Electronic Supplementary Information

Light-activated Drug Release From Prodrug Nanoassembles by Structure Destruction

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Experimental Section

Materials

Stearic acid, tert-butyl acrylate, 3-mercapto-1,2-propanediol, 1,7-octadiene, 1-hexylthiolan, mercapto acetic acid, thiodiglycolic acid, oxalyl chloride and ethylene glycol were purchased from TCI reagents (Tokyo, Japan). Dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) were purchased from Alfa Aesar (MA, USA). 7-Ethyl-10-hydroxycamptothecin (SN38), DCFH-DA and protoporphyrin IX (PpIX) were purchased from MEILUN Biology Technology Co., LTD. (Dalian, China). All solvents used in this study were analytical grade.

Synthesis of S_2C_{16}-COOH (Scheme S1)

5 ml of 1-hexylthiolan was mixed with 5.3 ml of 1,7-octadiene, the mixture was stirred under nitrogen at 80 °C for 8 h. The unreacted reactants were removed with a vacuum rotatory evaporator {Biermann, 2018 #939}. Then, the residue combined with mercapto acetic acid (1.2 eq.) and triethylamine (0.5 eq.) were dissolved in dichloromethane (DCM) with stirring at room temperature for 8 h. The mixture was washed with 0.1 M HCl (3× 50 ml) and 10 wt% NaCl (3 × 50 ml). The organic layer was collected and dried over anhydrous Na_2SO_4. Finally, the resultant product was filtered, evaporated and purified using a column chromatography (ethyl acetate/petroleum ether, 1/5) to provide S_2C_{16}-COOH as a white solid.

Synthesis of $2S_2C_{16}$-S/COOH (Scheme S1)

2 ml of tert-butyl acrylate, 2.5 ml of 3-mercapto-1,2-propanediol and 0.5 ml triethylamine were dissolved in 10 ml of methanol with stirring at 30 °C overnight. The mixture was then evaporated to remove methanol, and the residue was dissolved in the 50 ml of 10 wt% NaCl. Aqueous 1M HCl was added to neutralize the solution, followed by the washing with hexane (3× 30 ml). Next, the product was extracted with DCM (3× 30 ml). The combined organic phases were dried over anhydrous Na_2SO_4, and the solvent was removed with a vacuum rotatory evaporator to provide product 1 as a viscous oil.

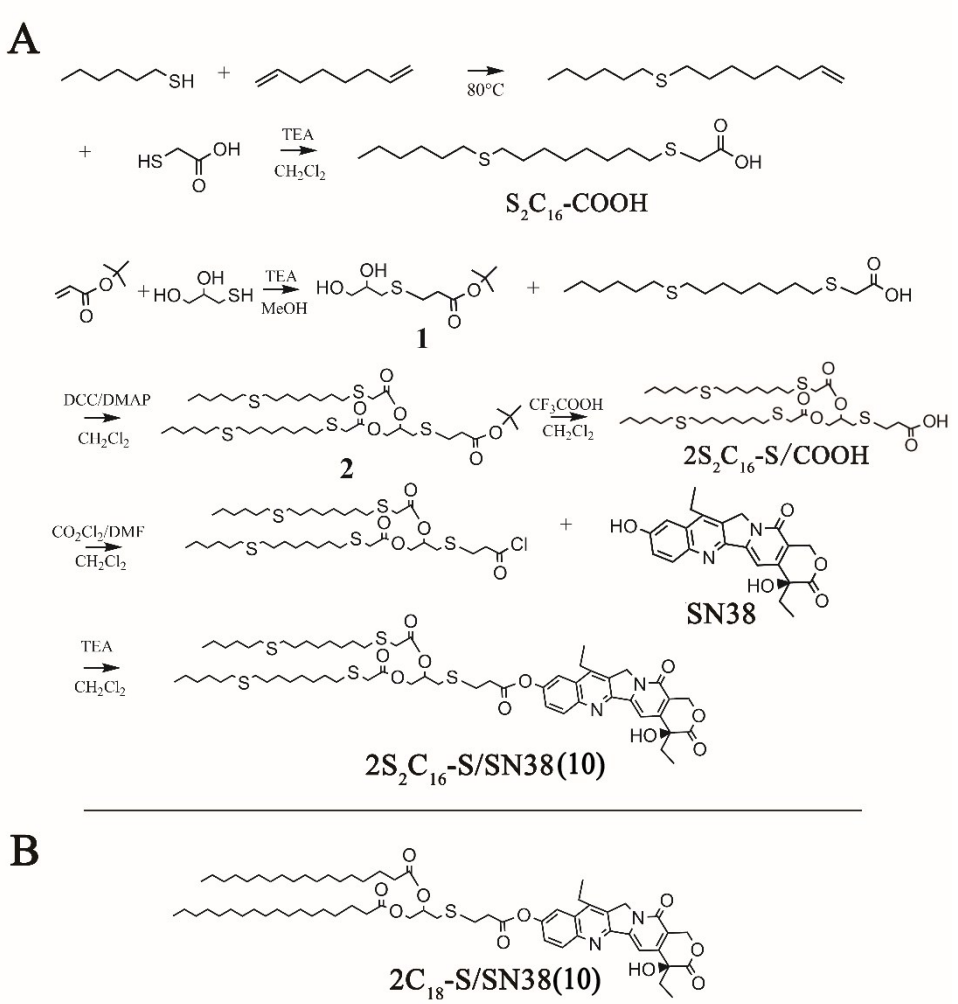
The S_2C_{16}-COOH (2 g) was dissolved and stirred at 0 °C in 20 ml anhydrous DCM for 5 min, the DCC (1.2 eq.) was added and stirred for 10 min at 0 °C. The product 1 and catalytic quantity of DMAP (~50 mg) were added and stirred at the room temperature overnight. After the DCM was removed, the 50 ml ethyl acetate was added and the solution was filtered to remove N,N'-dicyclohexylurea (DCU). The filtrate was reduced under the reduced pressure to obtain the oil products, which was further purified by silica gel column chromatography (ethyl acetate/petroleum ether, 1/20) to obtain the corresponding product 2.

The product 2 (1 g) was dissolved in a mixture of 5 ml DCM and 5 ml trifluoroacetic acid (TFA) and stirred for 8 h at room temperature. The solvent was removed under reduced pressure. The residue was dissolved in 25 ml DCM, and the solution was washed successively with 5 wt% sodium hydrogen carbonate aqueous solution and water. The organic phase was then dried over anhydrous Na_2SO_4, and DCM was removed under reduced pressure to obtain pure $2S_2C_{16}$-S/COOH

as a white solid.

Synthesis of $2S_2C_{16}$-S/SN38(10) and $2C_{18}$-S/SN38(10) (Scheme S1)

150 mg $2S_2C_{16}$-S/COOH was dissolved in 10 ml anhydrous DCM at 0 °C under nitrogen. The oxalyl chloride (1.1 eq.) and 10 μl dimethyl formamide (DMF, as a catalyst) were added into the solution, the solution was stirred at room temperature for 4 h. Then, the powder of SN38 (0.8 eq.) was added into the solution, followed by the triethylamine (3.0 eq.). The reaction was performed at room temperature overnight, and the $2S_2C_{16}$-S/SN38(10) was purified by silica gel column chromatography. $2C_{18}$-S/SN38(10) was synthesized according to the procedure similar to that of S_2C_{16}-S/SN38(10) described above.



Scheme S1. Synthesis of $2S_2C_{16}$-S/SN38(10) and $2C_{18}$-S/SN38(10)

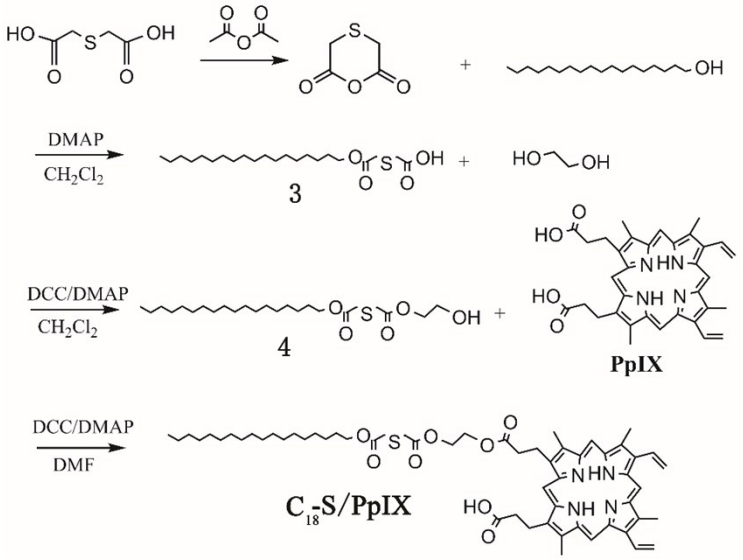
Synthesis of C_{18}-S/PpIX (Scheme S2)

Thiodiglycolic acid (2.0 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, and the residue was dissolved in 2 ml anhydrous DCM. The solution was then added into the stearyl alcohol (1.0 eq.) 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 (ethyl acetate/petroleum ether, 1/2) to give product 3.

Product 3 (1 g) was dissolved and stirred at 0 °C in 20 ml anhydrous DCM for 5 min, the DCC (1.2 eq.) was added and stirred for 10 min at 0 °C. Then, 1 ml ethylene glycol and catalytic quantity of DMAP (~50 mg) were 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 DCM was removed under the reduced pressure to obtain the crude products, which was purified by silica gel column chromatography to obtain the corresponding product 4.

100 mg PpIX was dissolved and stirred in 5 ml anhydrous DMF, DCC (0.5 eq.) was added and stirred for 10 min at 0 °C. Then, 4 (0.5 eq.) and catalytic quantity of DMAP (~1 mg) were added and stirred at the room temperature overnight in dark. The DMF was removed under reduced

pressure, and the residue was purified by silica gel column chromatography to obtain the corresponding C_{18}-S/PpIX.

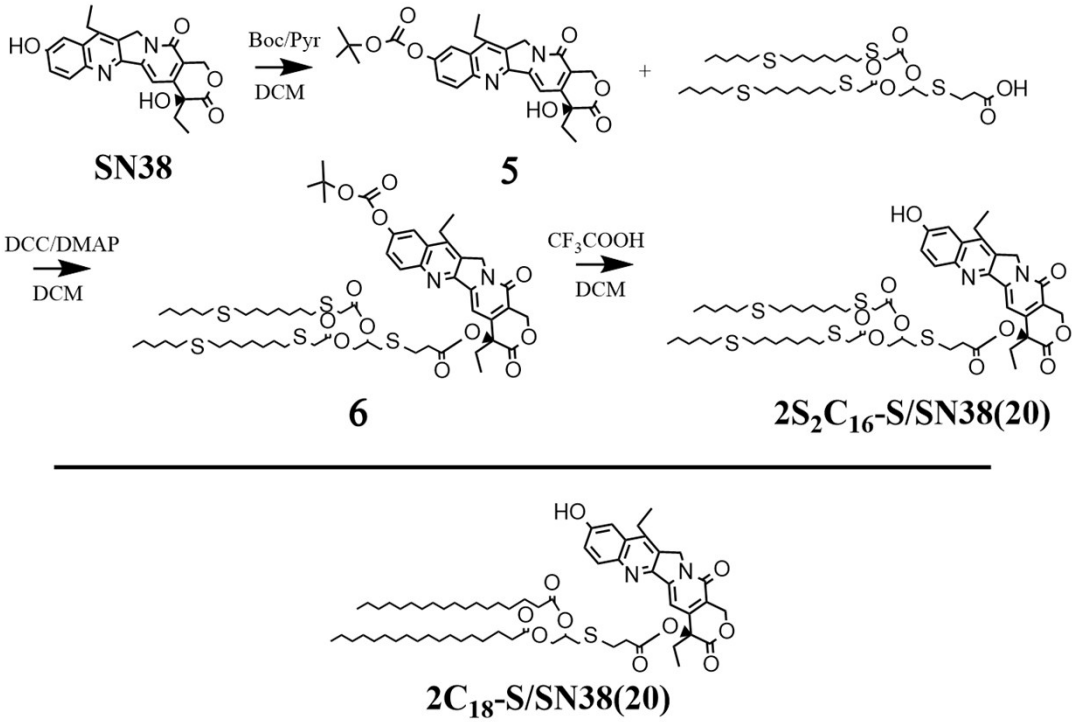


Scheme S2. Synthesis of C_{18}-S/PpIX

Synthesis of $2S_2C_{16}$-S/SN38(20) and $2C_{18}$-S/SN38(20) (Scheme S3)

200 mg of SN38 was dispersed in the 20 ml DCM containing 0.5 ml pyridine. Ditert-butyl dicarbonate (BoC, 1.2 eq.) was added and the mixture was stirred overnight at room temperature. The mixture was washed with three times with 30 mL of saturated citric acid and 50 mL of 5 wt% sodium hydrogen carbonate aqueous solution. The organic phase was dried over MgSO4, filtered and dried under reduced pressure to obtain Product 5.

150 mg $2S_2C_{16}$-S/COOH was dissolved and stirred in the 20 ml DCM, the DCC (1.2 eq.) was added and stirred for 20 min at room temperature. Product 5 (1.0 eq.) and catalytic quantity of DMAP (~1 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 concentrated under the reduced pressure and purified on silica gel plate chromatography (Methanol/DCM 1:20) to give Product 6. The pure product 6 was dissolved in a mixture of 3 ml DCM and 1 ml trifluoroacetic acid (TFA) and stirred for 2 h at room temperature. The solvent was removed under reduced pressure. The residue was dissolved in 25 ml DCM, and the solution was washed successively with 5 wt% sodium hydrogen carbonate aqueous solution and water. The organic phase was then dried over anhydrous Na_2SO4, and DCM was removed under reduced pressure to obtain $2S_2C_{16}$-S/SN38(20). Similarly, $2C_{18}$-S/SN38(20) was synthesized according to the procedure similar to that of S_2C_{16}-S/SN38(20) described above.



Scheme S3. Synthesis of $2S_2C_{16}$-S/SN38(20) and $2C_{18}$-S/SN38(20)

Preparation of self-assembled NAs

$2S_2C_{16}$-S/SN38(10) nanoaggregates (5S/SN38(10)-NAs), $2C_{18}$-S/SN38(10) nanoaggregates (S/SN38(10)-NAs) and C_{18}-S/PpIX nanoaggregates (S/PpIX-NAs) were prepared according to the nanoprecipitation method. Briefly, lipophilic prodrugs of SN38 or PpIX was dissolved in the DMF (10 mg/ml), which were then dispersed dropwise into distilled water under vigorous agitation. The resultant solution was dialyzed against distilled water for 24 h to eliminate residual organic solvents.

$2S_2C_{16}$-S/SN38(10) or $2C_{18}$-S/SN38(10) was co-assembled with C_{18}-S/PpIX at the SN38/PpIX molar ratio of 8/1 using the procedure as described above, thus to obtain corresponding 5S/SN38(10)-PpIX-NAs and S/SN38(10)-PpIX-NAs, respectively. The 5S/SN38(20)-PpIX-NAs and S/SN38(20)-PpIX-NAs were prepared using the same procedure as described above.

Characterization of NAs

The size and size distribution of NAs were measured using Zetasizer (Nano-ZS90, Malvern, England) at 25 °C. The NAs were diluted by the distilled water to adjust scattering light intensity to an acceptable level for measurement. Transmission electron microscopy (TEM, JEM-1200EX, Japan) was utilized to observe the morphology of NAs. Samples were negatively stained with phosphotungstic acid and dried at room temperature before observation.

Fluorescence spectroscopy

Emission spectra of NAs were determined by a fluorescence spectrometer (Thermo varioskan flash, Thermo scientific). The NAs were loaded in a 96-well plate, and was excited at 362 nm with the band-pass of 5 nm. The emission spectra were recorded in 2 nm intervals from 380 to 740 nm.

Irradiation-induced Destruction of NAs using FRET

The stability of 5S/SN38(10)-PpIX-NAs and S/SN38(10)-PpIX-NAs was estimated using Forster resonance Energy transfer (FRET) between fluorescent SN38 (FRET donor) and PpIX (FRET acceptor). 5S/SN38(10)-PpIX-NAs and S/SN38(10)-PpIX-NAs were diluted with 1 ml of 10 mM phosphate buffer (PB, pH 7.4) to reach a final SN38 equivalent concentration of 10 μg/ml in the sample vials. The diluted solutions were then irradiated by a 635 nm laser at various power densities (0.02, 0.05, 0.1, 0.2 and 0.4 W/cm^2) for 1min or at the fixed power density of 0.2 W/cm^2 for various times (1, 2, 3, 5 and 10 min). Then, 100 μl solution was withdrawn and loaded in a 96-well plate for fluorescence analysis (λ_{ex} = 480 nm and λ_{em} = 380-740 nm).

TEM and UV-vis spectra were further recorded to investigate the light-induced destruction of NAs. Briefly, 5S/SN38(10)-PpIX-NAs and S/SN38(10)-PpIX-NAs (50 μg/ml, SN38 equivalent concentration) were irradiated by a 635 nm laser at the power density of 0.2 W/cm^2 for 5 min. Then,

the irradiated NAs were then stained with phosphotungstic acid and observed under TEM. For measurement of UV-vis spectra, the 5S/SN38(20)-PpIX-NAs and S/SN38(20)-PpIX-NAs (10 μg/ml) were irradiated at 0.2 W/cm^2 for 0, 2 and 5 min before the absorption spectra were recorded.

Degradation of 5S/SN38(10)-PpIX-NAs and S/SN38(10)-PpIX-NAs under 635 nm light

5S/SN38(10)-PpIX-NAs and S/SN38(10)-PpIX-NAs were supplemented with 1 ml of 10 mM PB (pH 7.4) at the final SN38 equivalent concentration of 10 μg/ml in the sample vials. The sample was then irradiated by a 635 nm at power density of 0.2 W/cm^2. After irradiation for the given time (1, 2, 3, 5 and 10 min), 20 μl solution was withdrawn for HPLC analysis (1260 Infinity, Agilent, USA) at the detection wavelength of 362 nm. A photodiode array detector was also used to confirm the degradation products of $2S_2C_{16}$-S/SN38(10) or $2C_{18}$-S/SN38(10) according to their absorption spectra.

To evaluate the SN38 release from various versions of SN38-PpIX-NAs in culture medium, NAs were supplemented with 100 μl culture medium in the 2 ml Eppendorf tubes at the final SN38 equivalent concentration of 10 μg/ml. The samples were then irradiated by a 635 nm at power density of 0.4 W/cm^2 for 1 min. After irradiation for 24 h, 700 μl methanol (containing 1% acetic acid) was added, and the mixture was vortexed for 5 min and centrifuged at 10000 rpm for 5 min. The supernatants were moved into 1.5 ml Eppendorf tubes and dried with blowing nitrogen. The residue was re-dissolved in 100 μl methanol, and 20 μl solution was withdrawn for HPLC analysis.

For HPLC analysis, a DiKMA C_8 analytical column (25 × 4.6 mm, 5 μm) was used. The mobile phase comprised 10 mM ammonium acetate as buffer A and methanol as solvent B. Flow rate was 1 ml/min. Gradient elution was employed according to the following linear program: time zero, 50 % solvent B; 12 min, 100 % solvent B; 30 min, 100 % solvent B and 33 min, 50% solvent B.

SN38 released from 5S/SN38-PpIX-NAs and S/SN38-PpIX-NAs under 635 nm light

The diluted solution of 5S/SN38-PpIX-NAs and S/SN38-PpIX-NAs (10 μg/ml) in 10 mM PB were irradiated by a 635 nm laser at various power densities (0.02, 0.05, 0.1, 0.2 and 0.4 W/cm^2) for a given time (1, 2, 5 and 10 min). After irradiation, the solutions were incubated in a water bath at 37 °C. At the given time intervals which were determined by a preliminary experiment, 20 μl solution was withdrawn for HPLC analysis.

Detection of singlet oxygen

Singlet oxygen was determined using the previously reported method {Zeng, 2018 #622}. Briefly, 5 μL of DCFH solution (1×10^{-3} M) was mixed with 1 mL of 5S/SN38(10)-PpIX-NAs, S/SN38(10)-PpIX-NAs, PpIX-NAs and free PpIX at the PpIX equivalent doses of 1.8 μg/ml (equal to that in NAs). For blank control groups, 5 μL of DCFH solution (1×10^{-3} M) was mixed with 1 ml 10 mM PB (pH 7.4). The samples were then irradiated by a 635 nm laser at 0.2 W/cm^2. Afterward, the samples were withdrawn and loaded in a 96-well plate for fluorescence emission measurements at different time points (λ_{ex} = 480 nm and λ_{em} = 500-630 nm, where SN38 or PpIX displayed no fluorescence interference).

Cytotoxicity assay

The cell viability was assessed by MTT assay. Briefly, colorectal cancer cells (CT26) were seeded in a 96-well plate at a density of approximately 5000 cells per well. After 24 h of growth, the medium was replaced with the medium that contained 5S/SN38(10)-PpIX-NAs, S/SN38(10)-PpIX-NAs, 5S/SN38(20)-PpIX-NAs, S/SN38(20)-PpIX-NAs, free PpIX and PpIX-NAs at various concentrations. The cells were then irradiated by a 635 nm laser at 0.4 W/cm^2 for 1 min. The cells were further incubated for 48 h, and those without any treatment were utilized as the control.

Statistical Analysis

Data were presented with an average value and its standard deviation, shown as mean ± SD. Student's t-test and one-way analysis of variance (ANOVA) were utilized to analyze the differences of the groups, $p < 0.05$ was considered statistically significant.

Supporting Figures

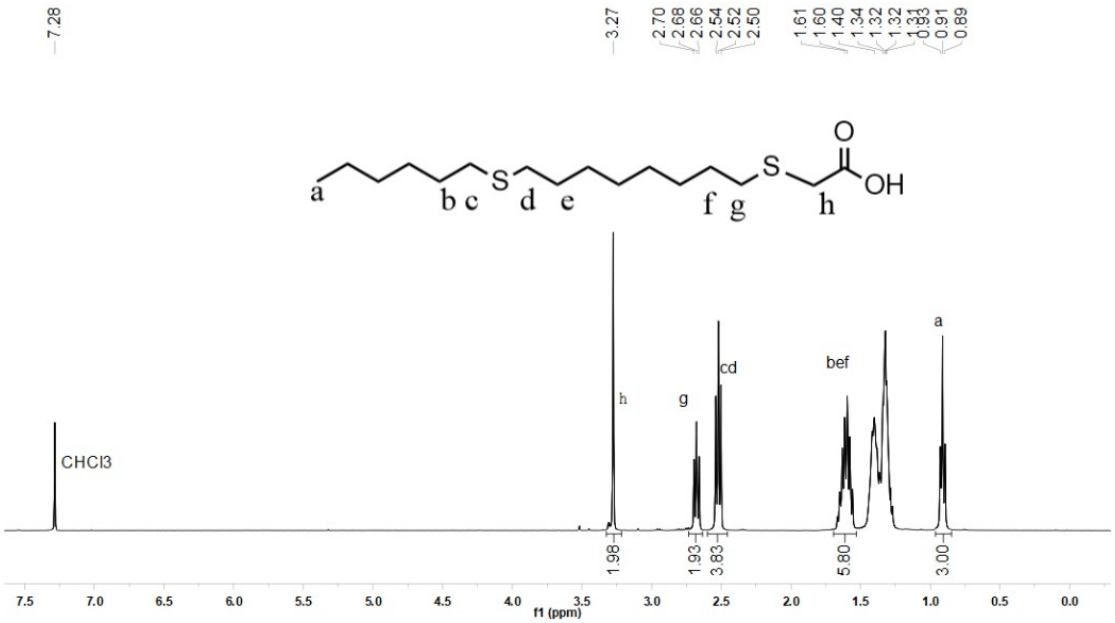


Figure S1. ^{1}H NMR spectrum of S_2C_{16}-COOH ($CDCl_3$)

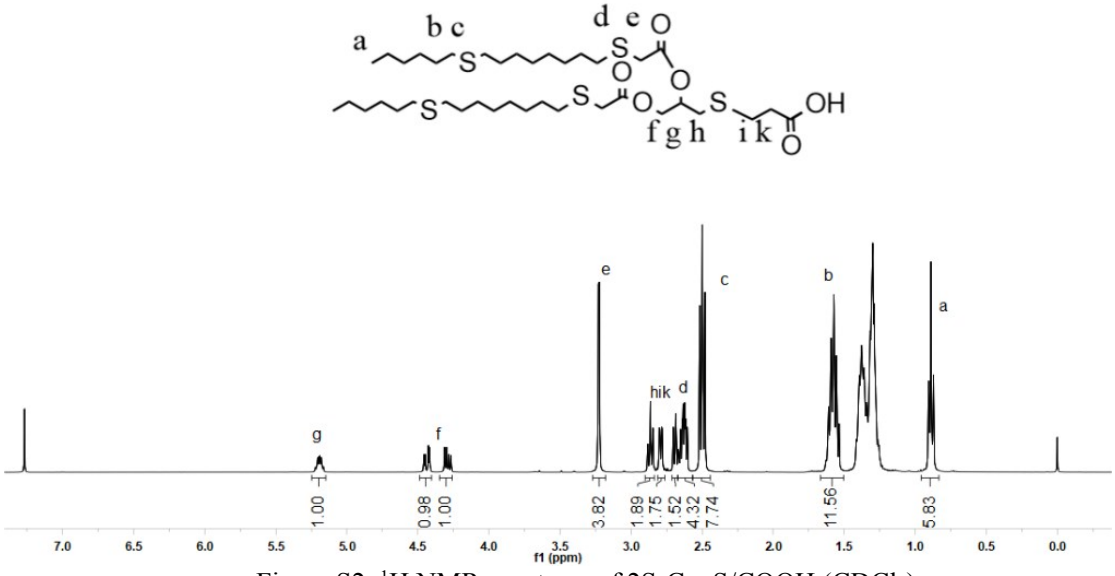


Figure S2. ^{1}H NMR spectrum of $2S_2C_{16}$-S/COOH ($CDCl_3$)

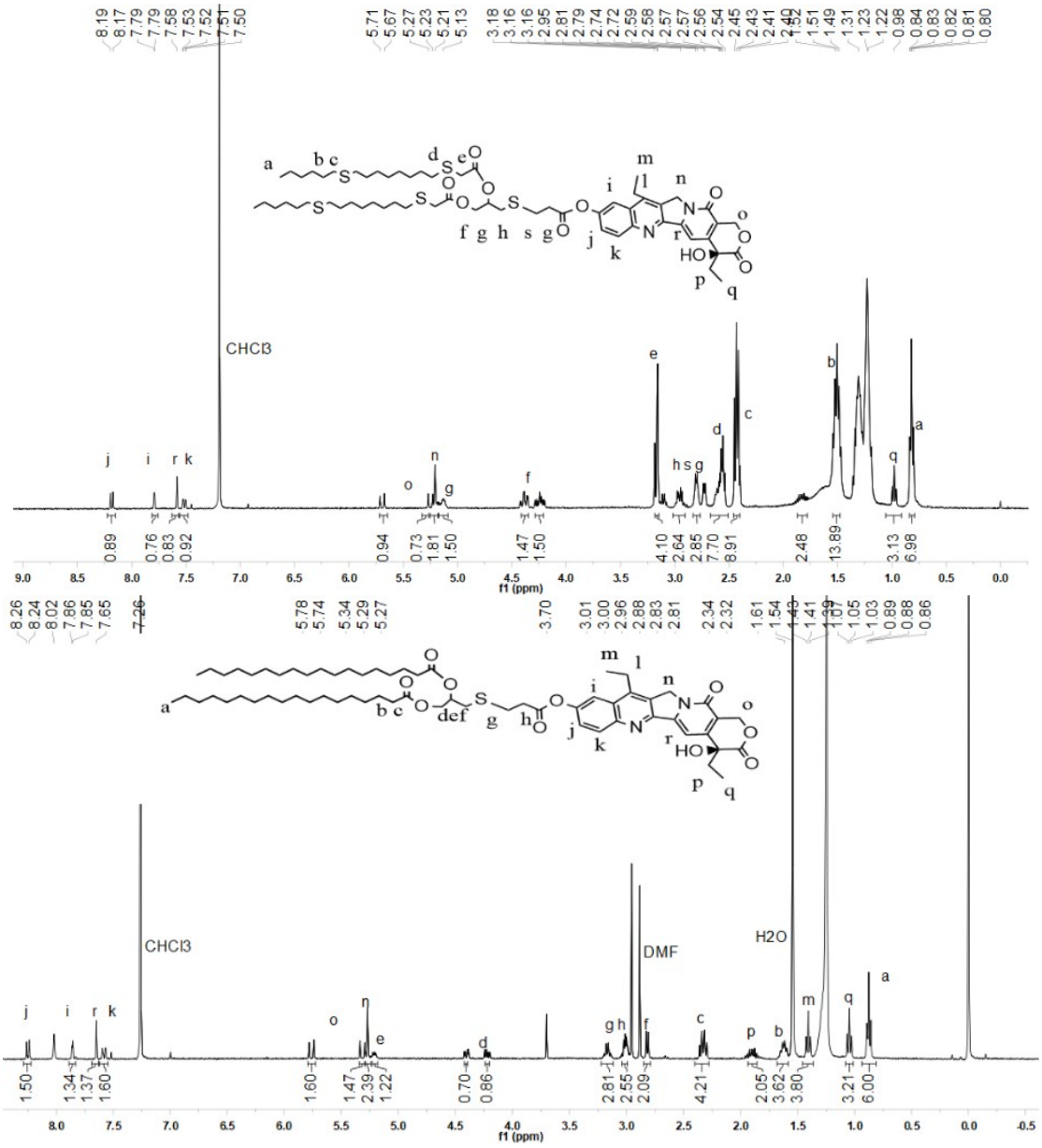


Figure S3. ^{1}H NMR spectrum of $2S_2C_{16}$-S/SN38(10) and $2C_{18}$-S/SN38(10) ($CDCl_3$)

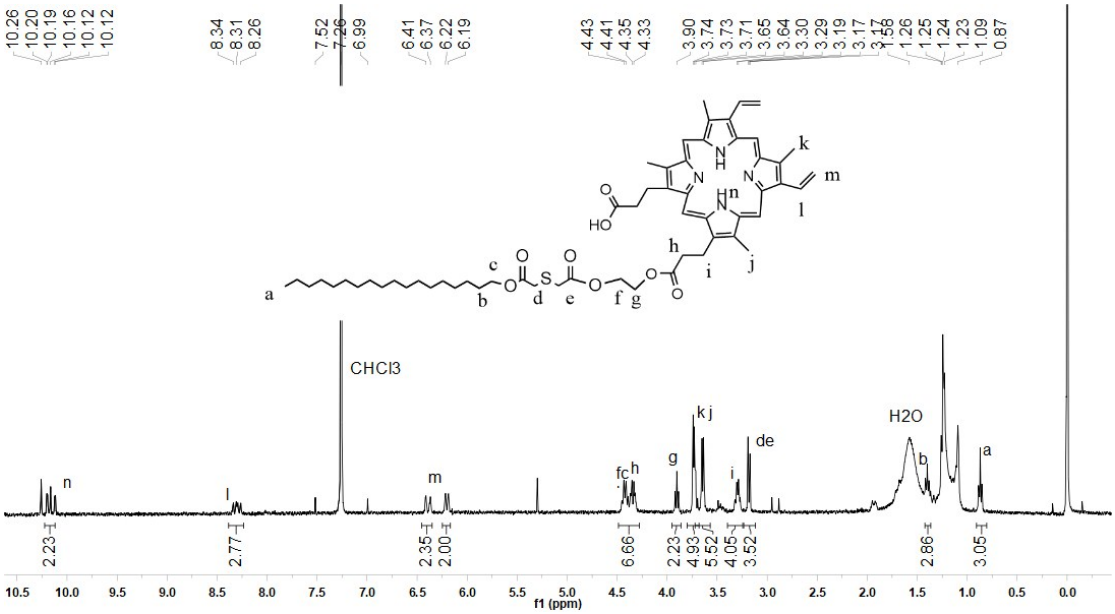


Figure S4. ^{1}H NMR spectrum of C_{18}-S/PpIX ($CDCl_3$)

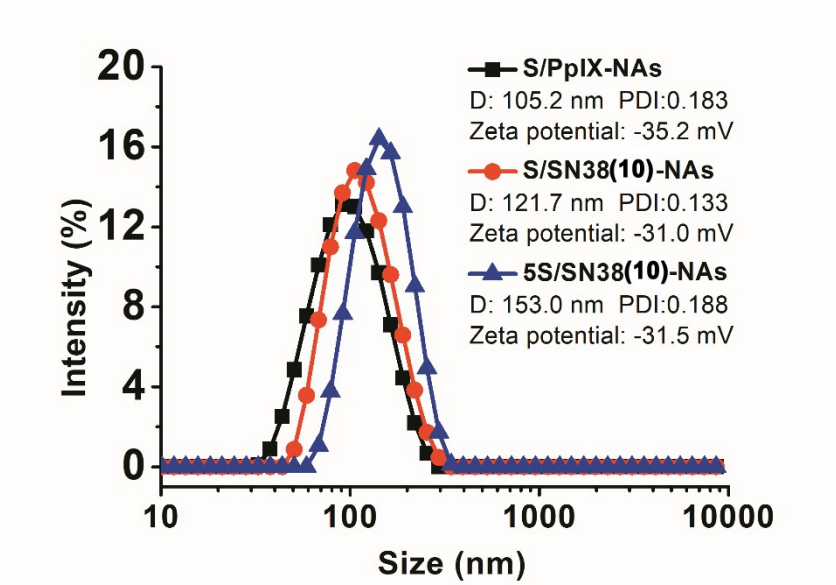


Figure S5. Size distributions, PDI and zeta potentials of S/PpIX-NAs, S/SN38(10)-NAs and 5S/SN38(10)-NAs.

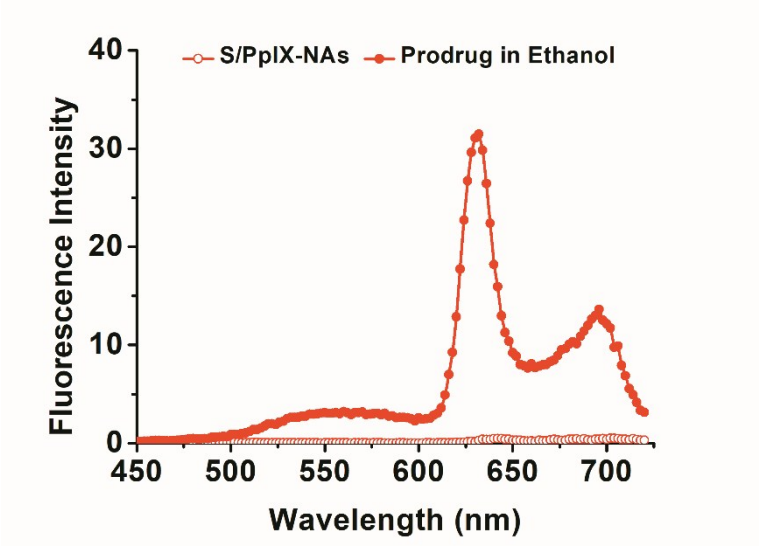


Figure S6. Emission spectra S/PpIX-NAs and corresponding ethanol solutions of C_{18}-S/PpIX with the excitation at 365 nm.

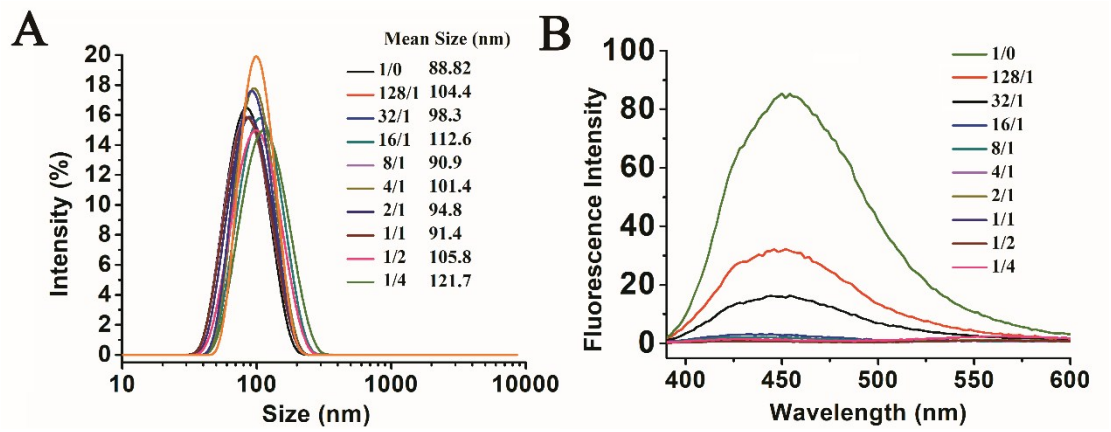


Figure S7. Size distributions and emission spectra of S/SN38(10)-PpIX-NAs prepared at various SN38/PpIX ratios (mole/mole).

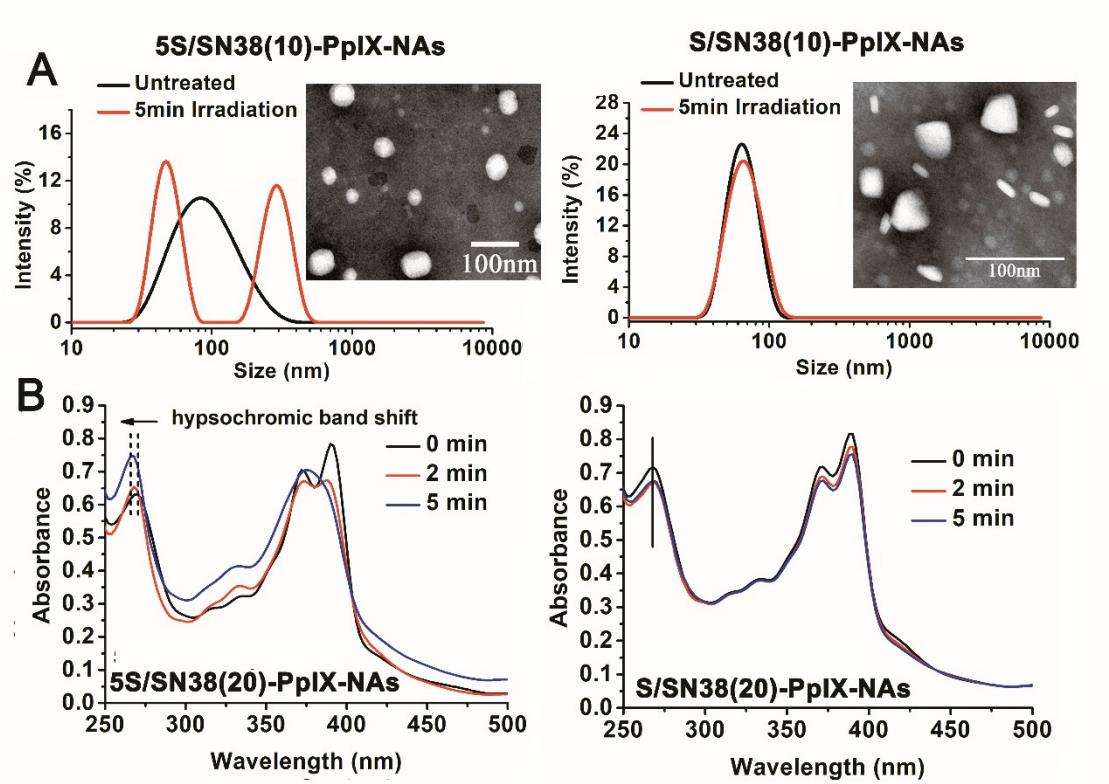


Figure S8. Size distributions of 5S/SN38(10)-PpIX-NAs and S/SN38(10)-PpIX-NAs before and after laser irradiation (635 nm, 0.2 W/cm^2) for 5 min (A). UV-vis spectra of 5S/SN38(20)-PpIX-NAs and S/SN38(20)-PpIX-NAs before and after laser irradiation (B).

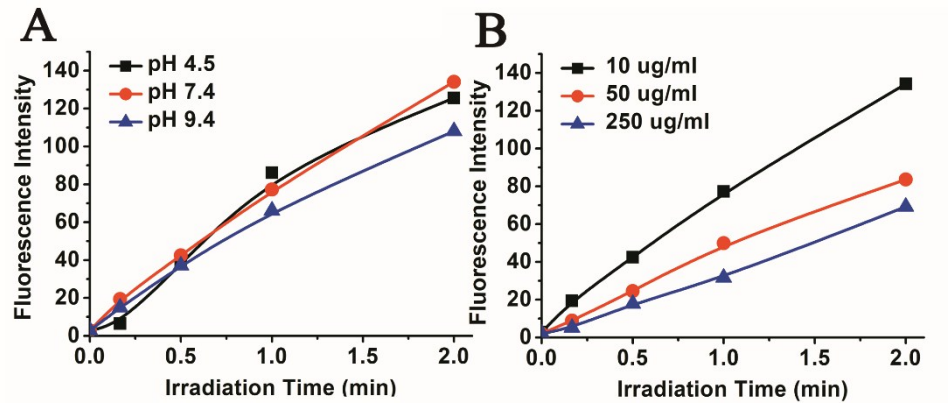


Figure S9. Effect of pH (A) and NAs concentration (B) on the recovering of SN38 fluorescence of 5S/SN38(10)-PpIX-NAs upon 635 nm laser irradiation at 0.2 W/cm^2.

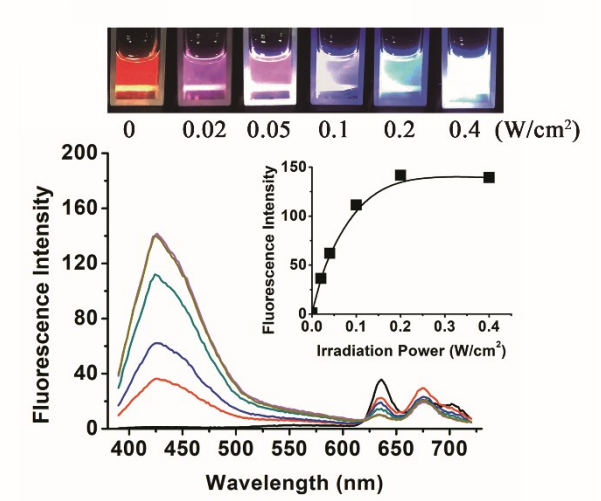


Figure S10 Kinetic changes of the appearance and emission spectra of 5S/SN38(10)-PpIX-NAs after 1 min laser irradiation at various power density.

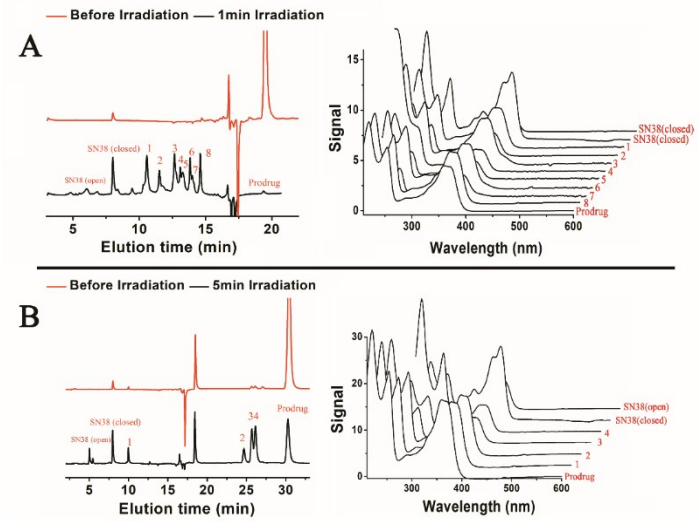


Figure S11. HPLC analysis of 5S/SN38(10)-PpIX-NAs (A) and S/SN38(10)-PpIX-NAs (B) before and after irradiation. The absorption spectra of chromatographic peaks were measured by a diode array detector, and emerged peaks displaying the SN38-like absorption spectrum were determined as the degradation products of lipophilic prodrug of SN38.

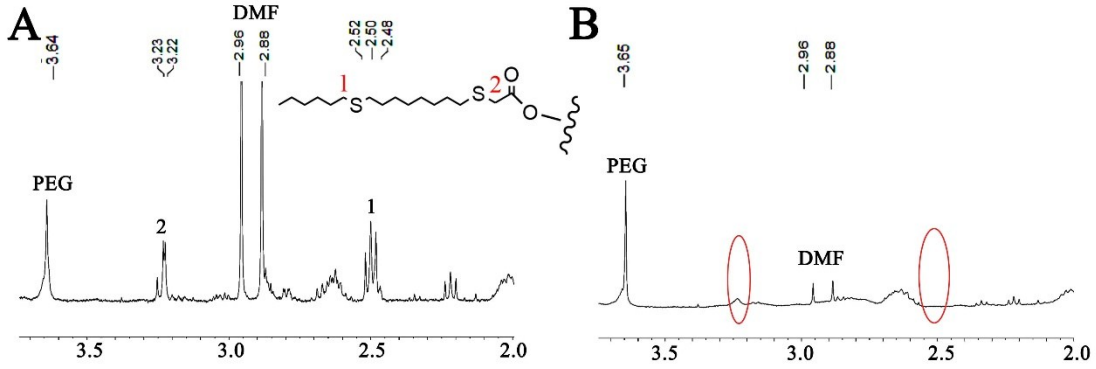


Figure S12. ^{1}H-NMR of 5S/SN38(10)-PpIX-NAs before (A) and after (B) laser irradiation (5 min, 0.2 W/cm^2): 5S/SN38(10)-PpIX-NAs (+/-) were extracted into DCM, which were dried under reduced pressure. The residues were re-dissolved in chloroform-d for ^{1}H-NMR measurement.

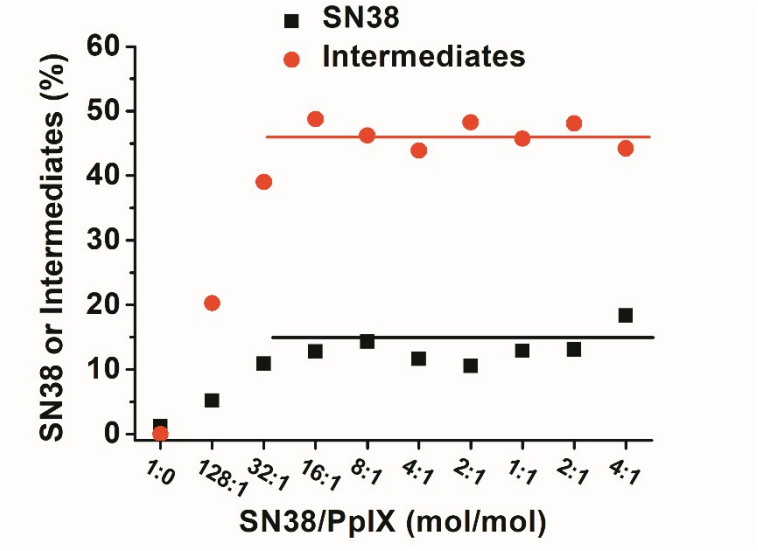


Figure S13. Effects of the SN38/PpIX ratio (mole/mole) on the generation of oxidized intermediates and SN38 from S/SN38(10)-PpIX-NAs after 5 min laser irradiation at 0.2 W/cm^2.

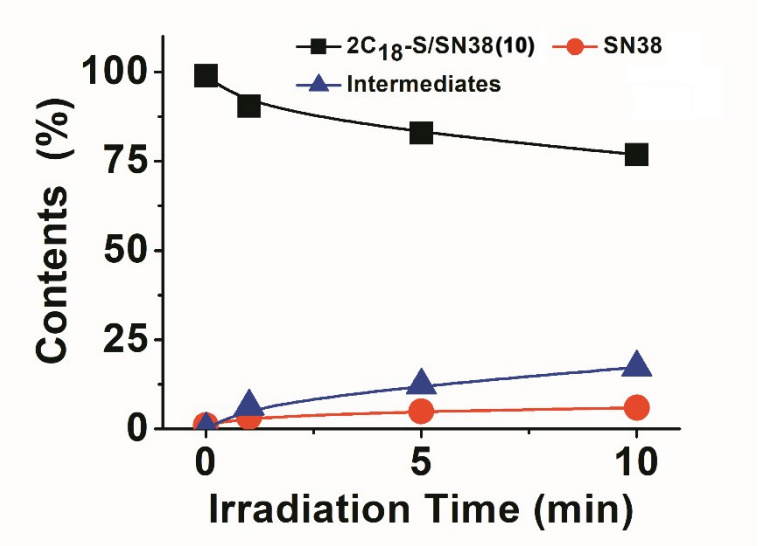


Figure S14. Kinetic change of peak areas of SN38, SN38 prodrug and intermediates derived from HPLC curve of light-irradiated mixture of S/SN38(10)-NAs and S/PpIX-NAs (0.2 W/cm^2).

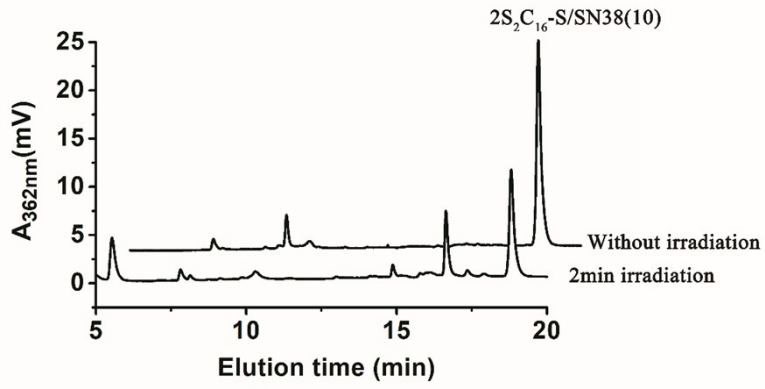


Figure S15. HPLC analysis of the mixture of 5S/SN38(10)-NAs and ICG (mole ratio, 8/1) upon 808 nm laser irradiation at 1.5 W/cm^2 for 2 min.

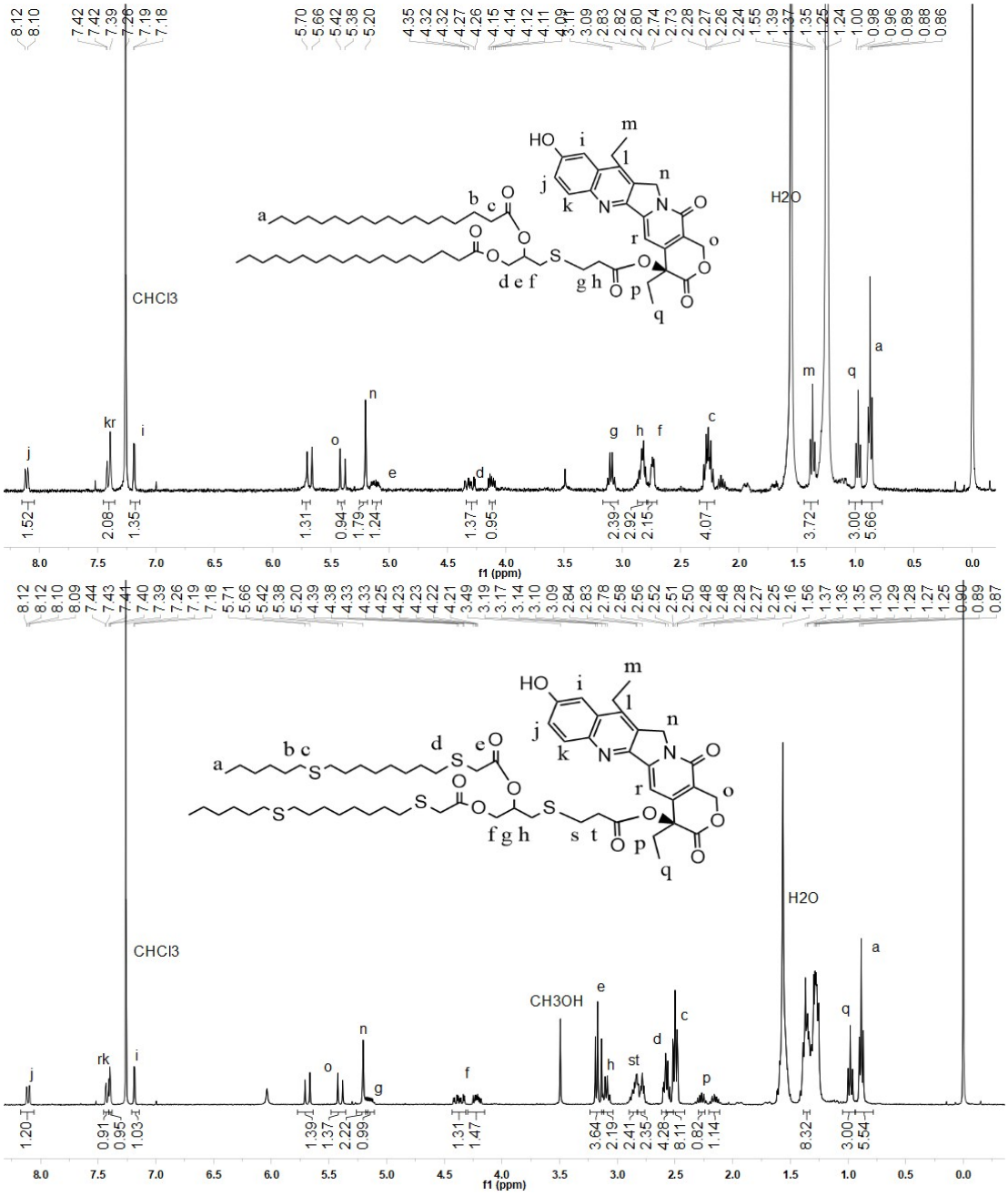


Figure.S16 ^{1}H-NMR spectra of lipophilic SN38 prodrugs ($2S_2C_{16}$-S/SN38(20) and $2C_{18}$-S/SN38(20)) synthesized by conjugating fatty acids at the C_{20} position of SN38.

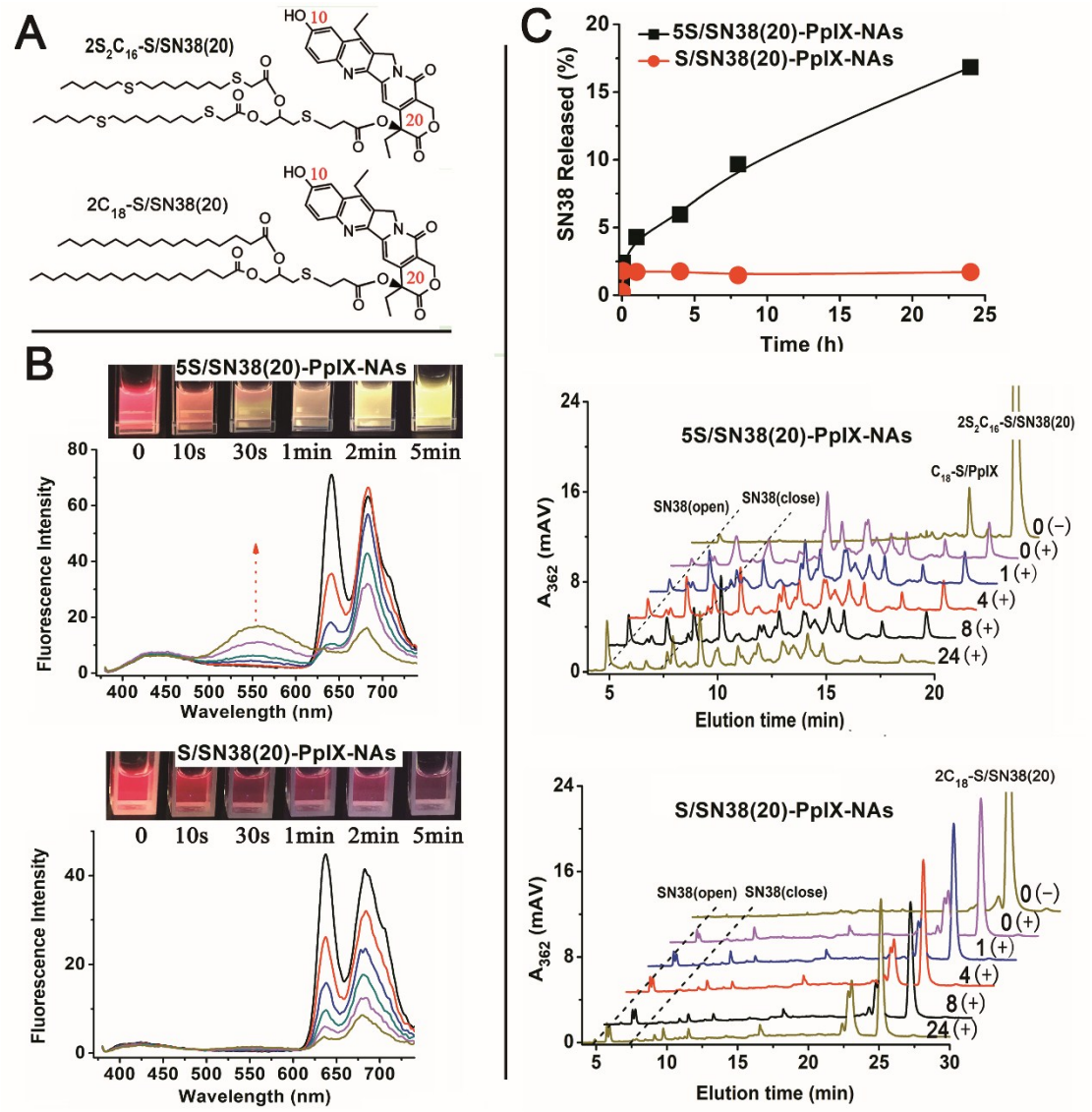


Figure.S17 Chemical structure of $2S_2C_{16}$-S/SN38(20) and $2C_{18}$-S/SN38(20) (A). Kinetic changes of appearance and emission spectra of 5S/SN38(20)-PpIX-NAs and S/SN38(20)-PpIX-NAs upon irradiation for different times (B). Cumulative SN38 release from 5S/SN38(20)-PpIX-NAs and S/SN38(20)-PpIX-NAs after 5 min irradiation at 0.2 W/cm^2 (C).

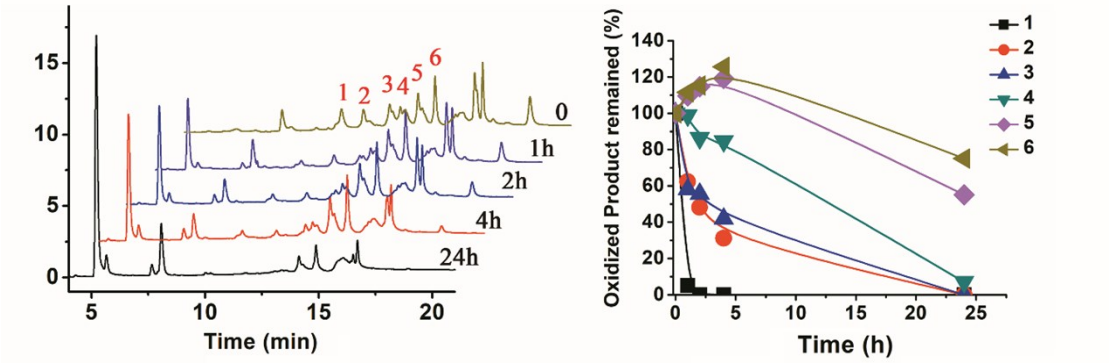
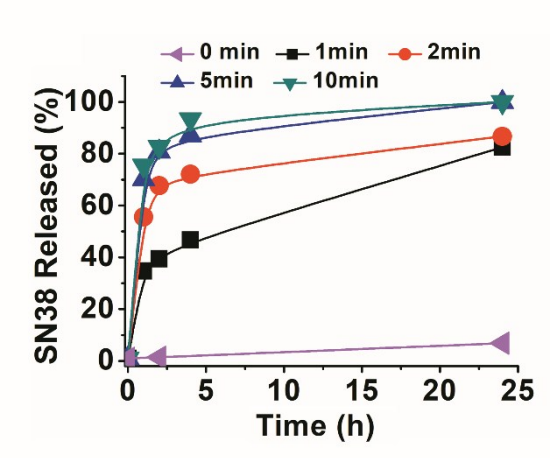


Figure S18. HPLC analysis of light-irradiated 5S/SN38(10)-PpIX-NAs (1 min, 0.2 W/cm^2) after incubation in 10 mM PB (pH 7.4) for different times (A). Kinetic change of peak areas of oxidized product of $2S_2C_{16}$-S/SN38(10) (expressed as percentages of the total area) (B).



279

280 Figure S19. Effects of irradiation time at the fixed power of 0.2 W/cm^2 on the SN38 release from
281 5S/SN38(10)-PpIX-NAs in 10 mM PB at pH 7.4.

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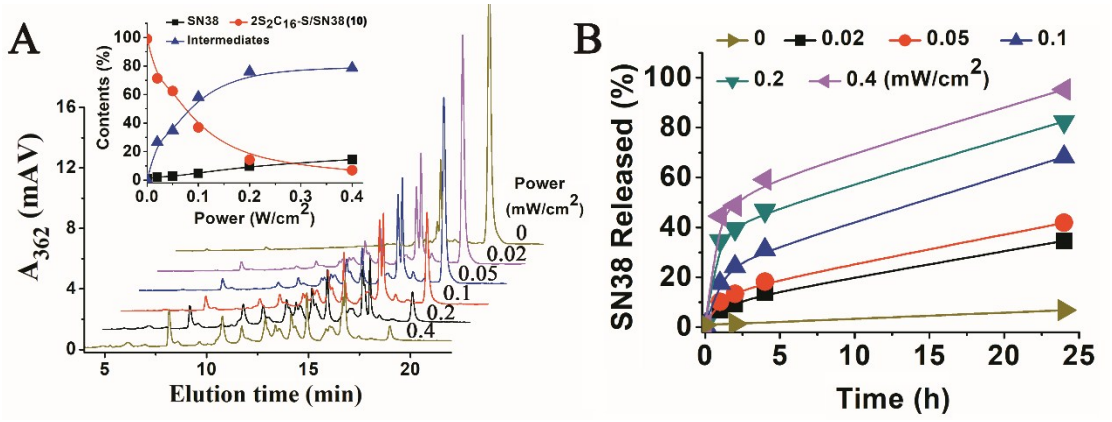
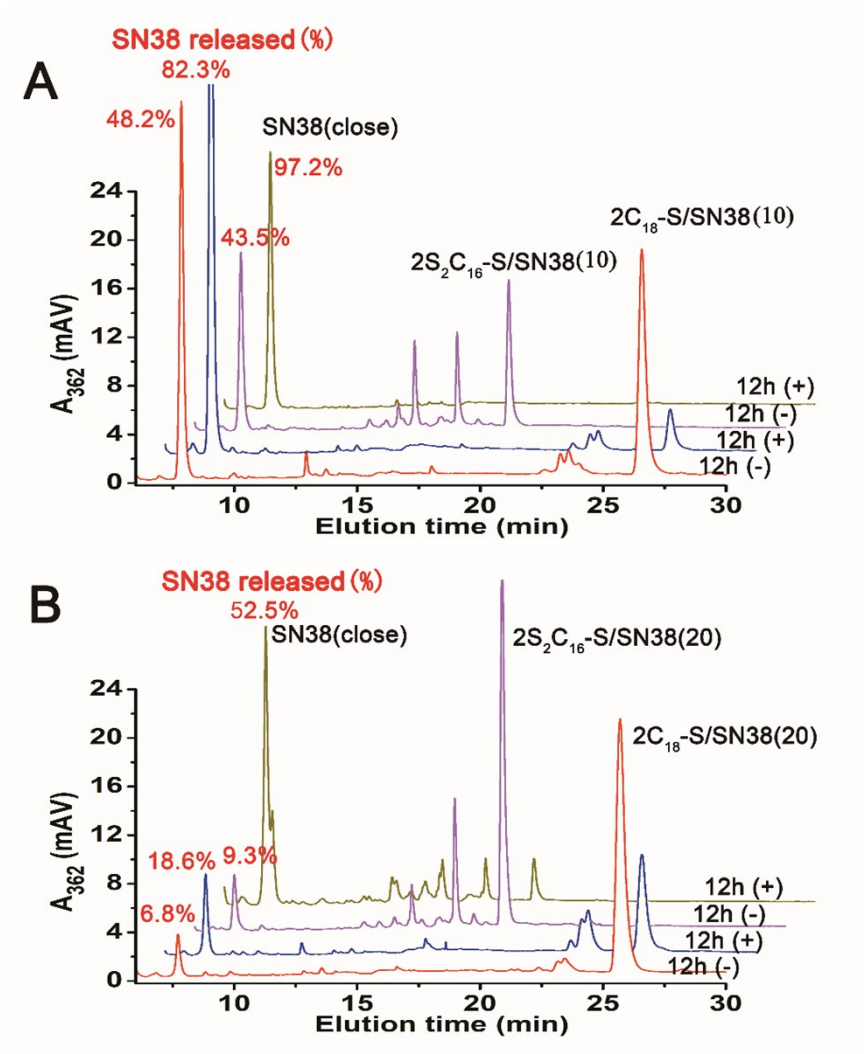


284

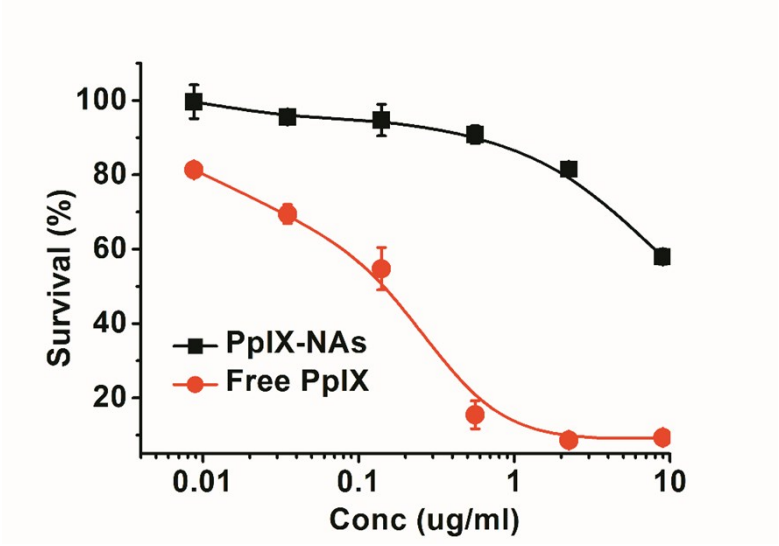
285 Figure S20. Analysis of the stability and degradation of 5S/SN38(10)-PpIX-NAs with 1 min of laser
286 irradiation at various irradiation power (A). Inset, the kinetic change of peak areas of SN38, SN38
287 prodrug and intermediates (expressed as percentages of the total area). Effects of irradiation power
288 on the SN38 release from 5S/SN38(10)-PpIX-NAs in 10 mM PB at pH 7.4 (B).

289



290

291 Figure S21. SN38 release from SN38(10)-PpIX-NAs (A) and SN38(20)-PpIX-NAs (B) within 24 h
292 in the culture medium without irradiation (-) or after 1min irradiation (+) at 0.4 W/cm^2. Only SN38
293 (close) was detected due to the presence of acetic acid in the extraction process.



294

295 Figure S22. Cytotoxicity of PpIX-NAs and free PpIX against CT26 cells after 1 min irradiation at
296 0.4 W/cm^2.

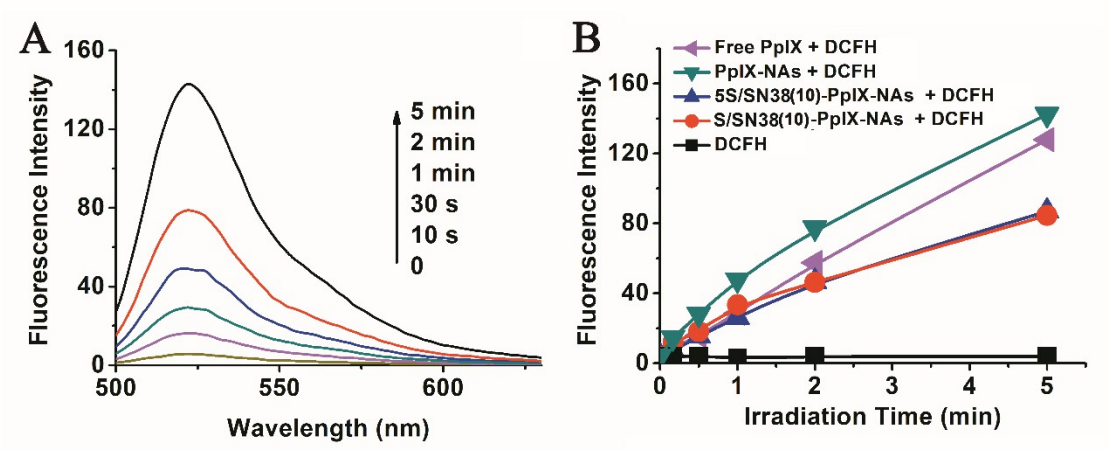


Figure S23. Typical changes of fluorescence spectra of DCFH caused by $^1O_2$ generated by PpIX-NAs under 635 nm laser irradiation (A). Detecting quantum yield of singlet oxygen of various NAs with DCFH under 635 nm laser irradiation (B, without NAs as control).