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SUPPLEMENTARY INFORMATION

Self-Assembly of a Robust, Reduction-Sensitive Camptothecin Nanotube

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Received ooth February 2015,
Accepted ooth February 2015

DOI: 10.1039/xoxxooooox

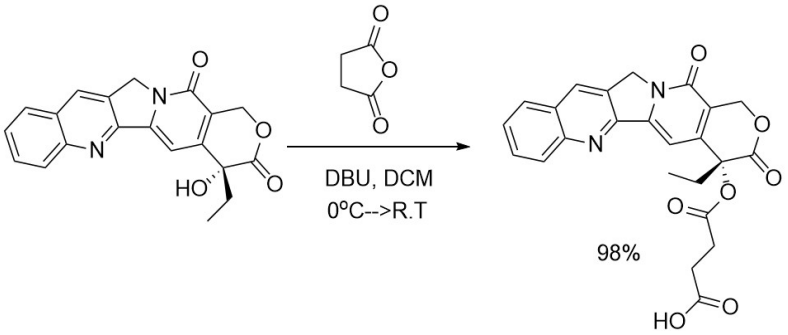
www.rsc.org/

General Methods

Transmission Electron Microscopy (TEM) was performed with Technai G2 Spirit instrument operating at 80 kV. All reactions were performed under nitrogen atmosphere. ^{1}H NMR was recorded at 400 MHz and ^{13}C NMR spectra at 100 MHz on a Bruker DPX-400 instrument. Water (pH 7.0), 2,2,2-trifluoroethanol (TFE) used for UV and TEM were of spectroscopic grade, and phosphate buffered saline (PBS, pH 7.4) was purchased from Gibco.

Synthesis of CPT-Succinic Acid (CPT-CO_2H)

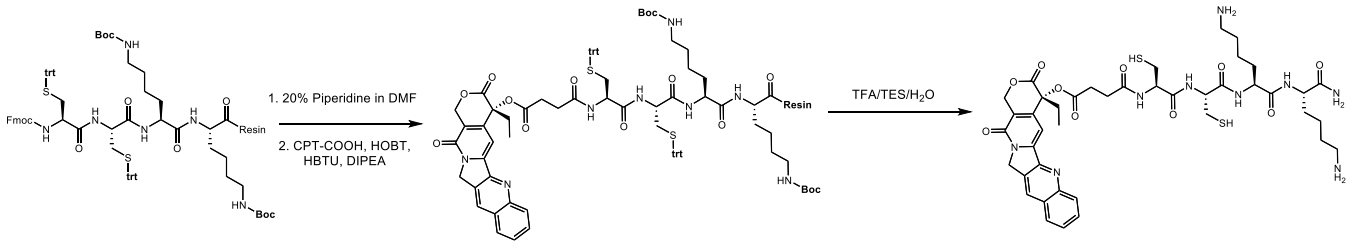
The synthesis of camptothecin-succinic acid was performed as reported previously.[1] Basically, 1,8-Diazabicycloundec-7-ene (DBU, 0.46 mL, 3 mmol) was slowly added to a mixture of (S)-(+)-camptothecin (348.35 mg, 1 mmol) and succinic anhydride (300.3 mg, 3 mmol) in 30 mL of dichloromethane at 0 °C. The reaction mixture was stirred at room temperature for 4 h, quenched with water (20mL), acidified with 1% aqueous HCl solution. The yellow precipitate was collected and washed with 1% aqueous HCl solution (10 mL× 3) and H_2O (10 mL× 3). The crude product was recrystallized with methanol to obtain the pale yellow crystalline product (439 mg, 98%). ^{1}H NMR (400 MHz; DMSO-d_6) δ 8.68 (1 H, s), 8.11-8.20 (2 H, m), 7.85-7.89 (1 H, m), 7.69-7.73 (1 H, m), 5.44-5.53 (2 H, m), 5.23-5.33 (2 H, m), 2.68-2.85 (2 H, m), 2.09-2.20 (2 H, m), 0.87-0.94 (3 H, m); ^{13}C NMR (100 MHz; DMSO-d_6) δ 172.93, 171.23, 167.13, 145.89, 145.24, 131.51, 131.36, 129.73, 128.98, 128.49, 127.93, 118.89, 95.09, 75.86, 66.28, 50.16, 30.39, 28.55, 28.37, 7.50.; ESI-MS for $C_{24}H_{20}N_2O_7$ $[M+Na]^+$ calculated 471.1163; found 471.1187.



Scheme S1. Synthesis of CPT-CO_2H ((R)-4-((4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-4-oxobutanoic).

Synthesis of Compound A (CPT-CCKK)

Compound **A** (CPT-CCKK) was prepared *via* on-resin modification of the *N*-terminus of peptide backbone (Scheme S2). Lysine protected with Fmoc and Boc protection groups and Cysteine protected with Trt group were manually linked on rink amide resin (loading 0.80 mmol/g) using solid-phase peptide synthesis. Amide-coupling steps were accomplished with standard techniques: Fmoc-Lysine(Boc)-OH or Fmoc-Cysteine(Trt)-OH, 1,3-diisopropylcarbodiimide (DIC), and 1-hydroxybenzotriazole (HOBt) (300 mol% each relative to resin) in 1:1 DMF/DCM for 1.5 h. A solution of 20% piperidine in DMF was used for Fmoc deprotection. A mixture of CPT-CO_2H, HBTU, and DIPEA (200 mol% each relative to resin) in DMF was added to the resin. The reaction mixture was shaken for 24 h at room temperature and then filtered through a fritted syringe. The resin was washed thoroughly (3 x DMF, 3 x CH_2Cl_2) and the final compound **A** was cleaved from the resin by the treatment with TFA/water/triethylsilane (95 / 1 / 4) at room temperature for 2 h. The crude product was precipitated with cold diethyl ether and purified by reversed-phased HPLC on preparative Varian Dynamax C18 column eluting with a linear gradient of CH_3CN/water containing 0.1 % TFA (10/90 to 100/0 over 30 minutes) and stored as lyophilized powers at 0 °C. Compound purity was assessed by analytical reverse-phase HPLC, and identity was confirmed using ESI-TOF mass spectrometry and NMR. ^{1}H NMR (400 MHz, DMSO-d^6) δ 0.86 (t, 3H, *J*= 7 Hz), 0.91-0.95 (t, 3H, *J*= 7.2 Hz), 1.28-1.32 (m, 4H), 1.47-1.53 (m, 6H), 1.63-1.67 (m, 2H), 2.15-2.17 (m, 2H), 2.36-2.42 (m, 2H), 2.71-2.81 (m, 10H), 4.13-4.24 (m, 2H), 4.37-4.39 (m, 2H), 4.48-4.50 (m, 2H), 5.32 (s, 2H), 5.50 (s, 2H), 7.04 (s, 1H), 7.13 (s, 1H), 7.34 (s, 1H), 7.69 (s, 6H), 7.72-7.76 (m, 1H), 7.86-7.90 (m, 2H), 8.03 (d, 1H, *J*= 7.6 Hz), 8.14 (t, 2H, *J*= 7.2 Hz), 8.22 (d, 1H, *J*= 8.4 Hz), 8.29 (d, 1H, *J*= 7.6 Hz), 8.72 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d^6) δ 8.06, 22.61, 22.71, 26.51, 27.05, 27.12, 29.38, 30.04, 30.86, 31.55, 31.94, 39.18, 39.38, 39.59, 39.80, 40.00, 40.21, 40.42, 40.63, 52.53, 52.99, 55.62, 66.78, 76.35, 95.55, 115.50, 118.44, 119.36, 128.21, 128.48, 129.04, 129.49, 130.30, 130.90, 132.09, 145.78, 146.44, 148.39, 152.88, 157.03, 158.40, 158.74, 167.71, 169.97, 170.46, 171.33, 171.55, 171.84, 173.80; ESI-MS: calculated for $C_{42}H_{55}N_9O_{10}S_2$; $[M+H]^+$ 910.3586, found 910.3521;



Scheme S2. On-resin synthesis of compound **A** (CPT-CCKK)

UV-Vis Studies

Experiments were performed in a quartz cell with a 1 mm path length over the range of 190-800 nm using a Shimadzu UV-2450 spectrometer. Samples were prepared from both PBS and TFE (20 mM for both) after 3 days incubation at room temperature and freshly diluted into 0.25 mM before the measurement.

Circular Dichroism (CD) Spectroscopy

CD spectra were recorded on a Jasco CD spectrometer under a nitrogen atmosphere. Experiments were performed in a quartz cell with a 1 mm path length over the range of 190-800 nm. Samples were prepared from both PBS and TFE (20 mM for both) after 3 day incubation at room temperature, and subsequently diluted to 0.25 mM before the measurement.

Infrared (IR) Spectroscopy

IR spectra were measured on an Shimadzu IRAffinity-1. Experiments were performed on samples assembled of **A** in Deuterated PBS using CaF salt plates. Sample aged at 20 mM in D-PBS for 72 h followed by 2 rounds of lyophilization and resuspension with D_2O. IR spectra was deconvoluted using Origin.

Transmission Electron Microscopy Measurement – Negative Stain TEM

The compound **A** in PBS (20 mM, pH 7.4) was prepared and aged for 3 days before the measurement. For the TEM microscopic studies, the samples were freshly diluted to 1 mM. Solutions (10 μL) in PBS and water were applied to carbon coated copper grid (Ted Pella, Inc.) for 2 min. After removing excess solution with filter paper, the grid was floated on 10 μL drops of 2 % wt uranyl acetate solution for negative staining for 1 min.

Crosslinking Cysteines within Self-assembled Nanotube

Compound **A** was aged at 20 mM in PBS for 3 days to allow for self-assembly into nanotubes. After 3 days, analytical Reverse Phase HPLC analysis was used to show no significant release of CPT during the aging time. The solution then underwent ultracentrifugation at 80,000 rpm at 4 °C for one hour to separate the nanotubes. The collected

nanotube pellet was then resuspended in the same amount of PBS, followed by the addition of 10 % DMSO with gentle stirring for 3 days. Ellman's test confirmed that no free thiol groups remain after 3 days of oxidation.

Ellman's Test

The oxidation of free thiol groups was monitored by Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB. A stock solution of DTNB was prepared at 2 mM in ultrapure H_2O and kept refrigerated. The working solution of DTNB was prepared by diluting DTNB stock solution to 0.1 mM with PBS. At each time point, 10 μL of oxidized compound **A** solution (10 mM) was taken and diluted with DTNB working solution to a concentration of 0.25 mM **A** (addition of 400 μL DTNB working solution). The mixture was allowed to stay at room temperature for 5 min before analysis by UV-Vis. The disappearance of UV absorption at 415 nm indicated the completion of oxidation.

Release of Free Camptothecin from Self-assembled Nanotubes

The release profile of Camptothecin from self-assembled nanotubes was measured by analytical reversed-phase HPLC (1 mL/min) under ambient temperature eluting with a linear gradient of CH_3CN/water containing 0.1% trifluoroacetic acid (TFA) in both H_2O and CH_3CN. The timeline was set to 0-60 min, 20-80% CH_3CN/Water. For crosslinked nanotube sample, small amount of DTT was added to cleave any existing disulfide bonds. The amount of released free drug was detected by UV-Vis detector at 360 nm and the percentage was determined by comparing the ratio of the peak area measured at each time point.

Cell Culture and Reagent

Cytotoxicity assays was performed using human non-small cell lung cancer cell lines A549 and NCI-H460 , obtained from American Type Culture Collection (ATCC, Manassas, GA). Human non-small cell lung cancer cell lines (A549 and NCI-H460) were grown in RPMI-1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and incubated in a humidified 37°C, 5% CO_2 environment. Compound **A** was prepared at 10 mM in sterile PBS and allowed to age 3 days prior to experiments. Immediately prior to experiments, *S*-camptothecin (95%, Sigma-Aldrich) was dissolved in dimethyl sulfoxide (2.5mM).

Cytotoxicity Assay

Human non-small cell lung cancer cell lines (A549 and NCI-H460) cells were seeded in 48 well plates (1.0×10^4 cells/well) 24 h prior to the addition of test compounds. Concentrations were 0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μM and cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2 for 96 h prior to MTT viability analysis. Absorbance was measured with SpectraMax M2 Microplate Reader (Molecular Devices) and the effects of compound activity were evaluated by determining the 50% inhibition values (IC_{50}) as an average of three replicas. Each experiment was repeated at least 3 times.

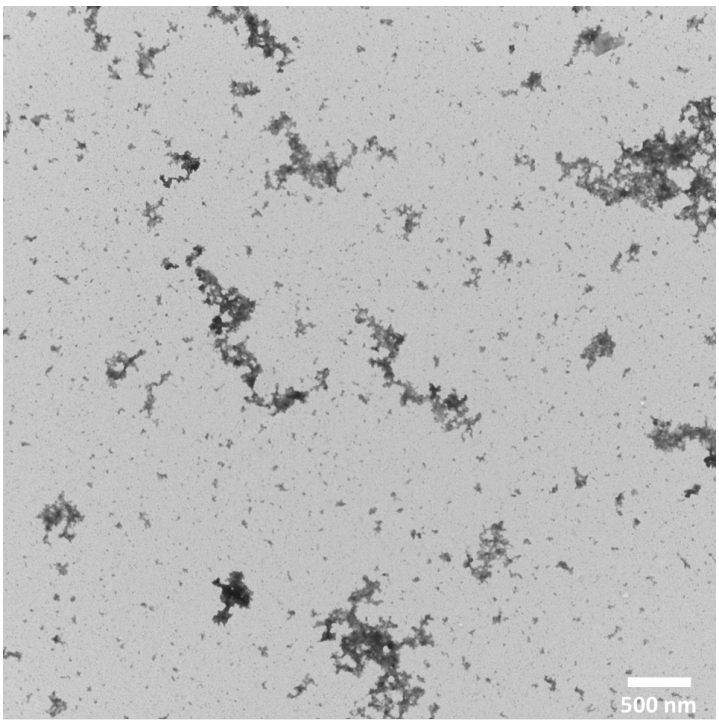


Figure S1. Transmission Electron Microscopy of compound **A** in PBS (pH = 7.4) at 0.2 mM showing no organized nanostructures.

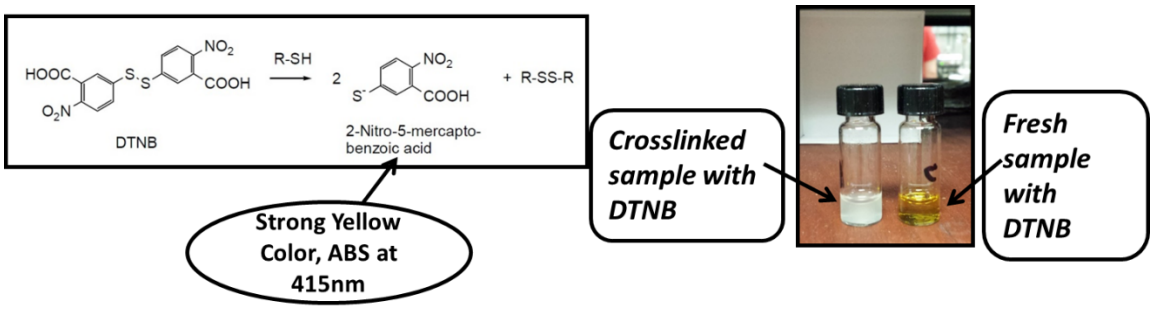


Figure S2. Ellman's test for free thiol groups.

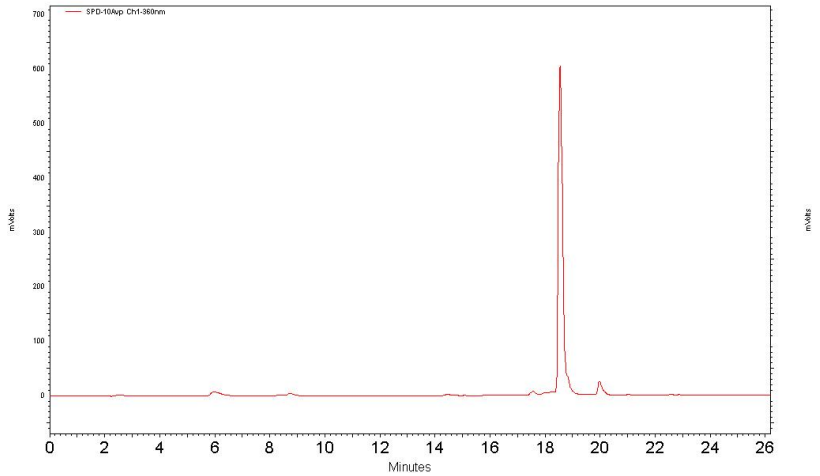


Figure S3. HPLC trace of purified **A** eluted with a linear gradient of CH_3CN/water containing 0.1% trifluoroacetic acid (TFA) in both H_2O and CH_3CN. The timeline was set to 0-60 min, 20-80% CH_3CN/Water.

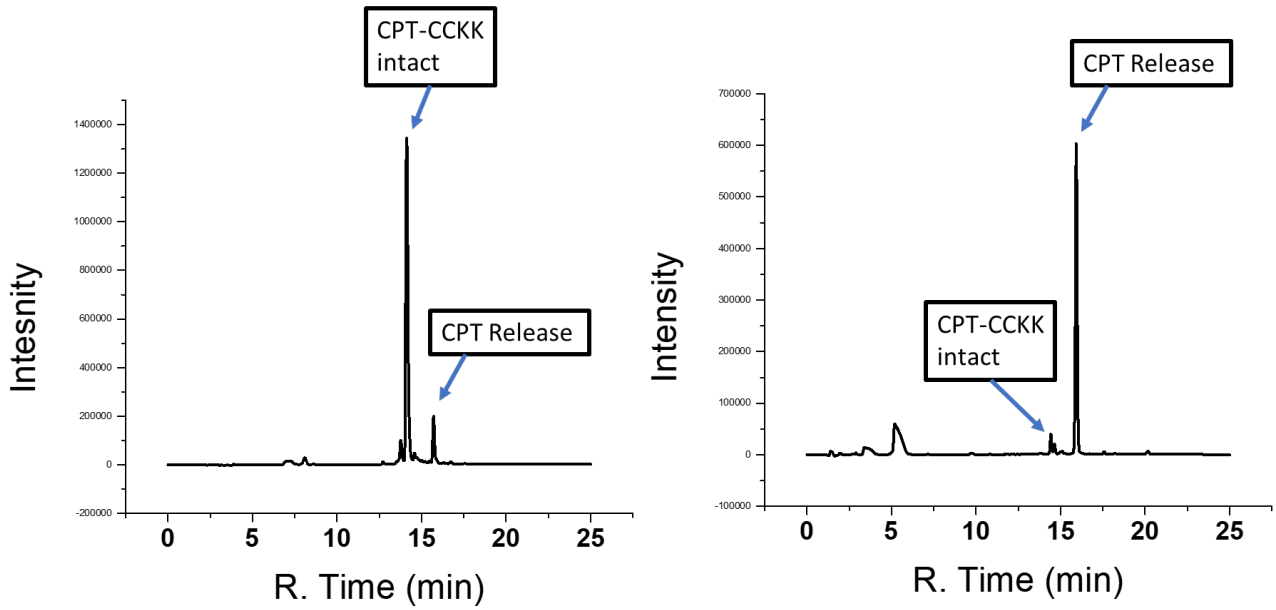


Figure S4. HPLC trace of CPT release from oxidatively crosslinked nanotubes of **A** (100 mM in PBS at 37.5°C) in presence of dithiothreitol (DTT, 10 mM) after 0 h (left) and 72 h (right). Samples were prepared for analysis by treating with DTT (123 mM) immediately prior to injection to dissociate the nanotubes. DTT treatment reduces the disulfide bonds and dissociates the nanotubes to allow hplc quantitation of CPT release. No hydrolytic release of CPT occurs during the short, elapsed time period (~30 sec) between sample treatment and hplc analysis. The peaks were eluted using the following progressively increasing gradient of CH_3CN in H_2O (with 0.1% trifluoroacetic acid (TFA)): 0-10 min:10-35% CH_3CN, 10-17 min: 35-45% CH_3CN, 17-25 min: 45-100% CH_3CN.

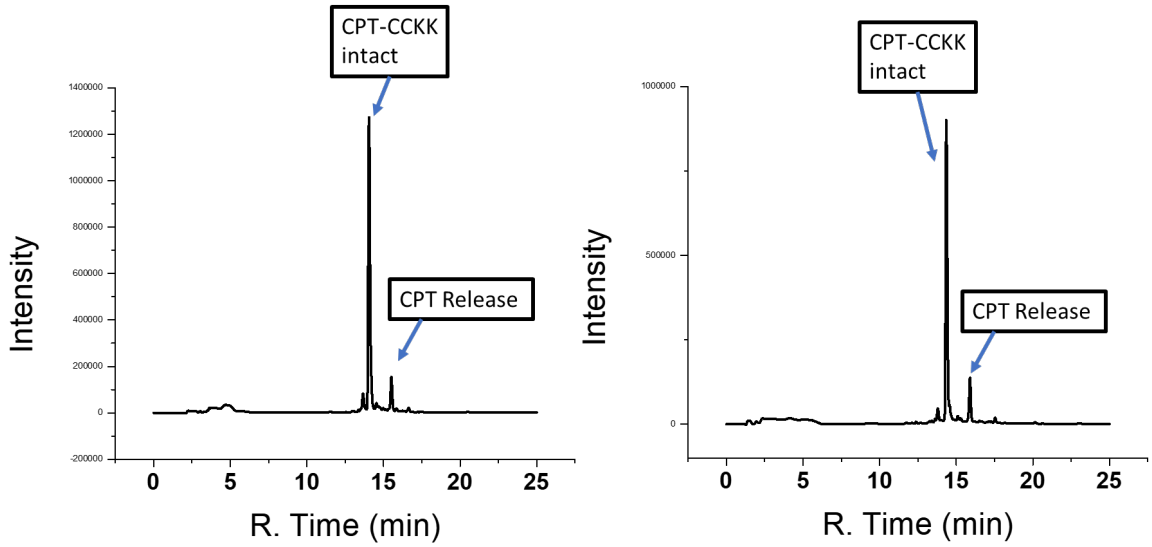


Figure S5. HPLC trace of CPT release from oxidatively crosslinked nanotubes of **A** (100 mM in PBS at 37.5°C) in the absence of DTT after 0 h (left) and 72 h (right). Samples were prepared for analysis by treating with DTT (123 mM) immediately prior to injection to dissociate the nanotubes. DTT treatment reduces the disulfide bonds and dissociates the nanotubes to allow hplc quantitation of CPT release. No hydrolytic release of CPT occurs during the short, elapsed time period (~30 sec) between sample treatment and hplc analysis. The peaks were eluted using the following progressively increasing gradient of CH_3CN in H_2O (with 0.1% trifluoroacetic acid (TFA)): 0-10 min:10-35% CH_3CN, 10-17 min: 35-45% CH_3CN, 17-25 min: 45-100% CH_3CN.

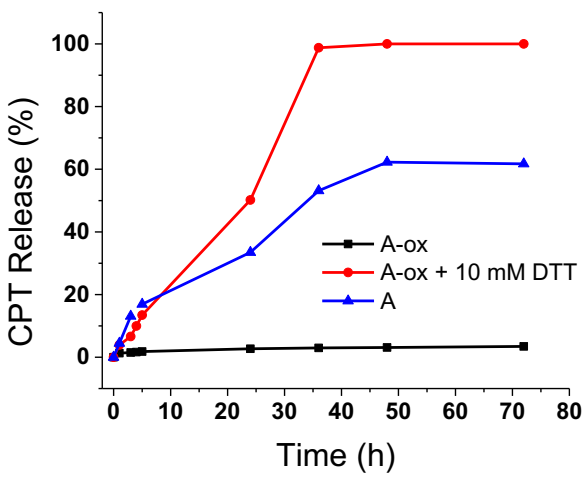


Figure S6. Release of CPT in PBS, as monitored by HPLC, from crosslinked nanotubes of **A** (100 μM in PBS at 37.5°C,) in presence (red) and absence (black) of dithiothreitol (DTT). For comparison, CPT release rate of assembled nanotubes (PBS, 7.4 pH, 20 mM, 3 days) after diluting to 100 μM, prior to oxidation with DMSO.

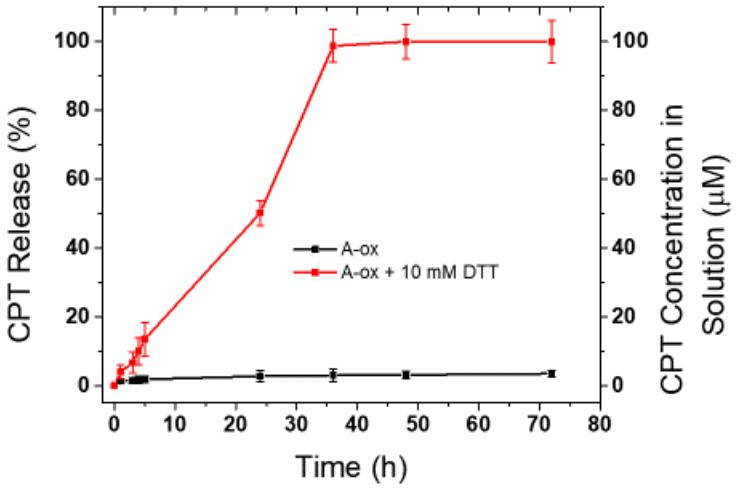
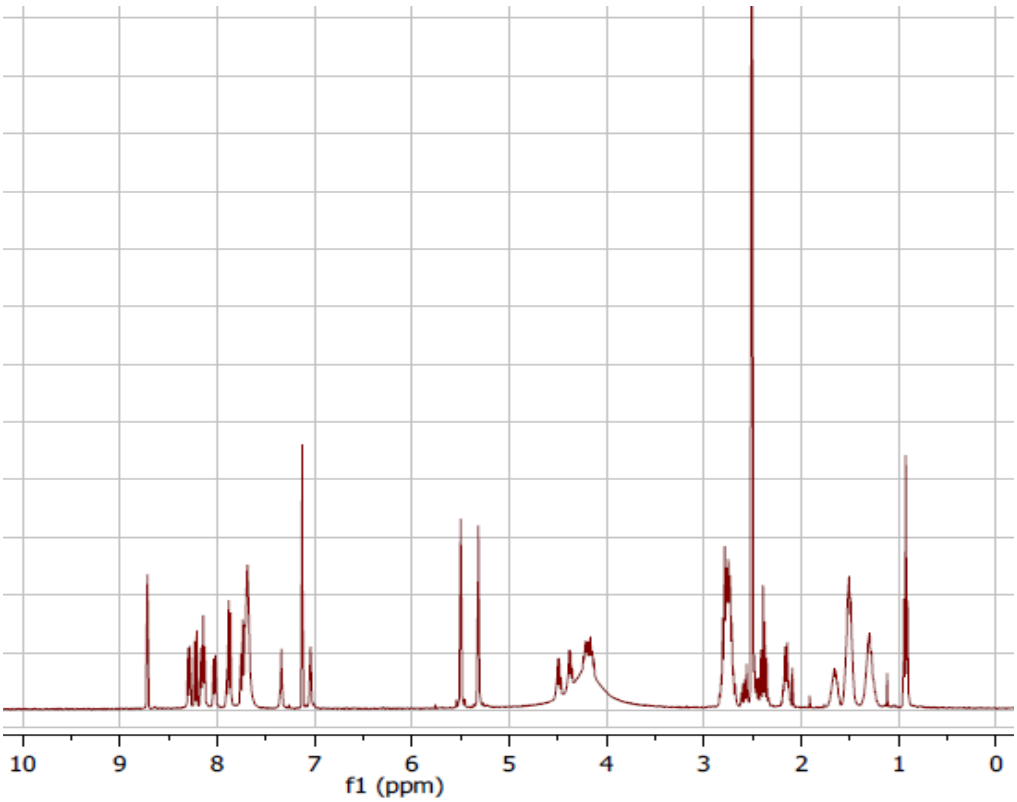
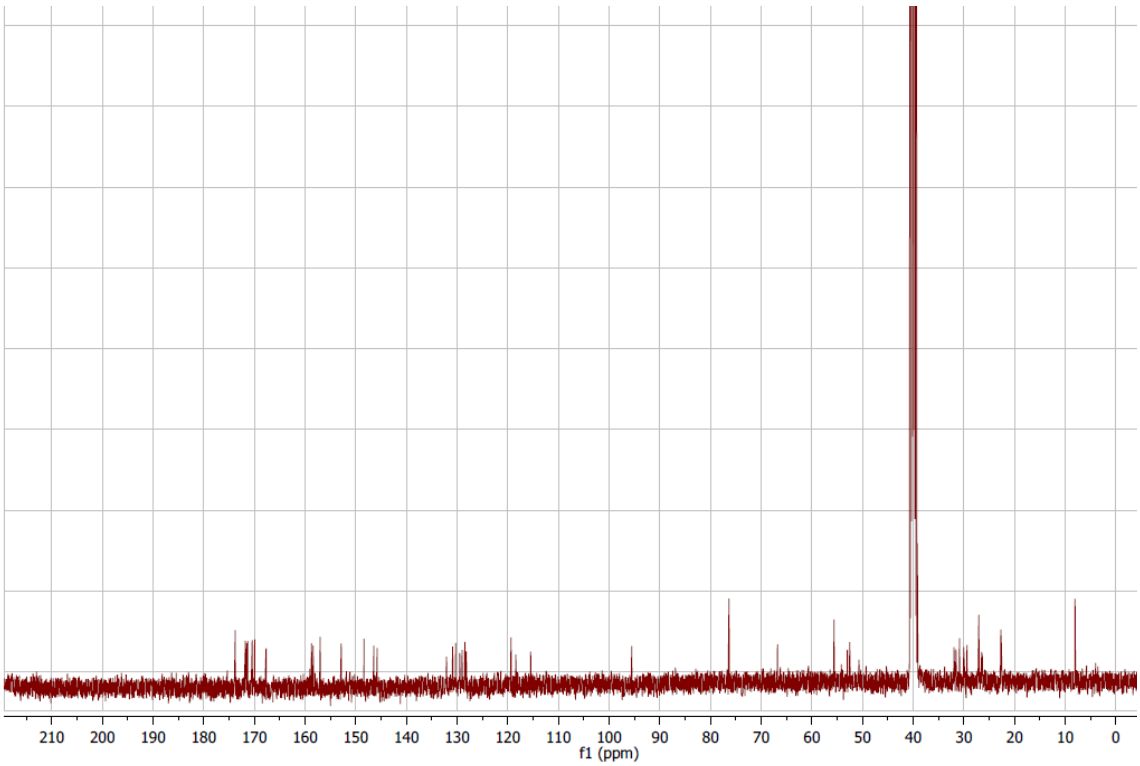


Figure. S7. Concentration of CPT released from crosslinked nanotubes of **A** over 80 h in PBS (100 μM, 37.5 °Ç) in the presence and absence of DTT (10 mM). The concentration of released CPT was estimated using the relative response factors of **CPT** and **A**, measured at 100 μM. Thus, we estimate that under the reducing conditions at an initial concentration of 100 μM, the concentration of CPT released after ~35 h would be ~100 uM.

^{1}H NMR of CPT-CCKK, **A**



^{13}C NMR of Compound **A**



Mass Spectrum of CPT-CCKK, **A**.

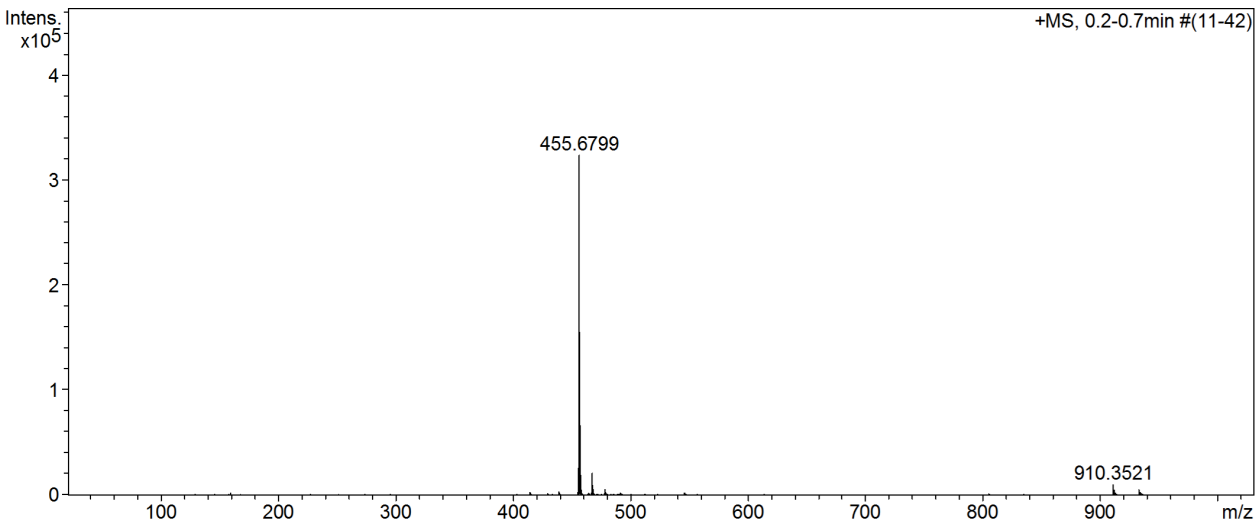


Figure S8. ^{1}H, ^{13}C NMR and high resolution mass spectra of purified peptide **A**.

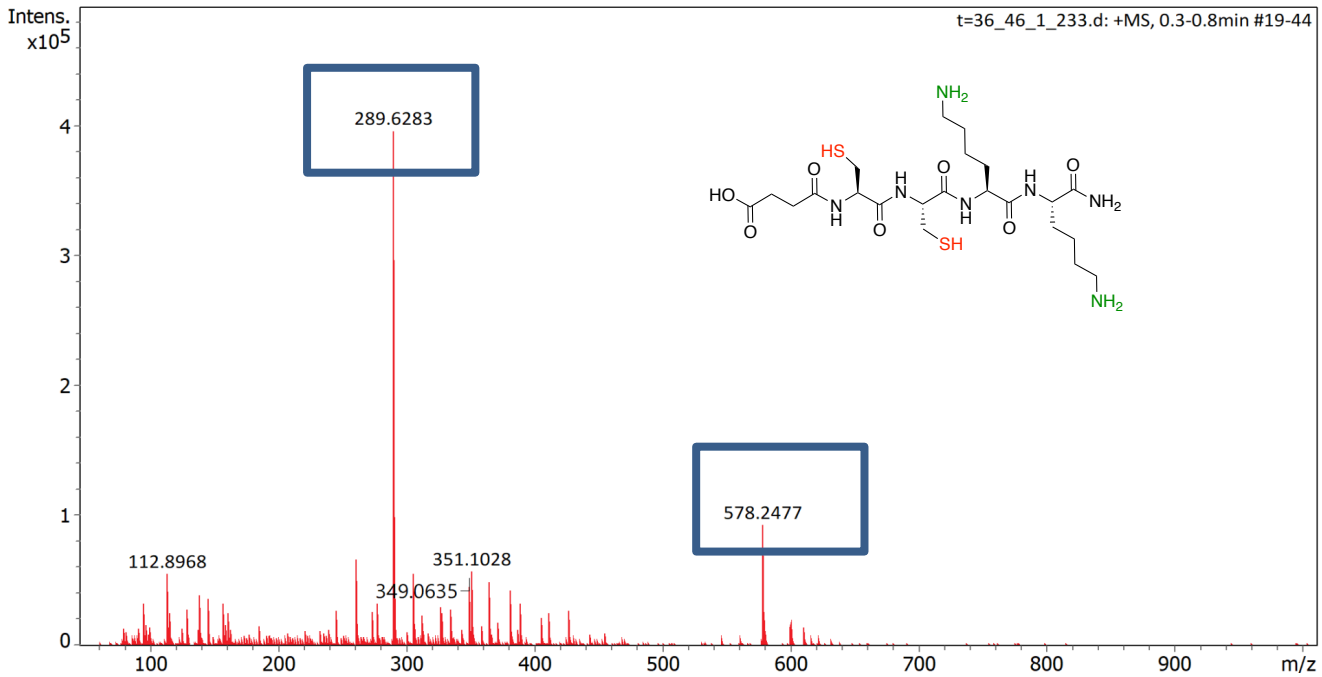


Figure S9. HRMS spectrum of peptide byproduct after hydrolytic release of CPT from **A**. A sample of **A** in PBS (100 µM) was incubated at 37.5 °Ç for 35 h to induce CPT hydrolytic release. The sample was subsequently analyzed by HRMS, which revealed peaks at m/e values of 578.2477 and 289.6283, corresponding to the +1 and +2 charge states of the (succinate)CCKK peptide byproduct, respectively. The peaks reflect disulfide bond formation that occurred under the conditions of analysis.

Molecular Dimensions of A in Extended Conformation.

Measures were taken by importing a ChemDraw structure into Chem3D and locking the coordinates. The length was measured from C(42) to N (13). The height was measured from N (31) to N (26).

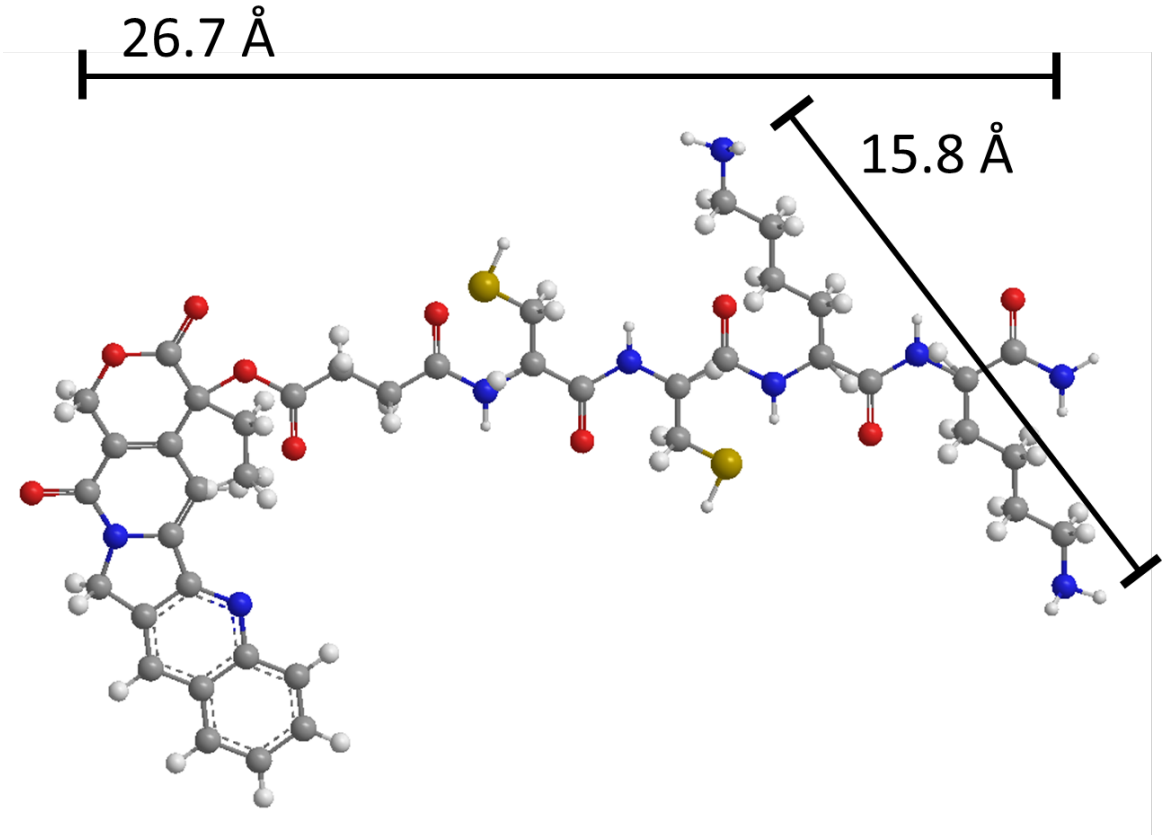


Figure S10: Dimensions of **A** in a fully extended conformation, estimated using Chem3D. The extended conformation was not energy optimized.

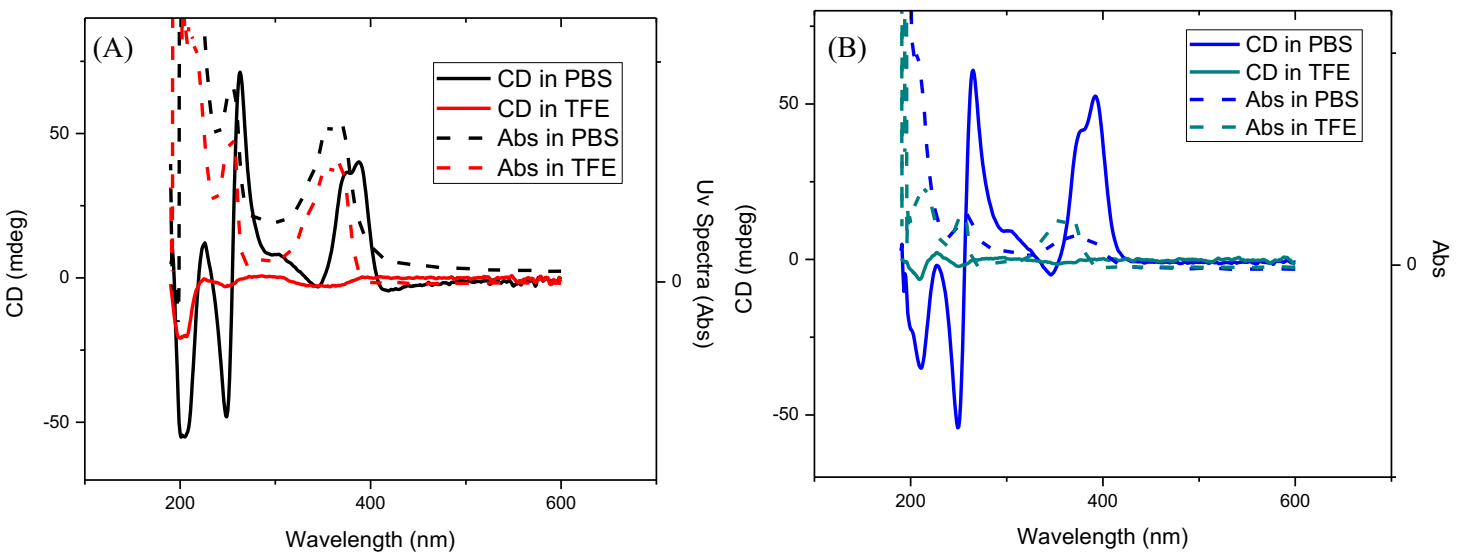


Figure S11: Circular dichroism and Ultraviolet-Vis spectra. (a) Non-crosslinked **A**, assembled in PBS (0.25 mM, black)) and in TFE (0.25 mM, red). A sample of **A** was aged at 20 mM in PBS (7.4 pH) for 72 h to induce nanotube assembly, then freshly diluted to 0.25 mM for CD and UV-Vis.(b) Oxidatively crosslinked nanotubes of **A**. **A-ox** samples were obtained by oxidation with 10% v/v DMSO, pelleting at 80,000 rpm for 1 h, then resuspension in PBS (blue) or TFE (green).

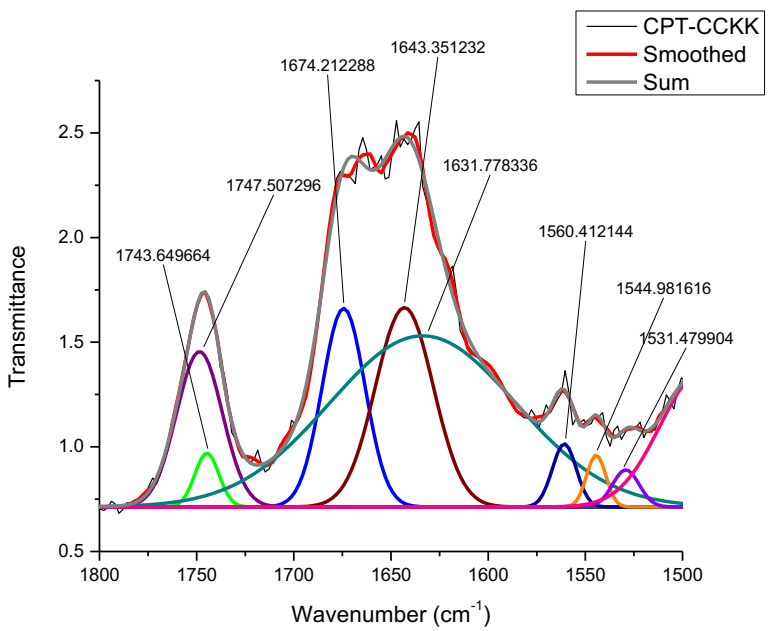


Figure S12: IR spectra taken of compound **A** assembled at 20 mM in deuterated PBS for 72 h followed by two cycles of lyophilization and resuspension in D_2O. The IR spectra reflect the presence of a random coil structure.

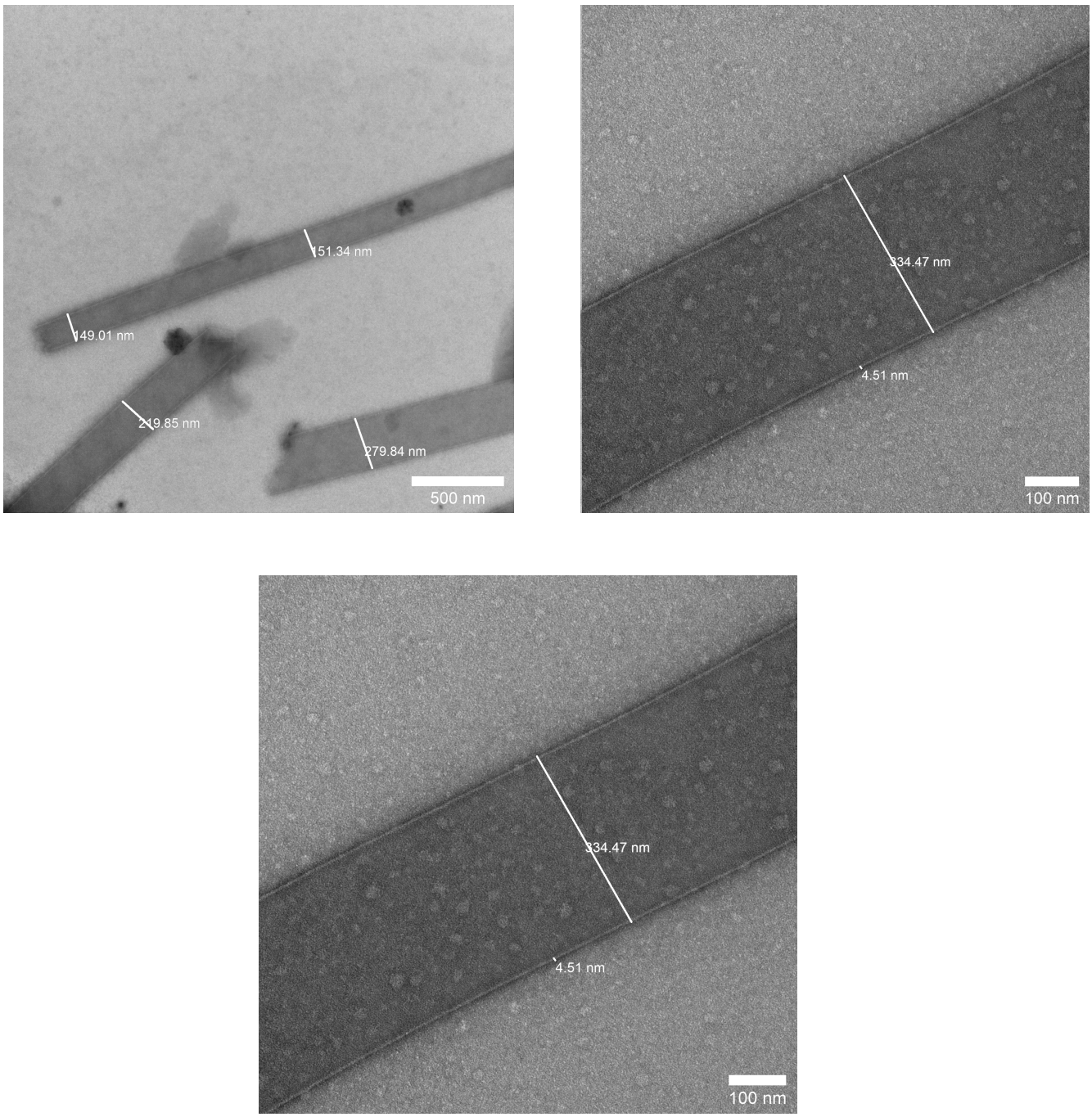


Figure S13: TEM images of compound **A** assembled in 20 mM in PBS (7.4 pH) for 72 h and freshly diluted to 1 mM for imaging. Dimensions of nantubes are indicated in images.

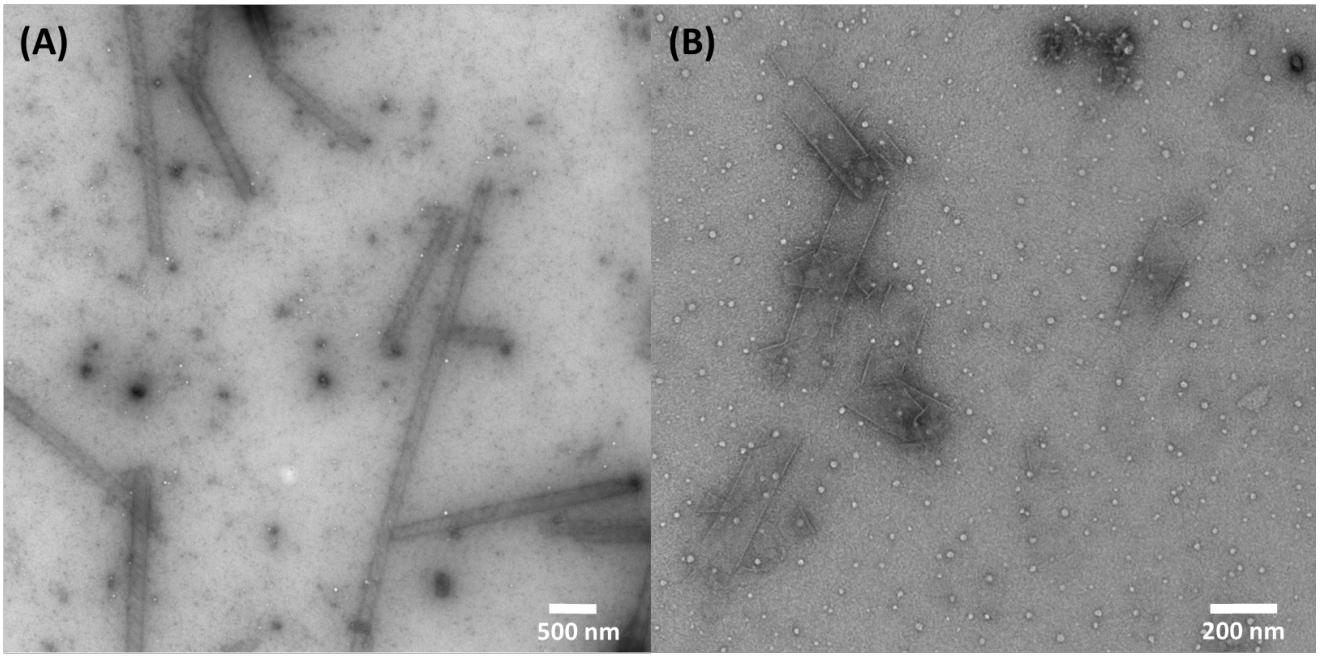


Figure S14: TEM images of **A** in (a) PBS (3 days, pH 7.4, 10 mM), (b) water (3 days, pH 7.0, 10 mM). Samples were prepared by dissolving **A** in PBS or H_2O (10 mM), then diluting to 1 mM after 3 days prior to imaging using a carbon coated copper grid and negatively stained with 2 % wt uranyl acetate for 1 min.

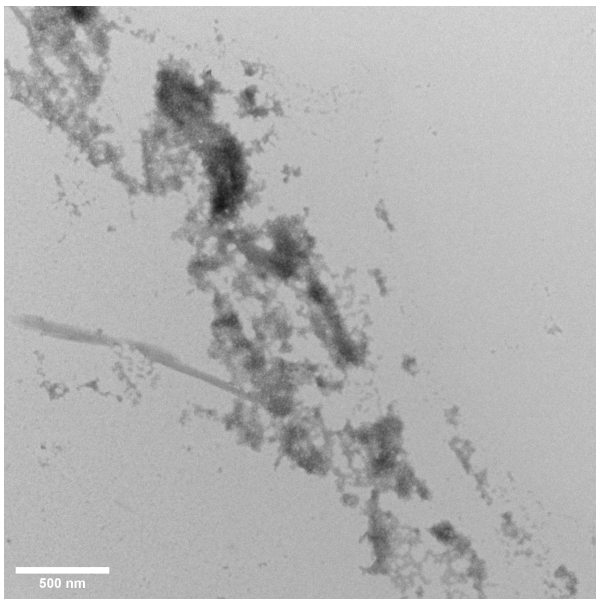


Figure S15. TEM images of **A** in water (3 days, pH 7.0, 20 mM). Samples was prepared by dissolving **A** in H_2O (20 mM), then diluting to 1 mM after 3 days prior to imaging using a carbon coated copper grid and negatively stained with 2 % wt uranyl acetate for 1 min. Due to poor solubility, no discrete structures were observed.

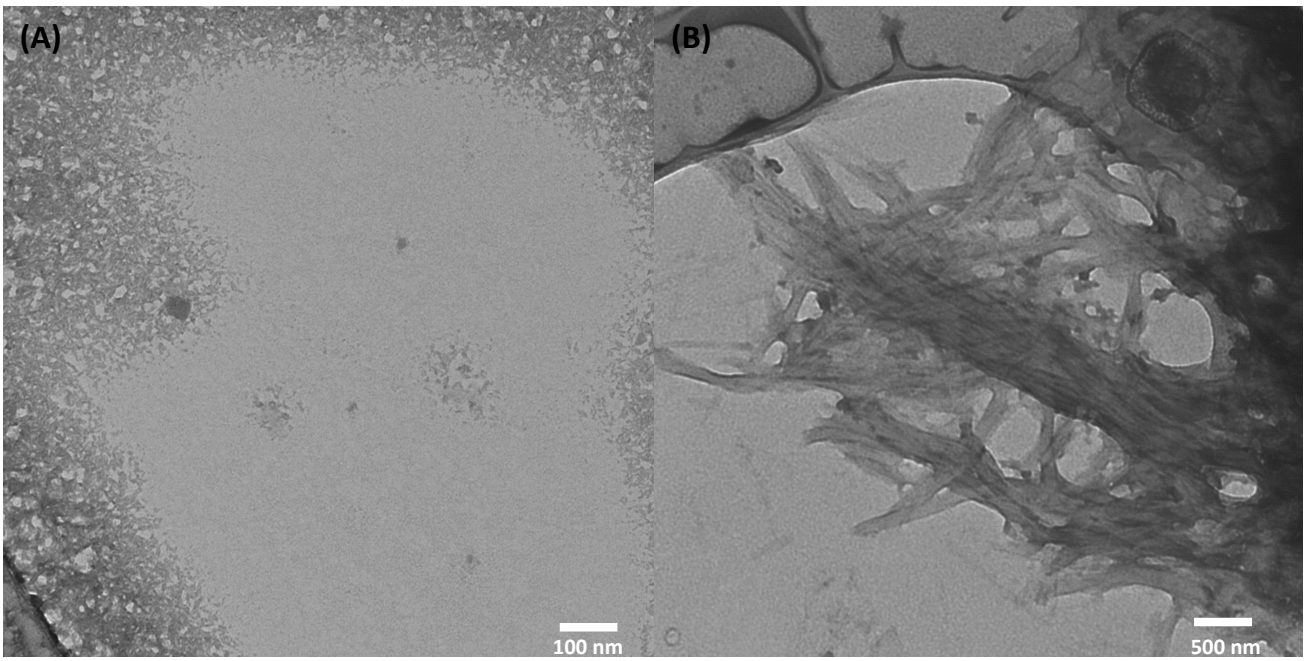


Figure S16. a) TEM image of compound **A** without crosslinking in TFE (10 mM); b) TEM image of crosslinked nanotubes of **A** (PBS, 10 mM, 3 days) after resuspending in TFE. Crosslinked samples of **A** were obtained by oxidation of **A**, preassembled in PBS (10 mM, 3 days), with 10% v/v DMSO, followd by pelleting at 80,000 rpm (278,835 g) for 1 h and resuspension in TFE (1 mM). The samples were imaged by TEM using a carbon coated copper grid and negatively stained with 2 % wt uranyl acetate.

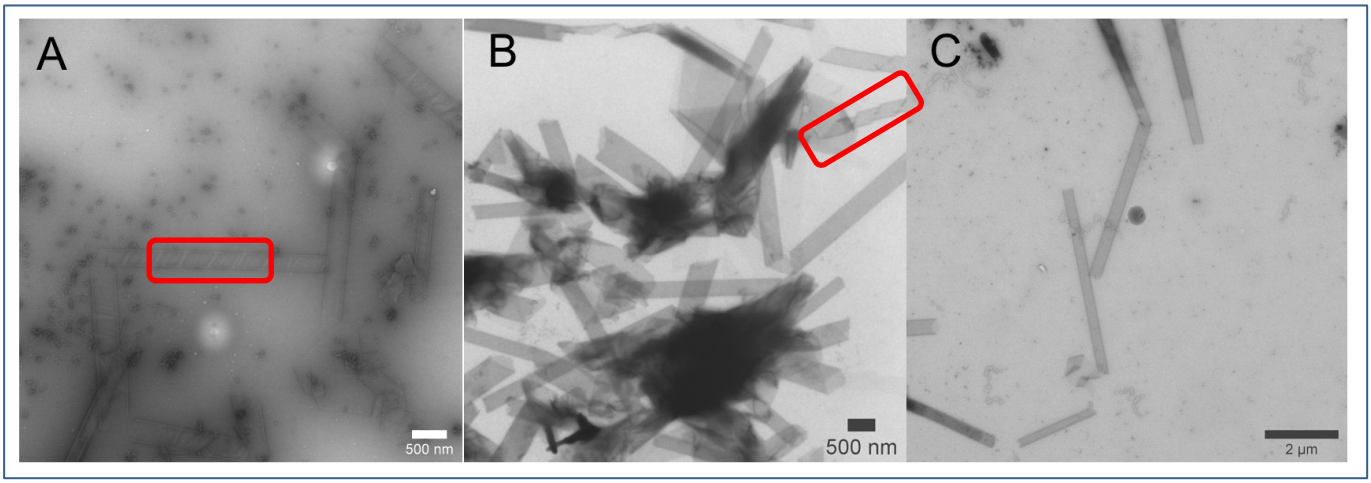


Figure S17. TEM images of **A** assembled in PBS (pH 7.4) at 20 mM for 24 h (A), 48 h (B), and 72 h (C). Samples were assembled at 20 mM, diluted to 1 mM immediately prior to deposition on a carbon coated copper grid then negatively stained with 2 % wt uranyl acetate for 1 min. Helical striations present on the nanotubes are highlighted with red boxes.

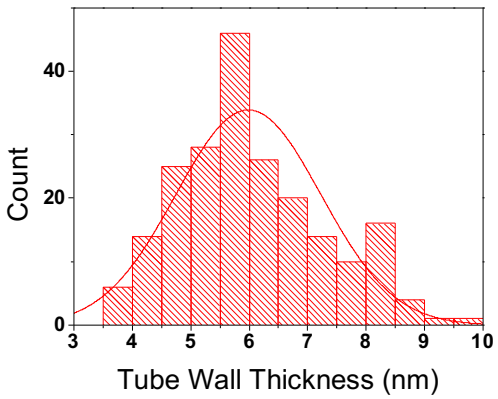


Fig. S18. Histogram of nanotube wall thicknesses based on TEM measurements of 211 nanotubes. Average wall thickness was 5.98 ± 1.2 nm, indicative of a bilayer wall structure.

1. S. H. Kim, J. A. Kaplan, Y. Sun, A. Shieh, H. L. Sun, C. M. Croce, M. W. Grinstaff and J. R. Parquette, *Chem. Eur. J.*, 2015, **21**, 101-105.