# Supporting Information for

# Redox responsive self-assembled chain-shattering polymeric therapeutics (CSPT)

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## MATERIALS AND METHODS

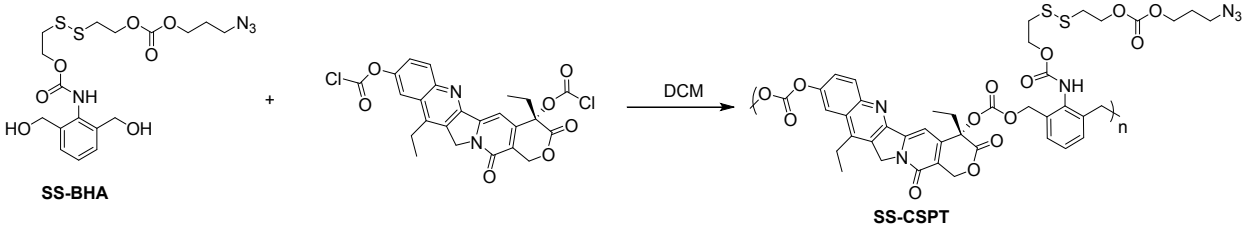
**Materials**. Chemicals were used as received unless otherwise specified. Anhydrous dimethylformamide (DMF) was dried with a column packed with 4Å molecular sieves. Tetrahydrofuran (THF) and methylene chloride (DCM) were dried with a column packed with alumina. Synthesis of SS-BHA and CPT-SS-CPT is reported elsewhere.[1] SN-38, sodium azide, *N,N*-dimethylamino pyridine, dithiothreitol (DTT), triphosgene, $mPEG_{2k}$-OH, CuBr, PMDETA (*N,N,N′,N″,N″*-Pentamethyldiethylenetriamine) were purchased from Sigma-Aldrich (St. Louis, MO,USA). Irinotecan hydrochloride trihydrate was purchased from LC Laboratory (Woburn, MA, USA). *In situ* cell death detection kit (TMR red) was purchased from Roche Diagnostics (Indianapolis, IN, USA). The HeLa cells (ATCC, Manassas, VA, USA) used in the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 1000 units/mL aqueous penicillin G, and 100 μg/mL streptomycin at 37 ℃ under 5% $CO_2$ humidified atmosphere.

**Animals.** Female athymic nude mice were purchased from the National Cancer Institute (NCI, Frederick, MD, USA) and ovariectomized at the age of 21 days by the vendor. After arrival, mice were single-cage housed and had free access to food and water. The study protocol was reviewed and approved by the Illinois Institutional Animal Care and Use Committee (IACUC) of the University of Illinois at Urbana Champaign.

**Instrumentation.** NMR spectra were recorded on Varian U500 (500 MHz) or VXR-500 (500 MHz) spectrometer. All chemical shifts were reported in part per million (ppm). Tandem gel

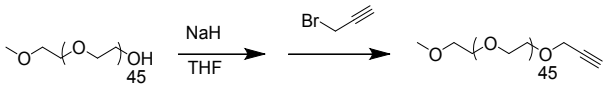
permeation chromatography (GPC) was performed on a system equipped with an isocratic pump (Model 1200, Agilent Technologies, Santa Clara, CA, USA), a DAWN HELEOS multi-angle laser light scattering detector (MALLS); (Wyatt Technology, Santa Barbara, CA, USA), and an Optilab rEX refractive index detector (Wyatt Technology). The detection wavelength of the HELEOS was set at 658 nm. Separations were performed on serially connected size exclusion columns (100 Å, 1000 Å, $10^4$ Å, $10^5$Å and $10^6$ Å Phenogel columns, 5 μm, 300 × 7.8 mm, Phenomenex, Torrance, CA, USA) at 60 °C with DMF containing 0.1 M LiBr as the mobile phase. The HELEOS detector was calibrated with pure toluene without using external polymer standards and was used for the determination of the absolute molecular weights. The molecular weight of polymer was determined from the $d_n/d_c$ value calculated assuming 100% mass recovery, and was processed by ASTRA software (Version 6.1.1, Wyatt Technology). Nanoparticle sizes and polydispersities were measured with a ZetaPlus dynamic light scattering (DLS) detector (15 mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY, USA). HPLC was performed on a Shimadzu HPLC system (LC-20AT, C18, 50 mm × 4.6 mm, 3 μm, Shimadzu) or Gold system (Beckman Coulter, Fullerton, CA, USA) equipped with a 126P solvent module, a System Gold 128 UV detector, and an analytical C18 column (Luna C18, 250 mm × 4.6 mm, 5 μm, Phenomenex). The UV wavelength for detecting camptothecin and SN-38 was set at 369 nm and 380 nm, respectively. TEM samples were prepared on 200 mesh carbon film supported copper grids. One drop of the nanoparticle solution (~ 10 μL, 100 μg/mL) was placed on the grid and allowed to stand for 10 min. Filter paper was then used to remove the

residual solution. The resulting sample was imaged using JEOL 2100 Cryo TEM at 200 kV. Lyophilization was conducted on a Labconco FreeZone lyophilizer (Kansas City, MO, USA). MTT absorption was measured on Perkin Elmer Victor3 multi-label readers at $\lambda_{abs}$ = 570 nm. Fluorescent images of tumor sections were collected by Nanozoomer Digital Pathology System (Hamamatsu). Infrared spectra were recorded on a Perkin Elmer 100 serial FTIR spectrophotometer calibrated with polystyrene film.




**Scheme S1.** Synthesis of **SS-CSPT**

**CSPT polymerization**. A DCM suspension (3 mL) of SN-38 (39.2 mg, 0.10 mmol), triphosgene (20.8 mg, 0.070 mmol, 0.70 equiv) and DMAP (61 mg, 0.50 mmol, 5.0 equiv) was stirred at room temperature for 15 minutes, during which the SN-38 dissolved gradually. Then 3 mL DCM solution of **4** (46.1 mg, 0.10 mmol, 1.0 equiv) was added once. The solution was stirred at room temperature for 48 hours. The solution was then poured into 10 mL ethyl ether. The light yellow precipitate was collected by centrifuge and washed with 1% HCl contaning methanol (10 mL) and methanol (5 mL × 3), dried under vacuum giving a light yellow powder (47 mg, yield 52 %). $d_n/d_c$ = 0.1430, $M_n$ = 3.0 kDa, PDI = 1.53.



**Scheme S2.** Synthesis of $mPEG_{2k}$-alkyne.

**Synthesis of $mPEG_{2k}$-alkyne.** To a THF solution of $mPEG_{2k}$-OH (2.0 g in 50 mL) was added 48 mg NaH (2.0 mmol, 2.0 equiv) in one portion and the suspension was stirred at room temperature for 45 minutes. Then propargyl bromide (238 mg, 172 μL, 2.0. mmol, 2.0 equiv) was added and the solution was stirred at room temperature overnight. MeOH was added to quench the reaction and the clear solution was subject to flash column (neutral alumina, EtOAc:MeOH 3:1) giving a colorless oil. The crude product was dissolved in 10 mL DCM and precipitated in 40 mL $Et_2O$, collected by centrifugation, reprecipitated in DCM/MeOH twice giving a white powder (1.05 g, yield 50 %) after being dried under vacuum. $d_n/d_c$ = 0.0352, $M_n$ = 1.96 kDa, PDI = 1.06.

**PEGylation of CSPT by click reaction**. 20.6 mg SS-CSPT was mixed with 8.2 mg $mPEG_{2k}$-alkyne, 3.0 mg CuBr and 3.0 μL PMDETA in 1.0 mL DMF in a glovebox and stirred at room temperature for 72 hours. The solution was then burbled with air for 10 minutes to fully oxidized the Cu(I) to Cu(II) salt. 4.0 mL $H_2O$ was added to dilute the solution and dialyzed against 2 L dilute EDTA (aq). The solution was changed to 2L $H_2O$ every 6-12 hours (3 times altogether). The final solution was lyophilized to obtain a yellow powder as the product (28 mg, yield 97 %). Due to distinct $d_n/d_c$ difference between the two GPC peaks, the absolute $M_w$ calculation is not accurate by combining light scattering (LS) and reflective index (dRI) results. Considering the high yield and quantitative modification efficiency shown by GPC, we calculated the $M_w$ from the fed CSPT-PEG ratio and the starting CSPT: $M_n$ = (2.5+1.0)/2.5*3.0 kDa = 4.2 kDa.

**Nanoparticle Preparation**. SS-CSPT-PEG polymer was first dissolved in DMF and 50 μL solution was added dropwise into 2.0 mL DI water (or phosphate buffered saline) with mild stirring using a magnetic bar. Nanoparticle size and distribution was analyzed by DLS directly without further purification.

**Nanoparticle stability test**. The freshly prepared CSPT NP solution was diluted with $H_2O$ or PBS 10 fold to test its stability upon dilution and salt effect. The size and distribution of the NPs was measured by DLS. No significant size change was observed over 24 hours.

**Drug loading measurement**. The freshly prepared NP solution was centrifuged at 1000 rpm for 10 minutes to remove large aggregates. Then 300 μL supernatant was mixed with 300 μL 2 M NaOH (aq) and incubated at 37 ℃ overnight. Then 300 μL 2 M HCl (aq) was added to neutralize the solution and the clear solution was subject to HPLC analysis after being diluted to around 5 μg/mL. Calibration curve was obtained by area integration of standard samples ($\lambda_{abs}$ = 380 nm). Drug loading of the PEGylated CSPT polymer was determined to be 18 % by the same method. Drug loading efficiency was calculated as apparent drug loading of nanoparticle divided by 18%.

**Camptothecin (CPT) release from free CPT-SS-CPT in DMF/PBS**. 8.0 mL 50 μg/mL CPT-SS-CPT in 1:1 DMF/PBS solution was added 22 mg DTT at room temperature and the CPT release was monitored by HPLC. CPT release was quantified by integration of the peak at the same elution time as free CPT. The experiments were carried out in triplicates and the release profile was presented as mean value ± standard deviation.

**SN-38 release from SS-CSPT NP**. The SS-CSPT NPs prepared from 10 mg/mL DMF solution

was diluted with PBS to 50 μg/mL (6.9 μg/mL equiv SN-38 according to drug loading and encapsulation efficiency calculation). Then specified concentration of DTT was added and the solution was separated into 1 mL volume in separate tubes at 37 ℃. At designated time, one tube was centrifuged at 15000 rpm for 5 minutes to remove particles and the supernatant was analyzed by HPLC and quantified by area integration ($\lambda_{abs}$ = 380 nm).

**Cytotoxicity evaluation of SS-CSPT NP**. Standard MTT protocol was followed to evaluate the cytotoxicity of the CSPT NPs. Briefly, HeLa cells were seeded in 96-well plate at 1000 cell/well in 100 μL medium and were allowed to attach overnight. Free SN-38 and irinotecan was first dissolved in DMSO and diluted with PBS to the concentration desired. For the highest drug concentration tested, the DMSO content was 5% for SN-38 and 1% for irinotecan. CSPT NPs were formulated in PBS from 10 mg/mL PEGylated CSPT DMF solution through nanoprecipitation, diluted with PBS and sterilized by filtration through 0.45 μm PVDF membrane. 10 μL drug/nanoparticle solution was added into the well to the designated final concentration and incubated at 37 ℃ for 72 hours. PBS and 5% DMSO-PBS were taken as 100% control. 20 μL 5 mg/mL MTT solution was added to the medium and incubated at 37 ℃ for 3 hours. Then the medium was carefully removed and the violet crystal was dissolved in 100 μL DMSO and quantified by absorption at $\lambda_{abs}$ = 570 nm.

***In vivo* efficacy studies in a xenograft tumor model**

*Acute antitumor efficacy study in athymic nude mice bearing subcutaneously implanted MCF-7 human breast tumors.* Female athymic nude mice, 8-week old, were prepared for implantation of

the tumor cells. MCF-7 cells were collected from culture, and $1 \times 10^6$ cells suspended in a 100 μL 1:1 mixture of Hank's Balanced Salt Solution (HBSS) buffer and matrigel were injected subcutaneously into the flanks of a mouse (four injection sites per mouse). After about 4 weeks when tumors had reached ~100 $mm^3$, mice were randomly divided into three groups that each had 2-3 mice, minimizing weight and tumor size difference among the groups. Tumor-bearing mice of the corresponding group were treated three times (every four days) by PBS (200 μL, i.v. injection), CSPT NPs (in 200 μL PBS, i.v. injection), irinotecan (in 200 μL PBS, i.p. injection), respectively. The experimental time line and injection protocol are summarized in Fig. S4. All the mice were sacrificed on Day 11. All the tumors were collected and embedded in OCT except that one piece of tumor in each mouse was excised and subject to H&E staining. Cell apoptosis was analyzed using *in situ* cell death detection kit (Roche Diagnostics GmbH, Mennheim, Germany) and TUNEL staining was performed following the manufacture's procedure. The cell nuclei of the sections were stained with Hoechst (blue fluorescence) and the positive apoptotic cell nuclei DNA fragments were stained by TUNEL (red fluorescence). The tissue sections were imaged with Nanozoomer Virtual Microscopy (Hamamatsu). The collected images were then created into stack using the InCell Translator software. The stacks were then analyzed using the GE InCell Analyzer workstation. The nuclei were identified using top-hat segmentation in the DAPI channel and the numbers of cells were then calculated. The identified nuclei were further segmented by multiscale top-hat in the red signal to determine the number of cells with positive red signals (apoptotic cells). The cell death number was then determined by dividing the number

of apoptotic positive cells by the total number of cells. Data are represented as average ± SEM (standard error of the mean) and are analyzed by two-tailed Student t test (*$p$<0.05).

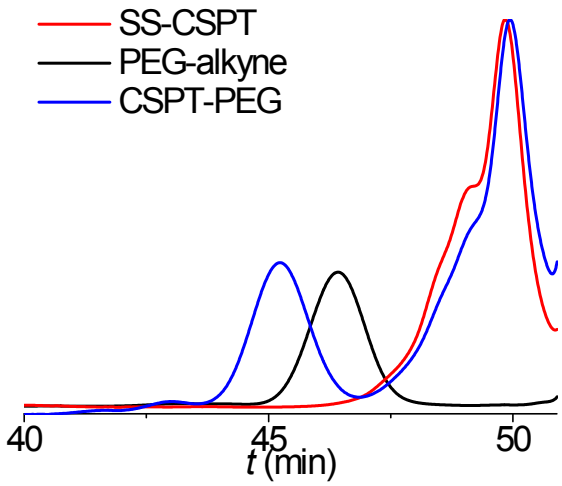



Figure S1. GPC trace of SS-CSPT and SS-CSPT-PEG.

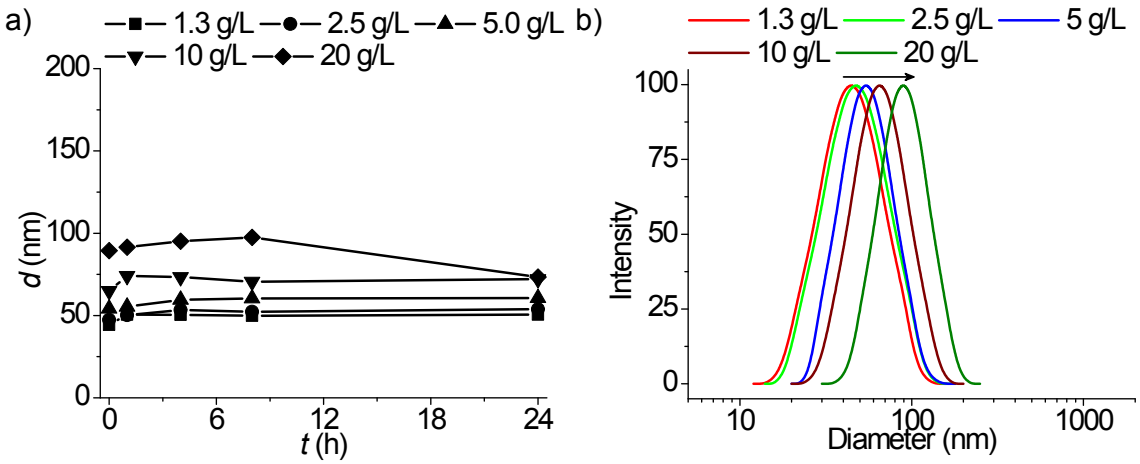



Figure S2. (a) CSPT nanoparticles size change over time as measured by DLS. (b) Size distribution of CSPT NP formulated at different concentrations. Particle size was monitored by DLS without further purification.

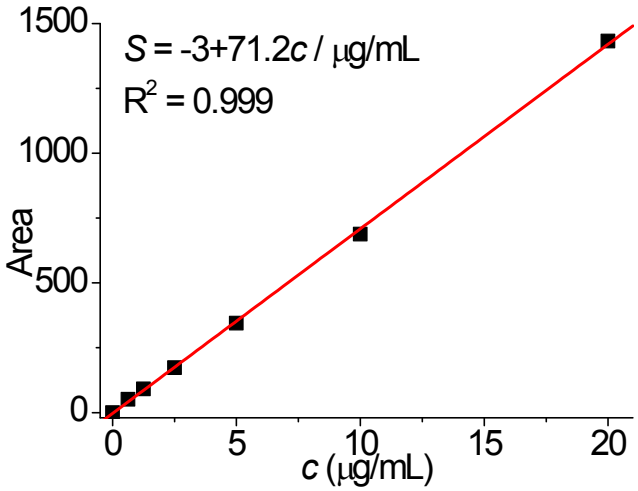



Figure S3. Standard HPLC calibration curve of SN-38 at $\lambda_{abs}$ = 380 nm.

| *c* (mg/mL) | *d* (nm) | PDI | EE (%)[a] |
|---|---|---|---|
| 1.25 | 44 | 0.22 | 87 |
| 2.5 | 47 | 0.23 | 89 |
| 5.0 | 54 | 0.15 | 79 |
| 10 | 65 | 0.16 | 78 |
| 20 | 89 | 0.13 | 86 |

Table S1. DLS characterization of CSPT NPs formulated from different DMF solution. [a]EE stands for encapsulation efficiency.

Figure S4. (a) Schematic illustration of the timeline of the acute antitumor efficacy study. (b) Body weight change of mice after administration of PBS (i.v.), Irinotecan (i.p. 50 mg/kg) and CSPT NPs (i.v. 20 mg/kg equiv SN-38). (c) Apoptosis index of subcutaneously implanted MCF-7 tumors after administration of PBS, irinotecan and CSPT NPs. * $p < 0.05$.

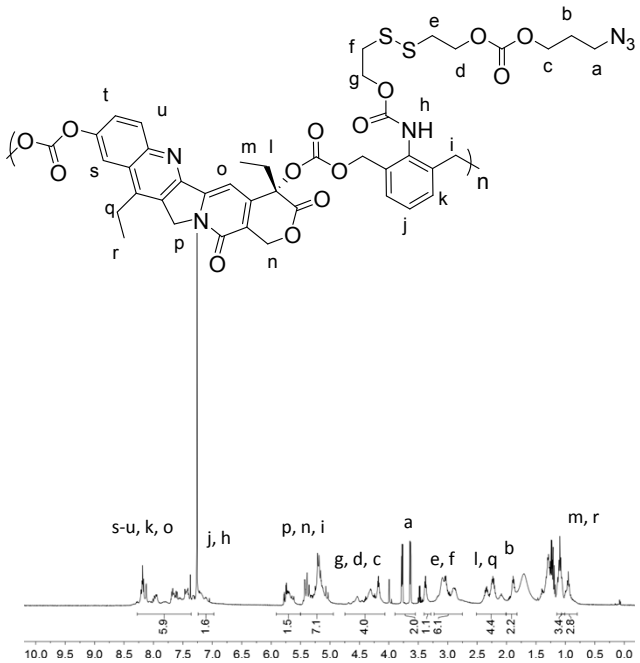



Figure S5. $^{1}$H NMR of SS-CSPT

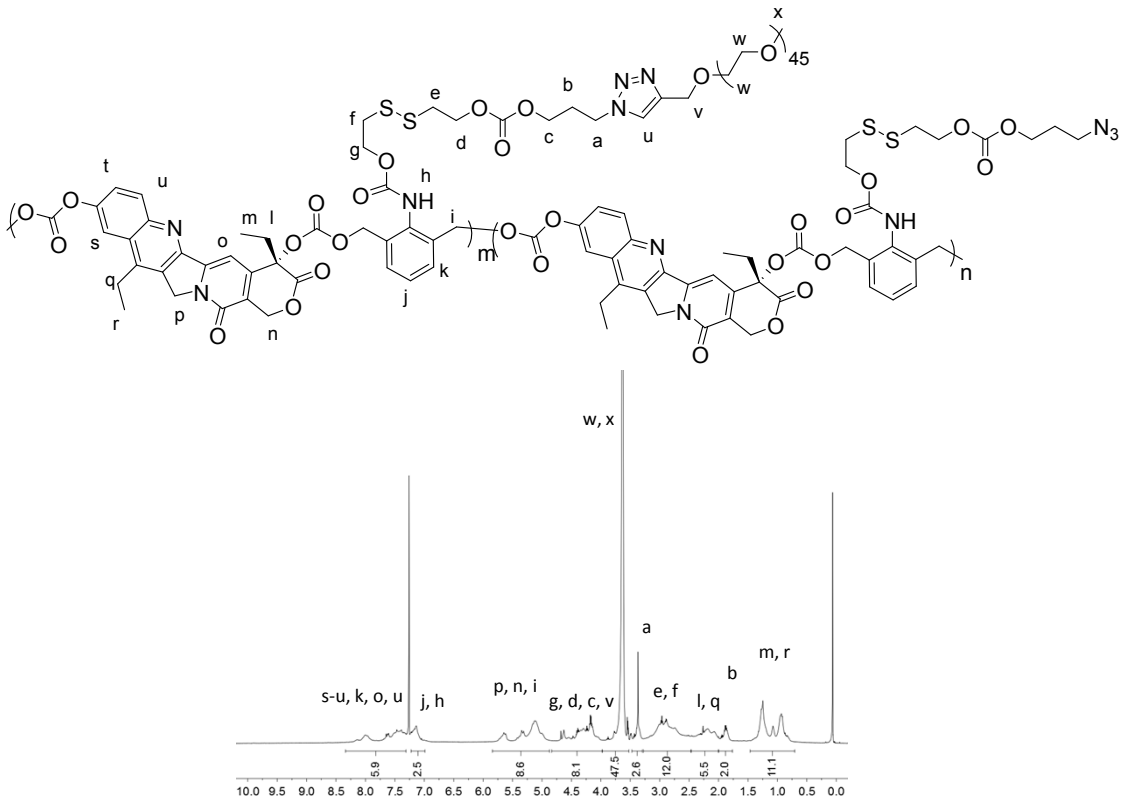



Figure S6. $^{1}$H NMR of SS-CSPT-PEG

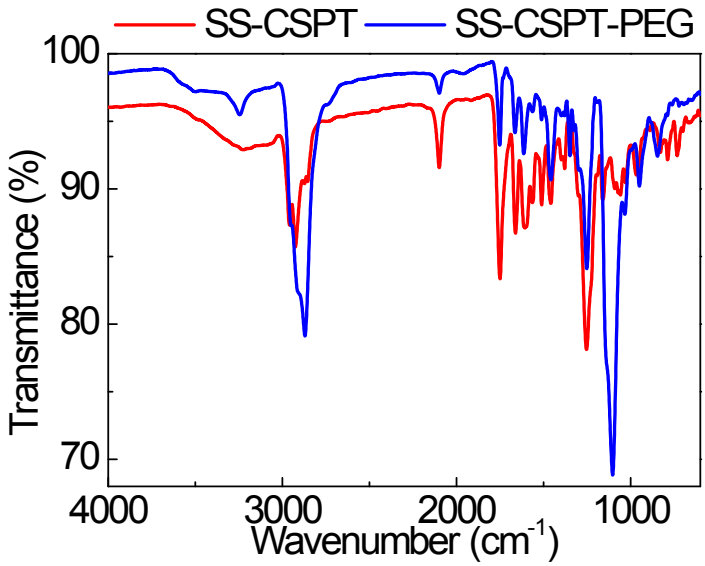



Figure S7. IR spectrum of SS-CSPT and SS-CSPT-PEG. Note the azide peak (2100 $cm^{-1}$) is present in both polymers while the characteristic peaks of triazole of SS-CSPT-PEG (~1600 $cm^{-1}$, 1470 $cm^{-1}$) overlap with the SS-CSPT peaks.

## REFERENCE

1. K. Cai, Y. Zhang, Z. Song, and J. Cheng, Dimeric Drug Polymeric Nanoparticles with Exceptionally High Drug Loading and Quantitative Loading Efficiency. Unpublished.