Supporting Information for:

Processible Cyclic Peptide Nanotubes With Tunable Interiors

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Materials & Methods

Fmoc-*D*-Ala-OH, Fmoc-*L*-Lys(Boc)-OH, Fmoc-*L*-Leu-OH, polystyrene-(2-chlorotrityl) resin (loading: 1.5 mmol/g), and 2-(6-Chloro-*1H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were purchased from Nova Biochem. 2-Propanephosphonic acid anhydride (T3P) in DMF was purchased from Advanced ChemTech. 3-amino-2-methylbenzoic acid (**B**), Carboxylic acid-terminated polyethylene glycol (PEG) (HO-PEG-NHCOCH$_2$CH$_2$COOH) (*Mw=3000 g/mol)* was purchased from Rapp Polymere, PEG-covered CPNs of Mba-8CP were prepared as described previously[1] using 2 equivalents of PEG to the Mba-8CP, *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu), and all other reagents were purchased from Aldrich without further purification. All solvents used were of HPLC grade.

Solid Phase Peptide Synthesis was performed on a Protein Technologies Prelude solid phase synthesizer using standard 9-fluorenylmethyl carbamate (Fmoc) protection chemistry.

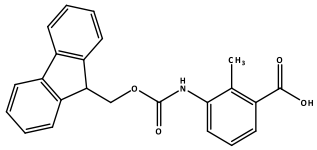
Analytical HPLC was performed on a C18 column (Vydac), 0-50% ACN in H_2O over 30 minutes at a flow rate of 1 mL min^{-1} using a Varian Prostar 335 CC UV detector. Peptides were purified by RP-HPLC (Beckman Coulter) on a C18 column (Vydac). The flow rate was 10 mL min^{-1} for semipreparative runs and peptides were injected at a concentration of 10 mg/mL. Peptide elution was monitored with a diode array detector at wavelengths of 220 and 280 nm. Water-soluble conjugates were eluted with a linear AB gradient, where solvent A consisted of MilliQ water containing 0.1% *v/v* TFA and solvent B consisted of acetonitrile containing 0.1% *v/v* TFA. ***UV-Vis*** spectra were recorded on a Varian 5000 UV-VIS-NIR spectrophotometer. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry has been performed on an Applied Biosystems 4800 MALDI-ToF/ToF Analyzer. All spectra were recorded in positive ion mode operating in reflector mode. ***Circular dichroism (CD)*** and UV-Vis spectra were recorded on a Jasco J-815 CD spectrophotometer equipped with a Jasco PTC-4245/15 cooling element. DLS measurements were performed on a Malvern Zetasizer ZS. ***FT-IR*** spectra were recorded on a Perkin Elmer Spotlight 200 FTIR Microscope System. ***Transmission electron microscopy (TEM)*** samples were prepared from solutions of the lyophilized materials in ACN at concentration of 0.25mg/mL by placing 5 µl of the peptide suspension on holey carbon-coated copper grid (TED Pella 01824) for 10 seconds before removing the excess solution by filter paper blotting. The sample on the grid was negatively stained with a 2% (w/v) phosphotungstic acid (adjusted to pH = 3 with NaOH) for 10 seconds. Excess stain solution was wicked off. TEM images were collected on a FEI Tecnai 12 transmission electron microscope at an accelerating voltage of 120 kV. ***Tapping mode AFM images*** were collected using silicon cantilevers (RTESP from Veeco, Inc.) with a resonant frequency of 255 Hz. ATR-FTIR spectra were collected using a NICOLET 6700 FT-IR Spectrometer. ***Molecular Modeling*** calculations were performed using HyperChem software, where the optimized molecular models were generated using the Molecular Mechanics method with the MM+ force field.[2] The geometry optimization was carried out using the Polak–Ribiere conjugate gradient, set to terminate at an RMS gradient of 0.01 kcal Å^{-1} mol^{-1}.

Molecular Dynamics Simulations: A wide range of computational approaches such as molecular dynamics (MD)[3] or quantum mechanics methods[4] have been employed in earlier studies of CPNs. Encouraged by earlier MD studies that corroborated experimental data such as pore size, intersubunit distances, and crystal structure lattice constants, we carried out MD simulations to obtain long time scale dynamical data on nanotube structure and stability in non-

periodic, explicit solvent conditions relevant to self-assembly in solution, taking into account short as well as long-range interactions.

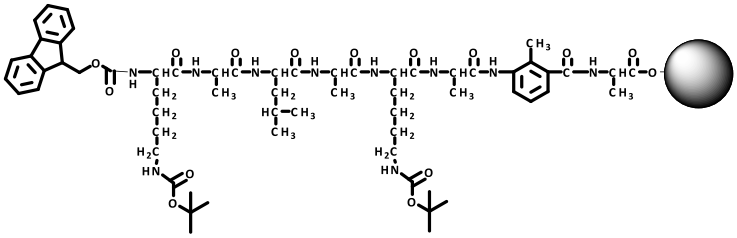
Initial coordinates for the CP subunit structures were taken from optimized geometries calculated using MM+ force field in HyperChem. Molecular Dynamics simulations were carried out in Materials Studio using the *ab-initio* based COMPASS[5] force field. In order to explore the conformational space and find the minimum energy configuration of the CP monomers in the tubular morphology, three subunits were stacked and subjected to five annealing cycles with a temperature range of 298K to 600K in the NVT ensemble, where each cycle was followed by minimization calculations. The final configuration of the central subunit was selected to build the CPNs used for equilibration simulations. The structures computed from these final equilibration runs were used for the comparative analysis. CPNs were composed of eight subunits stacked in an antiparallel fashion. These simulations were carried out in an isothermal-isobaric ensemble (NPT) with the Berendsen method and the Nose thermostat to control pressure and temperature at 1 atm and 298 K respectively. The system is solvated in explicit water and periodic boundary conditions are employed for a cubic box that is 50Åx30Åx30Å. To calculate the electrostatic interactions and the Van der Waals forces, we use the Ewald summation method with a repulsive cutoff of 6 Å and a cutoff distance of 12.5 Å. The time-step is fixed to 1 fs and the total duration of the simulations is 1.5 ns. Post-processing was done using Visual Molecular Dynamics[6] (VMD) and .tcl scripts. The cutoff distance for the hydrogen bonds was 3.4 Å and the minimum D-H-A angle for bond formation is taken as 130°. The inter-subunit distance was calculated as the center of mass distance between the alpha-carbon atoms of each subunit. For all calculations, measurements are taken every 2 ps, excluding the first 200 ps of the equilibration runs.

Compounds Synthesis & Characterization

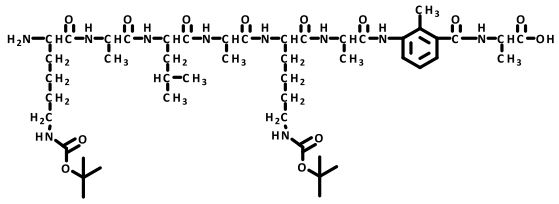


Synthesis of Fmoc-Mba-OH: Sodium bicarbonate (3.84 g, 45.7 mmol) was added slowly to an aqueous solution (50 mL) of 3-amino-2-methylbenzoic acid (γ-Mba) (10.0 g, 29.7 mmol). The resulting solution was cooled in an ice bath, and *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) (3.45 g, 22.8 mmol) was added slowly as a solution in *p*-dioxane (50 mL). The solution mixture was left to stir at 0 °C for 1 h, and then was left to stir at RT overnight. Water (50 mL) was added, and the aqueous solution was extracted 3 times with ethyl acetate (150 mL). The organic layer was washed twice with saturated solution of sodium bicarbonate (100 mL). The aqueous layers were combined, and the resulting solution was acidified to pH 1 by addition of 1 M HCl. The acidified solution was then extracted with 3 x 50 mL portions of ethyl acetate. The organic layers were combined, dried over sodium sulfate, filtered and solvent was evaporated in vacuum. The product was redissolved in minimal amount of THF, and dried under vacuum. The product was obtained as a white solid (6.80g, 80%). ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) 2.32 (s, 3H), 4.29 (s, broad, 1H), 4.44 (d, J = 8.8 Hz, 2H), 7.23 (t, J = 7.5 Hz, 1H), 7.34 (m, 3H), 7.43 (t, J =7.6 Hz, 2H), 7.56 (d, J =7.5 Hz, 1H), 7.72 (s, broad, 2H), 7.90 (d, J = 7.5 Hz, 2H), 9.16 (s, 1H), 12.96 (s, 1H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6): δ 15.5, 47.2,

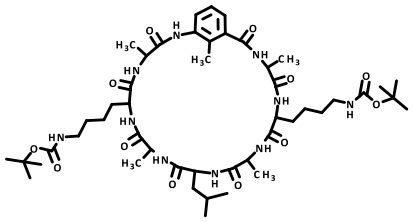
66.1, 120.6, 125.6, 126.0, 127.1, 127.5, 128.1, 129.4, 133.2, 133.6, 137.6, 141.2, 144.2, 154.9, 169.6. MALDI-TOF for $C_{23}H_{19}NO_4$ Calculated: 374.13 $[M+H]^+$; Found 374.21.



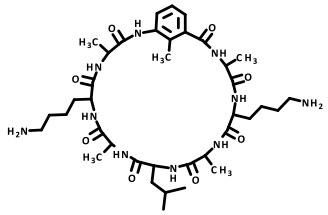
Procedure for solid phase peptide synthesis: 2-Chlorotrityl chloride resin was swelled for two hours in DMF. Fmoc-*D*-Ala-OH (2 eq.) was dissolved in DMF and DIPEA (4 eq.) was added. The amino acid mixture was added to the resin suspension and stirred for two hours. Methanol was then added to block remaining chloride residues. The resin was filtered and washed with DMF, DCM and methanol, and left to dry under vacuum. The Fmoc protecting group was then removed by stirring the resin in 20% *v/v* piperidine in DMF solution for 15 minutes. The liquid was removed and the mixing procedure repeated twice. The remaining solid was washed intensively with DMF. A solution of HCTU (5 eq.) and Fmoc-Mba-OH (5 eq.) in DMF was added to the loaded resin, followed by the addition of DIPEA (10 eq.). The mixture was left to stir for one hour, the liquids were then filtered out, and the reaction was repeated with a fresh reagents. The resin was then washed with DMF, DCM, and methanol, and left to dry under vacuum. The Fmoc protecting group was then removed as described above, and the same coupling procedure for Fmoc-Mba-OH was done using Fmoc-*D*-Alanine-OH. All consequent coupling reactions were performed using the automated prelude solid phase synthesizer using standard Fmoc protection chemistry. For the conventional CP, Fmoc-*L*-Lys(Boc)-OH was loaded first on the resin and all subsequent AA couplings were performed on the prelude solid phase synthesizer (PTI) using standard Fmoc protection chemistry. The Fmoc protecting group of the final linear sequence was removed as described earlier.



Procedure for resin cleavage of H_2N-*L*-Lys-*D*-Ala-*L*-Leu-*D*-Ala-*L*-Lys-*D*-Ala-γ-Mba-*D*-Ala-OH: The H_2N-*L*-Lys-*D*-Ala-*L*-Leu-*D*-Ala-*L*-Lys-*D*-Ala-γ-Mba-*D*-Ala-OH loaded resin (200 μmol) was gently stirred in a solution (20 mL) of 1% *v/v* TFA, 5% *v/v* TIS in DCM for 20 minutes. The solid residue was then removed by vacuum filtration and extensively washed with DCM. The filtrate volume was reduced under vacuum to ~5mL, and the product was precipitated using cold ether (50 ml). The mixture was centrifuged, and ether was then decanted. This step was repeated twice, resulting in an off-white precipitate. The solid residue was dried under vacuum to yield 0.119 g (60%). MALDI-TOF for $C_{48}H_{80}N_{10}O_{13}$ Calculated: 1005.51 $[M+H]^+$; Found 1005.56 $[M+H]^+$, 1027.64 $[M+Na]^+$, 1043.52 $[M+K]^+$.



Procedure for peptide cyclization, *cyclo*-(*L*-Lys-*D*-Ala-*L*-Leu-*D*-Ala-*L*-Lys-*D*-Ala-γ-Mba-*D*-Ala): H_2N-*L*-Lys-*D*-Ala-*L*-Leu-*D*-Ala-*L*-Lys-*D*-Ala-γ-Mba-*D*-Ala-OH (0.119 g, 118 μmol) was dissolved in DMF (90 mL). The solution was cooled to 0 °C in an ice bath. T3P (50% *w/w* in DMF), (375 mg, 589 μmol) was dissolved in DMF (30 mL), cooled to 0 °C, was slowly added to the solution mixture while stirring at 0 °C. DIPEA (218 μL, 1180 mmol) was added slowly to the mixture. The reaction mixture was left to stir for 6 hours at 0 °C and then at room temperature for two days. The same amounts of T3P and DIPEA were added, and left stirring for another two days at RT. DMF was removed by vacuum distillation at 55 °C, resulting in a gel-like residue. The crude product *cyc*- K(Boc)ALAK(Boc)ABA was used without further purification.

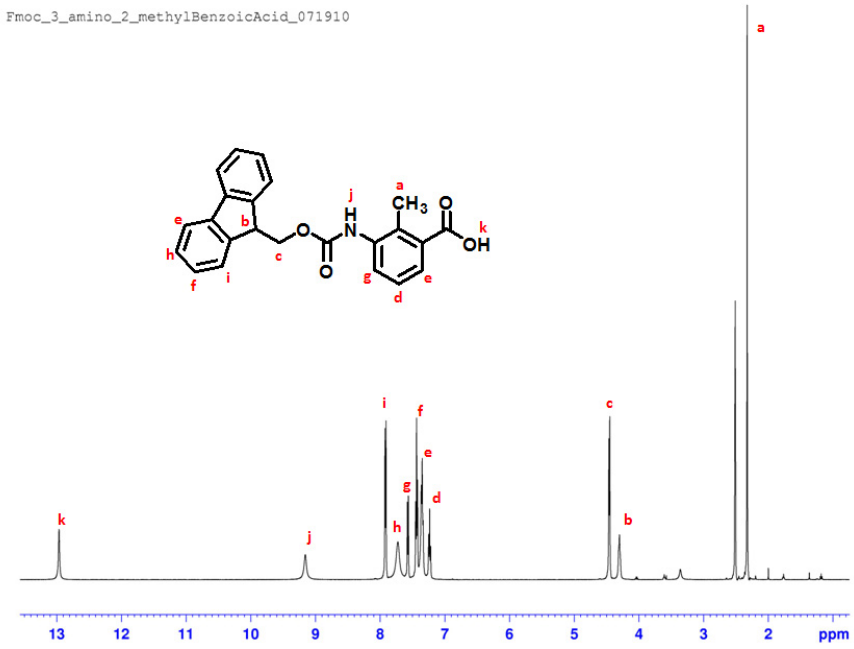


Procedure for Boc removal from *cyclo*-(*L*-Lys-*D*-Ala-*L*-Leu-*D*-Ala-*L*-Lys-*D*-Ala-γ-Mba-*D*-Ala): The cyclized product (*cyc*-K(Boc)ALAK(Boc)ABA) (118 μmol) was dissolved in 10 mL of 95% *v/v* TFA, 2.5% *v/v* TIS and 2.5% *v/v* H_2O. The mixture was left to stir for two hours at room temperature. The deprotected product was then precipitated using cold ether (100 mL). The mixture was centrifuged, and ether was then decanted. This step was repeated twice, resulting in an off-white precipitate. The product was then dissolved in H_2O (5 mL), and lyophilized giving an off-white fluffy solid. The yield of the cyclized and deprotected product was found to be 85% using analytical HPLC. The crude product was purified using preparative HPLC (0% to 50% ACN in H_2O in 30 minutes). The product fraction was lyophilized yielding a white solid (27.8 mg, 30%).

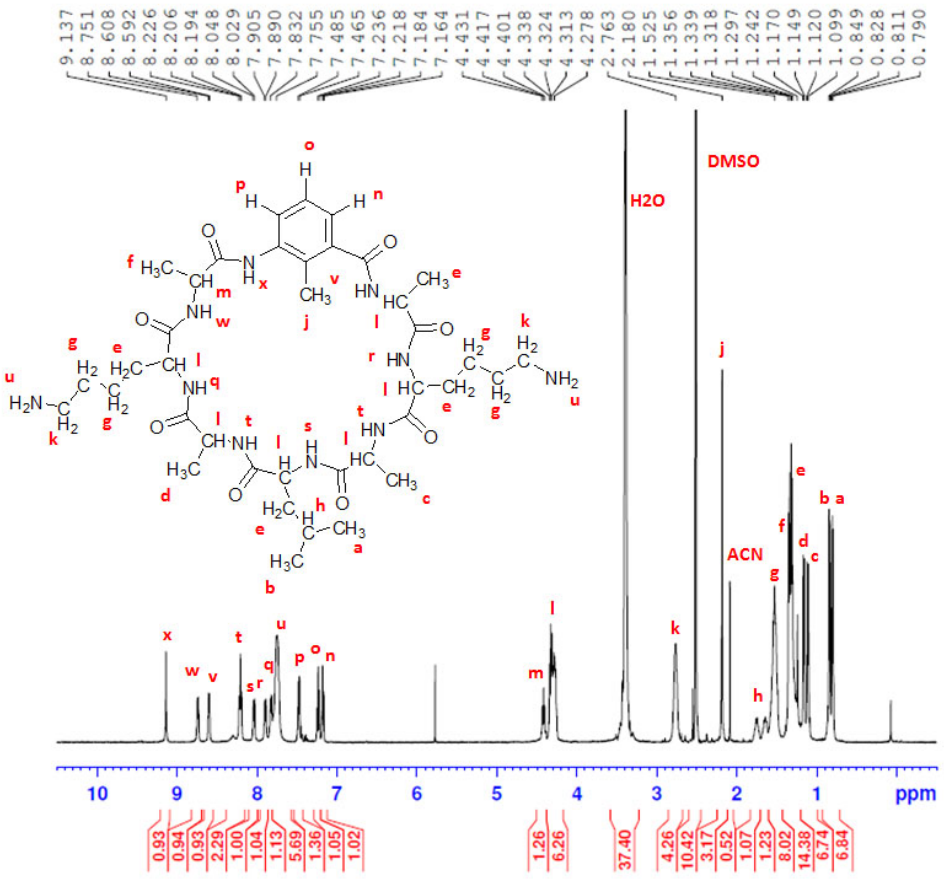
^{1}H NMR (500 MHz, DMSO-d_6) for Mba-CP: δ (ppm) 0.80 (d, J = 6.5 Hz, 3H), 0.84 (d, J = 6.6 Hz, 3H), 1.11 (d, J = 7.0 Hz, 3H), 1.16 (d, J = 7.0 Hz, 3H), 1.24 (s, 3H), 1.31 (dt, J = 16.8 Hz, 8.4, 12H), 1.34-1.53 (m, 8H), 1.65 (m, 1H), 1.76 (d, J = 7.0 Hz, 1H), 2.19 (s, 3H), 2.77 (s, broad, 4H), 4.28-4.33 (m, 6H), 4.42 (m, 1H), 7.17 (d, J = 6.9 Hz, 1H), 7.24 (t, J = 7.8 Hz, 1H), 7.48 (d, J = 7.9 Hz, 1H), 7.75 (s, broad, 6H), 7.82 (d, J = 7.4 Hz, 1H), 7.89 (d, J = 8.7 Hz, 1H), 8.02 (d, J = 7.4 Hz, 1H), 8.19 (t, J = 7.0 Hz, 2H), 8.58 (d, J = 5.7 Hz, 1H), 8.72 (d, J = 7.2 Hz, 1H), 9.12 (s, 1H). MALDI-TOF for $C_{38}H_{62}N_{10}O_8$ Calculated: 787.48 $[M+H]^+$; Found 787.65 $[M+H]^+$, 809.69 $[M+Na]^+$, 825.69 $[M+K]^+$.

^{1}H NMR (500 MHz, DMSO-d_6) for 8CP: δ (ppm) 0.82 (d, J = 6.5 Hz, 6H), 0.85 (d, J = 6.6 Hz, 6H), 1.10-1.70 (m, 30H), 2.74 (m, 4H), 4.20 (m, 2H), 4.35-4.50 (m, 6H), 7.65 (s, 6H), 8.10-8.20 (m, 6H), 8.30 (d, J = 7.7 Hz, 2H). MALDI-TOF for $C_{36}H_{66}N_{10}O_8$ Calculated: 767.51 $[M+H]^+$; Found 767.59 $[M+H]^+$, 789.63 $[M+Na]^+$, 805.65 $[M+K]^+$.

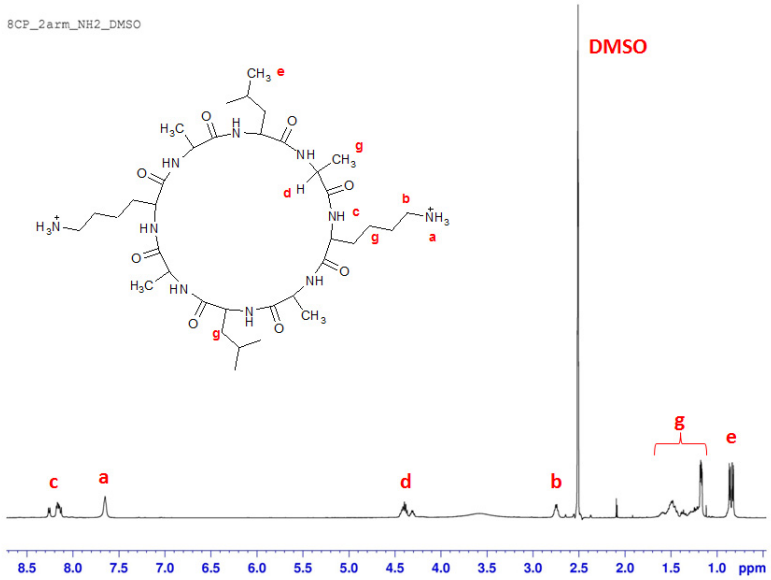
^{1}H NMR spectrum of Fmoc-Mba-OH in DMSO-d_6.



MR spectrum of Mba-8CP in DMSO-d_6.



^{1}H NMR spectrum of regular 8CP in DMSO-*d*$_6$.



Other Characterization of Nanotubes

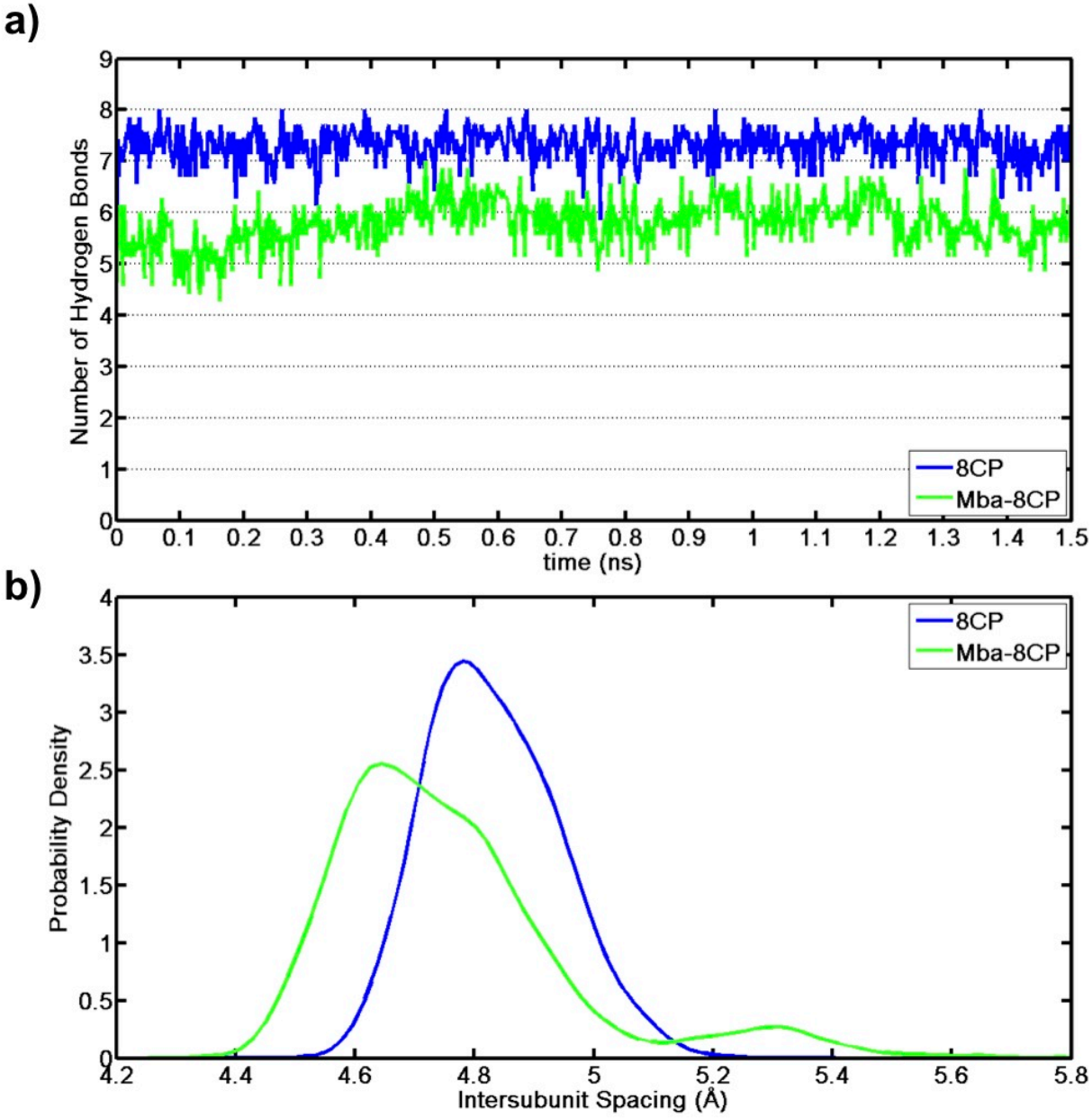


Figure S1. Time history of the average number of interring backbone hydrogen bonds. The number of H-bonds remains stable during the simulation for both cases, with a lower average value for Mba-8CP than the 8CP nanotubes. Intersubunit distance distribution (b) is comparable in both cases and agree with reported experimental values (4.7-4.8 Å) for CPNs.

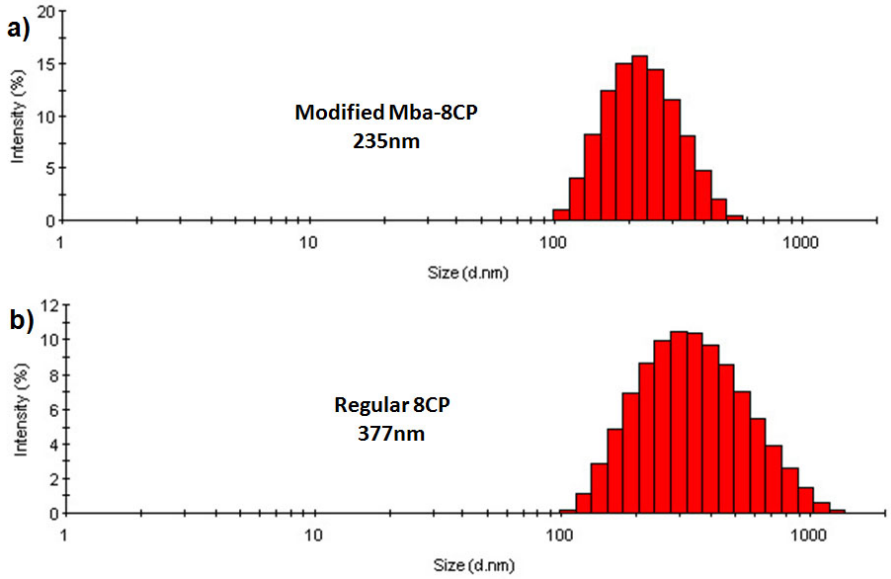


Figure S2. Dynamic Light Scattering (DLS) of bundles of cyclic peptide nanotubes derived from (a) Modified (Mba-8CP) and (b) conventional 8CP in ACN (0.25mg/mL).

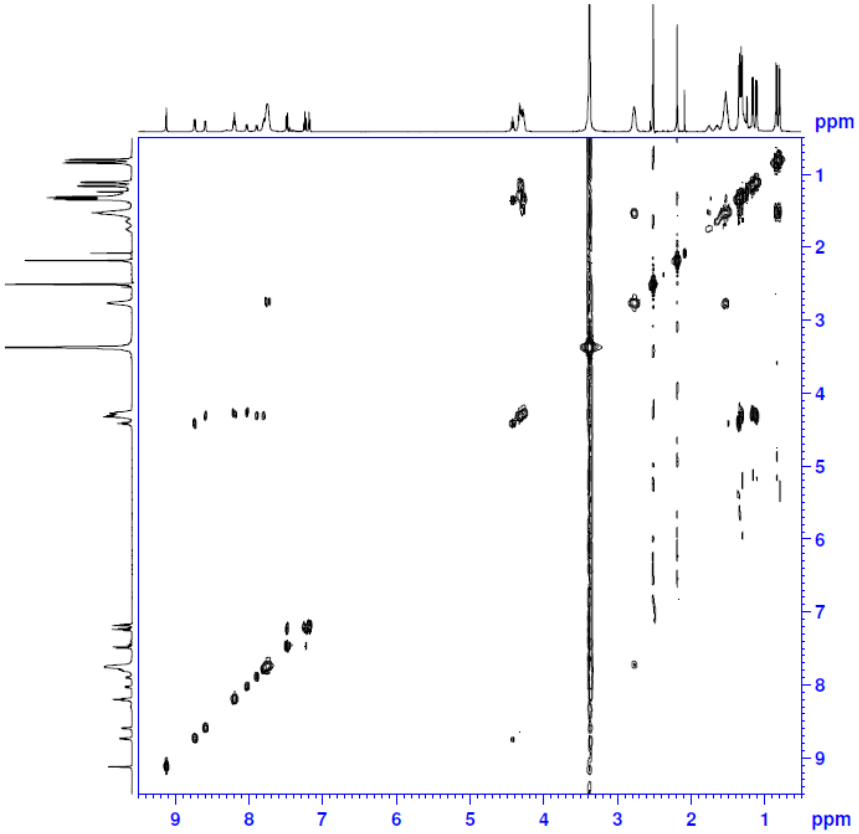


Figure S3. 2D COSY spectrum of molecularly dissolved Mba-8CP in DMSO-d_6 (~4mg/mL). Data were acquired over 1 h at room temperature.

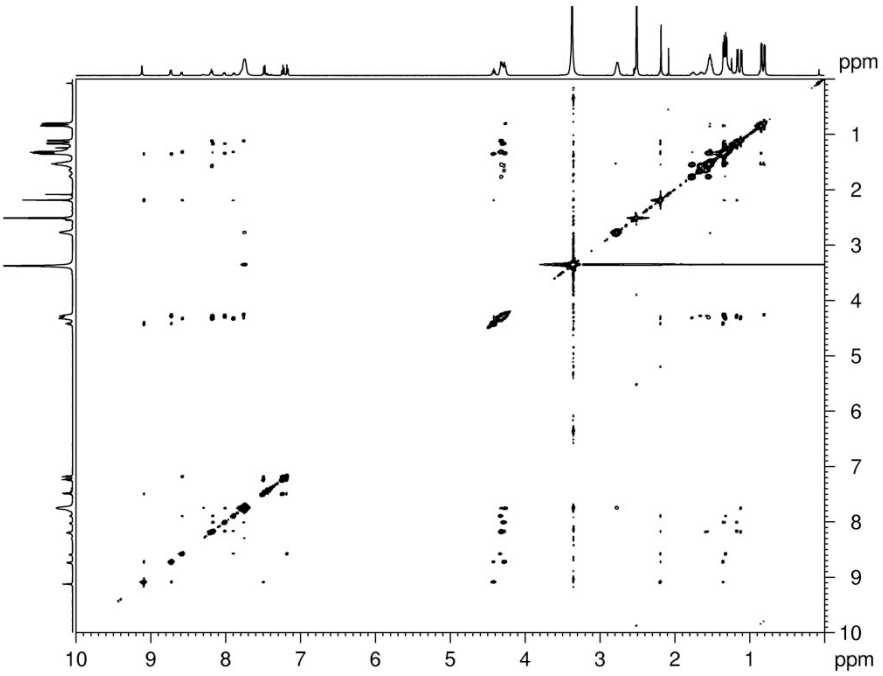


Figure S4. 2D NOESY spectrum of molecularly dissolved Mba-8CP in DMSO-d_6 (~4mg/mL). Data were acquired over 12 h at room temperature.

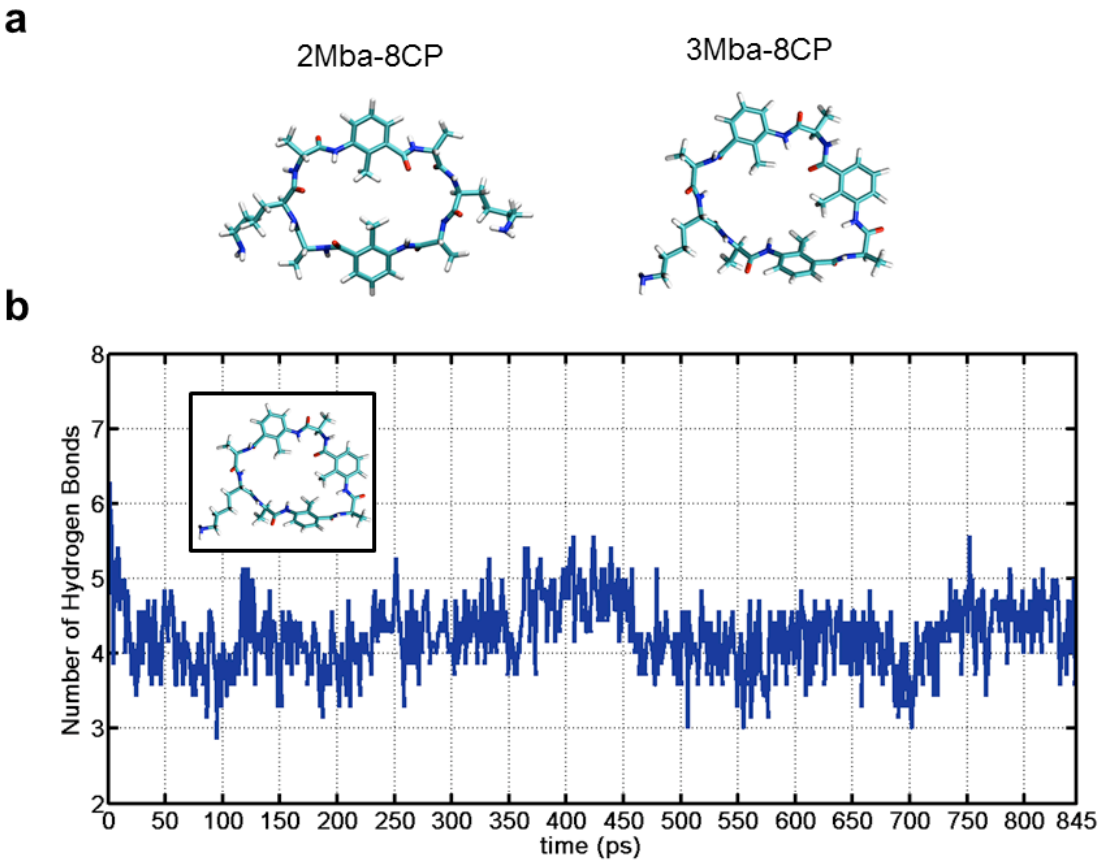


Figure S5. Stability of CPNs containing multiple modifications. a) Snapshots of a double and triple modified CP. A distorted shape is observed in both 2Mba-8CP and 3Mba-8CP cases. b) Number of hydrogen bonds in the triple modified CP. The preliminary studies show that the number of hydrogen bonds in the 3Mba-8CP is well below the optimum number of hydrogen bonds, 8, and much lower than in the cases of the Mba-8CP or the regular 8CP. Simulations suggest that due to the distorted structure of the rings, the H-bonds are generally less likely to be perpendicular to the plane of the ring, leading to less-stable assemblies.

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