Electronic Supplementary information

Carbonate Ester Turn Camptothecin-unsaturated Fatty Acid Prodrug into Nanomedicine for Cancer Therapy

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Experimental Section

Materials and animals

Oleic acid was obtained from Sigma-Aldrich (USA). Dithiodiglycolic acid, stearyl alcohol and hexanediol were purchased from TCI reagents (Tokyo, Japan). 2,2-Dithiodiethanol and dicyclohexylcarbodiimide (DCC) were purchased from Alfa Aesar (MA, USA). Oleic alcohol, linoleic acid, 4-dimethylaminopyridine (DMAP), triphosgene and stearic acid were purchased from Adamas Reagent, Ltd (China). The CPT and dithiothreitol (DTT) was purchased from MEILUN Biology Technology Co., LTD. (Dalian, China). The DSPE-mPEG$_{2000}$ was purchased from Lipoid GmbH (Ludwigshafen, Germany). All solvents used in this study were analytical grade.

Male C57 mice (6–8 weeks old) were purchased from the Laboratory Animal Center of Sichuan University (Chengdu, China). All studies involving mice were approved by the institute's animal care and use committee.

Synthesis of intermediate OA-PrSS-COOH

Intermediate OA-PrSS-COOH was synthesized according to the previous literature [1]. Dithiodipropionic acid (1.5 g) was dissolved and stirred in 20 ml anhydrous DMF at 0 °C under N_2 for 5 min, followed by addition of DCC (1.2 molar equiv.) and stirring for a further 20 min at 0 °C. Then catalytic quantity of DMAP (50 mg) and the oleic alcohol (1 molar equiv.) were added, and the reaction mixture was stirred at room temperature overnight. After the DMF was removed under vacuum, 50 ml ethyl acetate was added and the solution was filtered to remove dicyclohexylurea (DCU). The filtrate was reduced under the reduced pressure to obtain the crude products, which was purified by silica gel column chromatography (200 ml petroleum ether (PE), 100 ml PE/ethyl acetate (EA) 5:1, 100 ml 4:1 and 200 ml 2:1 (v/v)) to give OA-PrSS-COOH, yield 76 % (Fig.S1).

Synthesis of intermediate OA-acSS-COOH

Intermediate OA-acSS-COOH was synthesized according to the previous literature [2]. Dithiodiglycolic acid (1.5 g) was added in the 5 ml anhydrous acetic anhydride, the mixture was stirred for 3 h at 80 °C. The solution was then evaporated to dryness under high vacuum at 80 °C. The residue was dissolved in 2 ml anhydrous DCM. The solution was added into the oleic alcohol with a catalytic amount of DMAP (50 mg) in the 20 ml DCM, stirring at room temperature for 1 h. The OA-acSS-COOH was purified by silica gel column chromatography using the same procedure as described in "Synthesis of intermediate OA-PrSS-COOH", yield 81 % (Fig.S1).

Synthesis of intermediate LA-etcSS-OH, SA-etcSS-OH, OA-etcSS-OH and OA-hex-OH

The fatty acid (1.2 g) was dissolved and stirred at 0 °C in 20 ml anhydrous DCM under N_2 for 5 min, the DCC (1.2 molar equiv.) was added and stirred for 20 min at 0 °C. 2,2-Dithiodiethanol (90%, 2 molar equiv.) and catalytic quantity of DMAP (50 mg) was added and stirred at the room temperature overnight. After the DCM was removed, the 30 ml ethyl acetate was added and the solution was filtered to remove DCU. The filtrate was reduced under the reduced pressure to obtain the oil products, which was purified by silica gel column chromatography (400 ml PE/EA 5:1) to obtain the corresponding LA-SS-OH, SA-SS-OH and OA-SS-OH (yield ~30 %) (Fig.S1). The OA-hex-OH was synthesized using the above procedure by replacing the 2,2-Dithiodiethanol with 1,6-hexanediol.

Synthesis of CPT-prSS-OA, CPT-acSS-OA and CPT-est-OA

In a 25 ml round bottom flask, 100 mg intermediate OA-acSS-COOH, OA-PrSS-COOH and OA were dissolved in anhydrous DCM (10 ml) under N_2, respectively. DCC (1.2 molar equiv.) was added and stirred for 20 min at 0 °C. Then, CPT (1.0 molar equiv.) and catalytic quantity of DMAP (1 mg) were added and stirred 35 °C overnight. After the DCM was removed, the 20 ml ethyl acetate was added, and the solution was filtered to remove DCU. The filtrate was reduced under reduced pressure to obtain the crude products, which was purified by silica gel column chromatography (400 ml Methanol/DCM 1:80) to give corresponding CPT-prSS-OA, CPT-acSS-OA and CPT-est-OA (~yield 10 %) (Fig.S2).

Synthesis of CPT-etcSS-OA, CPT-etcSS-LA, CPT-etcSS-SA and CPT-hex-OA

CPT (100 mg) and DMAP (2.0 molar equiv.) were dispersed in 20 ml anhydrous DCM at 0 °C under N_2. Triphosgene (0.35 molar equiv.) in the anhydrous DCM was then added dropwise. After stirring at room temperature for 20 min, the LA-etcSS-OH, SA-etcSS-OH, OA-etcSS-OH and CPT-hex-OH (1.0 molar equiv.) was added into the solution. The solution was stirred at room temperature overnight. The solution was then evaporated to dryness and purified by silica gel column chromatography (400 ml Methanol/DCM 1:80) to give corresponding CPT-etcSS-OA, CPT-etcSS-LA, CPT-etcSS-SA and CPT-hex-OA (~yield 63 %) (Fig.S2).

Preparation of nanoaggregates based on CPT-lipids

CPT-lipid nanoaggregates were prepared according to the nanoprecipitation method. CPT-lipids were dissolved in the ethanol to obtain a clear solution. This solution was then added dropwise into distilled water under the agitation. Ethanol was then evaporated under vacuum at 40 °C. No addition of surfactant was done in any step of the preparation.

PEGylated nanoaggregates of CPT-lipid was prepared using the same procedure with the addition of DSPE-$mPEG_{2000}$ (10 % w/w) in ethanol solution.

Size and Zeta Potential

The size and zeta potential of various nanoaggregates were measured using dynamic light scattering (DLS) instrument (Nano-ZS90, Malvern, England) at 25 °C. Nanoaggregates were diluted by the distilled water before the measurement to adjust scattering light intensity to an acceptable level for measurement.

Morphology

Morphological evaluation of the nanoaggregate formed by CPT-lipid was done using scanning electron microscope (SEM, JSM-7500F, JEOL, Japan). The samples were coated with gold before observation.

Transmission electron microscopy (TEM, H-600, HITACHI, Japan) was utilized to further examine the nanoaggregate morphology of CPT-lipid. The diluted NP samples with CPT-lipid concentration of around 0.5 mg/ml were placed on a copper grid, stained with 2 % (w/v) phosphotungstic acid and dried at room temperature before observation.

In Vitro Hydrolysis Profiles of CPT from CPT-etcSS-OA

The ethanol solution of CPT-etcSS-OA were supplemented with 1 ml of ethanol-containing PBS (pH 7.4, 30 % ethanol (v/v), containing 10mM DTT or not) at the final CPT equivalent concentration of 20μg/ml in the sample vials. The solutions were incubated in a water bath at 37 °C. At the given time interval, 50μl solution was withdrawn for HPLC analysis (2695, Waters, America) with a photodiode array detector. The concentration of lactone and carboxylate form of CPT, relevant intermediate CPT analogue (CPT-SH) and CPT-lipid was determined.

Determination of critical aggregation concentration of CPT-lipid nanoaggregates

It is well known that there is sudden disappearance of scattered light once a nanoaggregates dissembled, therefore the intension of scattered light of CPT-lipid at various concentrations was detected by DLS to determine the critical aggregation concentration (CAC) below which the aggregations disassemble.

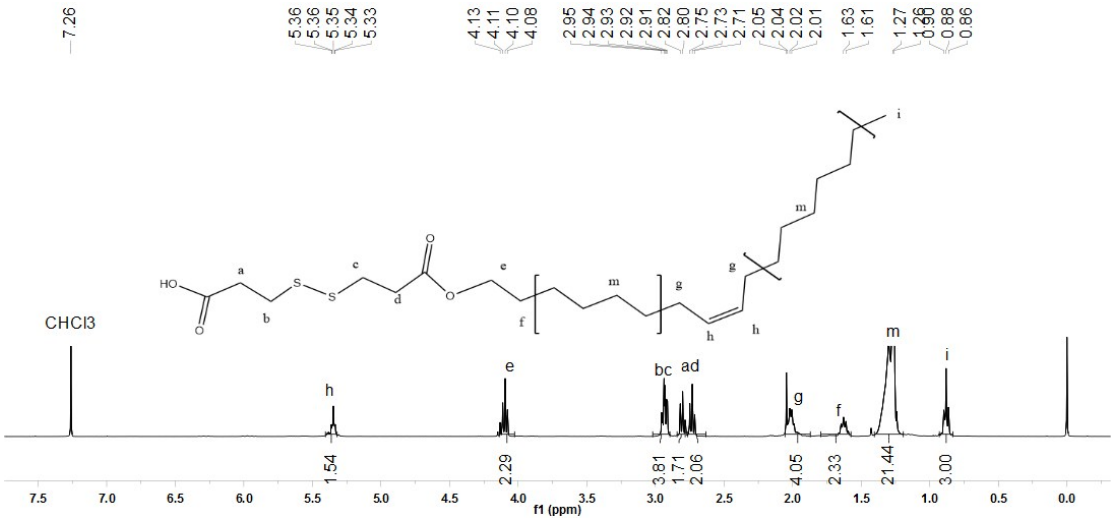
Cytotoxicity Assay

Lewis lung carcinoma (LLC) was maintained in DMEM containing 10 % FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5 % CO_2 at 37 °C. The cell viability was assessed by MTT assay. Briefly, LLC were seeded in a 96-well plate at a density of approximate 5000 cells per well. After 24 h of growth, the medium was exchanged for the medium that contained CPT and CPT-lipid nanoaggregate at various concentration. The cell was further incubated for 48 h, and these without any treatment were utilized as control.

In Vivo Antitumor Efficacy

A subcutaneous model of lung cancer was established by injecting LLC (2×10^6 cells per 100 μl) subcutaneously into the flank of each male C57 mouse. Six days later, the mice were randomly assigned to 3 groups (n = 6) and intravenously injected with saline, PEGylated CPT-etcSS-LA nanorod at a dose equivalent to 5 mg/kg and 10 mg/kg every two days for 5 times, respectively. Animal weight and tumor volume were measured every 2 days, and tumor volume was calculated using the formula (L × W2)/2, where L is the longest and W is the shortest diameter tumor. All the mice were sacrificed four day post the final injection, and all tumors were harvested and weighed.

Supporting Figures



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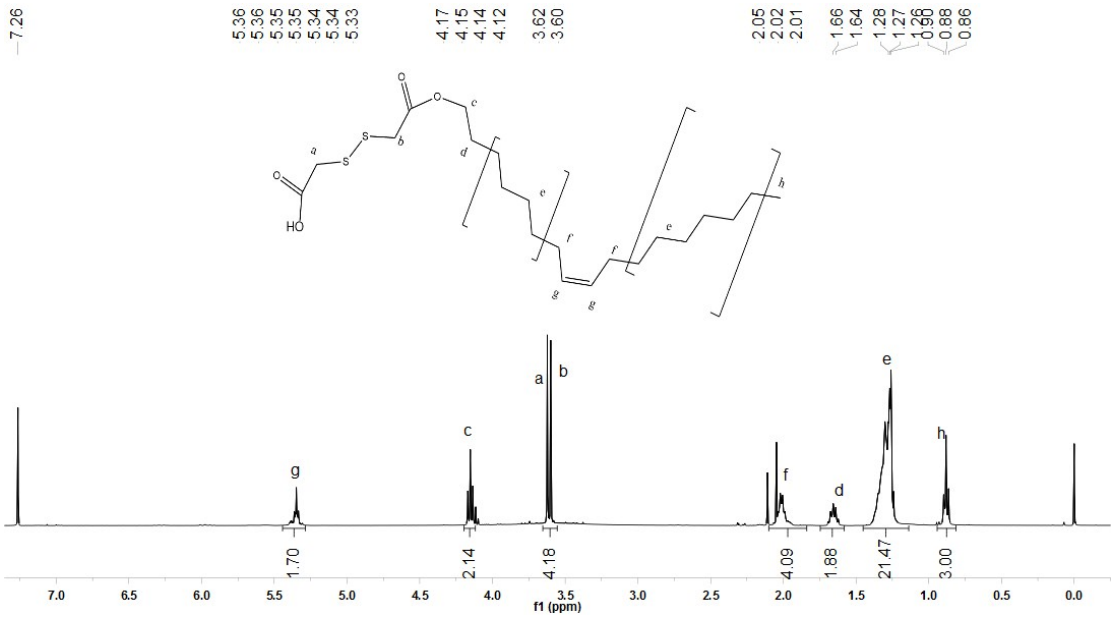

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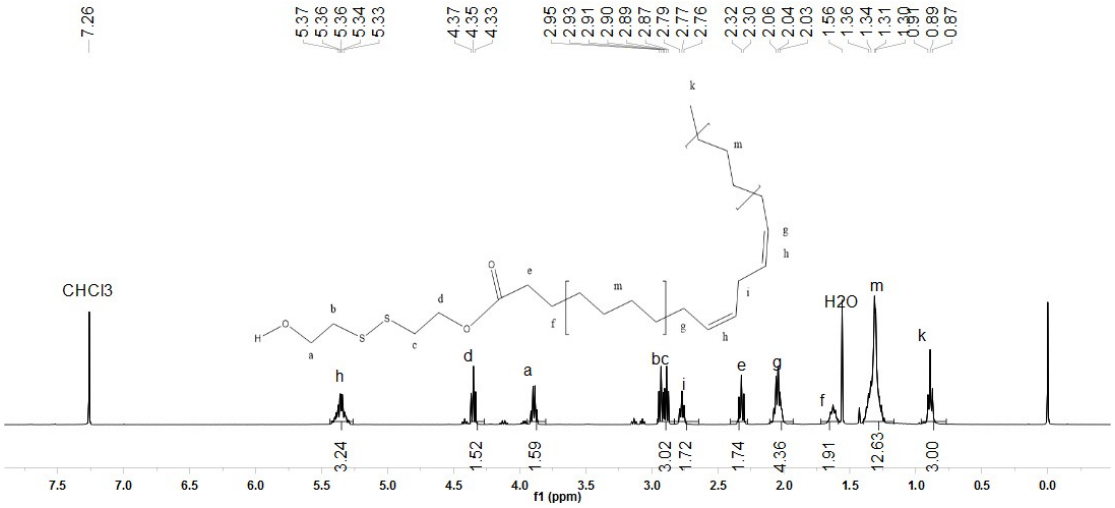

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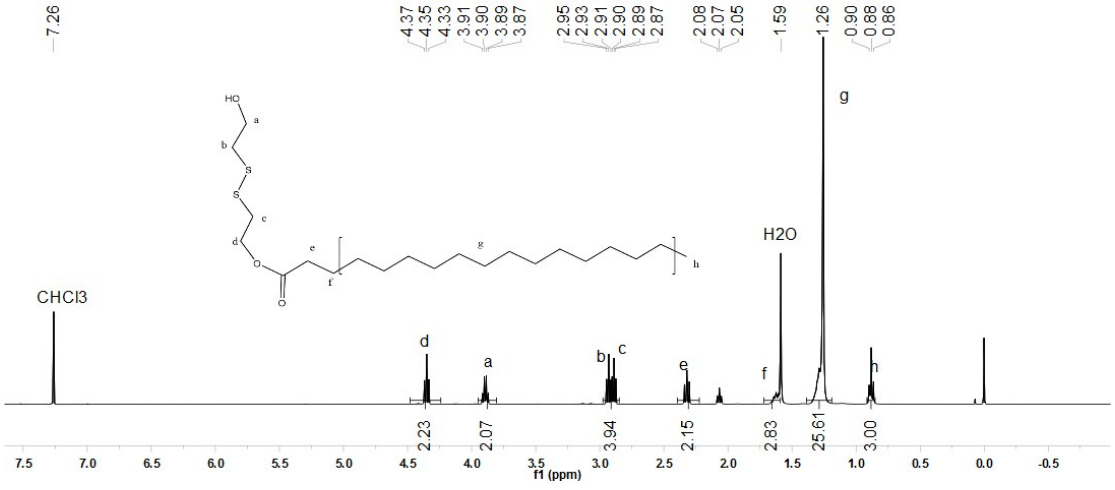

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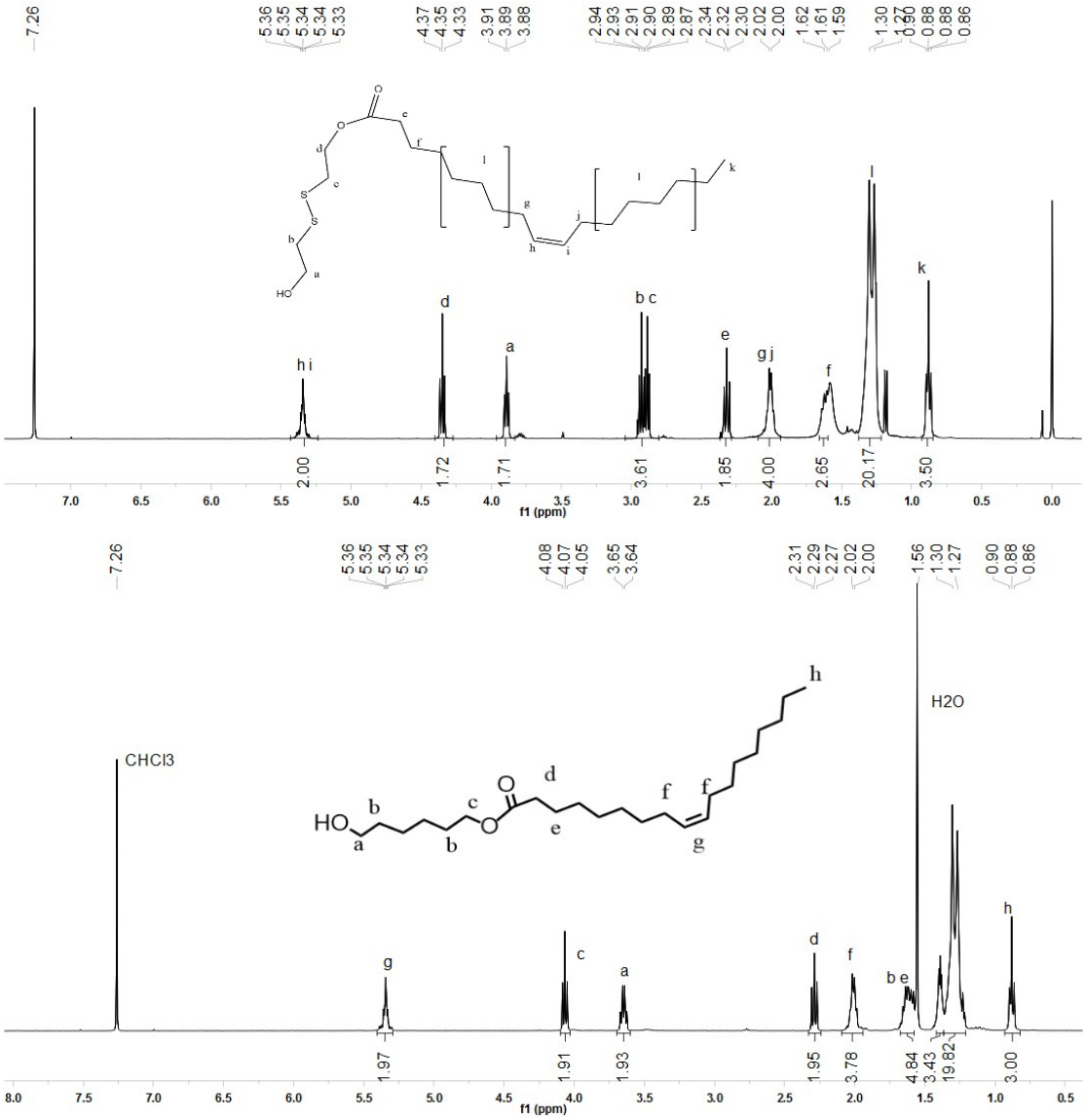
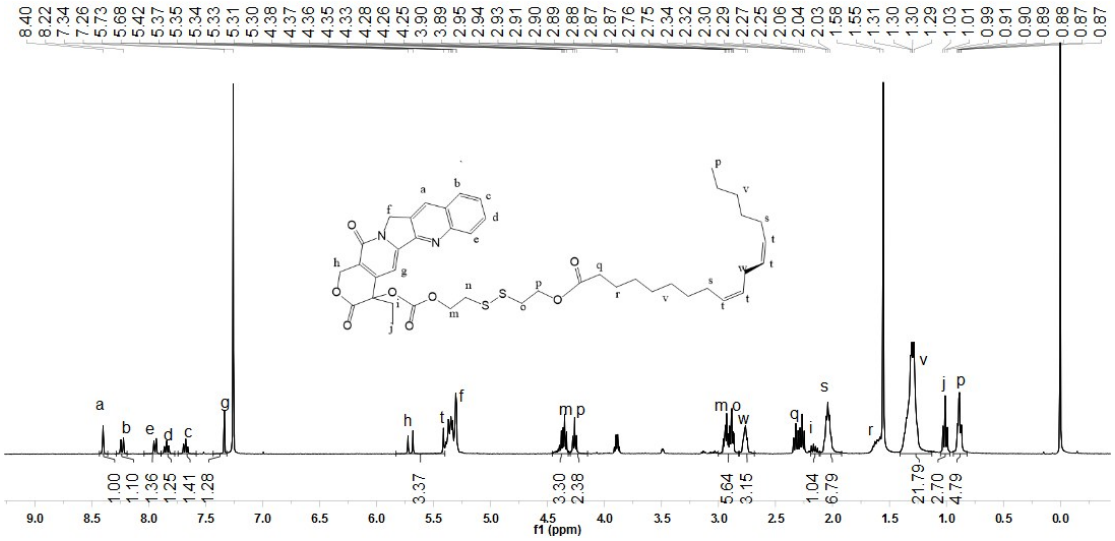


Fig.S1 ^{1}H NMR spectra of the OA-PrSS-COOH, OA-acSS-COOH, LA-etcSS-OH,SA-etcSS-OH，OA-etcSS-OH and OA-hex-OH in sequence.



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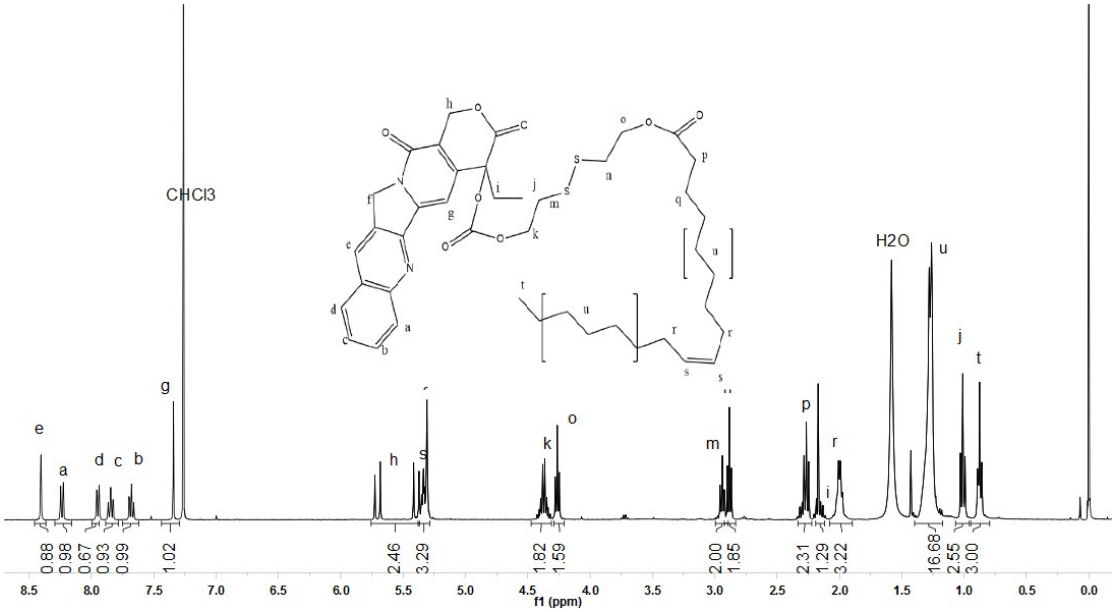

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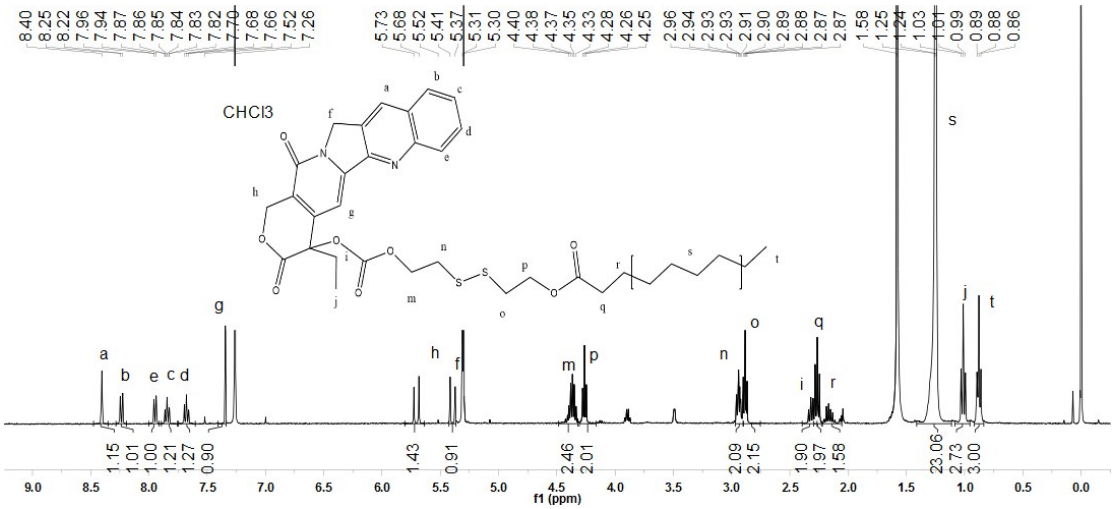

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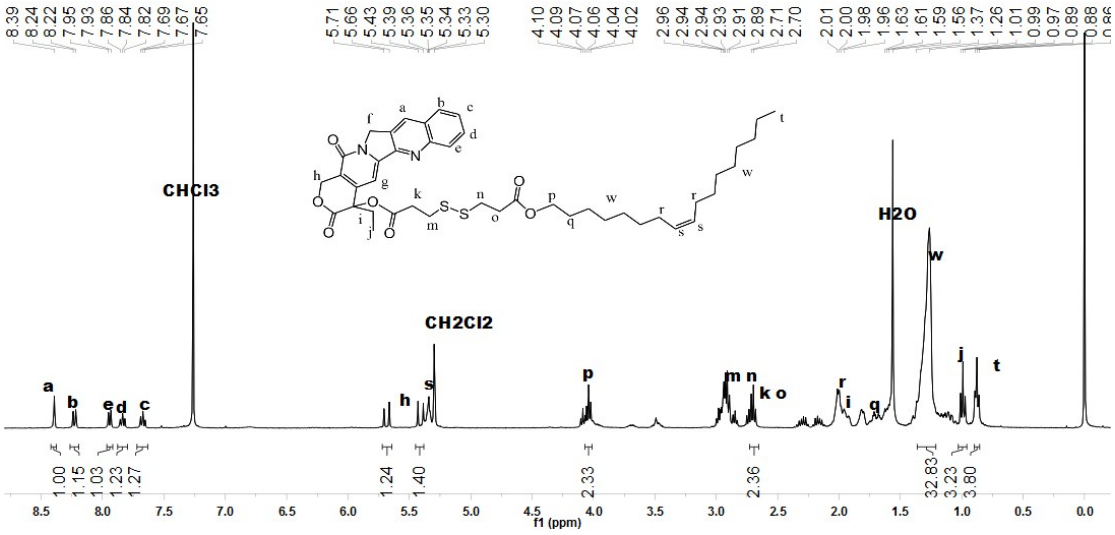

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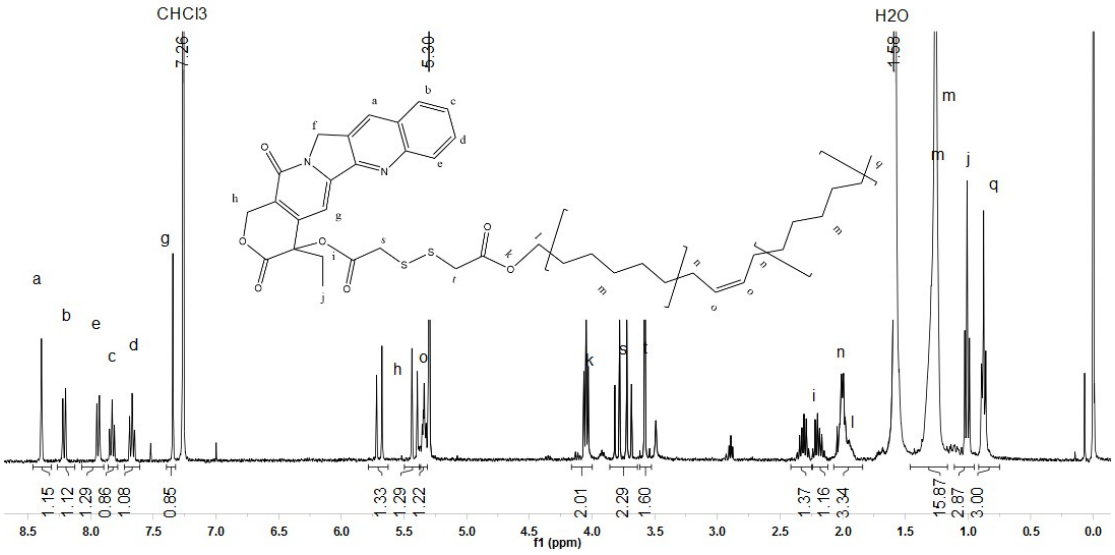

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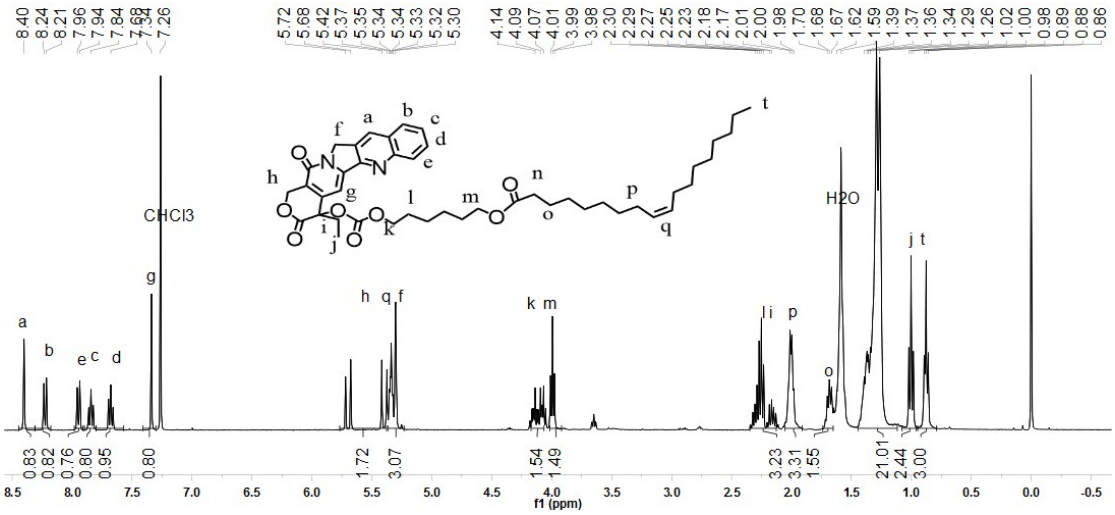

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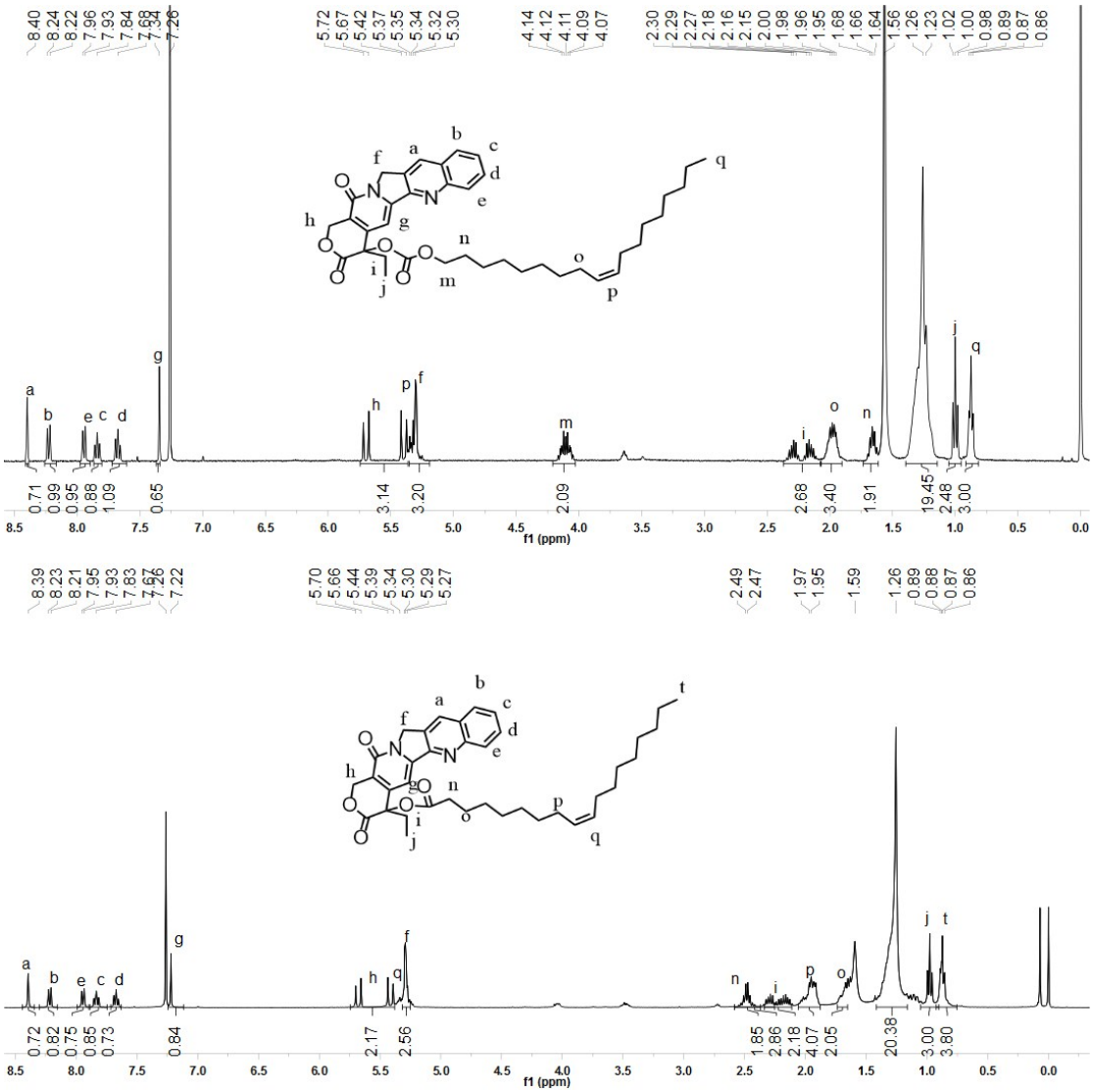


Fig.S2 ^{1}H NMR spectra of the CPT-etcSS-LA, CPT-etcSS-OA, CPT-etcSS-SA, CPT-prSS-OA, CPT-acSS-OA, CPT-hec-OA, CPT-car-OA and CPT-est-OA in sequence.

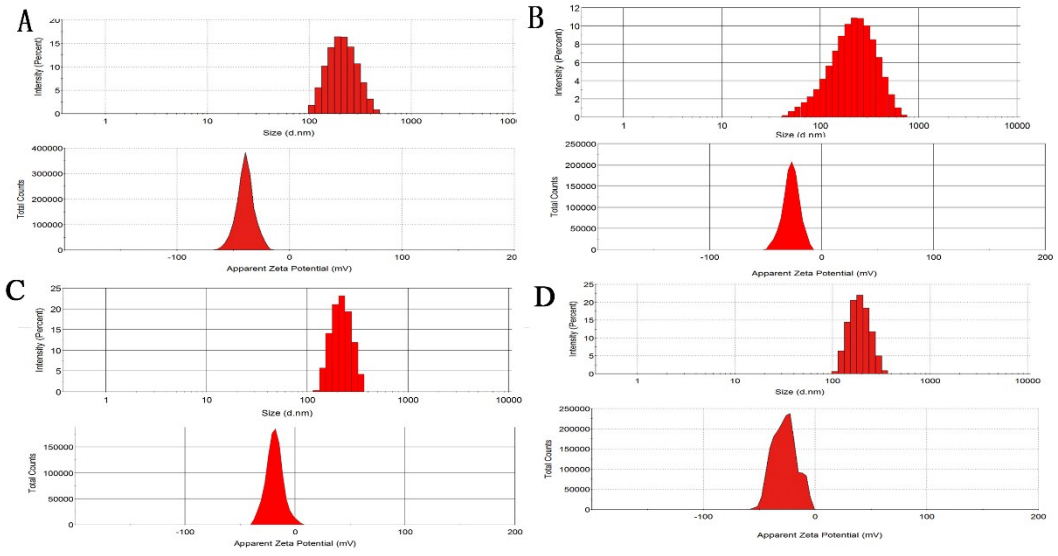
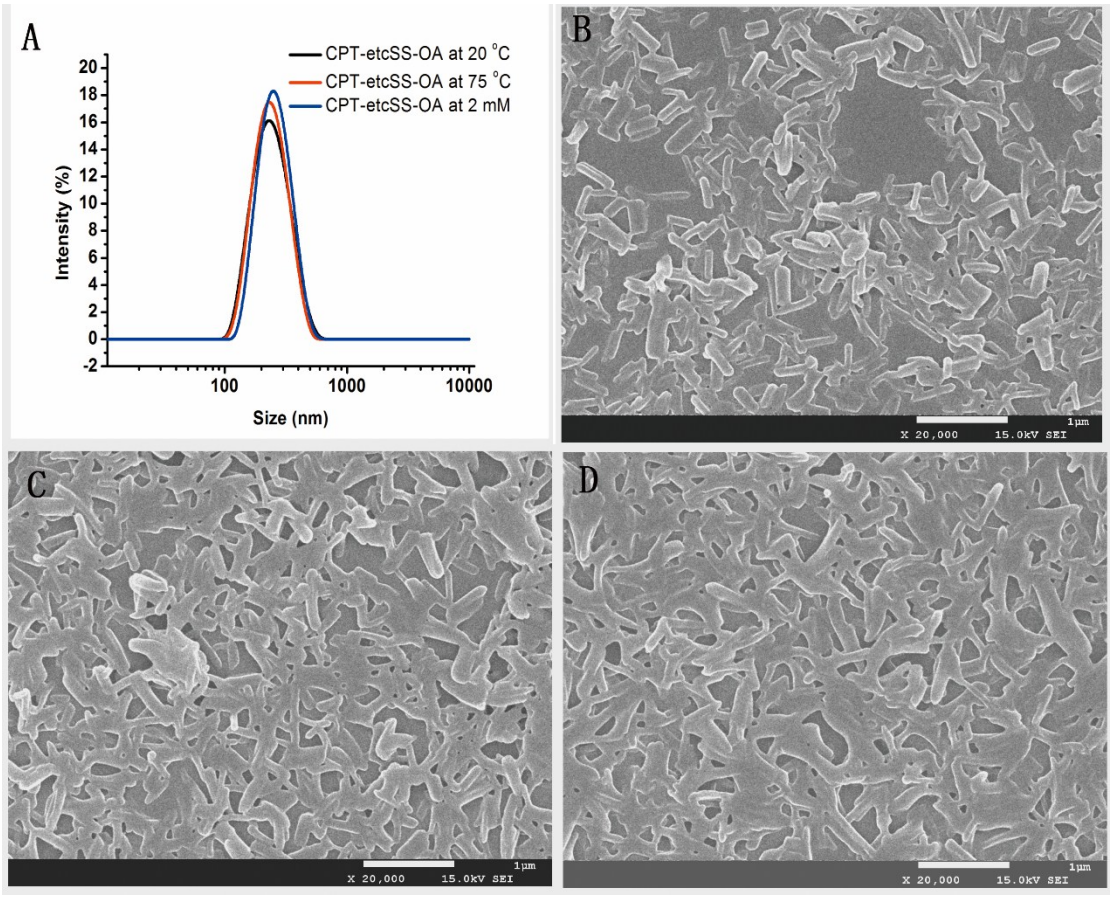


Fig.S3 Size distributions and zeta potentials of nanorod derived from the CPT-etcSS-LA (A), CPT-

143 hex-OA (B), CPT-car-OA (C) and CPT-etcSS-OA (D).



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145 Fig.S4 Size distribution (A) and SEM micrographs of CPT-etcSS-OA nanorod prepared by
146 dispersing the different concentrations of ethanol solution at the various temperatures: (B) 2
147 mM, 20° C; (C) 10 mM, 20° C (D) 10 mM, 75° C. It was revealed that the size distribution and
148 visual appearance of the nanorod obtained were almost the same irrespective of the different
149 concentration of the injected ethanol solution and temperature.

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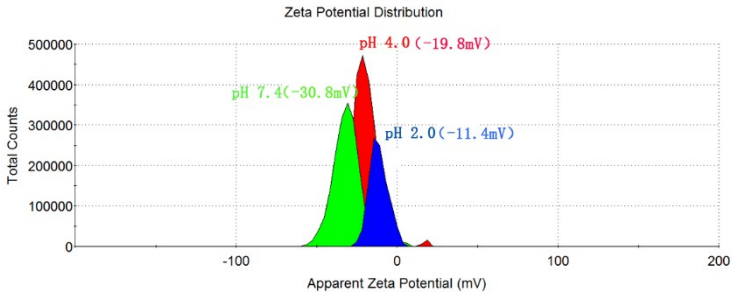

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152 Fig.S5 Effects of pH on zeta potentials of nanorod of the CPT-etcSS-LA.

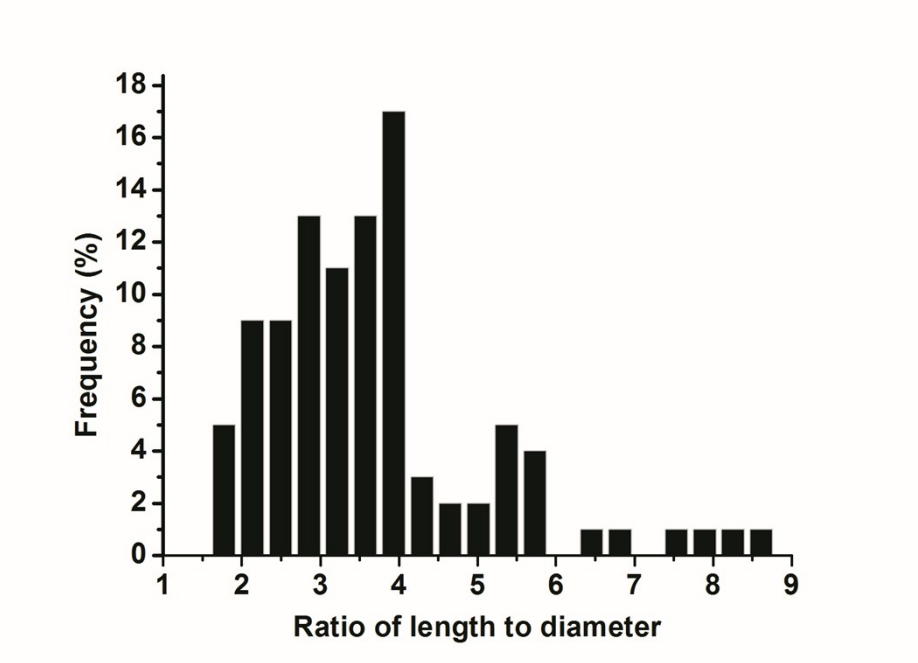

Fig.S6 The length/diameter ratio distribution of CPT-etcSS-LA nanorod, indicating that majority of nanorod displayed a ratio of length to diameter ranging 2 to 4.

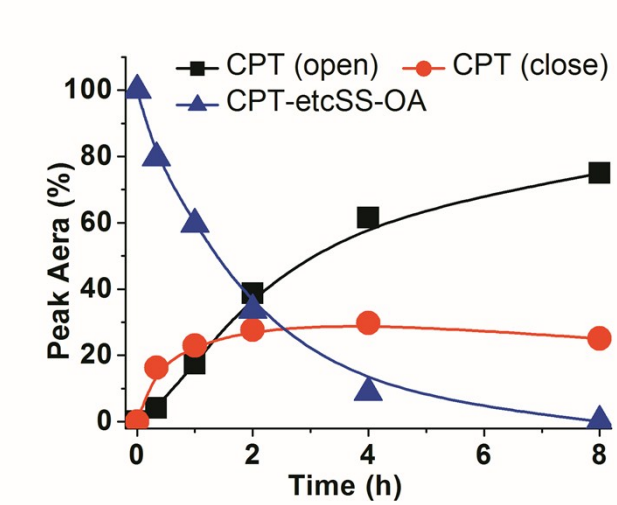

Fig.S7 HPLC analysis of level of CPT-etcSS-OA, CPT (open) and CPT (close) in the 10 mM PB at 37 ºC containing 10 mM DTT, the concentration of CPT (close) was higher than that of CPT (open) within the initial 1 h, suggesting that the CPT was mainly released in the active lactone CPT rather than the toxic and inactive carboxylate CPT.

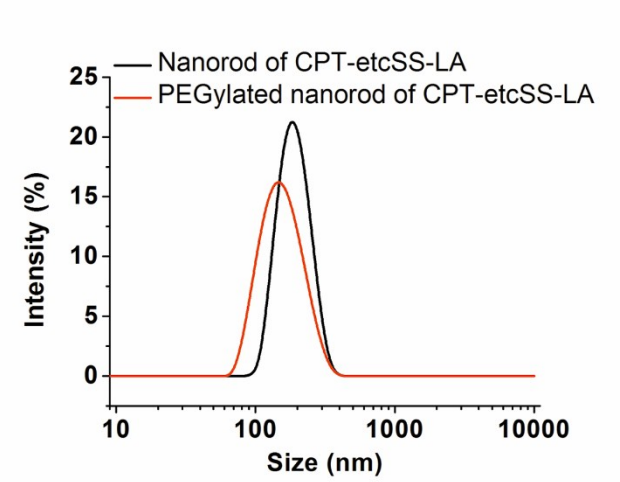

Fig.S8 The size distribution of CPT-etcSS-LA nanorod and PEGylated nanorod.

Table.S1 Comparison of tumor growth inhibition after intravenous injection of different CPT formulations.

Carrier[a]	Total dose (mg/kg)	Dose Schedule	Tumor Inhibition Rate	Weight Loss	Ref.
50%PDL-PPBS	20	10+1*5	90%	Not reported	[3]
EndoTAG®-2	15	6*2.5	80%	No significance	[4]
PLA-HPG/CPT	10	2*5	~85%	No significance	[5]
CPT-N-PLA10 NC	50	1*50	~ 62.5%	No significance	[6]
CPT-etcSS-LA nanorod	50	5*10	~85%	No significance	This work

[a]**50%PDL-PPBS:** CPT-loaded nanoparticles using poly(ω-pentadecalactone-co-butylene-co-succinate) containing 50% PDL units.
EndoTAGR®-2: DOTAP complexed camptothecin.
PLA-HPG/CPT: CPT-loaded hyperbranched polyglycerols (HPG)-PLA nanoparticles.
CPT-N-PLA10 NC: CPT-PLA conjugates linked by a facile hydrolysable amino ester.

Reference

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