is important to note that, the area exhibited sharp increase followed by gradual decrease during the reaction cycle and within 40 min, the area became close to the initial value i.e. zero, concluding the almost complete hydrolysis of the anhydride. HPLC data were recorded at 220 nm wavelength. n = 3 (mean ± SD).

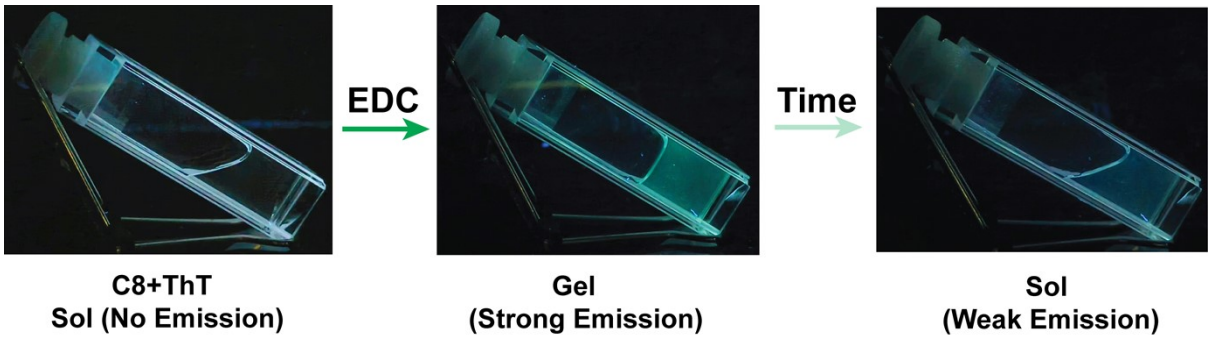


Figure S17. β-sheet formation assessed by Thioflavin T (ThT) Experiment. Left: Cuvette containing **C8-D** and ThT, demonstrating no emission. Middle Cuvette**:** Strong ThT emission (green) was observed from the gel after addition of EDC to **C8-D** solution. Right Cuvette: Emission intensity decreased as gel was transformed into sol. [**C8-D**] = 10 mM, [ThT] = 2 μM and [EDC] = 20 mM, solvent: MES buffer, pH 6, 50 mM, visualized under UV lamp.

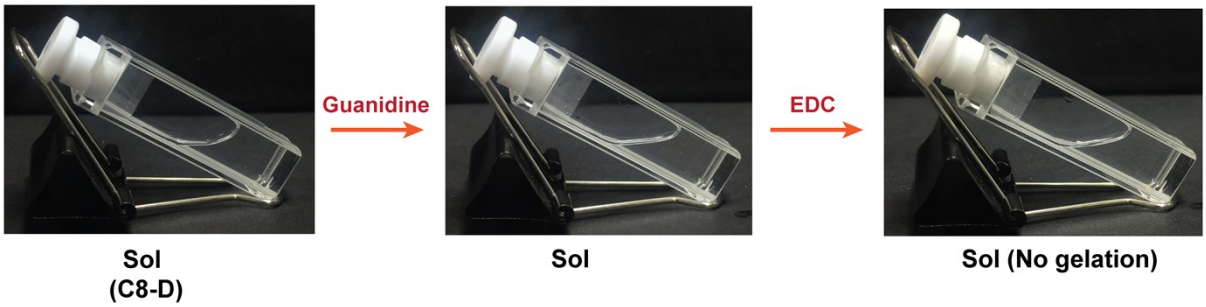


Figure S18. Addition of chaotropic agent, guanidine, prevents gelation. [**C8-D**] = 2 mM, [Guanidine] = 1 M.

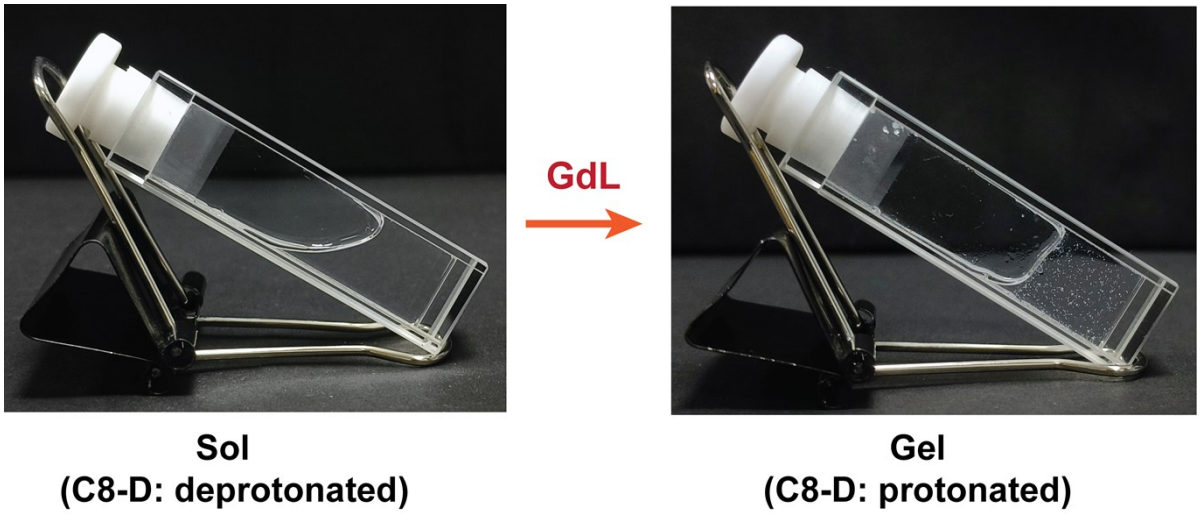


Figure S19. Charge neutralization of terminal carboxylates of **C8-D** peptide by adding GdL to **C8-D** resulted in hydrogelation, visualized under ambient light. The final pH of the system was 4, [**C8-D**] = 2 mM.

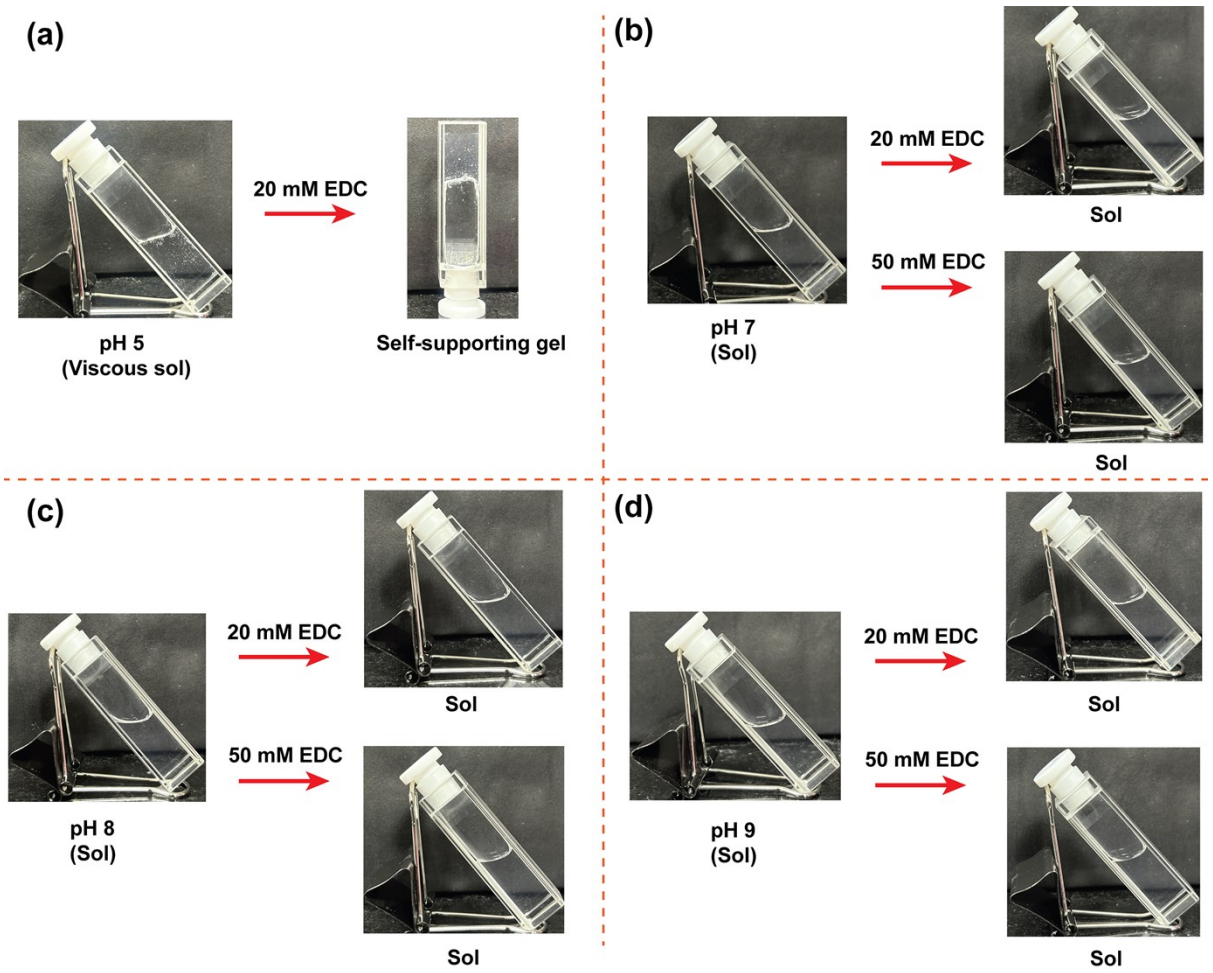


Figure S20. Investigation of EDC-fueled hydrogelation behaviour of **C8-D** on varying pH of the system. Acetate (for pH 5), MOPS (pH 7), AMPSO (pH 8 and 9) buffers were used to maintain the desired pHs. [**C8-D**] = 2 mM and [buffer]= 50 mM

Note: At pH 7 to 9, the peptide failed to form hydrogels because of the slower anhydride formation and rapid hydrolysis of the transient anhydride species. In contrast, at pH 5, gelation happened quickly. The resulting gel stayed stable for up to 48 hours, indicating prolonged stability of the assembled state in acidic conditions.

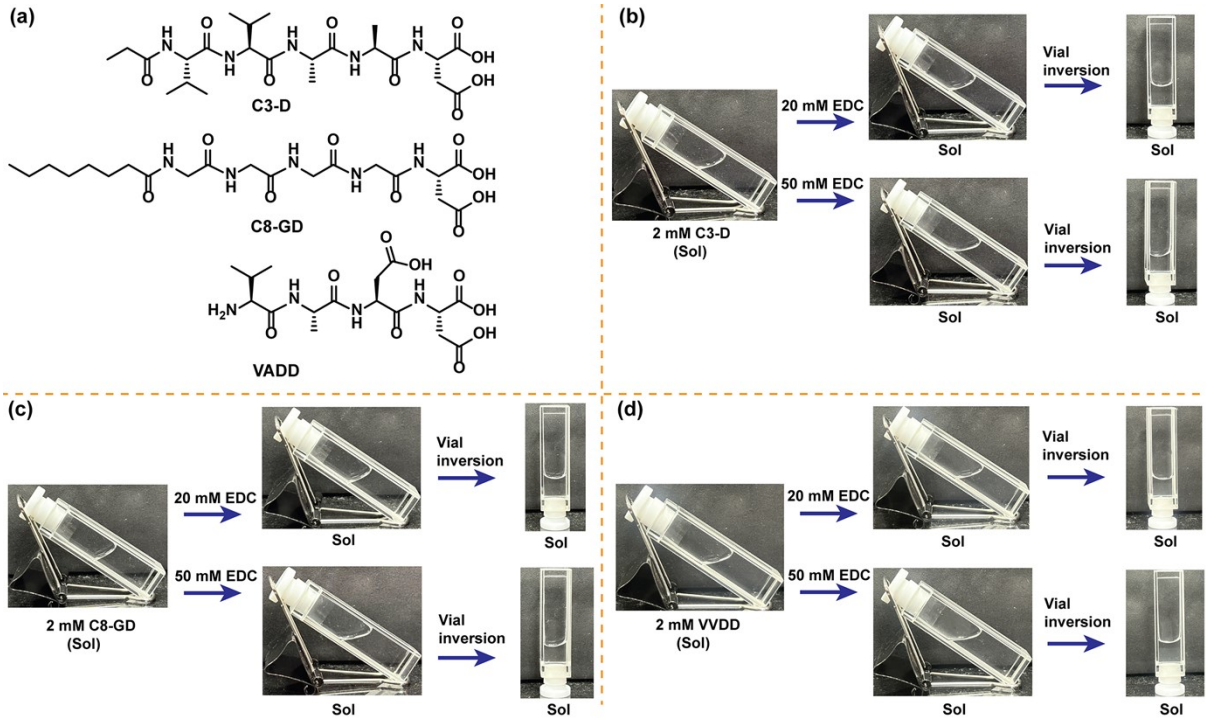
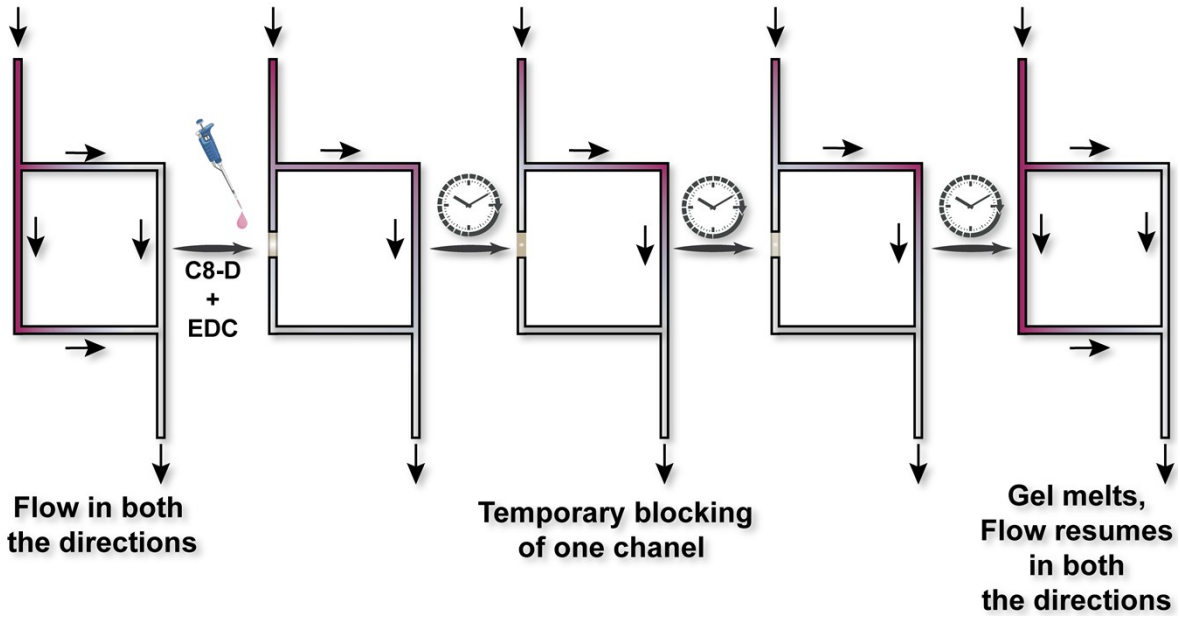


Figure S21. a) Chemical structure of the control molecules. EDC fueled gelation tests for b) **C3-D**, c) **C8-GD** and d) **VADD** at two different concentrations of EDC. [**Peptide**] = 2 mM. None of the peptides forms a gel on EDC fueling.

Note: The control molecules (i) **C3-VVAAD** (in short **C3-D**) has a shorter alkyl chain but same the β-sheet-forming sequence as of **C8-D** and (ii) **C8-GGGGD** (in short **C8-GD**), which has a much lower β-sheet-forming potential than the "VVAA" motif of **C8-D** peptide. Both control molecules dissolved well in aqueous buffer; however, neither formed a transient hydrogel upon EDC fueling under the same experimental conditions used for **C8-D**. These control experiments concludes that both the hydrophobic alkyl chain and the β-sheet-forming peptide sequence are crucial for gel formation.

Another arbitrarily selected molecule **VADD**, having two “D” units, when tested, did not form a gel too. Here, having two aspartic acid residues adds three carboxylate groups, increasing the risk of uncontrolled intermolecular anhydride formation in addition to the desired intramolecular cyclic anhydride formation. Thus, peptide design is very much important for creating an EDC-fueled transient hydrogel.



Scheme S3: Schematic illustration of time-programmed fluidic guidance.

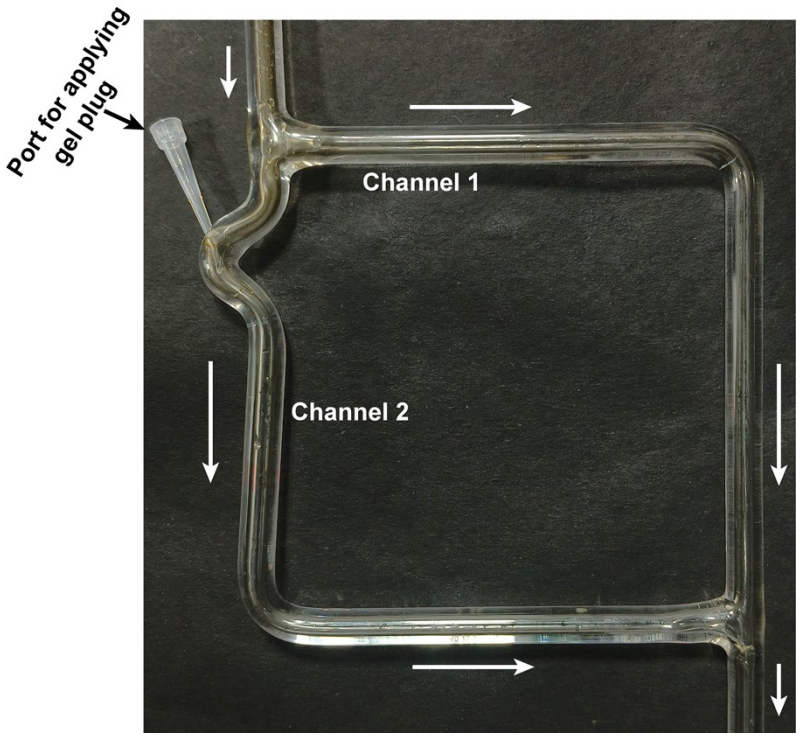


Figure S22. Device for fluidic guidance. In the actual experiment, a red dye was injected to visualize the direction of the flow.

Scheme S4: Schematic Illustration of encapsulation and time-programmed burst release of a drug.

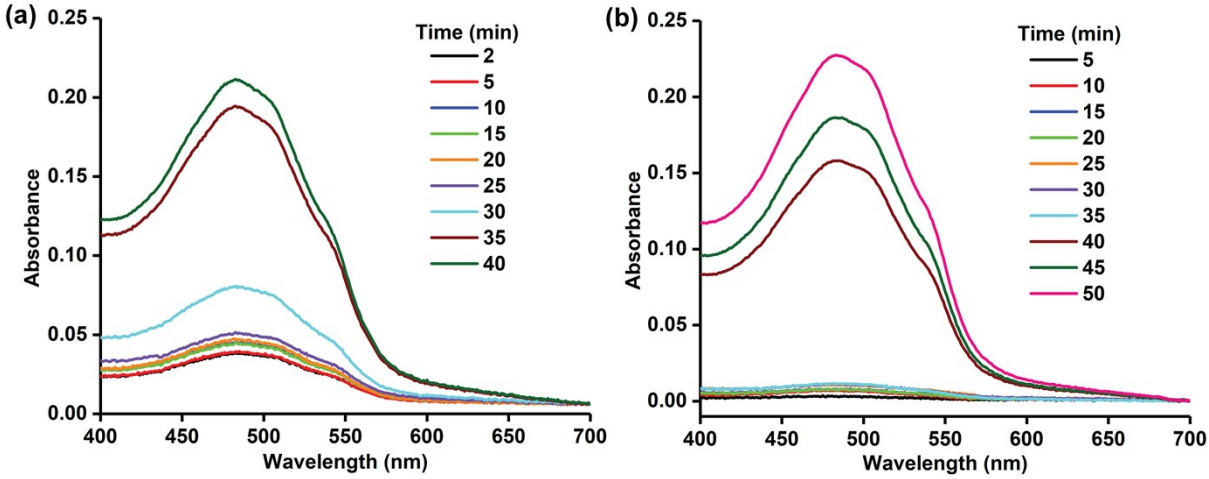


Figure S23. Absorption spectra of time course Dox release when a) 20 mM and b) 50 mM EDC were added.

Reference

1. S. Ahmed, S. A. Islam, M. K. Baroi, B. K. Das and B. Pramanik, *Adv. Mater.,* 2025, **37**, e09789.