Supporting information for

Supramolecular assembly of isomeric SN-38 prodrugs regulated by conjugation site

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1. Experimental Section

1.1 Materials

The following chemicals are used as received: 7-Ethyl-10-hydroxycamptothecin (Innochem, > 99%), Pentaethylene glycol monomethyl ether (Innochem, > 98%), (S)-(+)-Camptothecin (CPT) (Innochem, > 98%), Triphosgene (Adamas, > 99%), 4-Nitrobenzoyl chloride (Adamas, > 95%), Di-Tert-Butyl Dicarbonate (Adamas, > 99%), Triethylamine (Greagent, > 99%), fetal bovine serum (FBS, GIBCO), phosphate buffer saline (PBS, pH 7.2, GIBCO), petri dishes (bkmam Biotechnology Co., Ltd.). All of the solvent was obtained by OKINNO (Cheng du, China). Anhydrous dichloromethane (CH_2Cl_2) was purchased by Adamas (Chengdu, China).

1.2 Transmission electron microscopy (TEM)

SN38 SAPDs were dissolved in ultrapure water at concentrations of 1 mM and aged overnight at room temperature. The TEM samples were prepared by dropping 6 μL solution onto the grids (300 square mesh) and being wicked by filter paper.[1] The samples were negatively stained by uranyl acetate (20 mg/ml in water), and the procedures were similar to the previous step. The grids were dried at room temperature overnight before TEM imaging (Talos FEI 200i, Thermo Scientific, USA). The bright-field TEM imaging was obtained at an acceleration voltage of 200 kV.

1.3 Circular dichroism (CD) spectroscopy

Stock solutions were prepared at 1mM and aged overnight. The 10-OEG-SN38 solution was loaded into the 0.1 mm path length high-precision cell (Hellma Analytics) and measured from 190 nm to 450 nm with a 2 nm bandwidth. The 20-OEG-SN38 solution was diluted onsite to 200 μM instantly before measurements, using a 1 mm path length high precision cell (Hellma Analytics) and recording from 190 nm to 450 nm with a 2 nm bandwidth. A mean value of three measurements was used and a pure water background was subtracted to obtain the final result. The final spectra were normalized to the actual concentration of each sample.[2]

1.4 Critical micelle concentration (CMC)

Dynamic light scattering (DLS) was commonly utilized to determine the value of CMC.[3] The value of count rate will undergo a mutation as the free small molecules start to aggregate. Stock solutions were prepared at 1 mM, and diluted to 50, 20, 10, 5, 2, 1, 0.5, 0.2, and 0.1 μM for 20-OEG-SN38 and 900, 800, 700, 600, 500, and 100 μM for 10-OEG-SN38. After aged overnight, the solutions were loaded into a 10 mm path-length Marvin colorimetric dish, and the value of count rate was recorded by DLS (Malvern Nano ZS ZEN3690). The obtained count rates were plotted as a function of the concentration, which shows a transition in the data when the concentration exceeded the CMC (**Figure 3A, 3B**).

1.5 *In vitro* drug release

Briefly, a 400 μM solution SAPDs in deionized water was freshly prepared and allowed to age at room temperature for 24 h, before diluting to 200 μM with PBS (pH 7.4/5.4, 20 mM) at the start of the experiment. The solutions were incubated at 37 °C and sampled at 0, 1, 6, 12, 24, and 48 hours. The samples were flash frozen with liquid nitrogen and stored at -20 °C until

HPLC could be performed. The concentration of the residue ratio is determined by measuring the ratio of the area of the HPLC chromatographic peak to the initial peak area.

1.6 Cell culture

Colorectal cancer cells (CT26) and breast cancer cells (MDA-MB-231) were kindly provided by Prof. Yiwen Li (Sichuan University). CT26 was grown in a complete RPMI-1640 medium, containing 10% fetal bovine serum (FBS) and 1% of antibiotics. MDA-MB-231 was grown in a complete DMEM medium, containing 10% FBS and 1% of antibiotics. Both cells were incubated in a humidified atmosphere of 5% CO_2 at 37 ℃.

1.7 Cytotoxicity protocol

The cytotoxicity was evaluated by MTT assay. CT26/MAD-MB-231 cells was seeded onto a 96-well plate (5000 cells/well) and allow them to adhere overnight. Both SAPDs was diluted with fresh medium and incubated with cells immediately to achieve final conjugate concentrations of 1, 10, 100, 1000, 10000, and 100000 nM. Cells were also cultured in SN38 with the same concentration gradient of 0.1, 1, 10, 100, 500, 1000, 5000, and 10000 nM, with untreated cells (medium only) as the control group and medium (without cells) as blank control. In addition, irinotecan with concentrations of 10, 50, 100, 500, 1000, 5000, 10000, and 500000 nM was used as the second control. After 48 hours of incubation, all of cells were co-incubated with MTT solution at 37°C for 4 h, and the MTT solution was exchanged with 150 μL DMSO, and the absorbance of each well at 570 nm was determined by a microplate reader (Spark, TECAN, Switzerland) after shaken for 15 min. The cell viability was calculated by

$$\frac{(AbsSAPDs - Absblank)}{(Abscontrol - Absblank)} \times 100\%$$

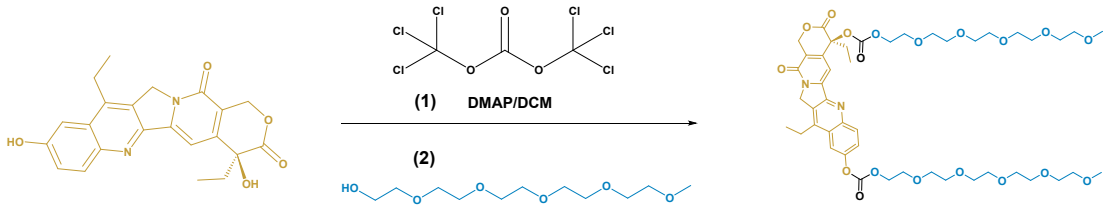
where the AbsSAPDs, Absblack, and Abscontrol are the absorbance of the SAPDs-treated cells, medium, and untreated cells, respectively.

1.8 *In vivo* antitumor efficacy

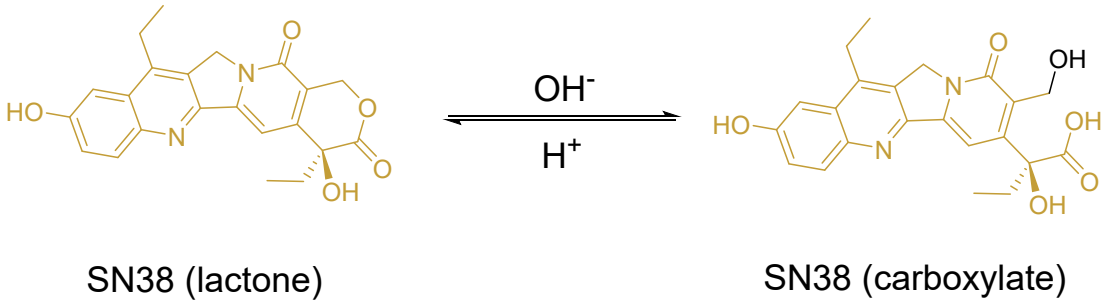
Female BALB/c mice (18~20 g) were obtained from Dashuo Laboratory Animal Co., Ltd. (Chengdu, China). In this study, all animal experiments follow the animal ethical standard from Animal Ethics Committee in West China Hospital, Sichuan University, Chengdu, China (KS20240281).

CT26 tumor model was established by injection of 1.0×10^6 CT26 cells on the right shoulder posterior of the female BALB/c mice. When the average tumor size reached ~100 mm^3, the mice were randomly divided into four groups, with 8 mice in each group. Both SAPDs were all dosed at 34.3 mg/kg (SN38 equivalent) with irinotecan (30 mg/kg) and physiological saline as the control group, on days 1, 3, 5, 7, 9, and 11, for a total of six times. During the experiment, mice were weighed using an analytical balance, and tumor volume was measured using a vernier caliper.

2. Figures



Scheme S1. Synthesis of diOEG-SN38 molecule.



Scheme S2: Hydrolysis of SN38 at different pHs.

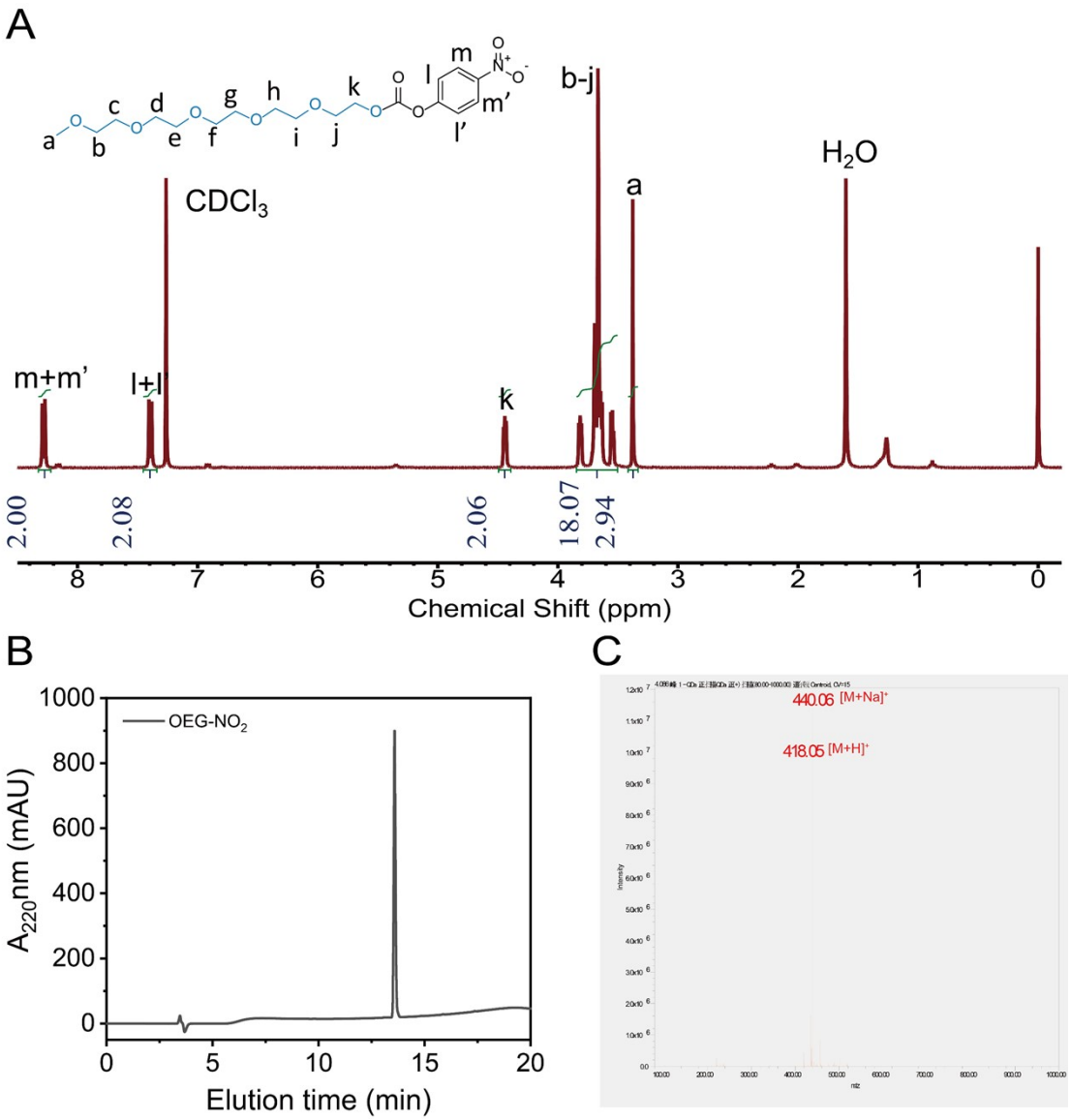


Figure S1. ^{1}H NMR (A), RP-HPLC chromatogram (B) and ESI-MS spectrum (C) of OEG-NO_2

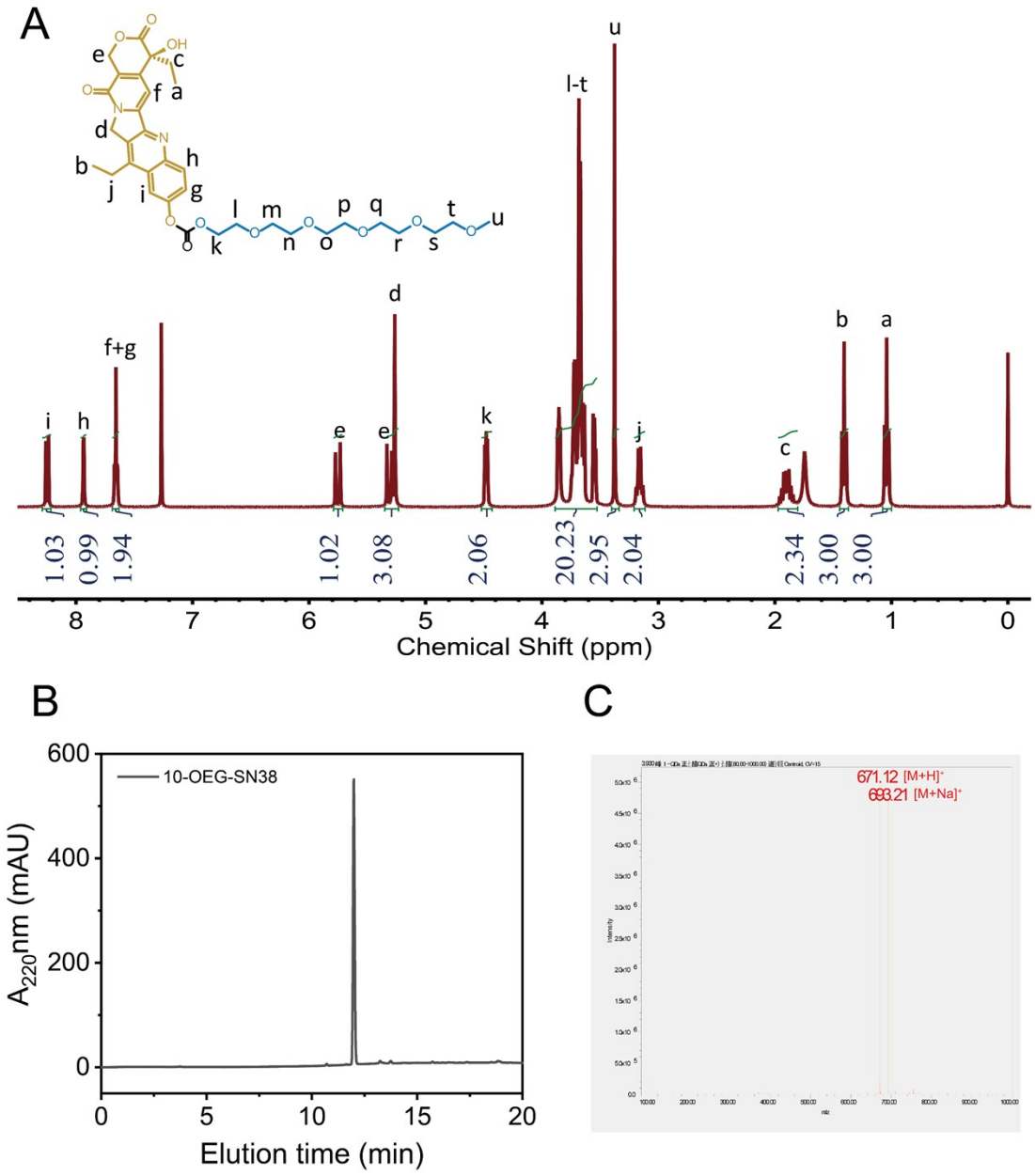


Figure S2. [1]H NMR (A), RP-HPLC chromatogram (B) and ESI-MS spectrum (C) of 10-OEG-SN38

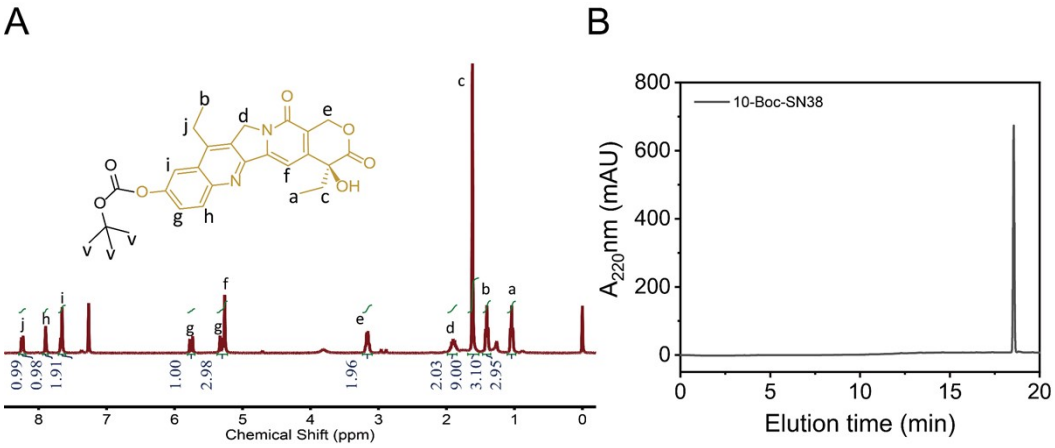


Figure S3. ^{1}H NMR (A) and RP-HPLC chromatogram (B) of 10-Boc-SN38.

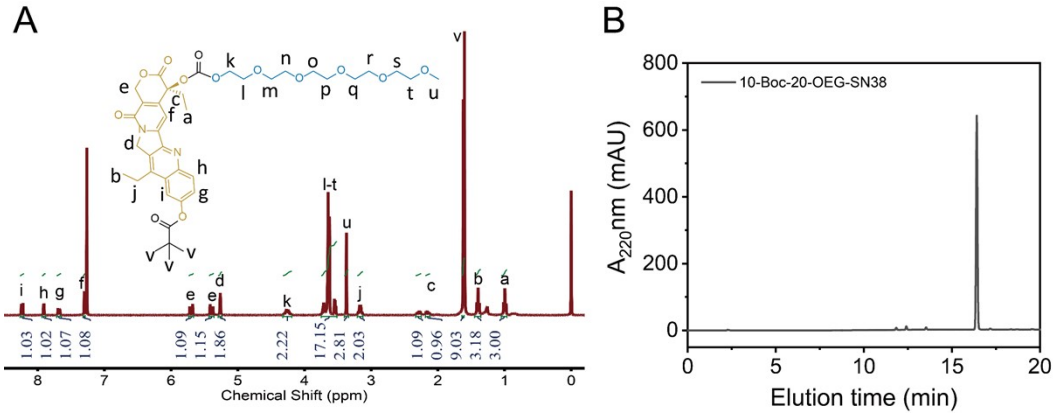


Figure S4. ^{1}H NMR (A) and RP-HPLC chromatogram (B) of 10-Boc-20-OEG-SN38.

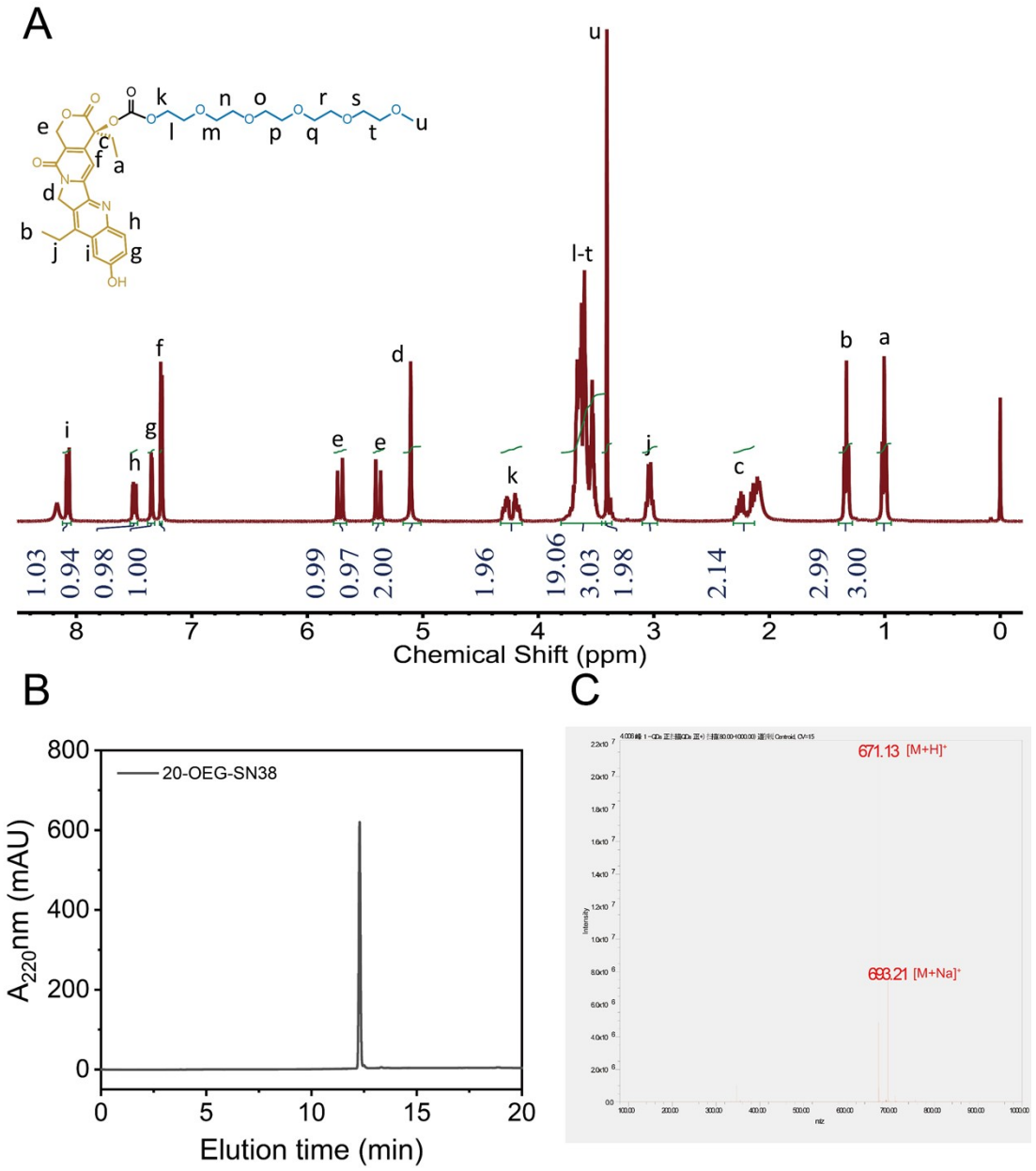


Figure S5. ^{1}H NMR (A), RP-HPLC chromatogram(B) and ESI-MS spectrum (C) of 20-OEG-SN38.

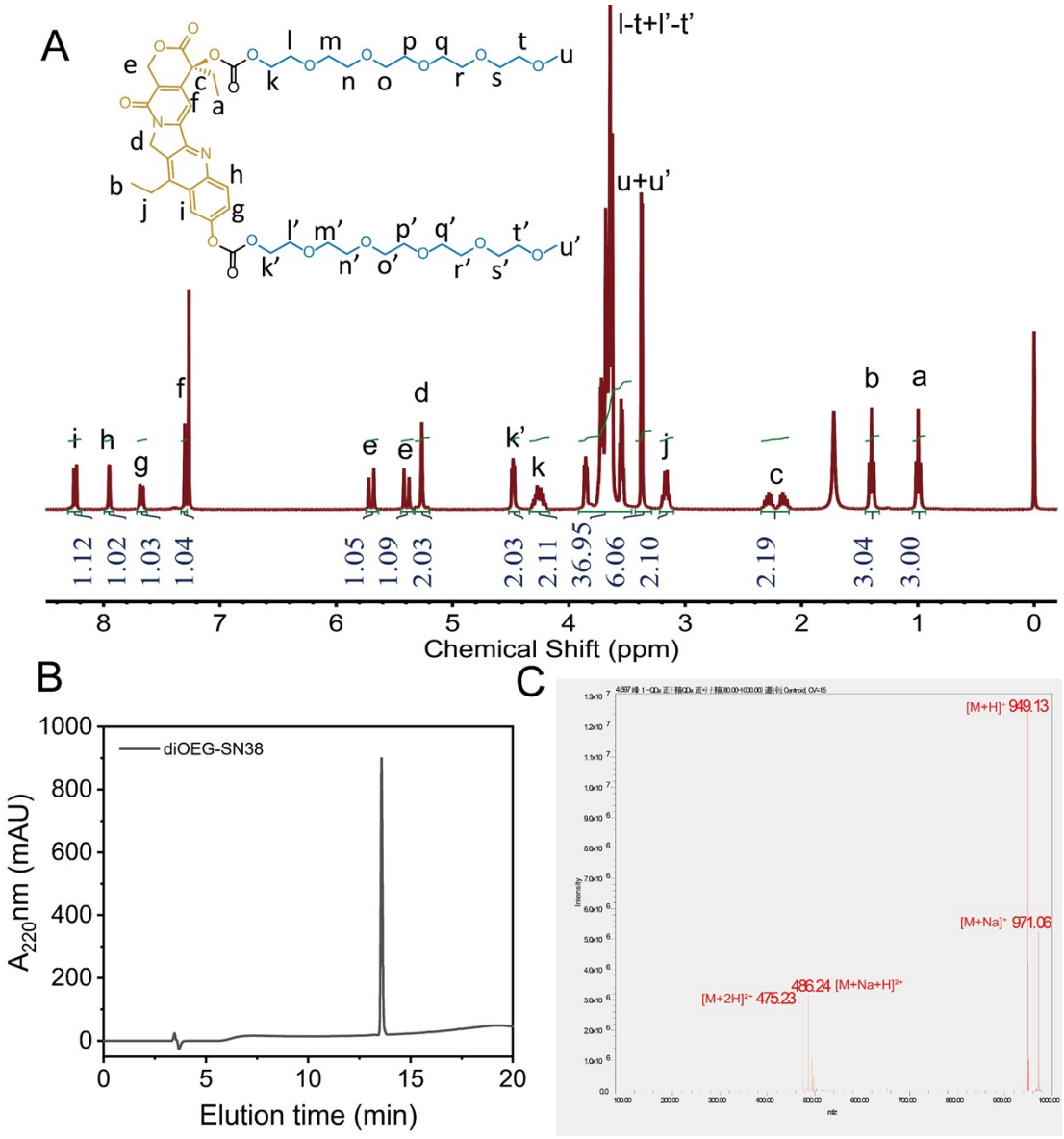


Figure S6. ^{1}H NMR (A), RP-HPLC chromatogram (B), and ESI-MS spectrum (C) of diOEG-SN38.

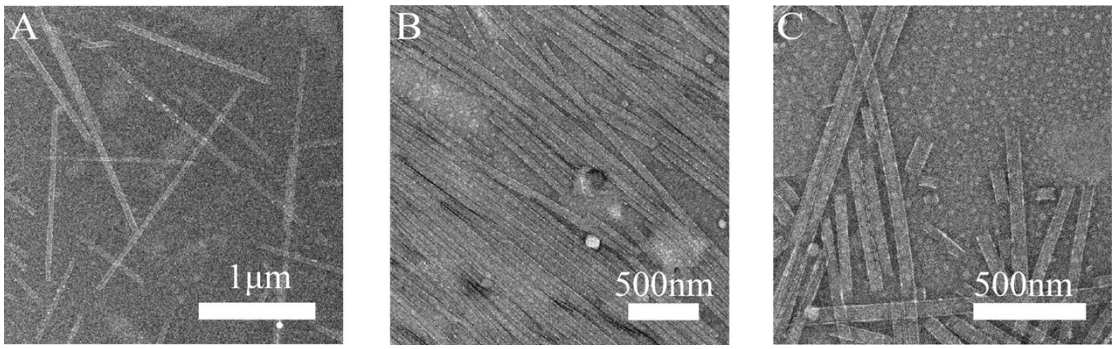


Figure S7. TEM micrographs of 10-OEG-SN38 at a concentration of 1 mM.

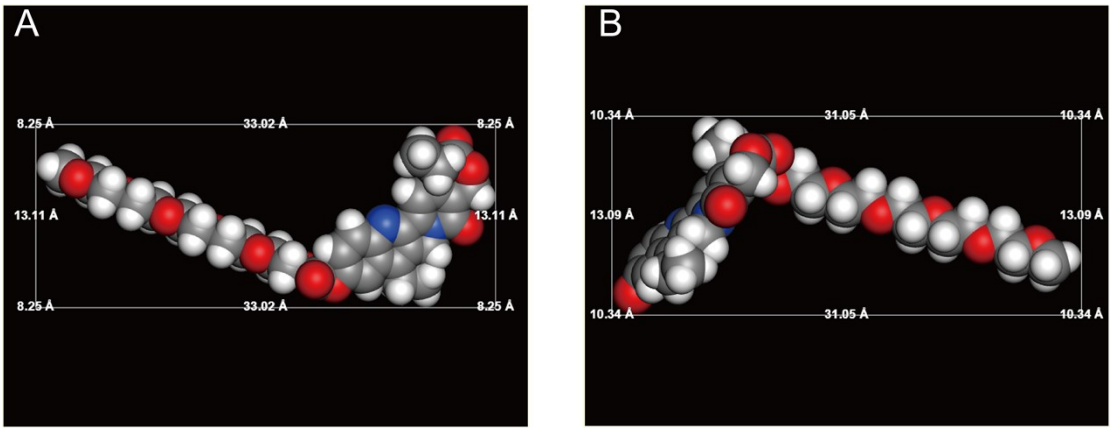


Figure S8. Molecular size of SAPDs: 10-OEG-SN38 (A) and 20-OEG-SN38 (B).

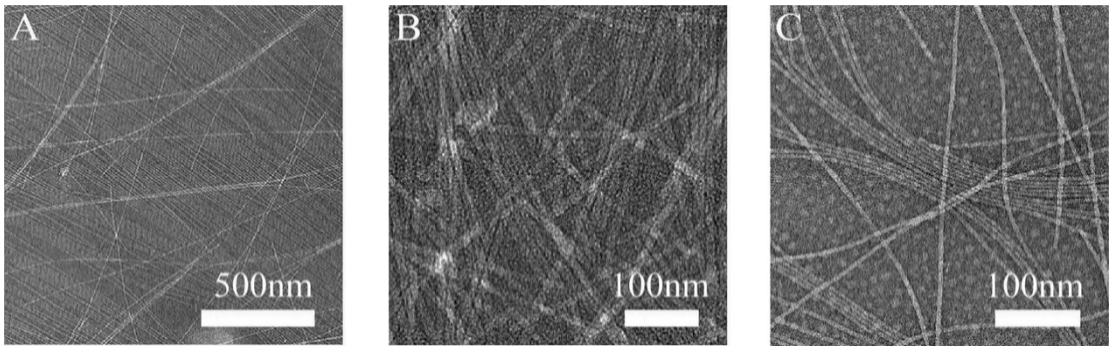


Figure S9. TEM micrographs of 20-OEG-SN38 at a concentration of 1 mM.

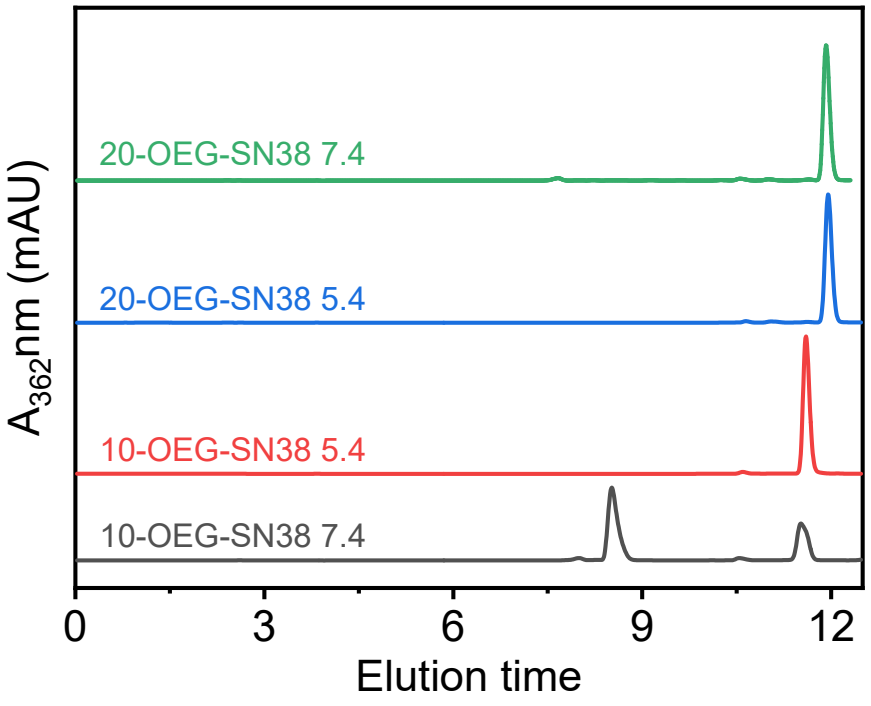


Figure S10. 10-OEG-SN38 and 20-OEG-SN38 after hydrolyzed for 1 h (pH 7.4/5.4).

References:

(1) Su, H.; Zhang, W.; Wang, H.; Wang, F.; Cui, H. Paclitaxel-Promoted Supramolecular Polymerization of Peptide Conjugates. *J. Am. Chem. Soc.* **2019**, *141* (30), 11997–12004.

(2) Cheetham, A. G.; Zhang, P.; Lin, Y.; Lock, L. L.; Cui, H. Supramolecular Nanostructures Formed by Anticancer Drug Assembly. *J. Am. Chem. Soc.* **2013**, *135* (8), 2907–2910.

(3) Liang, C. H.; Chen, Y. X.; Zheng, D. B.; Xu, T. Y.; Pu, G. J.; Chen, Y. M.; Wang, L.; Yang, Z. M.; Wang, H. M. Pathway-Dependent Supramolecular Polymerization of Camptothecin Derivatives into Filaments for Chemotherapy and Imaging. *Appl. Mater. Today* **2020**, *20*, 100787.