Supporting Information for:

Fine-Tuning the Linear Release Rate of Paclitaxel-Bearing Supramolecular Filament Hydrogels through Molecular Engineering

Rami W. Chakroun[§], Feihu Wang[§], Ran Lin[§], Yin Wang[§], Hao Su[§], Danielle Pompa[¥], Honggang Cui[§,‡,†]*

[§]Department of Chemical and Biomolecular Engineering, and Institute for NanoBiotechnology, The Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, United States.

[¥]Department of Biomedical Engineering, University of Utah, 201 Presidents Cir, Salt Lake City, Utah, 84112

[‡]Department of Oncology and Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, United States

[†]Center for Nanomedicine, The Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 North Broadway, Baltimore, Maryland 21231, United States

Corresponding Author E-mail: hcui6@jhu.edu

Table of Contents

S1. Molecular Synthesis

S1.1 Chemicals

Amino acids: AAPPTEC (Louisville, KY).

Arginine loaded Wang Resin: NovaBiochem (San Diego, CA).

Paclitaxel: Ava Chem Scientific (San Antonio, TX).

4-bromobutyric acid: Sigma-Aldrich (St. Louis, MO).

Thiourea: Sigma-Aldrich (St. Louis, MO).

N,N'-diisopropylcarbodiimide (DIC): TCI

4-dimethylaminopyridine (DMAP): TCI

All other reagents and solvents were sourced through VWR.

S1.2 Peptide Synthesis.

Through Fmoc solid phase peptide chemistry, an automatic peptide synthesizer (AAPPTEC Focus XC) was used to synthesize all four of the peptides used in this study. In summary, the procedure involves Fmoc-deprotection of the resin using 20% 4-methylpiperidine in dimethylformamide (DMF) prior to each amino acid conjugation. Amino acid conjugation was performed by adding a mixture of the Fmoc-amino acid, O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate (HBTU), and diisopropylethylamine (DIEA) in DMF in a 4:4:10 molar ratio to the resin. After the last amino acid cycle, the N-terminus was acetylated using 20% acetic anhydride in DMF. Finally, the peptide was cleaved by treating the resin with a mixture of 95% TFA, 2.5% TIS, and 2.5% H_2O for 2.5 hrs. After cleavage, cold diethyl ether was used to

precipitate the peptide out of the solvent. A preparative RP-HPLC column was used to purify the peptides with an increasing gradient of acetonitrile in water, both containing 0.1% TFA. Matrix assisted laser desorption-ionization (MALDI-ToF) mass spectrometry was used to analyze the fractions collected before the product was lyophilized. Peptides were stored in a freezer at −20 °C.[1-3]

S1.3 Manual Conjugation of the Alkly Chain and 5-FAM Dye

Before cleaving our peptide, we are able to manually conjugate specific compounds such as 5-FAM dye or a C_{12} alkyl chain using a similar procedure to amino acid conjugation. After Fmoc-deprotection of the N-terminus using 4-methylpiperidine in DMF, either compound was added to the resin beads along with HBTU and DIEA in DMF in a 4:4:10 molar ratio. The mixture was shaken overnight. After reacting, the resin was then cleaved/further processed using the standard procedure described in section S1.2.

S1.4 Paclitaxel-Conjugate Synthesis.

4-(pyridin-2-yldisulfanyl) butyric acid synthesis

4-Bromobutyric acid (2g, 12mmol) and thiourea (0.96 g, 12.6 mmol) were dissolved in ethanol (50 mL) and refluxed at 90 °C for 4 h. Following the dropwise addition of a NaOH solution (4.8 g in 5:1 H2O/ethanol), the mixture was refluxed for another 16 h and then cooled to room temperature. The white precipitate was collected and redissolved in water (40 mL). 4 M HCl was used to adjust the pH of the solution to 5 and the product was isolated using a diethyl ether liquid-liquid extraction. The organic phase was dried over anhydrous sodium sulfate to give 4-sulfanylbutyric acid as a colorless oil (310 mg, 15%), which was used in the next step without further purification. 4-sulfanylbutyric acid (105 mg, 0.87 mmol) and 2-aldrithiol (440 mg, 2.0

mmol, 2.3 eq) were dissolved in MeOH (1.3 mL) and stirred for 3 h. The solution was purified by RP-HPLC (5% to 95% gradient of acetonitrile in water with 0.1% TFA over 45 min), product fractions were combined and solvents were removed to give 4-(pyridin-2-yldisulfanyl) butyric acid as an oil (118 mg, 59%).[4]

Paclitaxel C2' ester synthesis

Paclitaxel (186 mg, 0.22 mmol), 4-(pyridin-2-yldisulfanyl)butyric acid (100 mg, 0.44 mmol), DIC (68 μL, 0.44 mol), and DMAP(26.7 mg, 0.22 mmol) were added to an oven dried flask equipped with a stirrer bar. The flask was evacuated and refilled with nitrogen three times to remove air, then the reactants were dissolved in anhydrous acetonitrile (12.7 mL). The reaction was allowed to stir in the dark at room temperature for 48 h. The solvents were removed under reduced pressure and the remaining residue was dissolved in chloroform and purified by flash chromatography (3:2 EtOAc/ hexane) to give the product as a white solid (108 mg, 47%).[4]

Paclitaxel-peptide conjugate synthesis

Previously synthesized peptide and paclitaxel C2' ester were added to an oven dried flask equipped with a stirrer bar. The reagents were then dissolved in DMF (2 mL) and the solution was allowed to stir for 24 hrs before purification by RP-HPLC (10% to 90% acetonitrile in water with 0.1% TFA over 30 min). Product fractions were combined and lyophilized to give a white powder.[4,2]

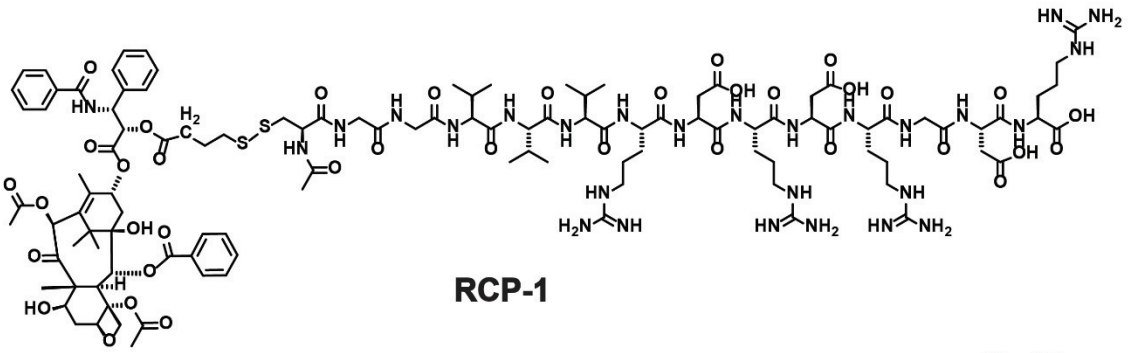
S1.5 MALDI-TOF

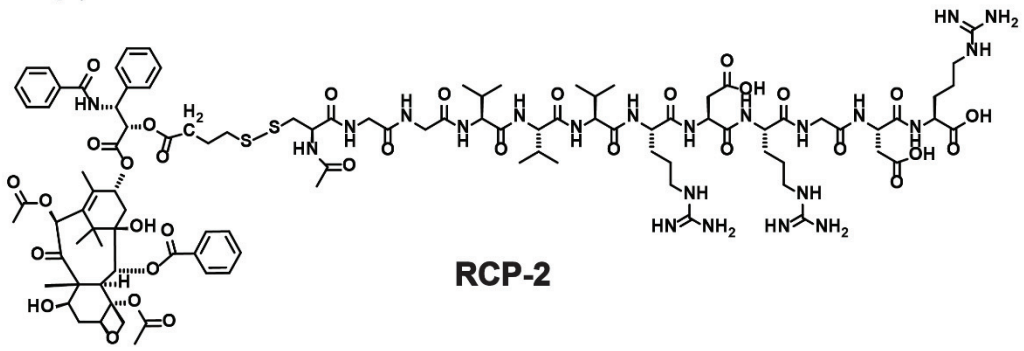
Molecular masses of the products were determined by MALDI-ToF mass spectrometry, using a BrukerAutoflex III MALDI-ToF instrument (Billerica, MA). 2 μL of sinapinic acid matrix (10

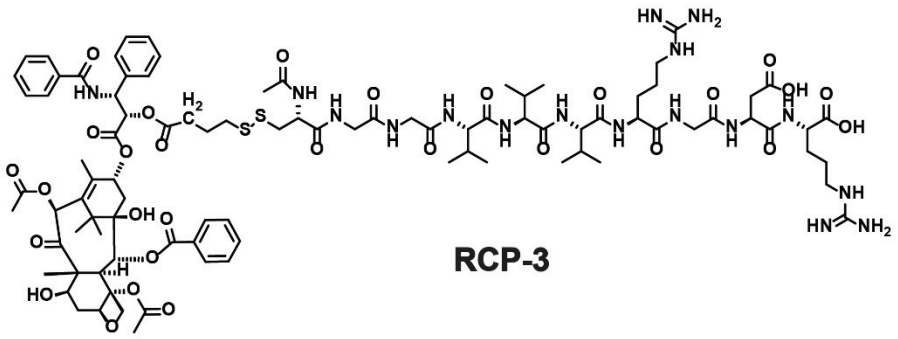
mg/ml in 0.05% TFA in H2O/MeCN (1:1), SigmaAldrich, PA) was deposited onto the MALDI plate. After 5 mins drying time, another 2 μL of sinapinic acid matrix was added to the corresponding spot followed by the immediate addition of 2 μL aqueous conjugate solution. The two substances were mixed and allowed to dry for 20 mins. Samples were irradiated with a 355 nm UV laser and analyzed in the reflectron mode.

S1.6 Analytical HPLC

Analytical reverse-phase HPLC was performed using a Varian polymeric column (PLRPS, 100 Å, 10 μm, 150 × 4.6 mm) with 20 μL injection volumes. A 10-90% water : acetonitrile gradient containing 0.1% v/v TFA at a flow rate of 1 mL/min was used. The chemical formulas and the respective MALDI and analytical RP-HPLC data for each molecule are displayed in Figures S1 and S2, respectively.







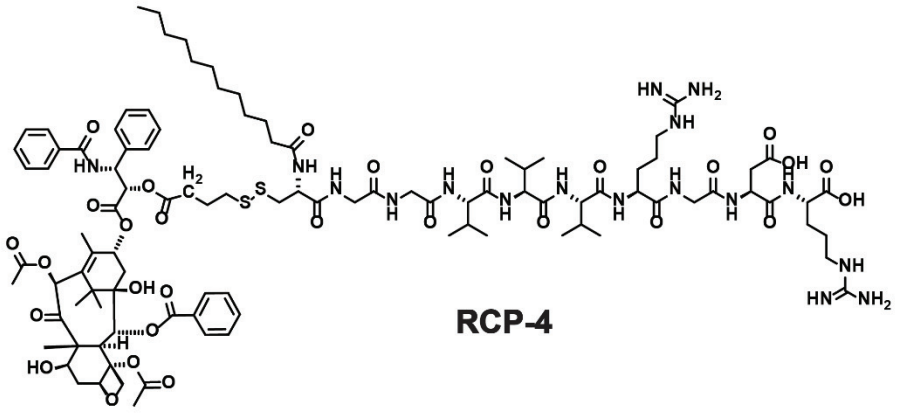


Figure S1. Chemical structures of the four molecules studied in this work.

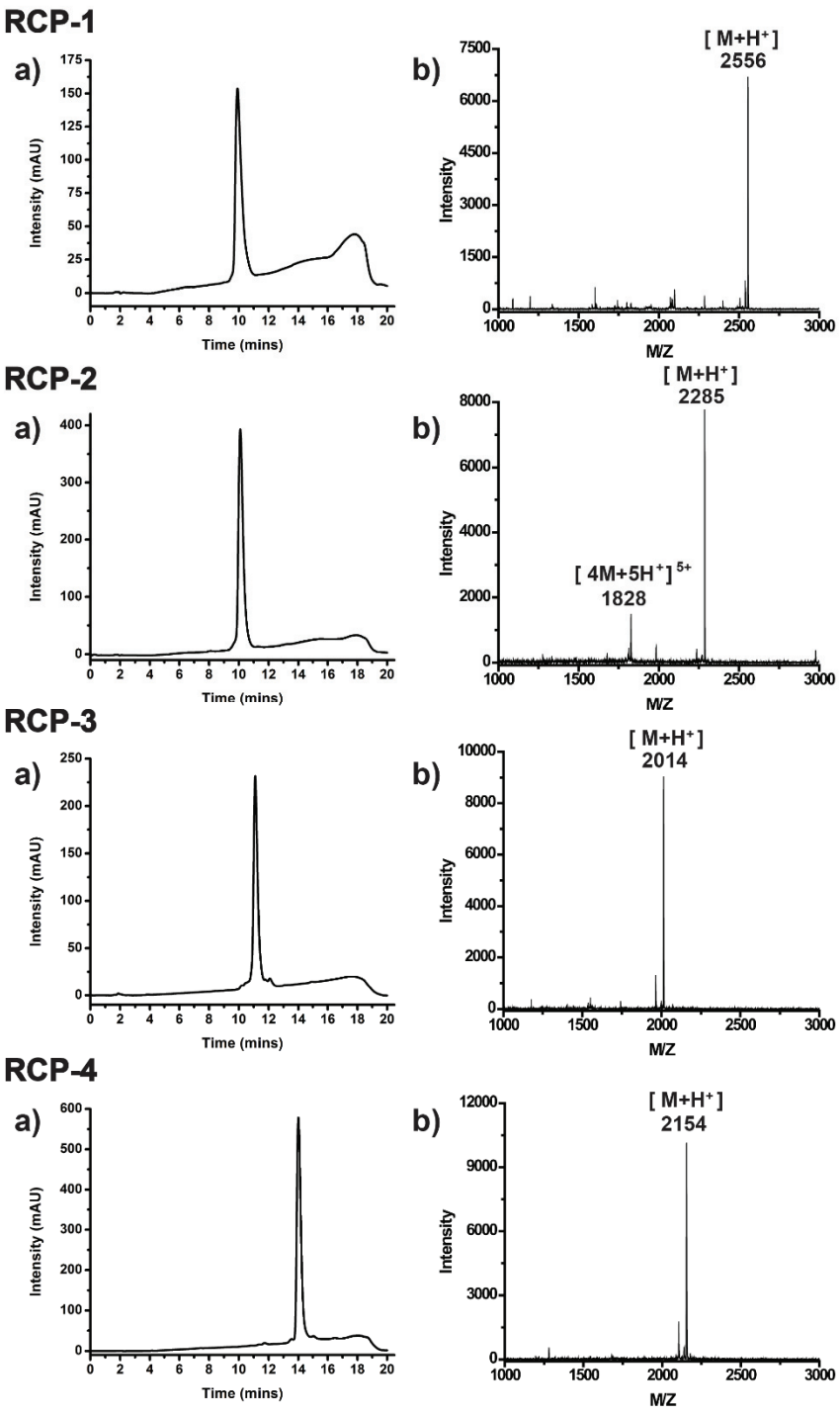


Figure S2. Respective Reverse-Phase HPLC chromatograms (a) and MALDI-ToF MS (b) for the studied four molecules.

S2. Self-Assembly Characterization

S2.1 Circular Dichroism (CD) Spectroscopy

100 μM sample dilution from a 1 mM drug conjugate stock solution that was aged for 1 week was used to obtain the CD spectra for each molecule. Data were recorded on a Jasco J-710 spectropolarimeter (JASCO, Easton, MD) using a 10 mm path length Spectrasil® quartz UV-Vis absorption cell (Starna Cells Inc., Atascadero, CA). A background spectra of water was acquired and subtracted from each sample spectra. The data collected was normalized with respect to the sample concentration[5].

S2.2 CAC measurement using Fluorescence Spectroscopy

A 500 μM Nile Red stock solution was prepared in acetone. 10 μL of solution was added to the bottom of each vial before allowing the acetone to evaporate. Drug conjugate solutions were prepared at different concentrations. 500 μL of each solution was added to the vials containing Nile Red. Samples were aged for two days. Emission spectra for each sample were then recorded on a Fluorolog spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ) between 580 and 720 nm, with an excitation wavelength of 550 nm[2]. Using the intensity ratio, the CMC value was calculated based on the transition point from 660 nm to 635 nm emission, as seen in Figure S4.

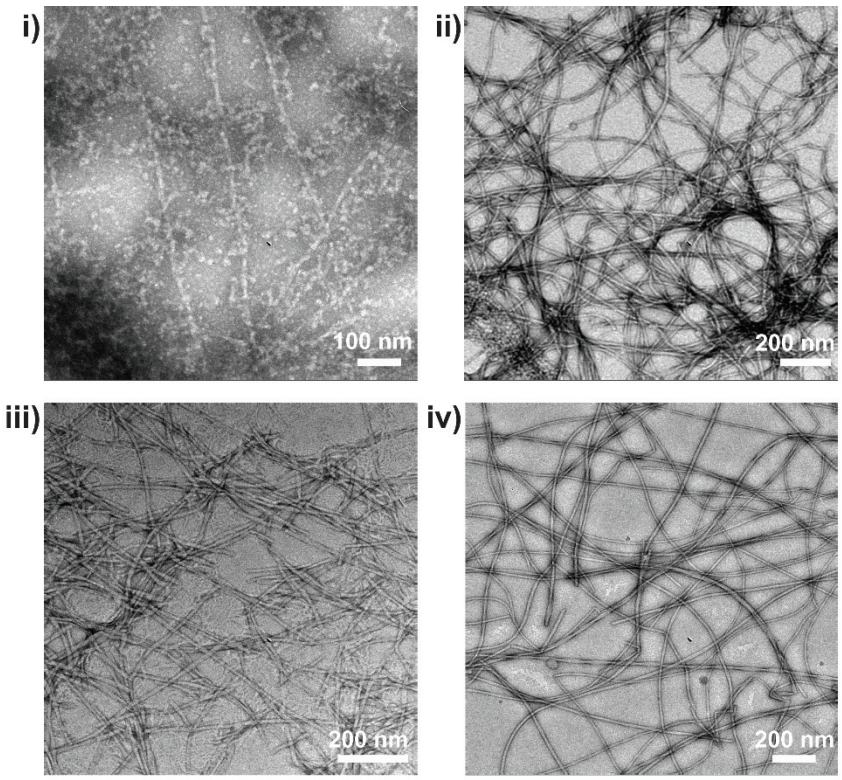


Figure S3. TEM images of RCP-1 (i), RCP-2 (ii), RCP-3 (iii), and RCP-4 (iv), aged for 1 day at 1 mM concentration. The results suggest that filamentous nanostructures can be observed after one day aging.

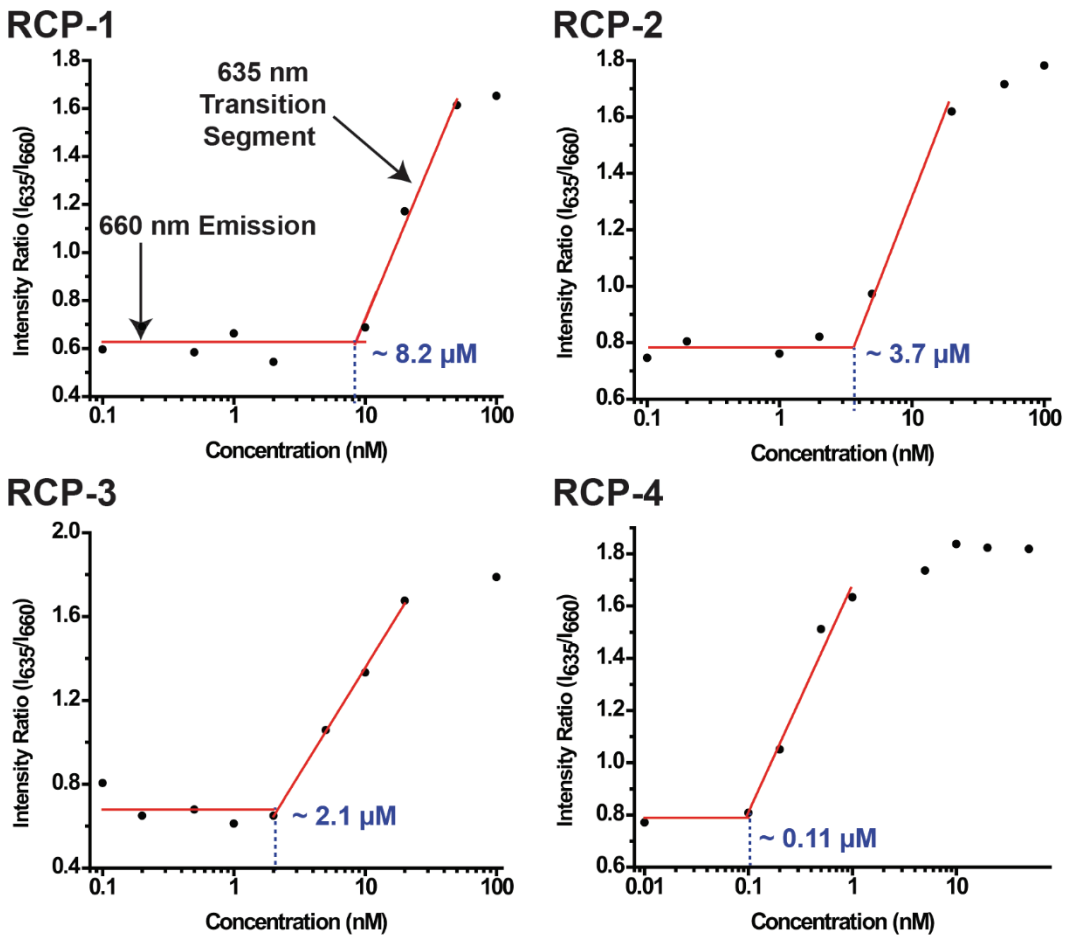


Figure S4. Intensity ratio (I_{635}/I_{660}) of the fluorescence spectroscopy data for each drug conjugate and a depiction of how the CAC values were calculated. These studies suggest that molecular engineering of the chemical structures of the PTX prodrugs represents an effective way to tune the stability/instability of their assemblies by having a different CAC.

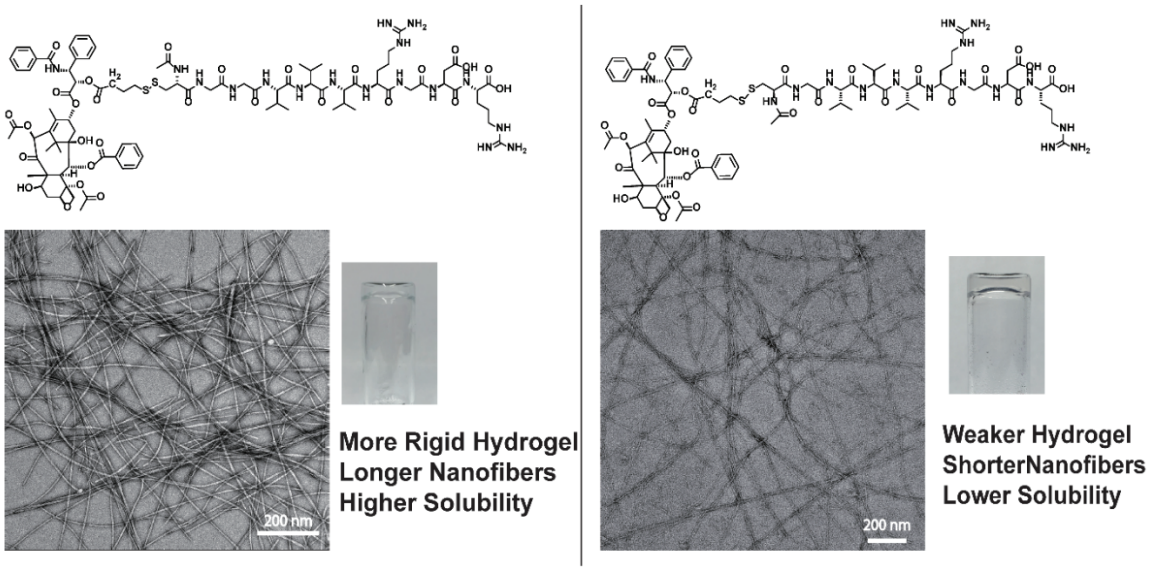


Figure S5. Chemical structures of two molecules that were designed and analyzed using TEM to compare the effect of using a larger spacer on DA gelation behavior.

S3. Gel Property and Release Study

S3.1 Critical Gelation Concentration

Drug conjugates were dissolved at different concentrations and aged for two days at room temperature. 100 µL of each solution was then aliquoted into 500 µL vials. 10 µL of 10x PBS was added to each solution to give a final 1x PBS concentration. Gel formation was assessed by inverting the vial.

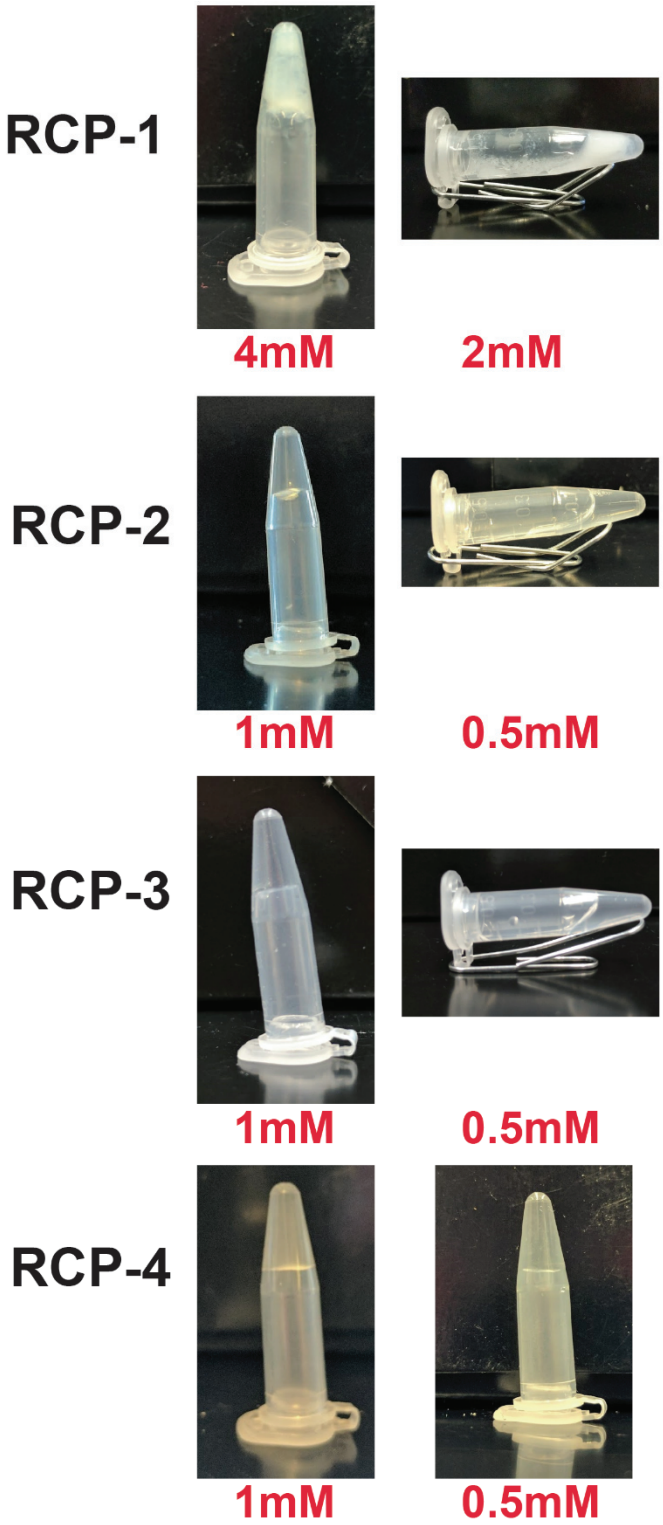


Figure S6. Vials containing different concentrations of drug conjugate were inverted following PBS addition in order to observe gel formation and determine the CGC.

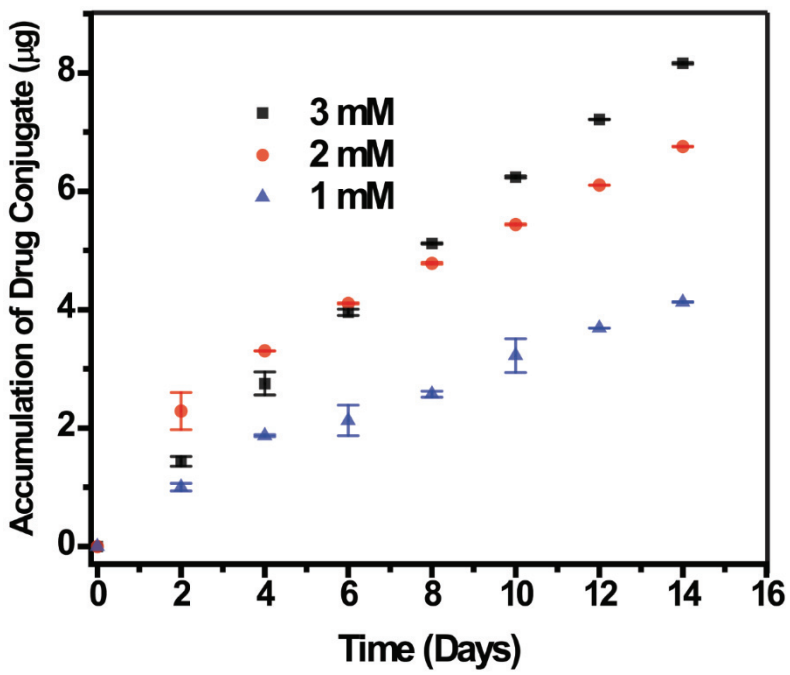


Figure S7. Drug release profiles of hydrogels made at 3 different concentrations of the RCP-3 molecule, given as conjugate accumulation in µg over time.

S4. Cytotoxicity studies

S4.1 Cell lines

U87 cell lines were gifted by Dr. Wirtz (ChemBE, JHU). 612 cells were provided by Dr. Quiñones-Hinojosa (Mayo Clinic). All cell lines were cultured according to providers' protocols. The U87 cell lines were grown in DMEM (Invitrogen) with 10% FBS (FBS, Invitrogen) and 1% antibiotics (Invitrogen). The 612 cell lines were grown on laminin coated plates in 88.97% DMEM/F12 HEPES (Invitrogen 11330-032), 1% Antimycotic (Invitrogen 15240-062), 10% B27 Serum Free Supplement (50x) (Invitrogen 17504-044), 0.02% hEGF (PeproTech AF-100-15), and 0.01% hFGF-Basic (PeproTech 100-18B)[2]. All cell types were incubated at 37 ℃ in a humidified incubator (Oasis, Caron, Marietta, OH, USA) set to a 5% CO_2 level.

S4.2 Cytotoxicity Data:

Control Peptide		
Concentration (nM)	*Cell Viability*	*Standard Error*
100000	0.97	0.01

Table S1. Cell viability ratio and standard error of the control peptide: C12-CGGVVVRDRGDR.

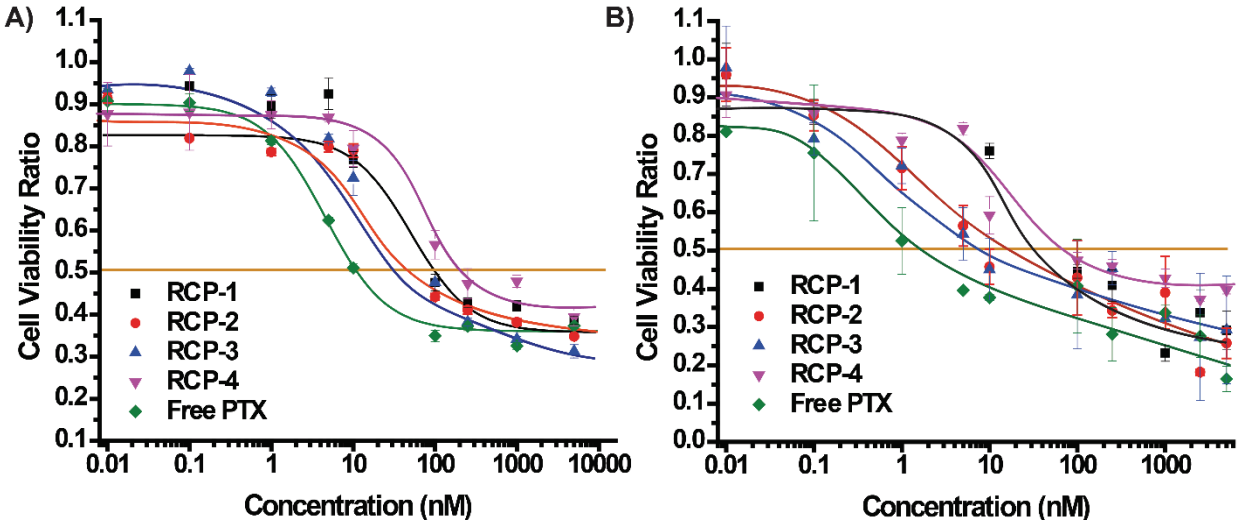


Figure S10. Cytotoxicity curves showing cell viability ratio as a function of concentration, which were used to obtain the IC50 values of the U87 cell lines (A) and Primary 612 cell lines (B).

References

S1 Cheetham, A. G.; Zhang, P.; Lin, Y.-a.; Lock, L. L.; Cui, H. Supramolecular Nanostructures Formed by Anticancer Drug Assembly. *J. Am. Chem. Soc.* **2013**, *135*, 2907-2910.

S2 Lin, R.; Cheetham, A. G.; Zhang, P.; Lin, Y.-a.; Cui, H. Supramolecular filaments Containing A Fixed 41% Paclitaxel Loading. *Chem. Commun.* **2013**, *49*, 4968-4970.

S3Lock, L. L.; Reyes, C. D.; Zhang, P.; Cui, H. Tuning Cellular Uptake of Molecular Probes By Rational Design of Their Assembly into Supramolecular Nanoprobes. *J. Am. Chem. Soc.* **2016**, *138*, 3533-3540.

S4 Dubikovskaya, E. A.; Thorne, S. H.; Pillow, T. H.; Contag, C. H.; Wender, P. A. Overcoming Multidrug Resistance of Small-Molecule Therapeutics through Conjugation with Releasable Octaarginine Transporters. *Proc. Natl. Aca. Sci. U. S. A.* **2008**, *105*, 12128-12133.