Photo-thermally Switchable Peptide Nanostructures towards Modulating Catalytic Hydrolase Activity

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1. Materials & Methods:

All Fmoc protected amino acids, activator (diisopropyl carbodiimide, DIC), piperazine, triisopropylsilane (TIPS), anisole, 1, 2-ethanedithiol (EDT) and *p*-Nitro phenyl acetate (*p*-NPA) were purchased from Sigma Aldrich. 4-methyl coumarin carboxylic acid was synthesized according to previous literature method.[1] Oxyma, Fmoc-Rink amide MBHA resin, HPLC grade acetonitrile, water, ethanol and DMF were obtained from Merck. Trifluoroacetic acid (TFA) and hexafluoroisopropanol (HFIP) was purchased from SRL and TCI chemicals respectively. HEPES, CHES, MES, CAPS, Tris HCl buffers were purchased from HIMEDIA.

Solid Phase Peptide Synthesis was performed in Liberty Blue CEM, Matthews, NC, USA and the synthesized peptide was characterized using reverse phase C18 column in Waters HPLC coupled with Q-TOF MS detector. UV spectra were recorded using Shimadzu UV 6000 UV-vis spectrophotometer in a wavelength range of 800 to 200 nm. CD spectra were recorded using JASCO J-1500 Circular Dichroism Spectrometer, Easton, MD, USA. The wavelength range was selected from 195 nm to 400 nm with a scan speed of 100 nm min^{-1} using 2 mm path length quartz cuvette. The samples were drop-casted on silicon wafer and AFM height images were recorded using tapping mode on a Bruker Multimode 8 scanning probe microscope with silicon cantilever and resultant files were analyzed using Nanoscope Analysis 1.5. The samples were drop-casted on 200 mesh carbon coated copper grids, negatively stained with 1w/v% uranyl acetate solution, dried for 1 day and the TEM images were recorded using JEOL JEM-2100 with a Tungsten filament at an accelerating voltage of 120 kV.

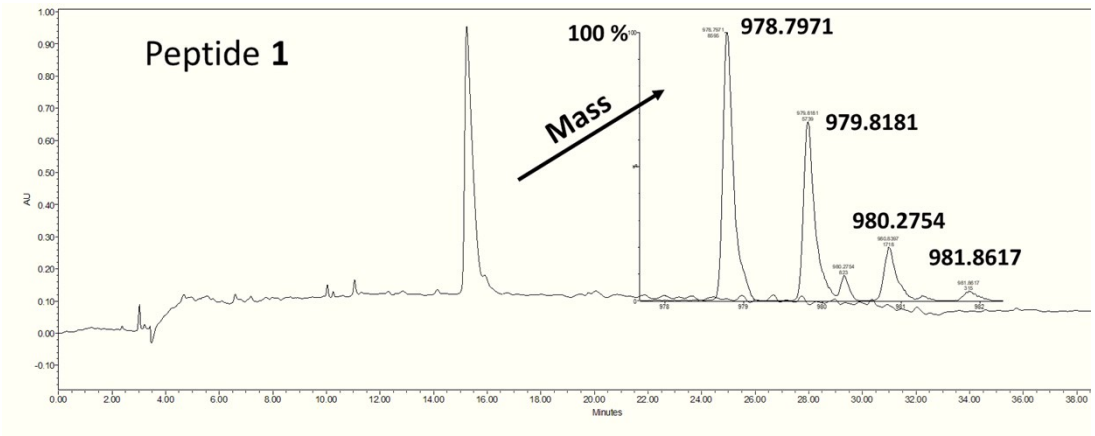
2. Synthesis and Characterization of peptide 1 and 2:

Peptide amphiphiles **1** and **2** were synthesized using Microwave Automated Solid Phase Peptide Synthesizer (Liberty Blue CEM, Matthews, NC, USA). Fmoc-Rink Amide MBHA Resin was swelled in DMF for 30 minutes. All Fmoc protected amino acids were weighed as per the desired scale of the reaction followed by dissolving in required DMF solution. Required mass of piperazine was vortexed in 10% of ethanol followed by addition of the remaining 90% of DMF for completely solubilization. DIC (activator) and oxyma (activator base) in DMF, were used as activators for the coupling reaction between acid and amine to form the peptide bond. Deprotection of Fmoc group from the amino acid was achieved by using 20% piperazine in DMF containing 10% ethanol in the microwave reactor. A cycle of coupling, deprotection steps was repeated to synthesize the required peptide anchored to the resin. The peptide was then cleaved from the resin upon a slow shaking in a cocktail solution, a mixture of TFA/TIPS/water/EDT (95: 2: 2: 1, v/v/v/v) for 3 h at room temperature. The resin was then filtered and the filtrate was collected, containing desired peptide in the cocktail solution. The excess TFA was evaporated using air blower. The peptide **1** and **2** was then precipitated from cold diethyl ether and precipitate were then washed using cold diethyl ether thrice. These were kept overnight to obtain white dried powder of the peptides. The peptides were purified by RP-HPLC using a Waters semi-preparative

binary HPLC system using a C18-reverse phase column with an acetonitrile-water mobile phase containing 0.1% TFA. Pure dried peptides were then dissolved in HFIP and was evaporated to erase self-assembly history of the peptides during purification/ freeze drying process. Synthesis of peptide **3** was already reported in literature.[2]

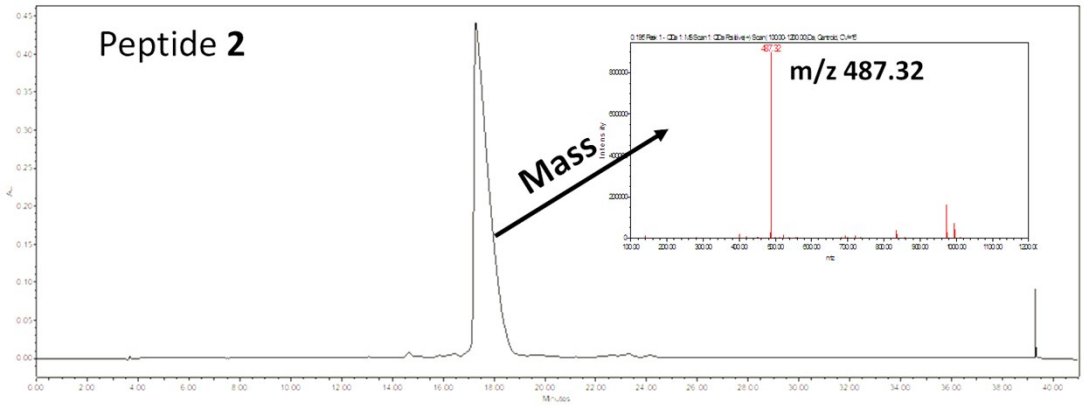
HPLC-Mass trace for **1:**

Calculated exact mass for **1** ($C_{53}H_{59}N_{11}O_8$) was 977.4548 and in positive mode mass found was 978.7971 [MH^+]



HPLC-Mass trace for **2:**

Calculated exact mass for **2** ($C_{50}H_{57}N_{11}O_{10}$) was 971.4290 and mass (m/z) found was 487.32 for doubly charge species $[MH_2]^{2+}$.



3. Pathway-driven self-assembly of peptide 1 and 2:

Nanoparticles: 20 mM stock solution of peptide **1** and **2** in HFIP were prepared at 10 °C. 6.25 μL of the stock solution was diluted in water or 10 mM HEPES buffer to result a final concentration of 0.25 mM of the peptides **1** and **2**. The ratios of HFIP and water (or 10 mM HEPES) were maintained at 1:3, 1:1 at 10 °C. AFM images showed nanoparticles ($\mathbf{1}_{NP}$ and $\mathbf{2}_{NP}$) that remained stable for 4 h at 10 °C (Figure S1).

Nanofibers: Upon increasing the temperature of the solutions containing $\mathbf{1}_{NP}$ and $\mathbf{2}_{NP}$ to 25 °C using a Stuart water bath (SWB15D), the nanoparticles were transformed to the nanofibers of $\mathbf{1}_{NF}$ and $\mathbf{2}_{NF}$ respectively.

Twisted bundles: Upon repeated cycle of annealing the nanofiber of $\mathbf{1}_{NF}$ to 80 °C, cooling down to 25 °C, it resulted in thermodynamically stable twisted bundle morphology.

Nanosheets: $\mathbf{2}_{NP}$ (0.25 mM, 250 μL) were added to γ-cyclodextrin (0.25 mM, 250 μL) in water or 10 mM HEPES buffer in 1:1 ratio to a final peptide concentration of 0.125 mM in 0.5 mL of quartz cuvette, followed by UV_B irradiation (λ = 320 nm, 8 Watts x 4 lamp) for 2 h. AFM analysis revealed the formation of 2D nanosheets of **(di-2□-CD)**$_{NS}$.

4. Microscopic Investigations:

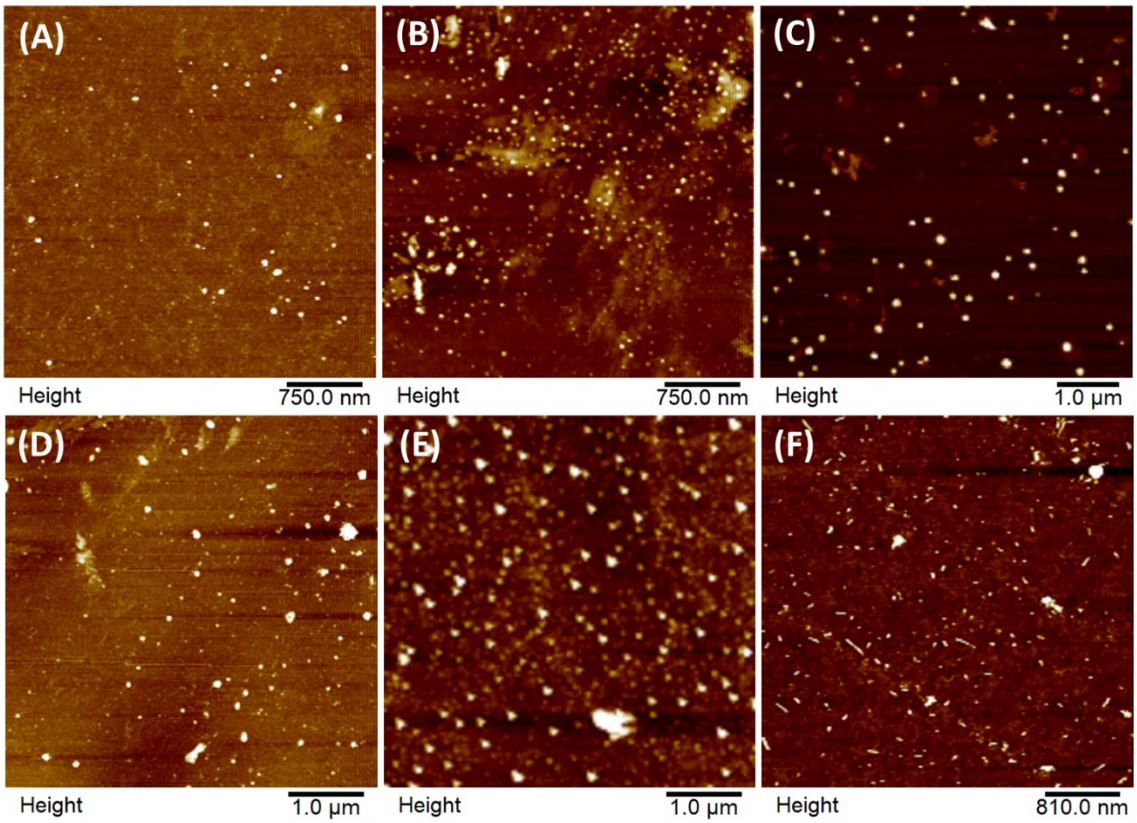


Figure S1. AFM height images for peptide **1** (top row) (A-C) and peptide **2** (bottom row) (D-F) at 10 °C after 30 min, 2 h, and 4 h respectively showing presence of $\mathbf{1}_{NP}$ and $\mathbf{2}_{NP}$.

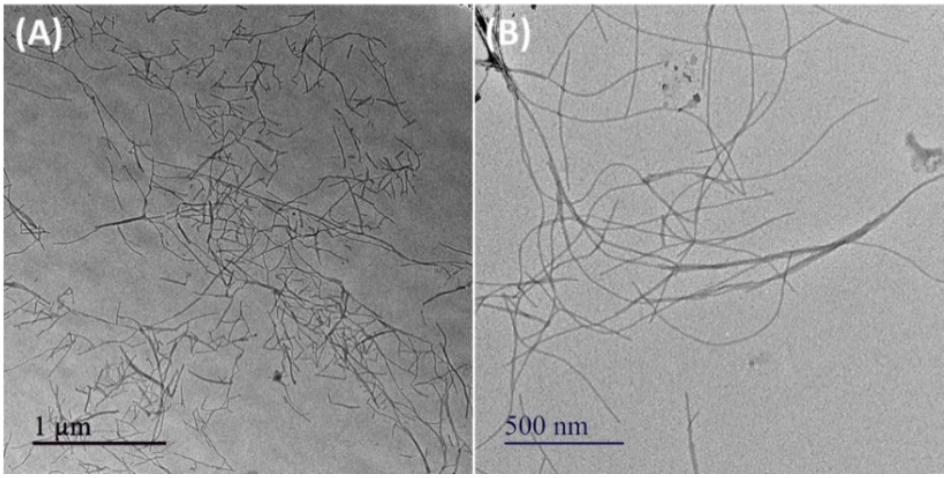


Figure S2. TEM images showing nanofibers of $\mathbf{1}_{NF}$ and $\mathbf{2}_{NF}$ at 25 °C, pH 7.4. (A) $\mathbf{1}_{NF}$ with a diameter of ~7-8 nm, (B) $\mathbf{2}_{NF}$ with a diameter of ~6-7 nm. The nanofibers $\mathbf{1}_{NF}$ and $\mathbf{2}_{NF}$ solutions of were negatively stained using freshly prepared uranyl acetate (1 w/v %) solution in water.

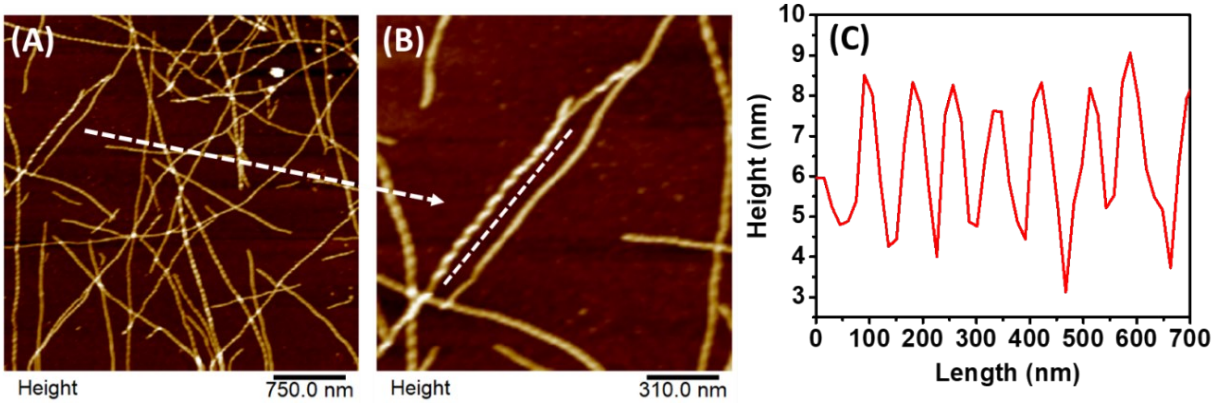


Figure S3. AFM height image showing (A) helical twisted bundle of $\mathbf{1}_{TB}$ upon annealing $\mathbf{1}_{NF}$ solution to 80 °C followed by cooling to 25 °C, (B) Zoomed area indicates intertwining of two nanofibers, and (C) Change in the height profile along the twisted bundle trajectory showing a height profile of ~4-5 nm.

5. Chemical cue and light mediated nano-structural transformation:

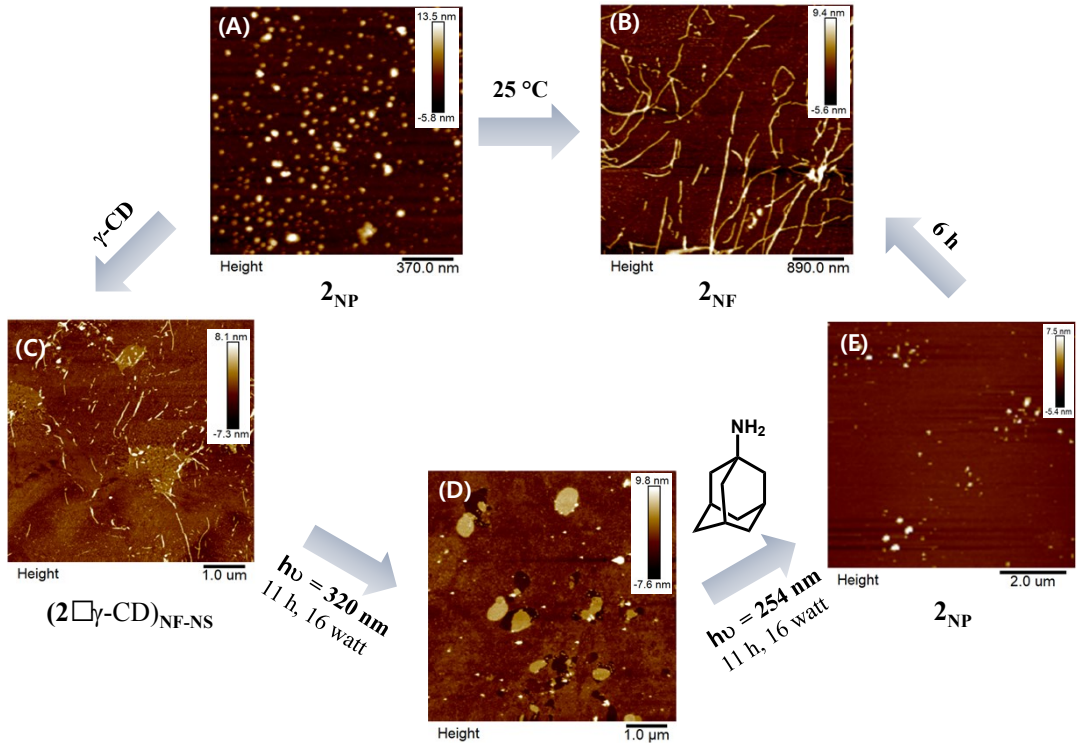


Figure S4. Nanostructures of (A) metastable nanoparticles, $\mathbf{2_{NP}}$ that gets converted to (B) nanofiber, $\mathbf{2_{NF}}$ upon incubating at 25 °C for 3 h. (C) Upon adding γ-CD to $\mathbf{2_{NP}}$ renders mixture of nanofiber and nanosheets of $\mathbf{(2\supset\gamma\text{-}CD)_{NF\text{-}NS}}$ that converts to (D) exclusive nanososheet of $\mathbf{(di\text{-}2\supset\gamma\text{-}CD)_{NS}}$ (height 4-6 nm) followed by UV_B light (λ_{max} = 320 nm, 16 watt) by the interplay of host-guest complexation with γ-CD and coumarin photodimerization. (E) The addition of 1-adamantylamine (chemical cue) and upon irradiating the $\mathbf{(di\text{-}2\supset\gamma\text{-}CD)_{NS}}$ solution with UV_c light (λ_{max} = 254 nm, 16 watt) resulted nanoparticles $\mathbf{2_{NP}}$ that upon standing for 6 h resulted in nanofibers.

6. Circular Dichroism Spectra:

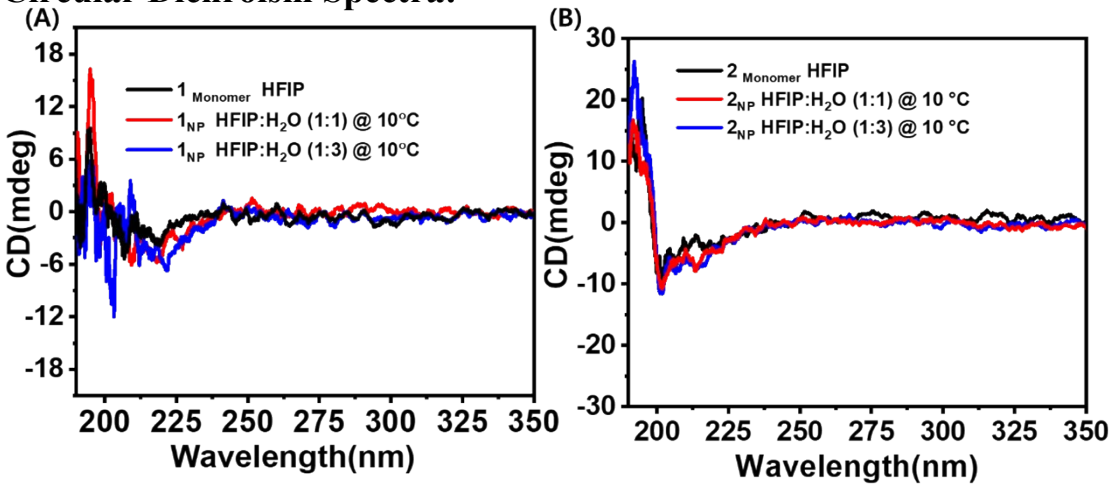


Figure S5. (A-B) CD spectra for peptide **1** and **2** at different volume percentages of HFIP and water at 10 °C (c = 0.125 mM). Monomerically dissolved **1** & **2** in HFIP showed random coil signature of $\mathbf{1_{NP}}$ and $\mathbf{2_{NP}}$ upon adding different v/v% of water.

7. Monitoring Host-Guest complexation by ^{1}H NMR Spectroscopy:

The host-guest interaction between **2** (guest) and γ-CD (host) to form **2**□γ-CD complex was monitored by ^{1}H NMR in D_2O (Figure S7). The peptide **2** was mixed with γ-CD host with variation of host: guest ratios from 0:10, 1:9, 3:7, 5:5, 7:3, 9:1, 10:0 keeping the total concentration constant (10 mM). With increasing the γ-CD concentration, there is an upfield shift in the coumarin protons *a, c, d, e* and *b* due to interaction of coumarin moiety in the hydrophobic inner cavity of γ-CD to form **2**□γ-CD complex.

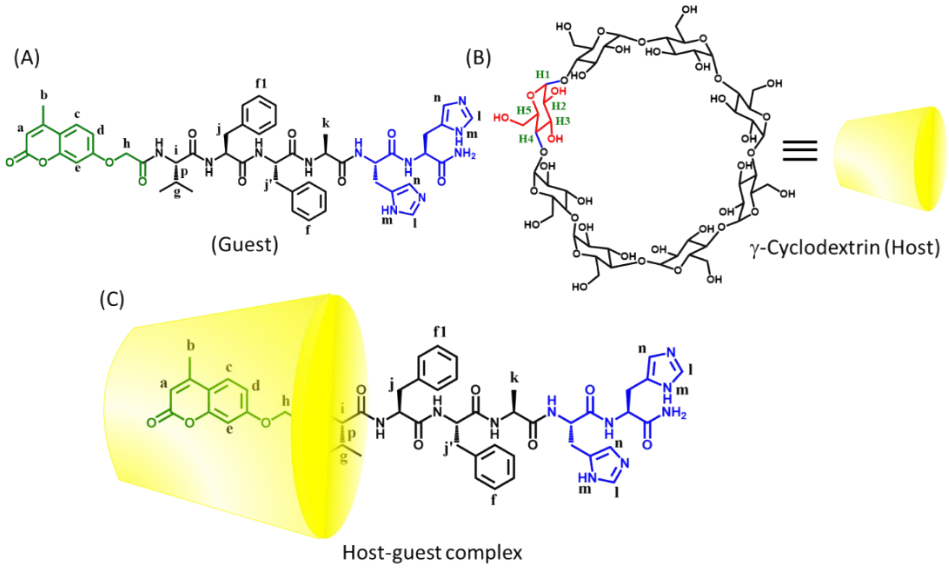


Figure S6. (A) Chemical structure of **2** (guest) with labelled protons, (B) γ-CD and (C) Inclusion complex as a result of host-guest interaction.

Further, the increase in splitting distance of *g* proton corresponding valine dimethyl protons (*g*) indicates that partial inclusion of the methyl groups in the the cyclodextrin cavity (Figure S8A). Moreover, the γ-cyclodextrin ring protons H_1 and H_3 also showed upfield shift with increasing amount of guest molecule which further proved the inclusion of coumarin moiety into the host cavity (Figure S8B-C). Job's plot analysis of the change in chemical shift ($\Delta\delta$) corresponding to the upfield shift of H_1 and H_3 protons of γ-CD with increasing molar ratio of host exhibited maximum change at 0.5 molar ratio indicating binding stoichiometry as 1:1 (Figure S8D).

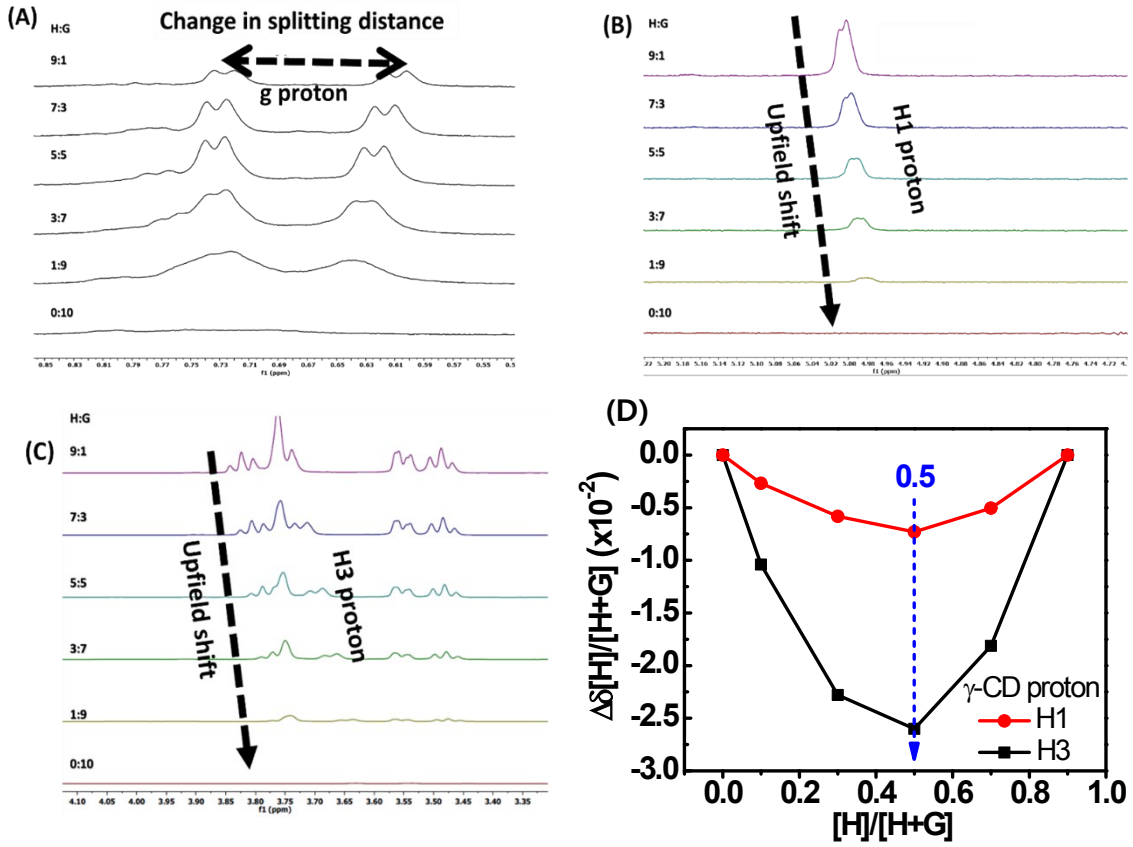


Figure S7. Partial ^{1}H NMR spectra of γ-CD titrated with **2** at different ratios with total concentration at 10 mM. (A) The separation among the doublet for H_g methyl protons of valine in **2** with increasing amount of host is ascribed to partial inclusion of methyl groups in the cavity. (B-C) H_1 and H_3 protons of the γ-CD showed upfield shift with increasing amount of guest. (D) Job's plot by monitoring H_1 and H_3 protons of γ-CD.

8. UV Spectroscopic Investigation & Association constant (K_a):

UV spectra for different morphological states for peptide **1** and **2** were recorded at 0.1 mM.

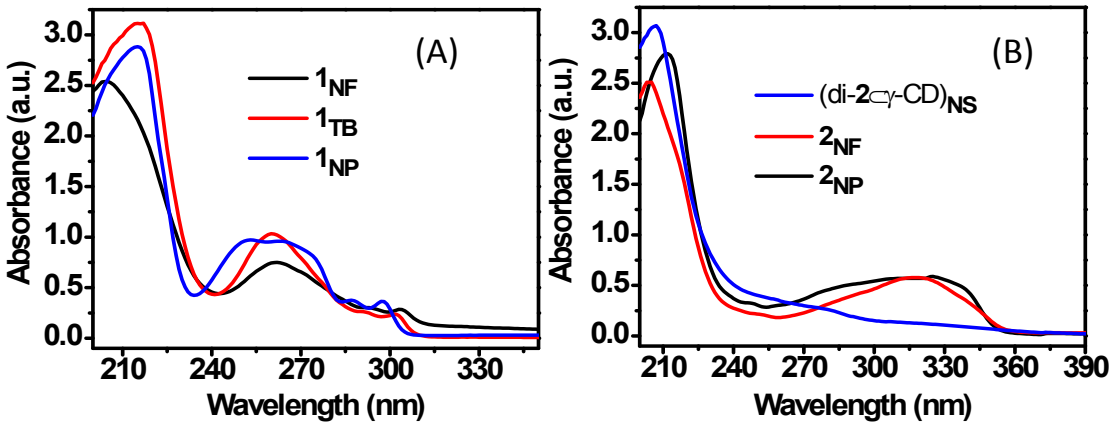


Figure S8. UV spectra of (A) peptide **1** (B) **2** upon converting to different nanostructures

UV spectra were recorded at a fixed concentration of 3 (0.25 mM) against a varied concentration of γ-CD. An increased in the absorbance value at 320 nm was observed with the addition of γ-CD followed by a saturation of the absorbance values. A reciprocal plot between inverse of change in absorbance (*1/ΔA*) against inverse in the concentration of γ-CD (*1/[H]*) furnished K_a ~3.5 x10^4 M^{-1}

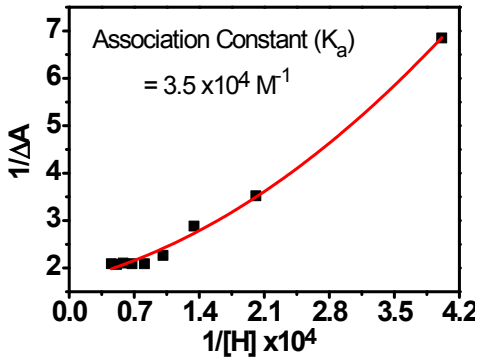


Figure S9. Reciprocal plot of change in absorbance (*1/ΔA*) against the varied concentration of γ-CD *(1/[H]*) to calculate the association constant.

9. Photodimerization Degree (PD):

To evaluate the photodimerization ability of 4-methyl coumarin moiety, UV spectra were recorded at various time intervals for the irradiated solutions of $\mathbf{2_{NF}}$ and (**2**⊂**γ-CD**) solutions at 320 nm. A gradual decrease in the characteristic peak at 320 nm with increase in the irradiation time which validated the photodimerization of the 4-methylcoumarin moiety.

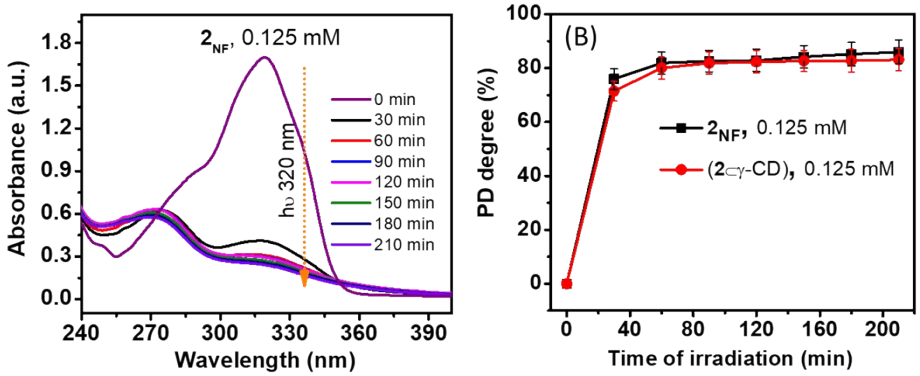


Figure S10. UV spectra show a decrease in the absorbance value upon UV_B irradiation of (A) $\mathbf{2_{NF}}$ (0.125 mM) at various time intervals. (B) Photodimerization degrees for $\mathbf{2_{NF}}$ and (**2**⊂γ-CD) were calculated to be ~85% and ~83% respectively.

10. Kinetic assays of para-nitrophenyl acetate (*p*-NPA) hydrolysis:

A stock solution of *p*-NPA (50 mM) was prepared in acetonitrile. 2 µL of the stock was added to a quartz cuvette containing $\mathbf{1_{NF}}$ solution (0.25 mM, 0.5 mL) in 10 mM HEPES buffer, pH 7.4 at 25 °C. At this stage, the final concentration of *p*-NPA was 0.2 mM. The kinetics spectra was recorded at 400 nm for 2 h. An increase in the absorbance value at 400 nm indicated the formation of *p*-nitrophenol (*p*-

NP) in course of time. Different substrate concentrations (2 to 12 μL) were added separately to $\mathbf{1}_{NF}$ (0.25 mM) to study the substrate dependence kinetics of hydrolysis. Similarly, solutions of $\mathbf{1}_{TB}$, $\mathbf{2}_{NF}$, and $(\mathbf{di\text{-}2\text{-}\gamma\text{-}CD})_{NS}$ were used against various substrate concentration to study the hydrolysis rates.

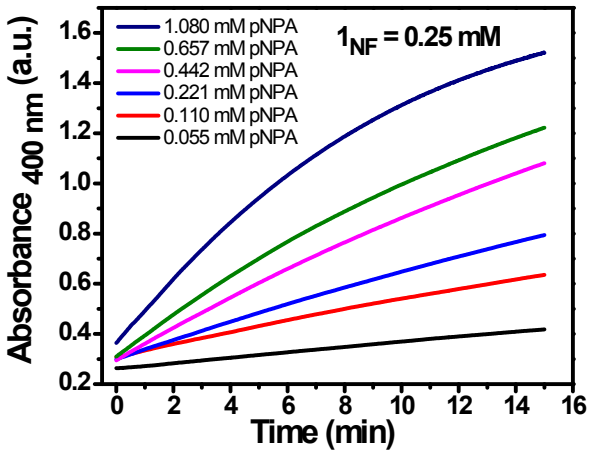


Figure S11. Kinetics for hydrolysis of *p*-NPA with different substrate concentrations. [$\mathbf{1}_{NF}$] = 0.25 mM.

Fitting to Michaelis-Menten's equation: The absorbance values till 300 s were subtracted from that of $\mathbf{1}_{NF}$ solution with substrate. Using the resulting values of the absorbance at different intervals of time, initial slopes were calculated for different substrate concentrations of *p*-NPA at a fixed concentration of $\mathbf{1}_{NF}$ (0.25 mM). These slope values with respect to their substrate concentration values were fitted in Michaelis-Menten's equation to get the kinetic parameters k_{cat}, K_M, V_{max}, and k_{cat}/K_M.

11. Consecutive addition of substrate:

Catalytic activities upon sequential addition of the substrate to catalyst solution were studied to understand the efficacy of active catalytic sites over multiple cycles. 4 μL of the stock solution of *p*-NPA (5 mM in acetonitrile) was added to 0.5 mL of $\mathbf{1}_{TB}$ solution of 0.25 mM and kinetic spectra was recorded at 400 nm for 3 h. The saturation plateau indicating no further formation of *p*-NP suggests completion of cycle 1. Similarly, 4 μL of the *p*-NPA was added to monitor the catalytic activity with increase in the absorbance value followed by saturation plateau to complete cycle 2 and 3.

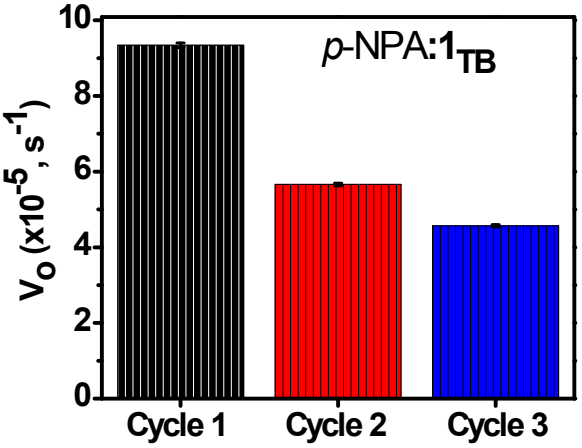


Figure S12. Initial hydrolysis rates for $\mathbf{1}_{TB}$ over three cycles of sequential addition of *p*-NPA.

12. Heterogeneous catalysis using embedded peptide hydrogel networks:

A stock solution of $\mathbf{1}_{NF}$, $\mathbf{1}_{TB}$, and $\mathbf{(di\text{-}2\square\gamma\text{-}CD)}_{NS}$ was prepared in water and then incubated for 24 h. A solution of sodium phosphate buffer (SPB, c = 10 mM) was added to it with a final concentration of the peptide is 4.14 mM (pH 7.4). The addition of SPB to the free flowing solution of the $\mathbf{1}_{NF}$, $\mathbf{1}_{TB}$, and $\mathbf{(di\text{-}2\square\gamma\text{-}CD)}_{NS}$ turned into a viscoelastic mass, that was injected in 0.22 μm nylon filter followed by drying to form embedded nanostructures.

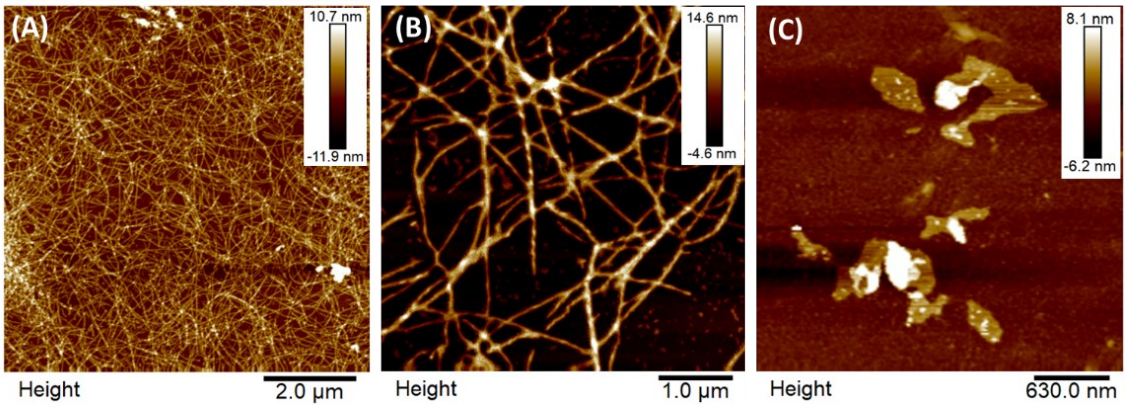


Figure S13. AFM image of the peptide hydrogels upon incubating sodium phosphate buffer (pH = 7.4, 10 mM) owing to the crosslinking network of (A) $\mathbf{1}_{NF}$, (B) $\mathbf{1}_{TB}$, and (C) $\mathbf{(di\text{-}2\square\gamma\text{-}CD)}_{NS}$.

A stock of *p*-NPA (500 mM in acetonitrile) was further in 10 mM HEPES buffer, pH 7.4 to make a final concentration of 0.4 mM. 1.5 mL of the subtrate solution was passed through modified catalyst embedded filter with a flow rate of 1.0 mL/min. The filtrate solution was collected and repeatedly passed through the same filter, to get product *p*-NP in the filtrate solution that was monitored at 400 nm. The enhancement in the absorption value followed by a saturation plateau suggested completion of the hydrolysis of *p*-NPA to the product *p*-NP. This was repeated for $\mathbf{1}_{TB}$, and $\mathbf{(di\text{-}2\square\gamma\text{-}CD)}_{NS}$ solution to compare the catalytic activity of the flow catalyst and turn over number was compared.

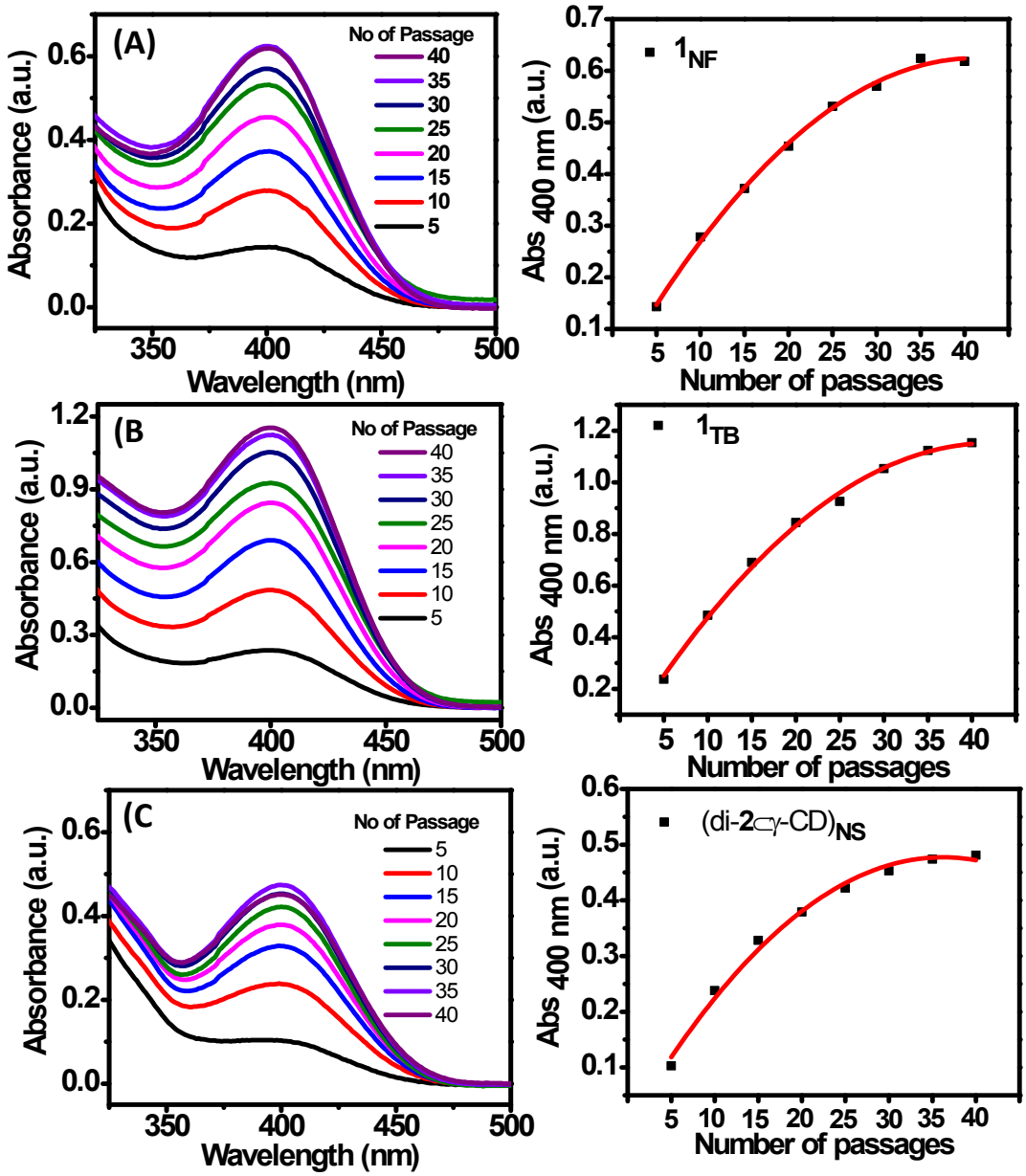


Figure S14. UV spectra (left) showing the catalytic rate of conversion of *p*-NPA to *p*-NP using embedded peptide catalyst and absorbance value at 400 nm was plotted against number of passage of the solution mixture (right) for (A) $\mathbf{1}_{NF}$, (B) $\mathbf{1}_{TB}$, and (C) **(di-2□γ-CD)**$_{NS}$.

13. References:

1. R. H. Huyck, S. R. Trenor, B. J. Love and T. E. Long, *J. Macromol. Sci. Part A*, 2007, **45**, 9-15.

2. A. Singh, J. P. Joseph, D. Gupta, I. Sarkar and A. Pal, *Chem. Comm.*, 2018, ***54***, 10730–10733.