Electronic Supplementary information

Interfacial Cationization to Quicken Redox-responsive Drug Release

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1. Experimental

1.1 Materials and animals

Oleic acid (OA) and 2,2-Dithiodiethanol were purchased from Sigma-Aldrich (USA). Tert-butyl acrylate and 3-mercapto-1,2-propanediol were purchased from TCI reagents (Tokyo, Japan). 4-dimethylaminopyridine (DMAP) and dicyclohexylcarbodiimide (DCC) were purchased from Alfa Aesar (MA, USA). Triphosgene, 3-amino-1,2-propanediol and succinic anhydride were purchased from Adamas Reagent, Ltd (China). The camptothecin (CPT), curcumin (CUR) and dithiothreitol (DTT) was purchased from MEILUN Biology Technology Co., LTD. (Dalian, China). All solvents used in this study were analytical grade.

1.2 Synthesis of CPT-SS-2OA

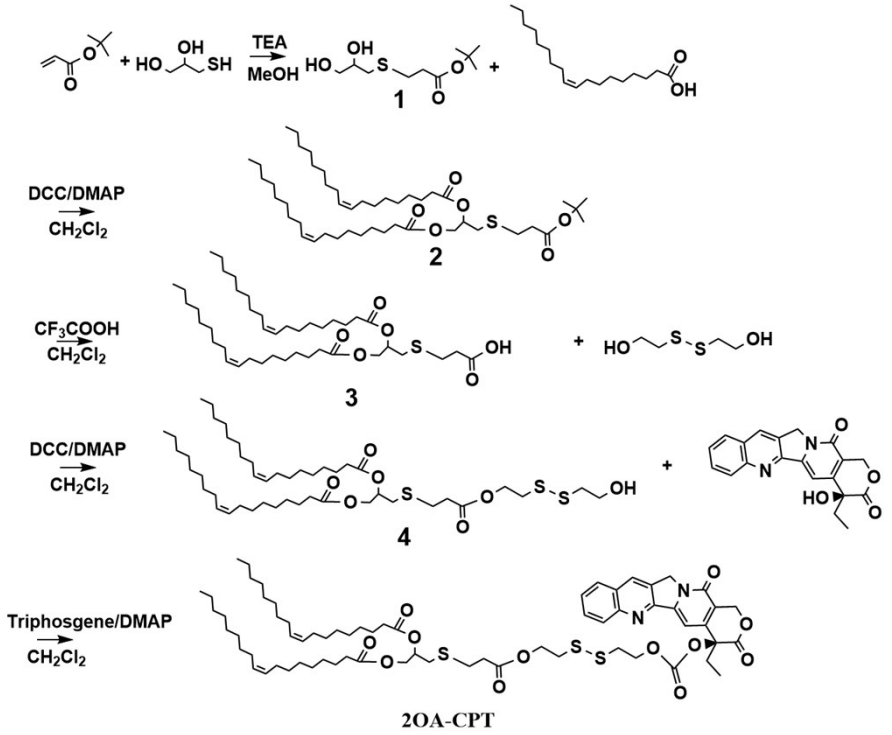
2 ml of tert-butyl acrylate, 2.5 ml of 3-mercapto-1,2-propanediol and 0.5 ml triethylamine were dissolved in 10 ml of methanol with stirring at 30 °C overnight. The mixture was then evaporated to remove methanol, and the residue was dissolved in the 50 ml of 10 wt% NaCl. Aqueous 1M HCl was added to neutralize 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 OA (4 g) was dissolved in 20 ml anhydrous DCM, the DCC (1.2 eq.) was added and stirred for 10 min. 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 to obtain the corresponding product 2.

The product 2 (1 g) was dissolved in a mixture of 2 ml DCM and 2 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 solvent was then removed under reduced pressure to obtain product 3. The product 3 (2 g) was then dissolved and stirred in 20 ml DCM, the DCC (1.2 molar equiv.) was added and stirred for 5 min. 2,2-Dithiodiethanol 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 to obtain the product 4.

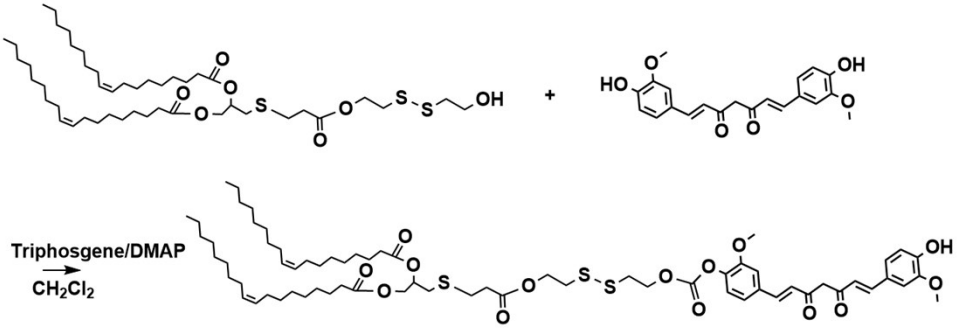
The CPT (100 mg) were dispersed in 20 ml anhydrous DCM at 0 °C under N_2. Triphosgene (0.35 eq.) in the anhydrous DCM was then added dropwise, followed addition of DMAP (2.0 eq.). After stirring at room temperature for 10 min, the product 4 (1.0 eq.) was added into the solution. The solution was stirred at room temperature overnight, and the solution was then evaporated to dryness and purified by silica gel plate chromatography (Methanol/DCM 1:30) to give corresponding CPT-SS-2OA.



Scheme 1 Synthesis of CPT-SS-2OA

1.3 Synthesis of CUR-SS-2OA

The product 4 (~200 mg) were dispersed in 20 ml anhydrous DCM at 0 °C under N_2. Triphosgene (0.35 eq.) in the anhydrous DCM was then added dropwise, following the addition of DMAP (2.0 eq.). After stirring at room temperature for 10 min, the CUR (1.0 eq.) was added into the solution. The solution was stirred at room temperature overnight, following the purification by silica gel plate chromatography (Methanol/DCM 1:30) to give corresponding CPT-SS-2OA.



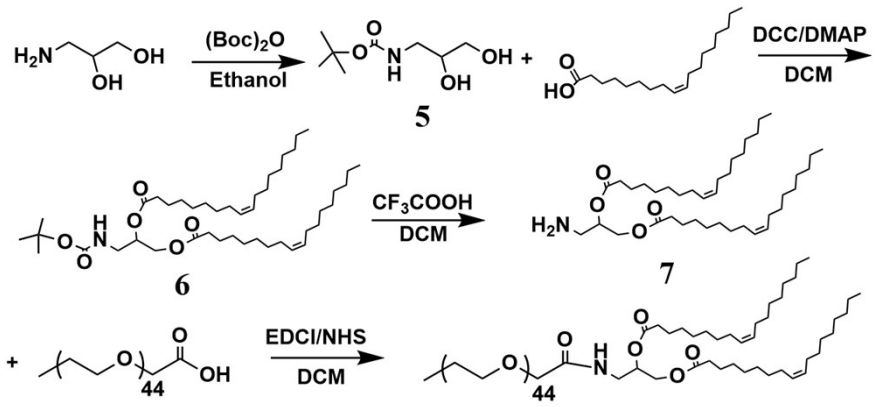
Scheme 2 Synthesis of CUR-SS-2OA

1.4 Synthesis of DON-PEG$_{2000}$

5 g of 3-amino-1,2-propanediol was dissolved in the 50 ml ethanol, Di-tert-butyl decarbonate (1.05 eq) was added and stirred overnight. The ethanol was removed under the reduced pressure to obtain the oil products (product 5), which directly used for the next synthesis.

The 10 g of OA was dissolved in DCM, DCC (1.2 eq) was added and stirred for 10 min. The product 5 and catalytic quantity of DMAP (~50 mg) were added and stirred at the room temperature overnight. After the DCM was removed, the ethyl acetate was added and the solution was filtered to remove DCU. The filtrate was reduced to obtain the oil products, which was further purified by silica gel column chromatography to obtain the corresponding product 6. The product 6 was dissolved in a mixture of 2 ml DCM and 2 ml TFA and stirred for 8 h at room temperature. The solvent was removed under reduced pressure to obtain the product 7.

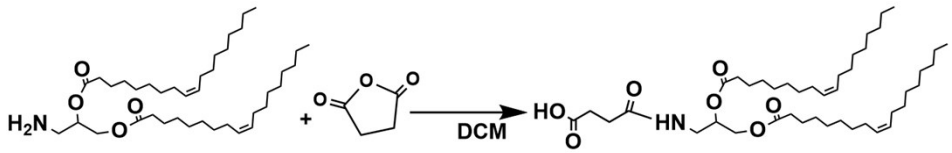
mPEG$_{2000}$-COOH was dissolved in DCM. EDCI (1.1 eq) and NHS (1.5 eq) were added and stirred at the room temperature for 4 h. Then, the product 7 (1.0 eq) and triethylamine (1.5 eq) was added in the above solution, and the solution was stirred at the room temperature overnight. The solution was washed with water for three times. The organic phases were dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The residue was subsequently subjected to the column packed with octadecylsilyl silica gel to get the pure DON-mPEG$_{2000}$.



Scheme 3 Synthesis of DON-PEG$_{2000}$

1.5 Synthesis of DON-COOH

The prodrug 7 and succinic anhydride (1.5 eq) was dissolved in DCM, and the reaction was performed at 50 °C under reflux conditions for 8 h. The DON-COOH was then purified by silica gel plate chromatography.



Scheme 4 Synthesis of DON-COOH

1.6 Preparation of self-assembling nanoaggregates (NAs) of lipophilic prodrugs

CPT/CUR-NAs were prepared according to the nanoprecipitation method. Briefly, CPT-SS-2OA and CUR-SS-2OA and DON-PEG$_{2000}$ (10 % by weight) were co-dissolved in the ethanol to obtain a clear solution. The solution was added dropwise into distilled water under the vigorous stirring to obtain CPT/CUR-NAs (CPT/CUR, 1/2, mol/mol). Ethanol was then removed under vacuum at 50 °C.

The cationic CPT/CUR-NAs (+CPT/CUR-NAs) was prepared by co-assembling

DOTAP with the lipophilic prodrugs of CPT and CRU using the above procedure. The +CPT-NAs and +CUR-NAs were prepared by dispersing the ethanol solutions of CPT-SS-2OA and CUR-SS-2OA in the water in the presence of DOTAP, respectively.

1.7 Characterization of Size, Zeta Potential and Morphology of NAs

The size and zeta potential of NAs were measured using dynamic light scattering instrument (Nano-ZS90, Malvern, England) at 25 °C. NAs were diluted by the distilled water before the measurement to adjust the scattering light intensity to an acceptable level for measurement. Transmission electron microscopy (TEM, H-600, HITACHI, Japan) was utilized to examine the morphology of various NAs. The diluted NA samples were placed on a copper grid, stained with 2 % uranyl acetate and dried at room temperature before observation.

To detect the size and zeta potentials of +CPT/CUR-NAs after the incubation with plasma (30 min, CPT 100 μg/ml), +CPT/CUR-NAs were separated on a column packed with Sepharose CL-4B (Pharmacia) and diluted by the distilled water before the measurement.

1.8 CPT release from CPT/CUR-NAs detected by a FRET method

CPT release from CPT-CUR/NAs or +CPT-CUR/NAs was monitored by detecting the kinetic change of CPT fluorescence. Briefly, the CPT-CUR/NAs were supplemented with PB (pH 7.4, containing 10 mM DTT) at the final CPT equivalent concentration of 10 μg/ml. The emission spectra or fluorescence intensity at 426 nm of CPT-CUR/NAs were determined using a fluorescence spectrometer (Thermo varioskan flash, Thermo scientific). The change of CPT fluorescence was also observed under a UV lamp with the excitation at 365 nm.

1.9 Reduction-triggered prodrug degradation by HPLC

The cationic CPT-NAs and CUR-NAs were supplemented with 1ml of 10 mM DTT (PB, pH 7.4) at the final CPT equivalent concentration of 10 μg/ml. At the given time, 20 μl solution was withdrawn for HPLC analysis. A photodiode array detector was used to confirm the degradation products of CPT-SS-OA and CUR-SS-OA according to their absorption spectra.

For HPLC analysis, a DiKMA C8 analytical column (25 × 4.6 mm, 5 μm) was used. The mobile phase comprised 10 mM ammonium acetate (for CPT detection) or 2% acetic acid (for CUR detection) 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, 20 % solvent B; 7 min, 100 % solvent B; 25 min, 100 % solvent B and 28 min, 20% solvent B.

1.10 Cytotoxicity assay

The cytotoxicity of NAs against CT26 cells and one normal cell (mouse fibroblast, 3T3 cells, containing lower level of GSH [1]) were evaluated by MTT assay. Briefly, CT26 cells and 3T3 cells were seeded in a 96-well plate at a density of approximate 5000 cells per well, respectively. After 24 h of growth, the medium was exchanged for the medium that contained CPT/CUR-NAs, +CPT/CUR-NAs, +CPT-NAs and +CUR-NAs at various concentrations. The cell was further incubated for 48 h, and these without any treatment were utilized as control to calculate cell viability.

1.11 Cell apoptosis analysis

CT26 cells or 3T3 cells in 24-well plates (5×10^4 cell per well) were incubated with CPT/CUR-NAs or +CPT/CUR-NAs at the CPT equivalent doses of 1 μg/ml for 48 h at 37 °C. The cells were then collected consecutively by trypsinization and centrifugation and washed with cold PBS four times. The cells were stained with annexin V-FITC and propidium iodide (PI) using the AnnexinV-FITC apoptosis detection kit, and apoptosis analysis was performed using a flow cytometer (NovoCyte 3130, ACEA, USA).

1.12 Cellular uptake of CPT/CUR-NAs and +CPT/CUR-NAs

CT26 cells (10^5) were seeded into 35 mm microscopy dishes. After incubation at 37 °C for 24 h, the cells were incubated with 10 μg/ml of CPT/CUR-NAs and +CPT/CUR-NAs (CPT equivalent) for 0.5 h at 37 °C, respectively. The cells were then washed with PBS three times and incubated with Lysotracker red for lysosome staining at a concentration of 50 nM. After 0.5 of staining, cells were washed with PBS twice and imaged using a confocal laser scanning microscopy (CLSM, Nikon, USA) with excitation at 403 nm for CPT and 560 nm for Lysotracker red.

To quantitatively evaluate the cellular uptake of NAs in tumor cells, CT26 cells or 3T3 cells were incubated with CPT/CUR-NAs for 2 h at a CPT equivalent concentration of 10 μg/ml, and CPT prodrugs entering into the cytoplasm were extracted by ethanol and analyzed by a fluorescence spectrophotometer (λ_{ex} = 362 nm, λ_{em} = 426 nm).

1.13 Real-time evaluation of drug release in cells

CT26 cells (10^5) were seeded into 35 mm microscopy dishes and incubated at 37 °C for 24 h. The microscopy dish was fixed onto the observation platform of the CLSM instrument. The CPT/CUR-NAs or +CPT/CUR-NAs was carefully added into the culture medium at the final CPT equivalent concentration of 10 μg/ml, and the images were continuously acquired immediately. As the CPT fluorescence was completely quenched in the FRET CPT/CUR-NAs, drug release could be readily reflected by the recovering of blue fluorescence.

To compare the drug release within CT26 and 3T3 cells, the cells were incubated with CPT/CUR-NAs or +CPT/CUR-NAs at an equivalent CPT concentration of 10 μg/ml for 2 h, respectively. The cells were then rinsed with PBS four times and fixed in 4 % paraformaldehyde. The fixed cells were examined using a fluorescence microscope.

1.14 Hemolytic test and aggregation of erythrocytes

Hemolysis was investigated at different CPT equivalent concentrations of CPT/CUR-NAs and + CPT/CUR-NAs. The blood from a healthy rat was centrifuged at 2000 g for 10 min to obtain cells, which then washed 4 times with normal saline. The cells were next diluted with saline to obtain 2% cell suspension. The CPT/CUR-NAs and +CPT/CUR-NAs were diluted to various CPT equivalent concentrations (80, 40, 20, 10, 2 μg/ml). 1ml of cell suspension was mixed with 1ml of CPT/CUR-NAs and + CPT/CUR-NAs (v/v, 1/1) at various concentrations, and the mixtures were incubated at 37 °C for 2 h in a shaker. After incubation, the mixtures were centrifuged at 3000 g for 15 min, the absorbance at 570 nm was measured by a UV/VIS spectrometer. The double distilled deionized water was used as positive control (100 % hemolysis), and saline was used as negative control (0 % hemolysis). Percent hemolysis was calculated as follows: hemolysis (%) =(As-An)/(Ap-An) × 100, where As is the absorbance of samples, Ap and An are absorbances of positive and negative controls, respectively.

To study the aggregation of erythrocyte, 0.5 ml of cells suspension (4 %) was incubated with CPT/CUR-NAs and +CPT/CUR-NAs at the final CPT equivalent concentrations 20 μg/ml. After 0.5 h incubation at 37 °C, the erythrocytes were checked by an optical microscope.

2. Supporting Figures

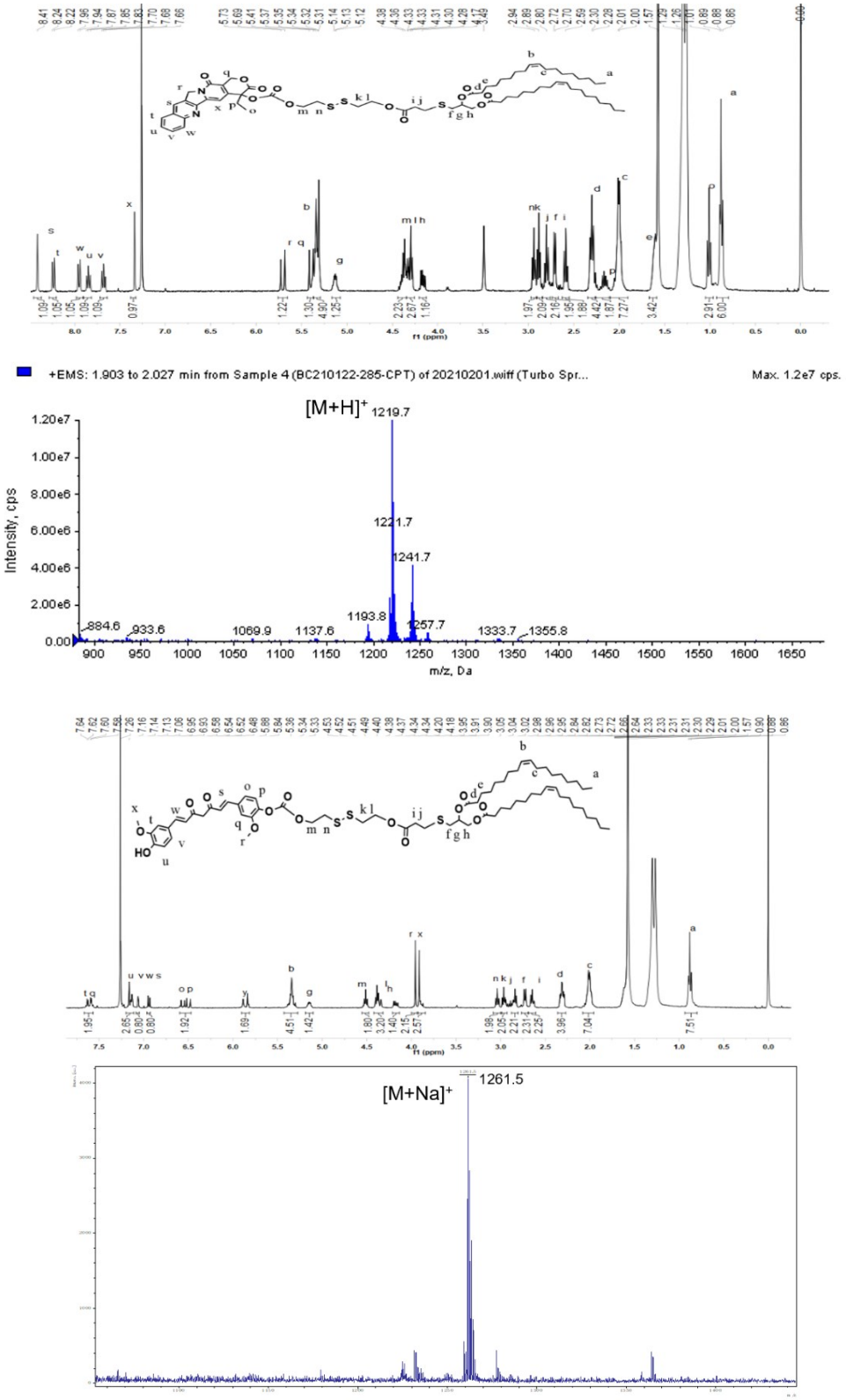


Fig.S1 ^{1}H-NMR and MS of CPT-SS-2OA (ESI) and CPT-SS-2OA (MALDI).

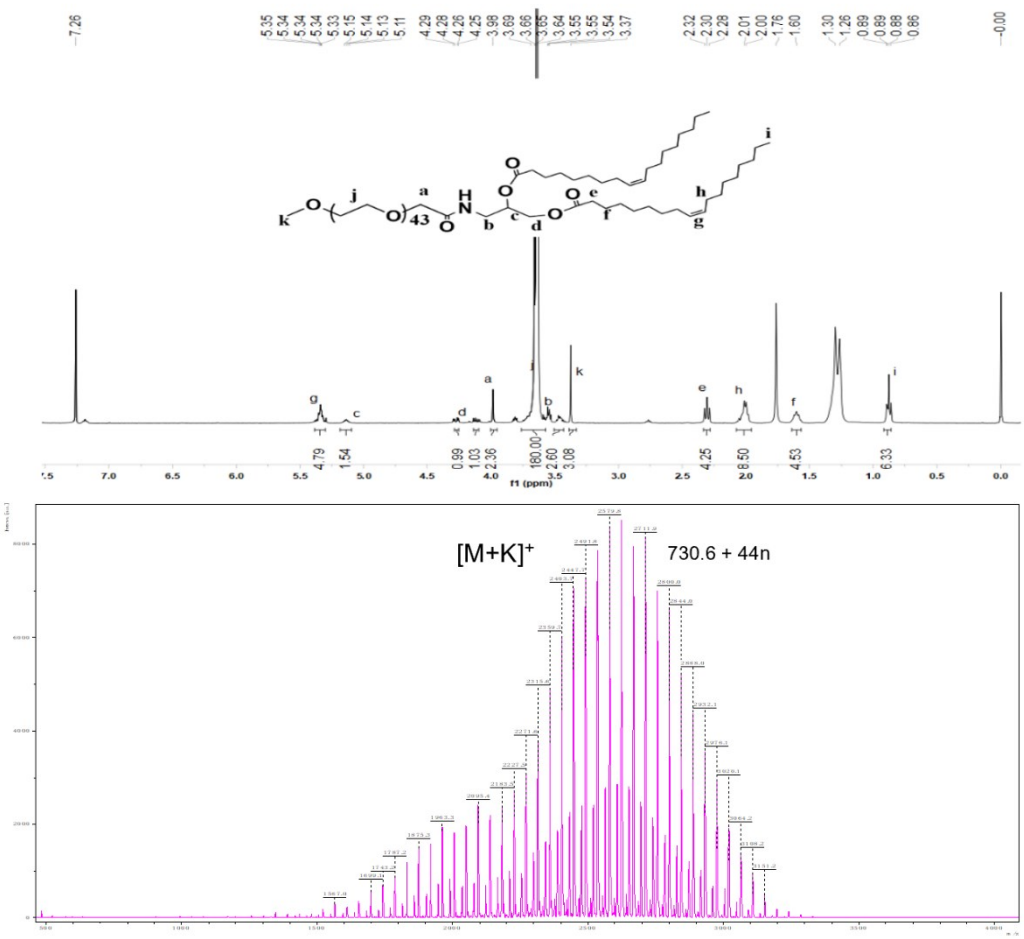


Fig.S2 ^{1}H-NMR and MS (MALDI) of DON-PEG$_{2000}$

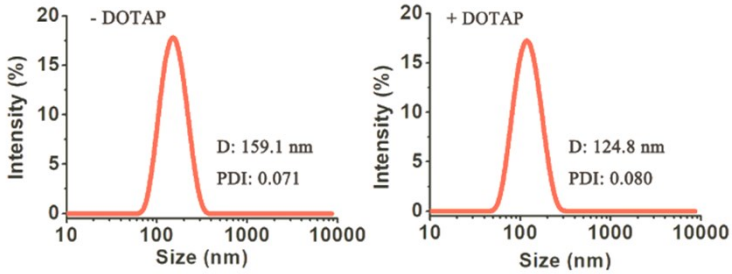


Fig.S3 Size distributions of unmodified CPT/CUR-NAs and +CPT/CUR-NAs.

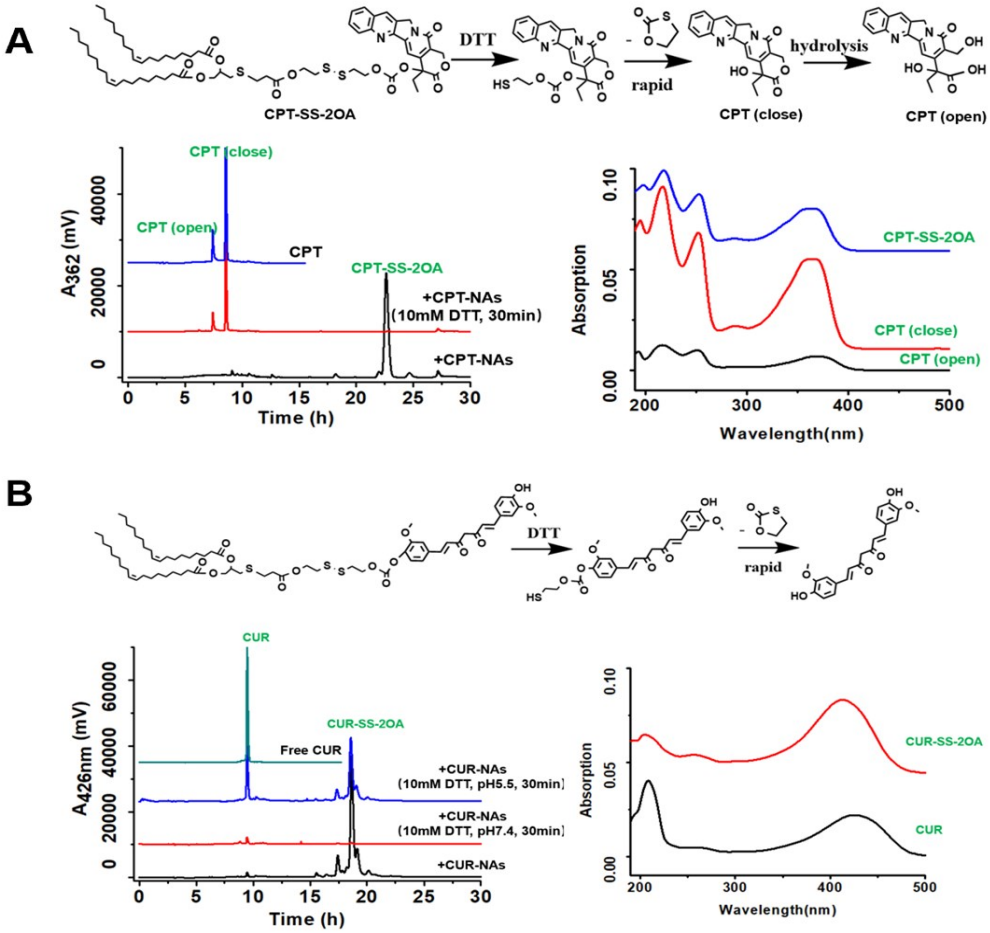


Fig.S4 HPLC analysis of +CPT-NAs (A) and +CUR-NAs (B) after 30 min incubation in 10 mM DTT. The absorption spectra of chromatographic peaks were measured by a diode array detector, and emerged peaks displaying the drug-like absorption spectrum were determined as the degradation products of prodrugs. It is shown that both CPT-SS-2OA and CUR-SS-2OA were directly converted into active drugs in the 10 mM DTT, without other CPT or CUR intermediates detected during this process. Note that the degradation of CUR-SS-2OA were not accompanied with the generation of CUR in 10 mM DTT at pH 7.4, which was ascribed to the poor stability of CUR at pH 7.4 [2]. By contrast, the CUR released from CUR-SS-2OA could be detected in 10 mM DTT at pH 5.5, where CUR has a good chemical stability.

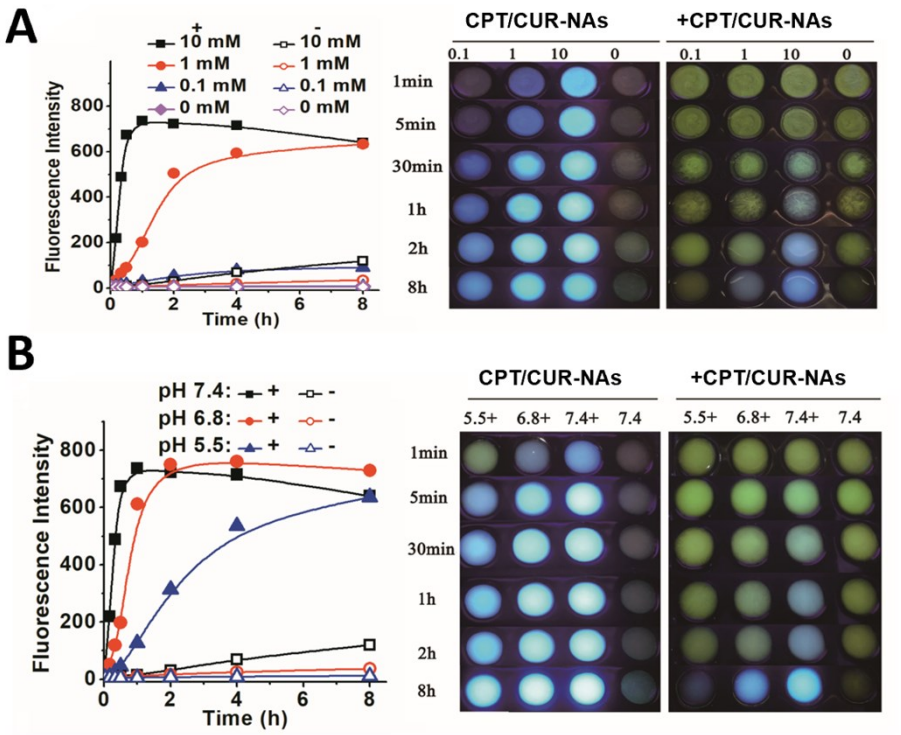


Fig.S5 Effects of DTT concentration (A) and pH (B) on the fluorescence response of unmodified and cationic CPT/CUR-NAs.

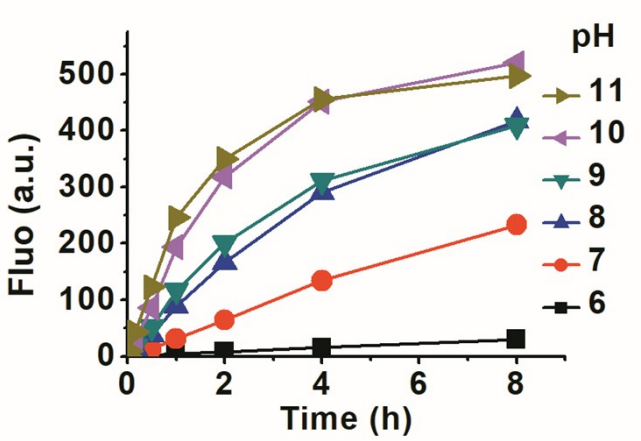


Fig.S6 Kinetic change of CPT fluorescence at different pH in the presence of 10 mM DTT.

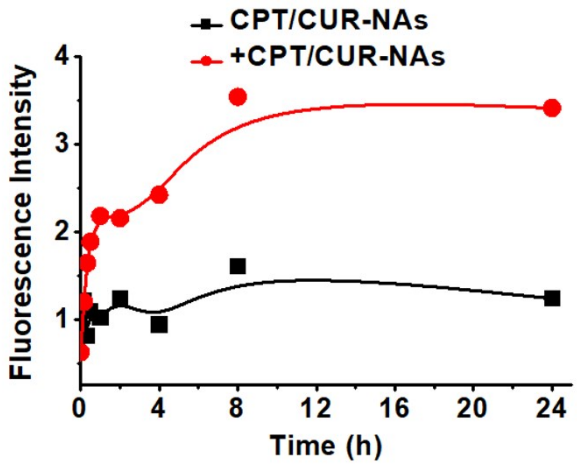


Fig.S7 Kinetic change of CPT fluorescence of unmodified and cationic CPT/CUR-NAs at 37 °C in 10 mM PB (pH 7.4).

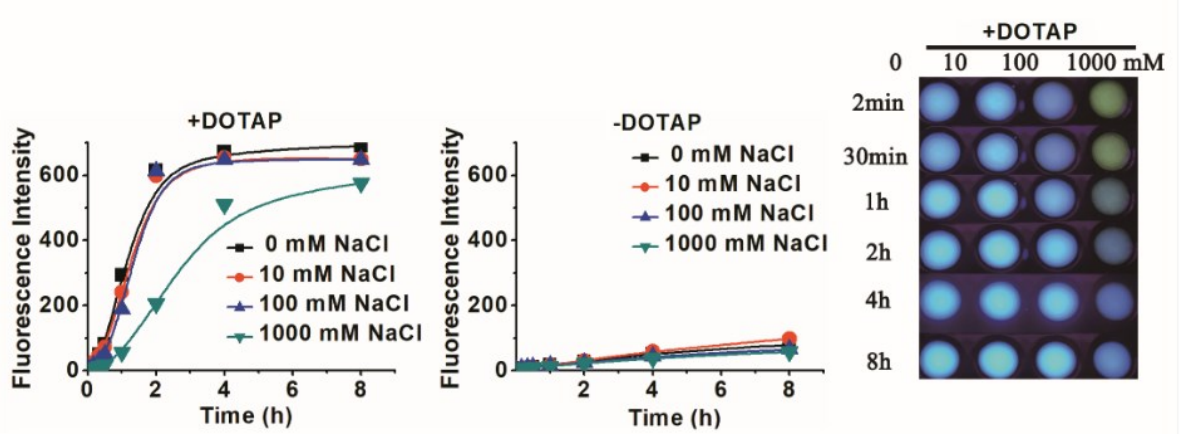


Fig.S8 Effect of ion concentration on the CPT fluorescence of unmodified and cationic +CPT/CUR-NAs at 37 °C in 10 mM PB (pH 7.4).

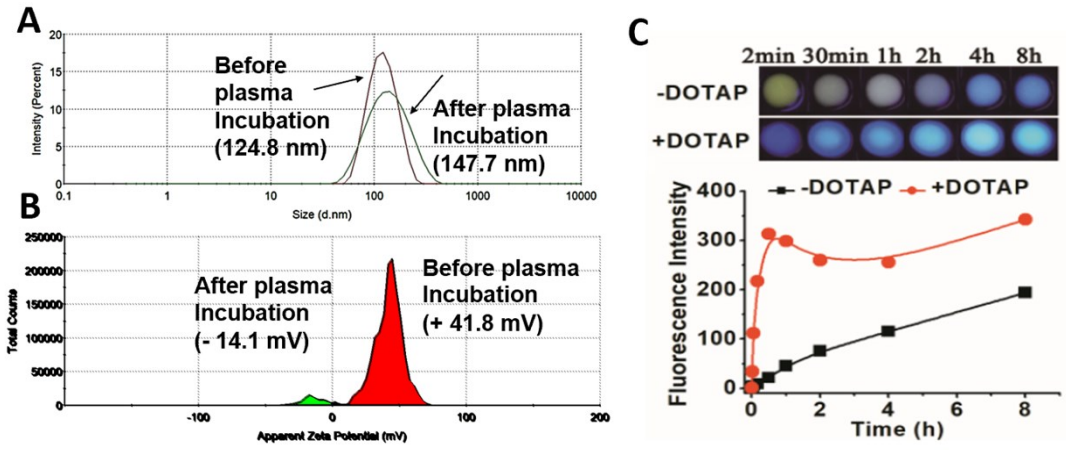


Fig.S9 Size distributions (A) and zeta potentials (B) of cationic +CPT/CUR-NAs before and after 30 min incubation with mice plasma at 37 °C. The kinetic change of CPT fluorescence of unmodified and cationic +CPT/CUR-NAs in the 50 % mice plasma (C).

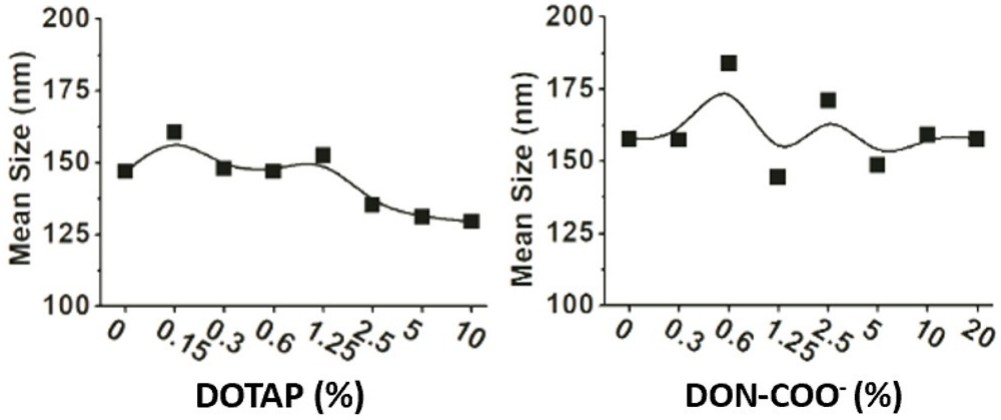


Fig.S10 Effects of weight proportions of DOTAP and DON-COOH on the particle size of CPT/CUR-NAs.

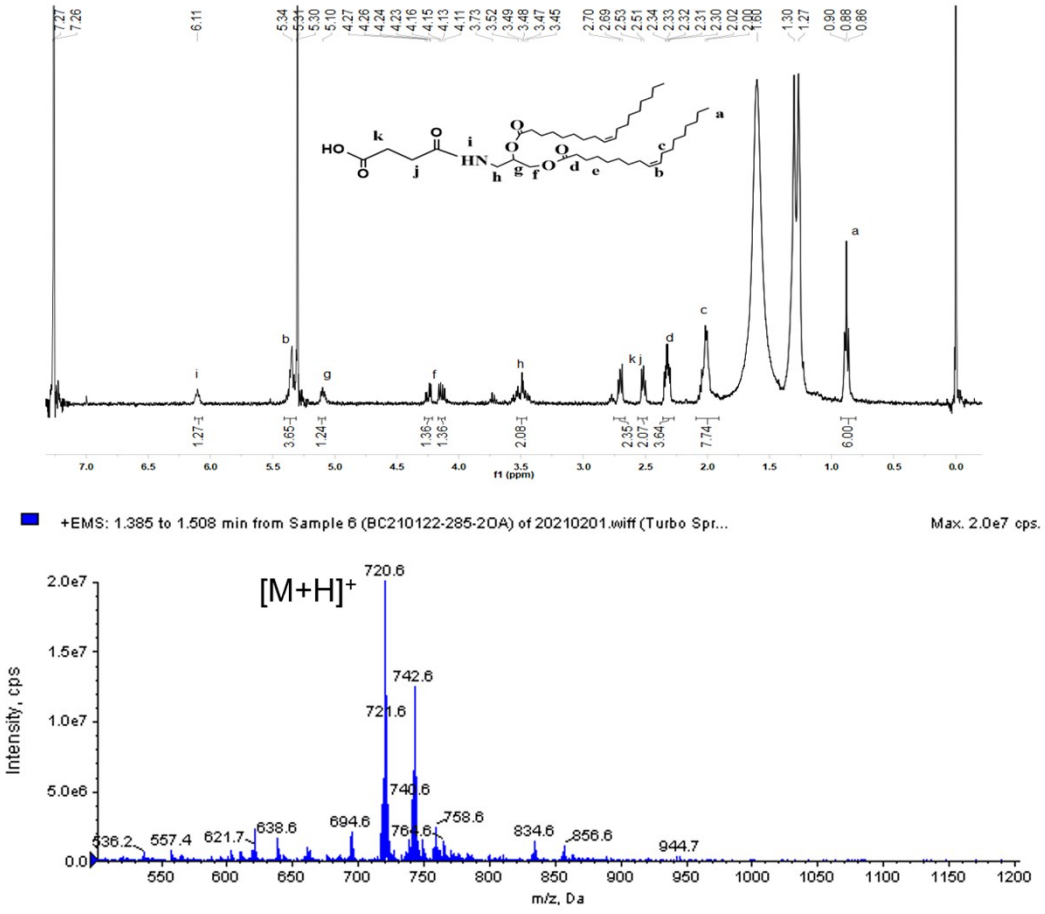


Fig.S11 ^{1}H-NMR and MS (ESI) of DON-COOH

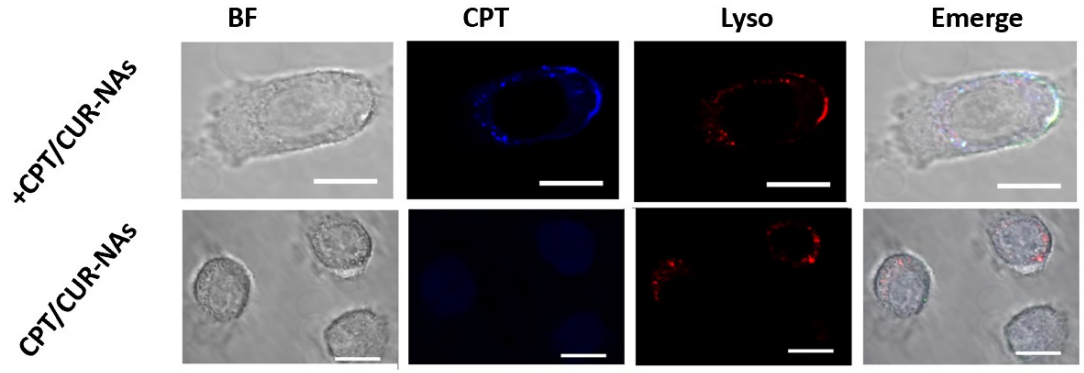


Fig.S12 Cellular uptake of the unmodified and cationic CPT/CUR-NAs in CT26 cells, lysosomes were labeled with lysotracker red, scale bar is 10 μm.

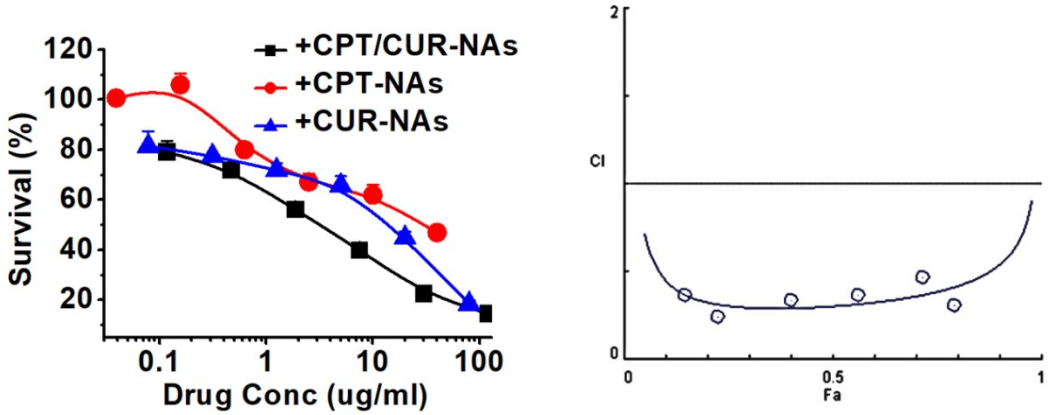
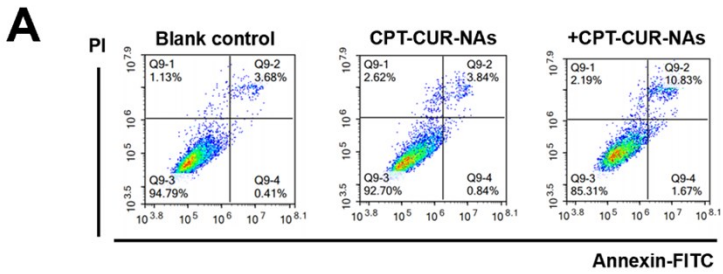


Fig.S13 MTT assay of CT26 cells after treated with various concentrations of +CPT/CUR-NAs, +CPT-NAs and +CUR-NAs. The combination index (IC) versus fraction affected (Fa) plot was drawn using the compysyn software [3].



B

Drug	IC_{50} (μg/ml)		Selectivity index
	CT26	3T3	
Free CPT	0.2	0.8	4.0
Free CUR	2.9	6.6	2.3
+CPT-NAs	30.5	47.9	1.5
+CUR-NAs	4.2	9.6	2.2
+CPT/CUR-NAs	0.9	6.5	7.2
CPT/CUR-NAs	3.2	11.9	3.7

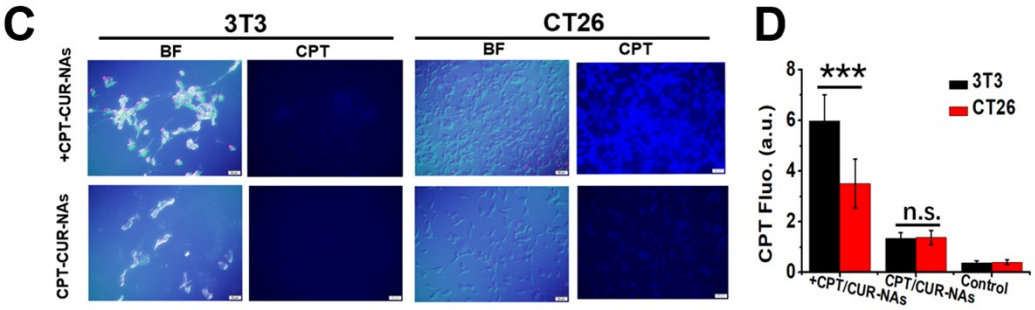


Fig.S14 Apoptotic analysis of 3T3 cells after the treatment of unmodified and cationic CPT/CUR-NAs (A). The IC_{50} and selectivity index of various formulations against CT26 and 3T3 cells (B). Visualization of the intracellular CPT release by observing the CPT fluorescence in cells using a fluorescence microscope (C). Cellular uptake of NAs on cultured CT26 and 3T3 cells (D).

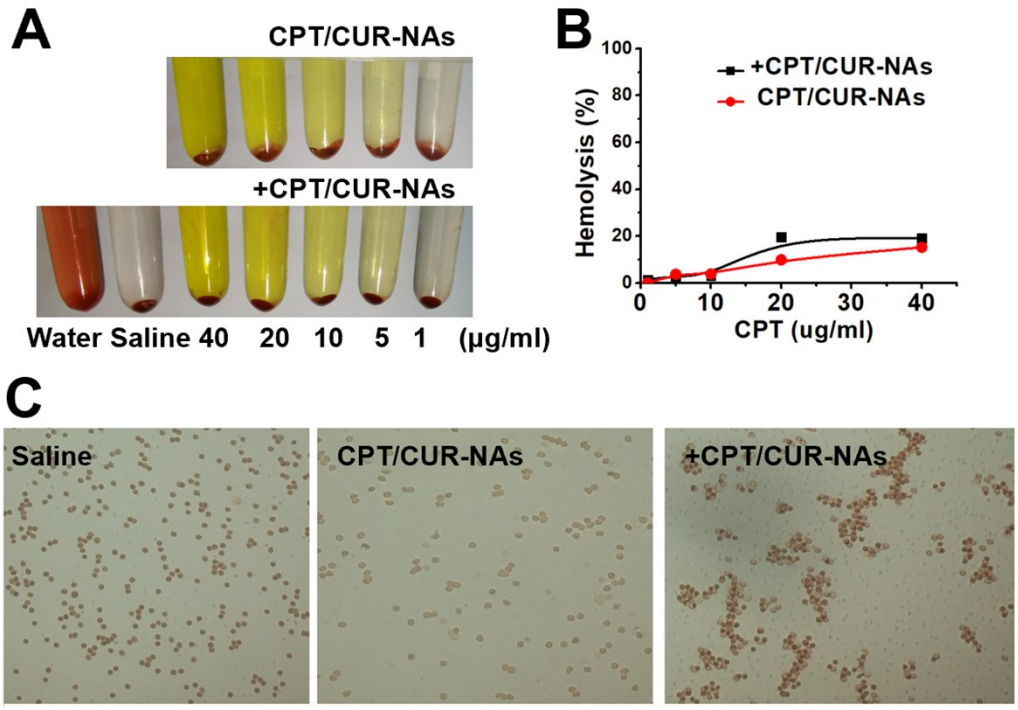


Fig. S15 Hemolytic test (A and B) and aggregation of erythrocytes (C) of CPT/CUR-NAs and +CPT/CUR-NAs

Reference for electronic supplementary information

1. Tang, Q., et al., Developing chemically modified redox-responsive proteins as smart therapeutics. Chemical Communications, 2019. **55**(35): p. 5163-5166.
2. Kharat, M., et al., Physical and Chemical Stability of Curcumin in Aqueous Solutions and Emulsions: Impact of pH, Temperature, and Molecular Environment. Journal of Agricultural and Food Chemistry, 2017. **65**(8): p. 1525-1532.
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