Supplemental Information.

Influence of Linker Design on the Stability, Folding, and Assembly of Tethered Collagen-Mimetic Peptides

Debasis Ghosh, Anthony R. Perez, S. M. Mobin Sikder, Jesus R. Vasquez, Claire Zhang, Anjan Maity, Tao Ye, and Andrea D. Merg*

Materials and Methods

All chemical reagents were purchased from Sigma Aldrich Chemical Co (St. Louis, MO) or Thermo Fisher Scientific Inc. (Waltham, MA) unless otherwise stated. Fmoc amino acids and Oxyma Pure were acquired from CEM (Matthews, NC). Peptide resins were purchased from Anaspec (Fremont, CA).

Peptide Synthesis, Purification, and Characterization

All peptides were synthesized using standard microwave-assisted solid-phase peptide synthesis (SPPS) protocols using a CEM Liberty Blue automated microwave peptide synthesizer. Peptides were synthesized on Fmoc-Gly-HMP tentaGel resin. Fmoc-deprotection was achieved by treatment with 4-methylpiperidine (20% v/v) in dimethylformamide (DMF), and Fmoc amino acids were activated using N, N'-Diisopropylcarbodiimide (DIC) and Oxyma Pure. All amino acids were double-coupled. For synthesis of tethered CMPs, Fmoc-Lys(ivDde) side chains were deprotected using 2% hydrazine solution in DMF. After deprotection of both Lys(ivDde) residues, standard SPPS methods were reemployed. After synthesis, the peptidyl resins were filtered and rinsed with acetone and air-dried. The crude peptides were cleaved from the resin for 4 hours at room temperature with a 92.5% trifluoroacetic acid (TFA), 2.5% H_2O, 2.5% 3,6-dioxane 1,8-octane-dithiol (DODT), 2.5% triisopropylsilane (TIPS) cleavage solution, precipitated with cold diethyl ether, and centrifuged at 4000 rpm for 10 min at 4 °C. After centrifugation, the supernatants were discarded, and the pellets were dried under vacuum overnight. Crude peptides were purified by an Agilent 1260 Infinity II high-performance liquid chromatography (HPLC) instrument that was equipped with a preparative scale Phenomenex Kinetex XB-C18 column (250 x 30 mm, 5 μm, 100 Å). Peptides were eluted with a linear gradient of acetonitrile-water with 0.1% TFA. The target fractions were collected, rotovaped, and lyophilized. Purified peptides were analyzed by mass spectrometry using a Bruker Microflex LRF mass spectrometer (positive reflector mode). Samples were mixed with α-cyano-4-hydroxycinnamic acid (CHCA) as the ionization matrix (1:1). We obtained yields of 5.5%, 6.9%, 7.0%, and 7.2% for **CTH**, **CTH-PEG2**, **CTH-PEG4**, and **CTH-PEG6**, respectively. A yield of 13.7% and 2.0% were obtained for **CTH-HEX** and **CTH-GSG**, respectively.

Assembly Protocol

Assembly experiments were heated and cooled using a Bio-Rad T-100 thermal cycler. Peptide solutions (200 μM for tethered CMPs; 600 μM for **CMP322**) were prepared using 20 mM MOPS

buffer (pH 7.0), and were heated to 90°C for 5 min. After heating, the solutions were slow-cooled to 4°C (0.2°C/min.). Upon reaching 4°C, the solutions were stored at 5°C for at least one week prior to characterization.

Circular Dichroism (CD) Spectroscopy

CD experiments were collected on a JASCO J-1500 spectrophotometer. Samples were equilibrated for 10 minutes at 5°C in a 0.02 cm pathlength cuvette, and three spectra were recorded and averaged at a scanning rate of 100 nm/min with a bandwidth of 2 nm. Measurements were baseline-corrected with blank 20 mM MOPS buffer (pH 7.0). Thermal denaturation and refolding experiments were conducted in triplicate (except for **CTH-GSG**). Samples were monitored at 224 nm using a 0.1 cm pathlength cuvette. Prior to heating, samples were equilibrated at 5°C for 10 minutes. The temperature was increased from 5°C to 65°C at a rate of 40°C/hour with 30 s equilibration time. After 10 minutes of incubation at 65°C, the samples were cooled to 5°C at the same rate. Melting temperatures (T_m) and refolding temperatures (T_r) were determined by plotting the first derivatives of the unfolding and refolding curves and utilizing 3rd order Savitzky-Golay smoothing feature in OriginPro.

Transmission Electron Microscopy

TEM images were collected with a Talos F200C G2 transmission electron microscope at an accelerating voltage of 200 kV. TEM specimens were prepared by briefly mixing 2.5 µL of peptide nanosheet solutions with 2.5 µL of aqueous uranyl acetate stain solutions (1%) directly on 200-mesh carbon-coated copper grids. After 30 s, excess liquid was removed from each sample using filter paper and allowed to air-dry.

Atomic Force Microscopy

AFM characterization of nanoribbons was performed on an Asylum Research Cypher AFM instrument (Oxford Instruments) that was operated in tapping mode in air. For sample preparation, a 20 µL aliquot of **CTH** assembly solution was deposited onto freshly cleaved mica, incubated for 5 minutes, gently wicked off with a Kimwipe, and dried under a stream of nitrogen gas. After drying, the sample was washed with 30 µL Ultrapure distilled water and nitrogen-dried. Imaging was performed directly on these substrates using AppNano ACTA-20 probes (silicon, Al reflex coating; vendor specifications: spring constant 13-77 N/m and resonance frequency 200-400 kHz) with thermal tune calibration yielding a spring constant of 22.95 N/m and a resonance frequency of 328.1 kHz. Scan sizes typically ranged from 2 to 20 µm for visualization of assemblies across multiple length scales, with image resolutions of 512-1024 pixels, a scan rate of 2 Hz, and a setpoint of 85-90% of free amplitude. Thickness measurements were obtained from height cross-sections extracted from Cypher/Igor Pro. For each labeled assembly, a line profile was drawn perpendicular to the long axis of the structure, extending from the mica substrate onto the nanoribbon plateau, and the resulting step height was taken as the assembly thickness. Nanostructures exhibiting different extended lengths and morphologies were included in the analysis to obtain a representative average thickness.

Supplemental Figures.

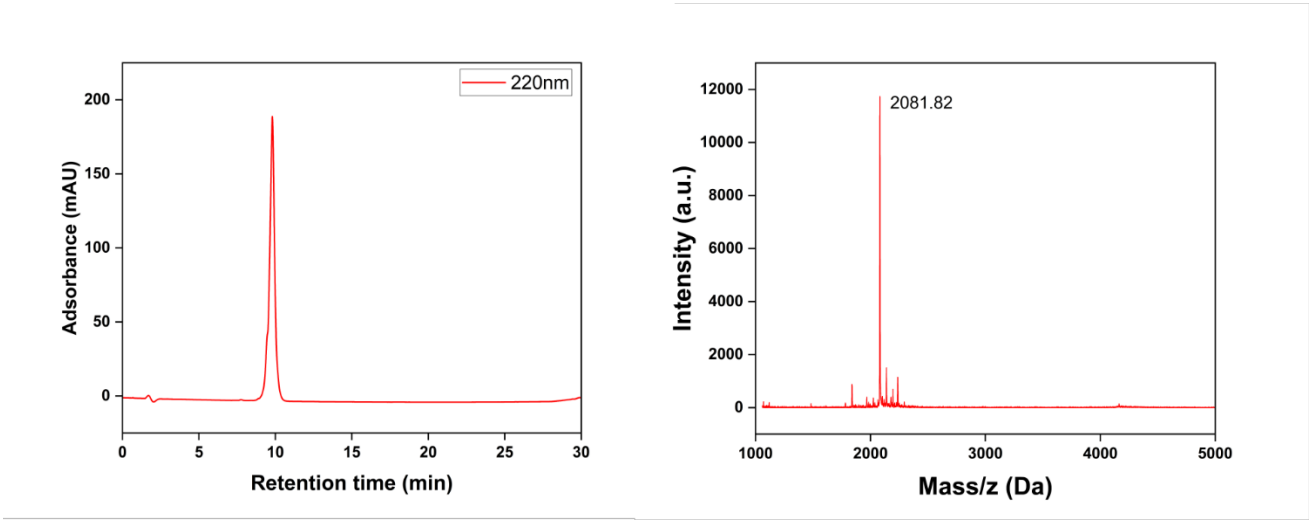


Figure S1. HPLC trace and MALDI-TOF MS spectrum of **CMP322**.

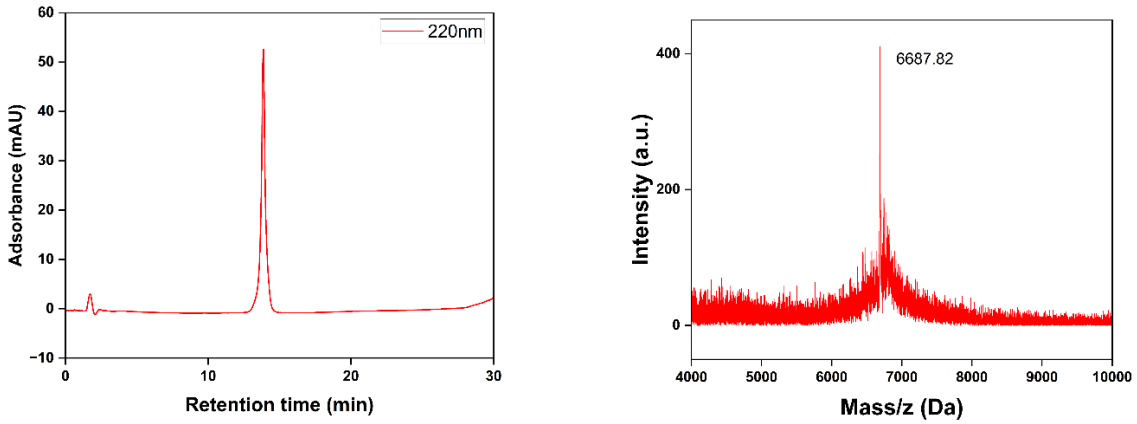


Figure S2. HPLC trace and MALDI-TOF MS spectrum of **CTH**.

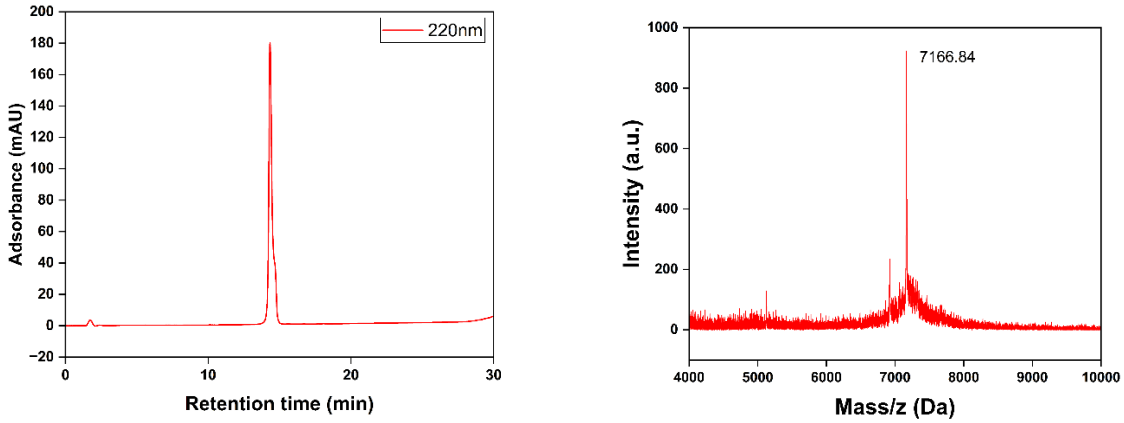


Figure S3. HPLC trace and MALDI-TOF MS spectrum of **CTH-PEG2**.

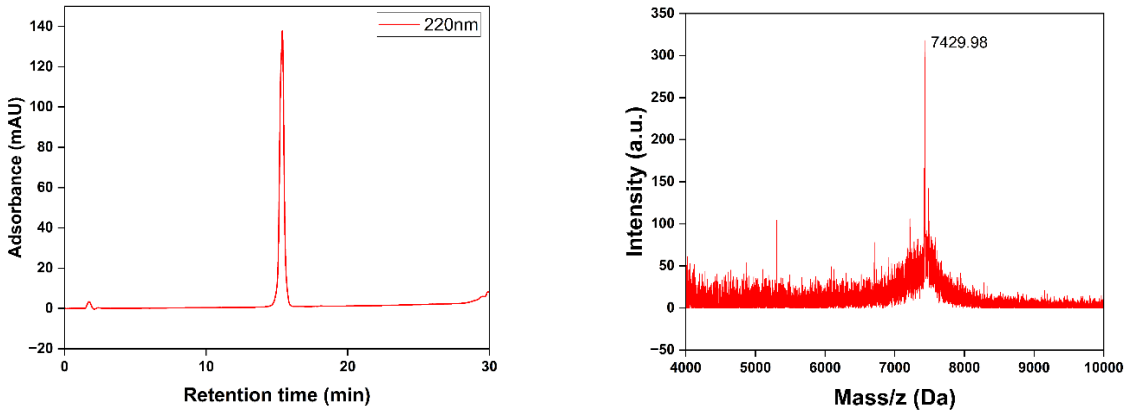


Figure S4. HPLC trace and MALDI-TOF MS spectrum of **CTH-PEG4**.

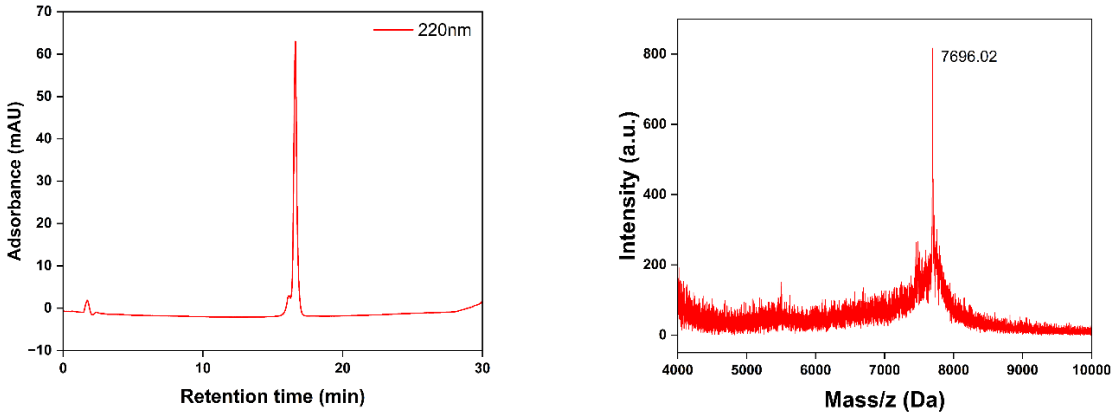


Figure S5. HPLC trace and MALDI-TOF MS spectrum of **CTH-PEG6**.

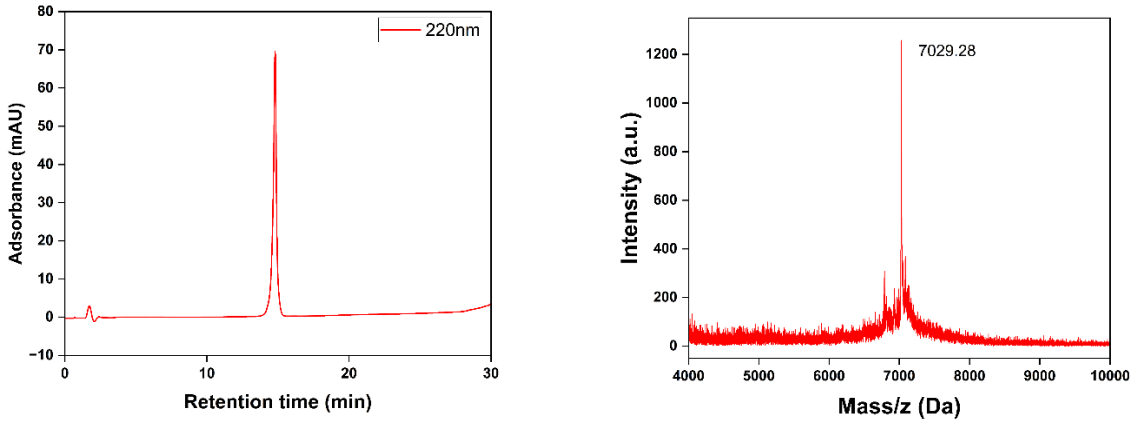


Figure S6. HPLC trace and MALDI-TOF MS spectrum of **CTH-HEX**.

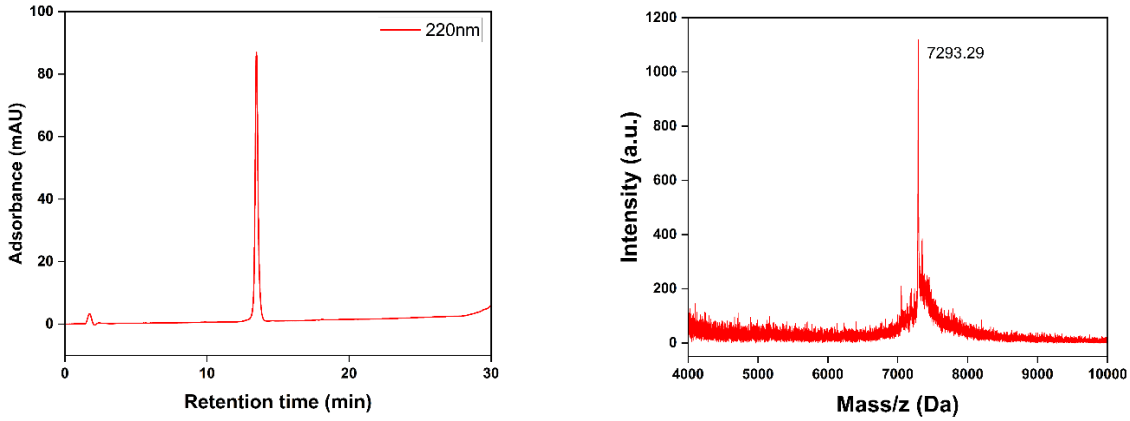


Figure S7. HPLC trace and MALDI-TOF MS spectrum of **CTH-GSG**.

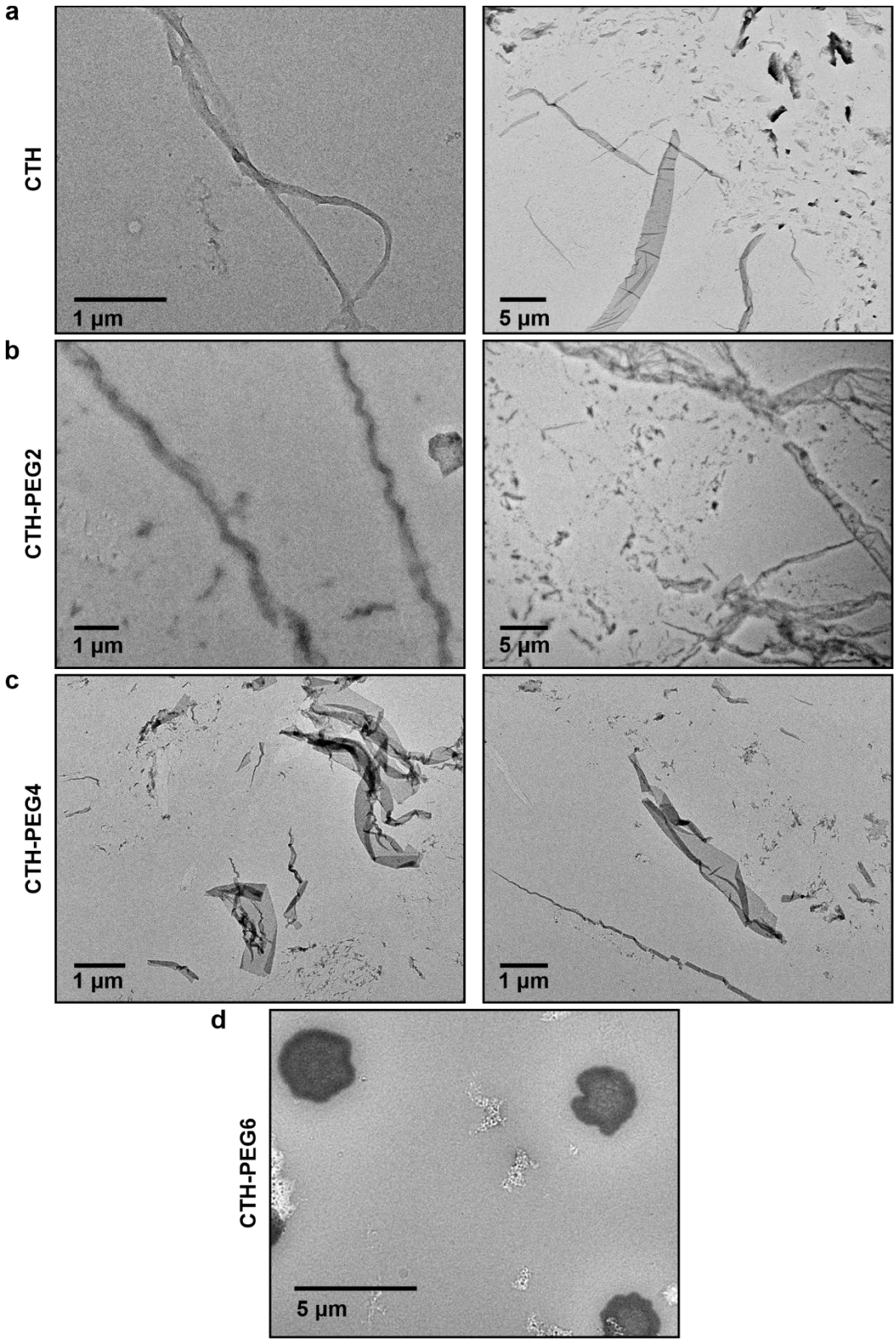
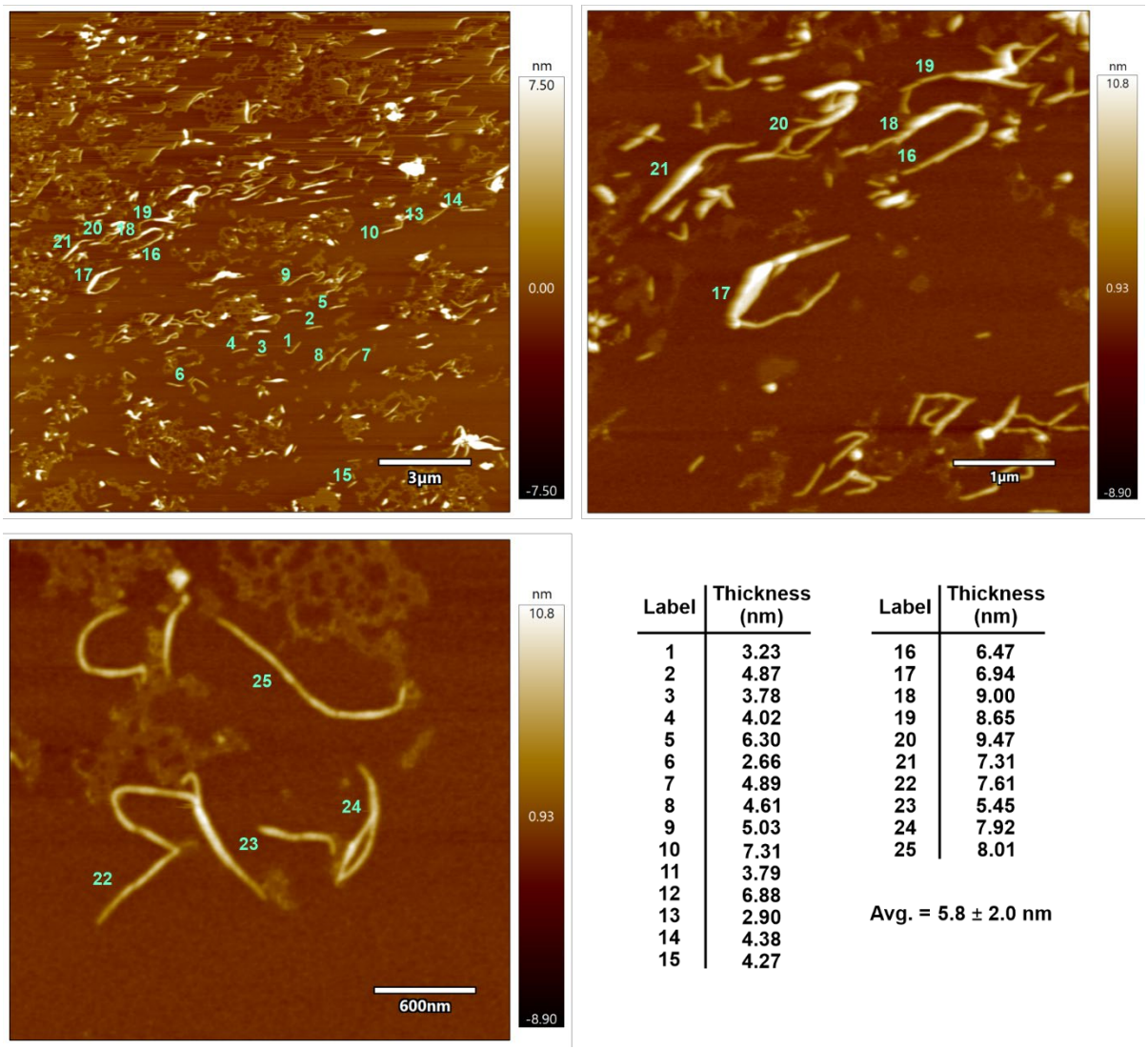


Figure S8. TEM images of (a) **CTH**, (b) **CTH-PEG2**, (c) **CTH-PEG4**, and (d) **CTH-PEG6** after one week of assembly. All peptides (200 µM) were assembled in 20 mM MOPS buffer (pH 7.0).



Label	Thickness (nm)
1	3.23
2	4.87
3	3.78
4	4.02
5	6.30
6	2.66
7	4.89
8	4.61
9	5.03
10	7.31
11	3.79
12	6.88
13	2.90
14	4.38
15	4.27

Label	Thickness (nm)
16	6.47
17	6.94
18	9.00
19	8.65
20	9.47
21	7.31
22	7.61
23	5.45
24	7.92
25	8.01

Avg. = 5.8 ± 2.0 nm

Figure S9. AFM images of **CTH** nanoribbons. Thickness values (nm) for the labeled assemblies are shown in the table.

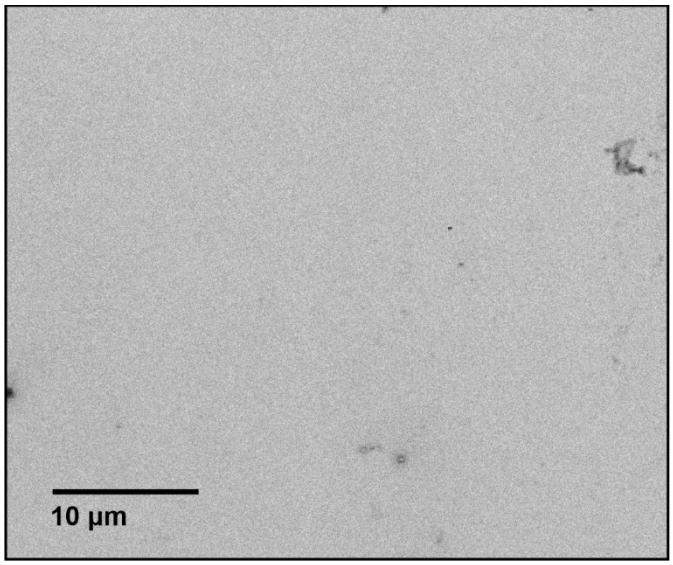


Figure S10. TEM image of **CMP322** (600 µM) in 20 mM MOPS buffer (pH 7.0) after one week of assembly time. No assemblies were observed.

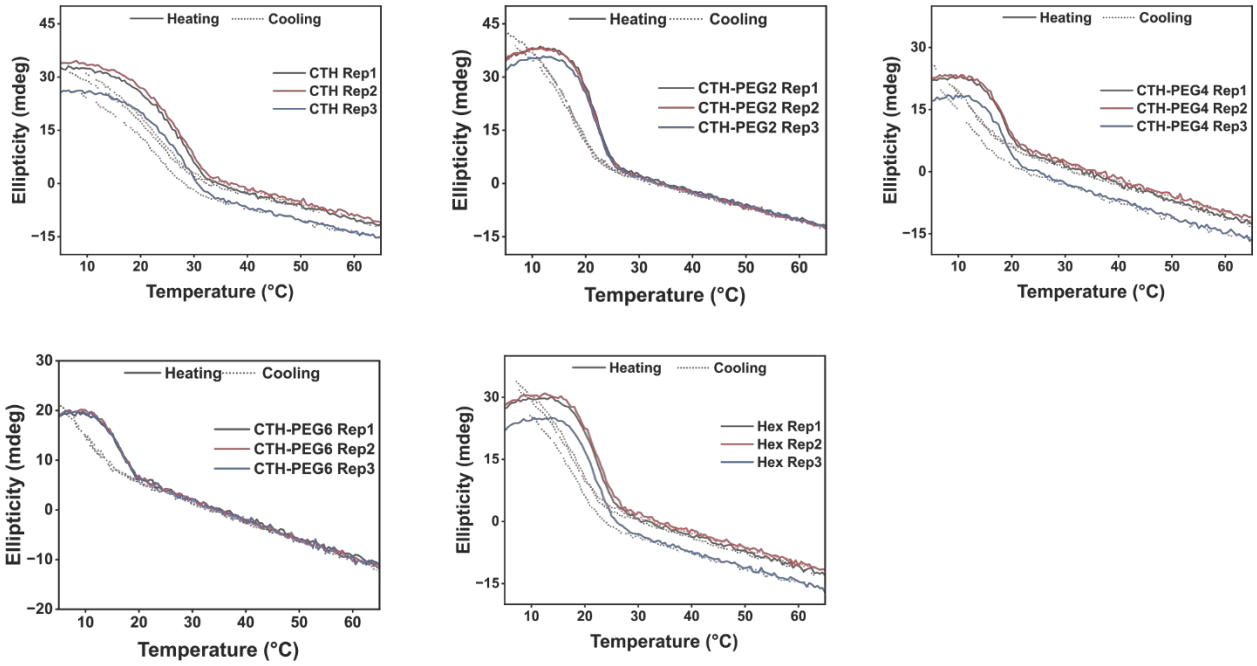


Figure S11. CD thermal denaturation and refolding curves for **CTH**, **CTH-PEG2**, **CTH-PEG4**, **CTH-PEG6**, and **CTH-HEX** (200 μM) in 20 mM MOPS buffer (pH 7.0) after one week. CD thermal denaturation and refolding experiments were carried out using three separate samples (with the exception of **CTH-GSG**).

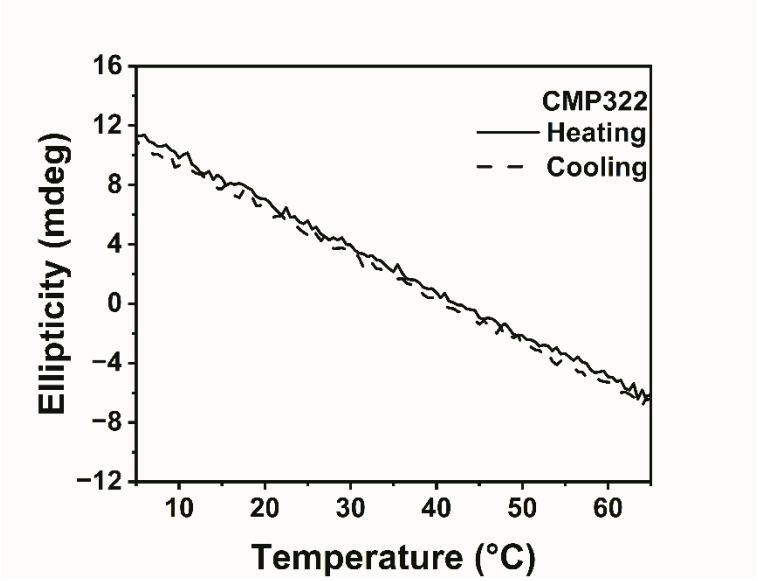


Figure S12. CD thermal denaturation and refolding of **CMP322** (200 μM) in 20 mM MOPS buffer (pH 7.0) after one week.

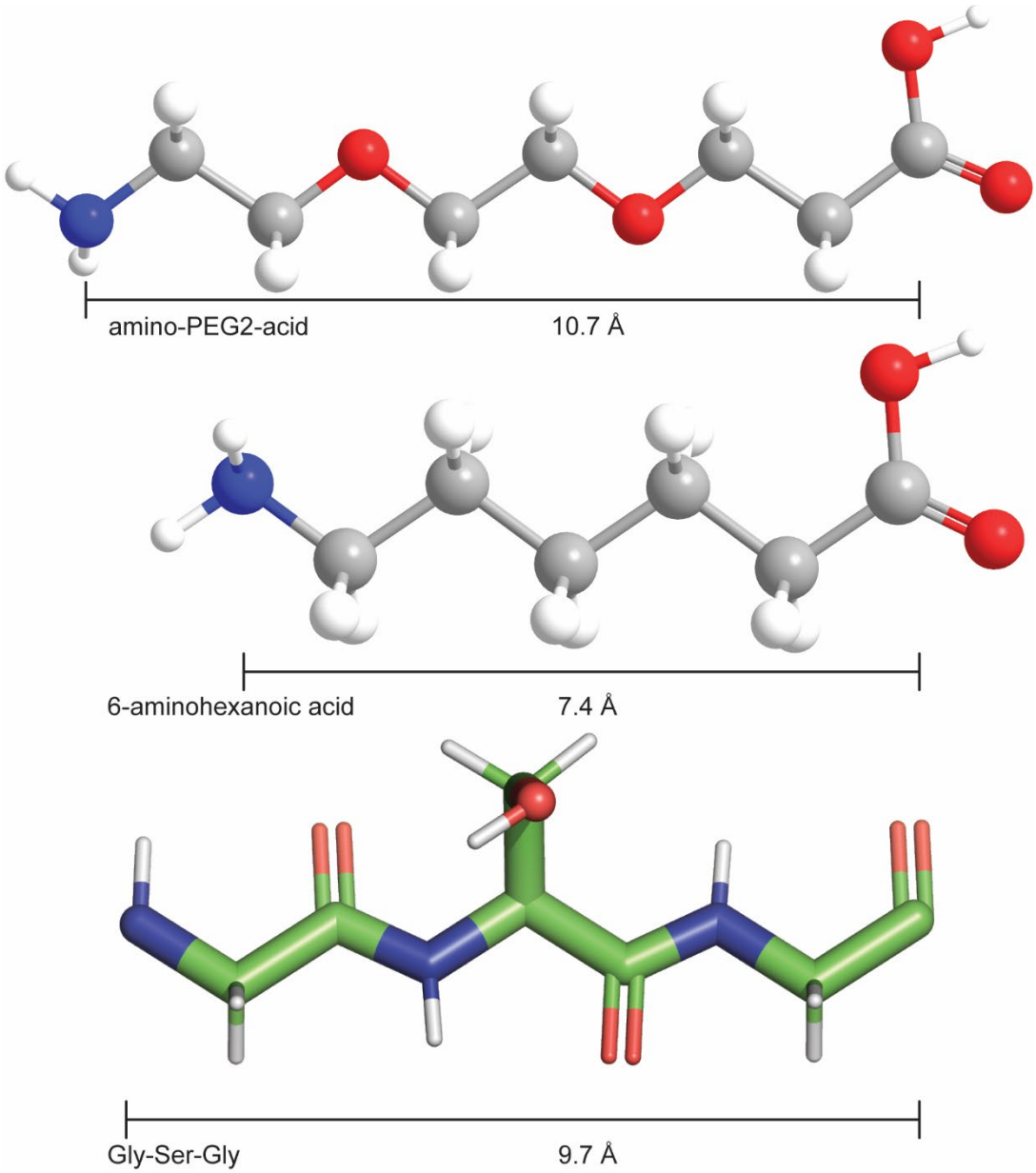


Figure S13. Contour lengths of PEG_2, 6-aminohexanoic acid (Ahx), and GSG. PEG_2 and Ahx were built in Chem3D and GSG was built in Pymol using the fab command.

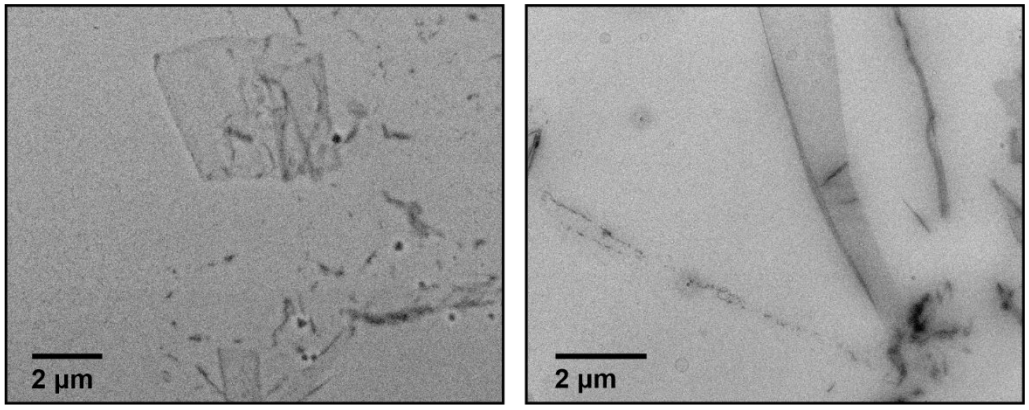


Figure S14. TEM images of **CTH-HEX** (200 µM) in 20 mM MOPS (pH 7.0) after one week of assembly.

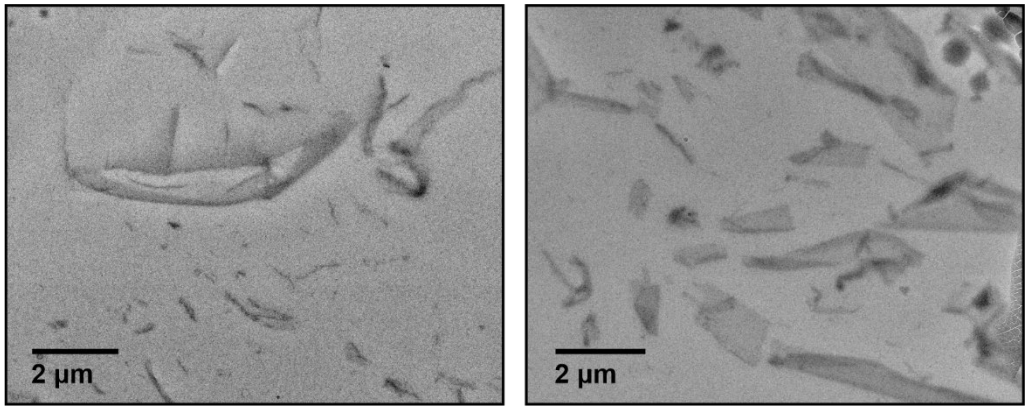


Figure S15. TEM images of **CTH-GSG** (200 µM) in 20 mM MOPS (pH 7.0) after one week of assembly.

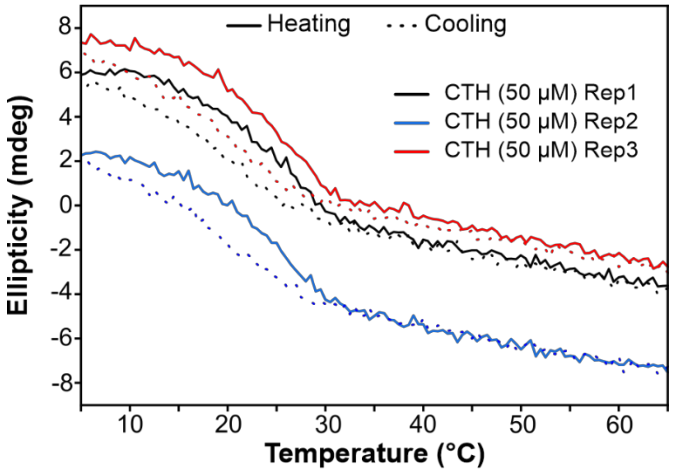


Figure S16. Triplicate CD thermal denaturation and refolding curves for **CTH** (50 μM) in 20 mM MOPS buffer (pH 7.0) after one week.