Supporting Information

An Albumin-binding Dimeric Prodrug Nanoparticle with Long Blood Circulation and Light-triggered Drug Release for Chemo-photodynamic Combination Therapy against Hypoxia-induced Metastasis of Lung Cancer

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Materials

Dihydroartemisinin (DHA), D-α-tocopheryl polyethylene glycol succinate (Vitamin E-TPGS, or simply TPGS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 6-maleimidohexanoic acid, N-hydroxysuccinimide (NHS), and pyrene were purchased from Aladdin Industrial Corporation. (Shanghai, China). Carboxymethyl chitosan (CMCTS) was obtained from D&B Biotech Co. Ltd. (Shanghai, China). Thiodiglycolic anhydride, N,N-Diisopropylethylamine (DIPEA), 1,3-diphenylisobenzofuran (DPBF), and 2-nitroimidazole (2-NI) were bought from Energy Chemical Corporation. (Shanghai, China). 4-Dimethylaminopyridine (DMAP) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Glutaric anhydride was obtained from Adamas-beta Chemical Reagent. (Shanghai, China). 1-Hydroxybenzotriazole (HOBT) was bought from Bidepharm Co. Ltd. (Shanghai, China). Ethyl 6-bromohexanoate, L-cysteine hydrochloride monohydrate, 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), and Nile red (NR) were obtained from Macklin Biochemical Co., Ltd. (Shanghai, China). β-Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Chlorin e6 (Ce6) was obtained from meilunbio. (Dalian, China). Bovine Serum Albumin (BSA) was bought from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) was purchased from BI. (Biological Industries, USA). Image-iT™ Green hypoxia reagent was bought from Thermo Fisher Scientific (Waltham, USA). Calcein-AM/PI assay kit, 4',6-diamidino-2-phenylindole (DAPI), and Hoechst33342 were obtained from KeyGen biotech. (Jiangsu, China). Rhodamine 123 (Rh123), N-acetyl-L-cysteine (NAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Reactive Oxygen

Species Assay Kit, and Bicinchoninic Acid Protein Assay Kit were purchased from Beyotime. (Shanghai, China). All other chemicals and solvents mentioned in the article were of analytical grade.

Synthesis of DHA Dimers

DHA (227.48 mg, 0.80 mmol) and DMAP (9.77 mg, 0.08 mmol) were dissolved in anhydrous dichloromethane (5 mL) followed by the addition of thiodiglycolic anhydride (126.85 mg, 0.96 mmol) drop by drop. After 4 h, HOBT (181.60 mg, 1.34 mmol), DIPEA (352.02 μL, 2.13 mmol), EDCI (204.16 mg, 1.06 mmol), and DHA (154.68 mg, 0.54 mmol) were added in order and the reaction proceeded for another 12 h. The product (DHA-S-DHA) was obtained by the purification of column chromatography and the structure was confirmed by nuclear magnetic resonance spectroscopy (NMR, Brucker Avance, Switzerland) and high-resolution mass spectrometry (HRMS, Agilent 6520, Agilent Technologies, USA). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.79 (d, 2H), 5.44 (s, 2H), 3.48 (s, 4H), 2.66-2.51 (m, 2H), 2.44-2.30 (m, 2H), 2.08-1.98 (m, 2H), 1.95-1.84 (m, 2H), 1.83-1.68 (m, 4H), 1.67-1.54 (m, 6H), 1.43 (s, 6H), 1.37-1.22 (m, 6H), 0.96 (d, 6H), 0.88 (d, 6H). ESI-MS: m/z = 700.3355 ($[M+NH_4]^+$) and m/z = 705.2914 ($[M+Na]^+$).

The synthesis process of DHA-C-DHA was similar to that of DHA-S-DHA by replacing thiodiglycolic anhydride with glutaric anhydride (109.54 mg, 0.96 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.78 (d, 2H), 5.43 (s, 2H), 2.62-2.50 (m, 2H), 2.5-2.43 (t, 4H), 2.40-2.29 (m, 2H), 2.08-2.01 (m, 2H), 2.01-1.95 (m, 2H), 1.93-1.83 (m, 2H), 1.81-1.67 (m, 4H), 1.66-1.56 (m, 6H), 1.43 (s, 6H), 1.33-1.23 (m, 6H), 0.96 (d, 6H), 0.84 (d, 6H). ESI-MS: m/z = 682.3800 ($[M+NH_4]^+$) and m/z = 687.3357 ($[M+Na]^+$).

Synthesis of CMCTS-MAL&NI (CMN)

6-(2-nitro-1H-imidazoyl) hexanoic acid (NHA) was synthesized following the methods in the literature.[1] Ethyl 6-bromohexanoate (H1, 1.04 g, 4.65 mmol), 2-nitroimidazole (0.50 g, 4.43 mmol), and potassium carbonate (4.90 g, 35.40 mmol) were dissolved in acetonitrile and reacted at 60 ℃. 6 days later, pH was adjusted to 7-8 by adding potassium carbonate. After appropriate amounts of ethyl acetate and water were added, the mixture was separated by extraction. The organic phase was evaporated to obtain ethyl-(2-nitroimidazolyl) hexanoate (H2). The above product H2 (1.13 g, 4.43 mmol) was added into concentrated hydrochloric acid drop by drop and the reaction proceeded overnight. The solvent was removed by rotary evaporation to obtain 6-(2-nitroimidazole) hexanoic acid (NHA). ^{1}H-NMR (300 MHz, DMSO-*d*6): δ 7.17-7.12 (d, 1H), 7.11-7.06 (d, 1H), 4.47-4.32 (t, 2H), 2.42-2.28 (t, 2H), 1.96-1.79 (m, 2H), 1.75-1.61 (m, 2H), 1.47-1.33 (m, 2H). ESI-MS: m/z = 228.0983 ($[M+H]^+$) and 250.0803 ($[M+Na]^+$).

NHA (227.22 mg, 1.00 mmol), 6-maleimidohexanoic acid (211.21 mg, 1.00 mmol), EDCI (766.8 mg, 4.00 mmol), and NHS (460.36 mg, 4.00 mmol) were dissolved in formamide and activated for 30 min. The above solution was added dropwise to carboxymethyl chitosan (190.18 mg) in formamide/water (5: 1, v/v) solution and the reaction was continued for 24 h. After that, the reaction mixture was added dropwise to acetone. The precipitate was collected by centrifugation and washed with acetone 3 times to obtain CMN.

Determination of Critical Micelle Concentration

The critical micelle concentration was determined by using pyrene as a fluorescent probe.[2] Pyrene is sensitive to the change in the polarity of solutions. There are five

characteristic peaks in the fluorescence emission spectrum of pyrene solution (excited at 337 nm), which are 370, 379, 385, 394, and 480 nm. Peak fluorescence intensity of the first emission/peak fluorescence intensity of the third emission (I1/I3) is often used to reflect the change in the polarity of the local environment. In the graph of intensity ratio I_{370}/I_{385} against the logarithmic concentration of micelles, an obvious turning point can be observed which represents the critical micelle concentration. Briefly, CMN was diluted in ultrapure water ranging from 1 to 800 μg/mL, respectively. Then the pyrene solution (a final concentration of 5.93×10^{-7} mol/L) was added to the above solution. The fluorescence spectras were obtained by a fluorescence spectrophotometer (RF-6000, Shimadzu).

Determination of Degree of Amino Substitution

CMN consists of carboxymethyl chitosan modified with maleimide and 2-nitroimidazole group. Thus, we measured the degree of substitution of maleimide and 2-nitroimidazole group by indirect Ellman's assay and UV method, respectively. Since the stoichiometry of Michael addition reaction between maleimide and thiol is 1:1 by mole, the amount of maleimide can be quantitated by a reverse Ellman's assay as previously described.[3, 4] Specifically, the sample reacted with an excess thiol and the amount of unreacted thiol was determined using Ellman's method, thereby the substitution degree in CMN was calculated indirectly. Briefly, 3 mg CMN was dissolved in 1 mL of PBS, then L-cysteine hydrochloride monohydrate was added and incubated for 10 min. 0.5 mL of the above solution was mixed with 2.5 mL of Ellman's reagent (0.1 mM DTNB) and the reaction was allowed to proceed for 30 min. Then the reaction was measured spectrophotometrically at 412 nm immediately. The standard curve was obtained by Ellman's assay of gradient concentration of L-cysteine

hydrochloride monohydrate.

The quantitation of 2-nitroimidazole was spectrophotometrically determined from the characteristic absorption at 325 nm on UV-vis spectra. Briefly, 2 mg 2-nitroimidazole was dissolved in 2 mL of PBS solution containing 0.1% Tween 80 and diluted to 100, 50, 20, 10, 5, 2, 1 μg/mL. Then the absorbance of different concentrations at 325 nm was measured by UV-Vis spectrophotometer (UV-2450, Shimadzu, Japan) to obtain the standard curve. Besides, sample determination was conducted by dissolving 2 mg CMN in 4 mL of PBS solution containing 0.1% Tween 80 and measuring the absorbance at 325 nm.

Characterization of Dimer Prodrug Nanoparticles

The particle size and size distribution of the nanoparticles were determined by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments, U.K.) at a concentration of 0.5 mg/mL. The nanoparticles was counterstained with 0.1% phosphotungstic acid and observed by transmission electron microscopy (TEM, HT7700, Hitachi, Japan).

Colloidal Stability Assay

Ce6&DHA-S-DHA@NPs (CD NPs), Ce6&DHA-S-DHA@CMN NPs (CDC NPs), Ce6&DHA-S-DHA@TPGS NPs (CDT NPs) (2 mg/mL of Ce6 equivalent) were dispersed in phosphate buffer saline (PBS, pH = 7.4) and PBS containing 4 mg/mL of BSA for 24 h. The size of nanoparticles at various points (0, 1, 2, 4, 6, 8, 12, 24 h) was subjected to DLS characterization. Besides, long-term stability was monitored by storing the drug at 4 °C for 3 months.

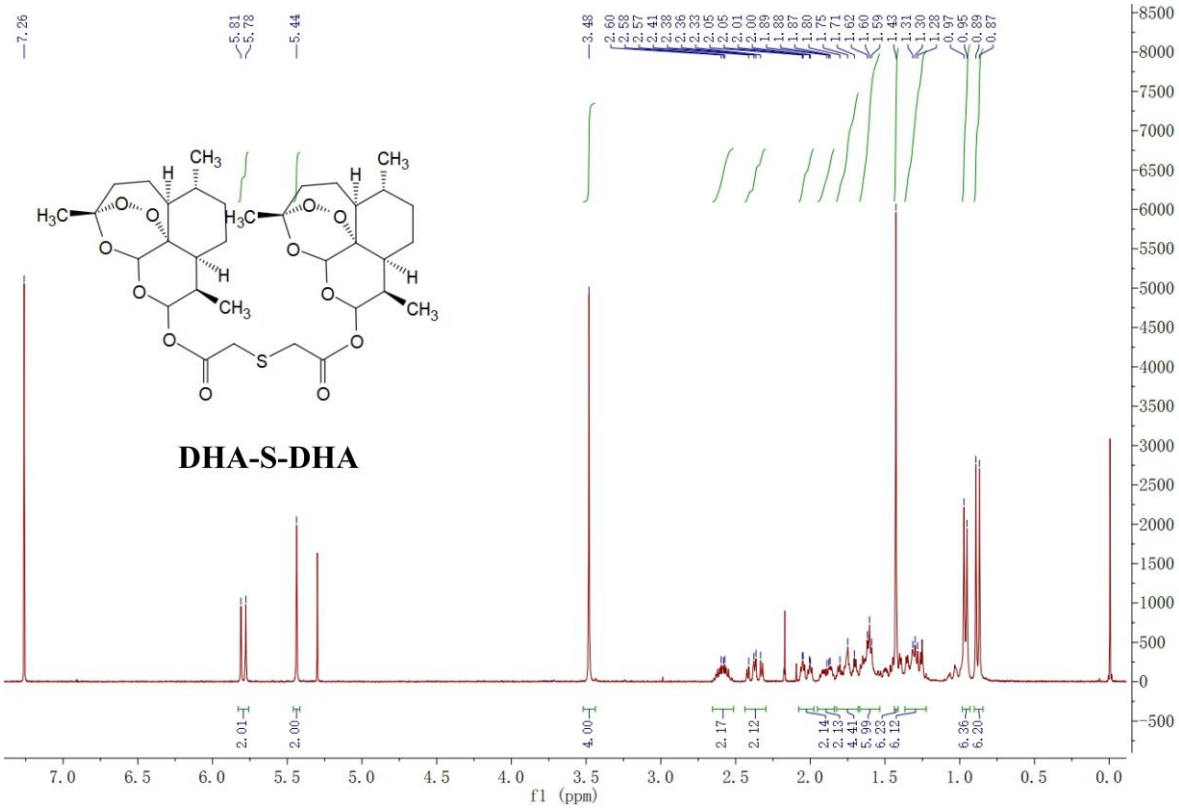

Figure S1. ^{1}H NMR spectrum of DHA-S-DHA.

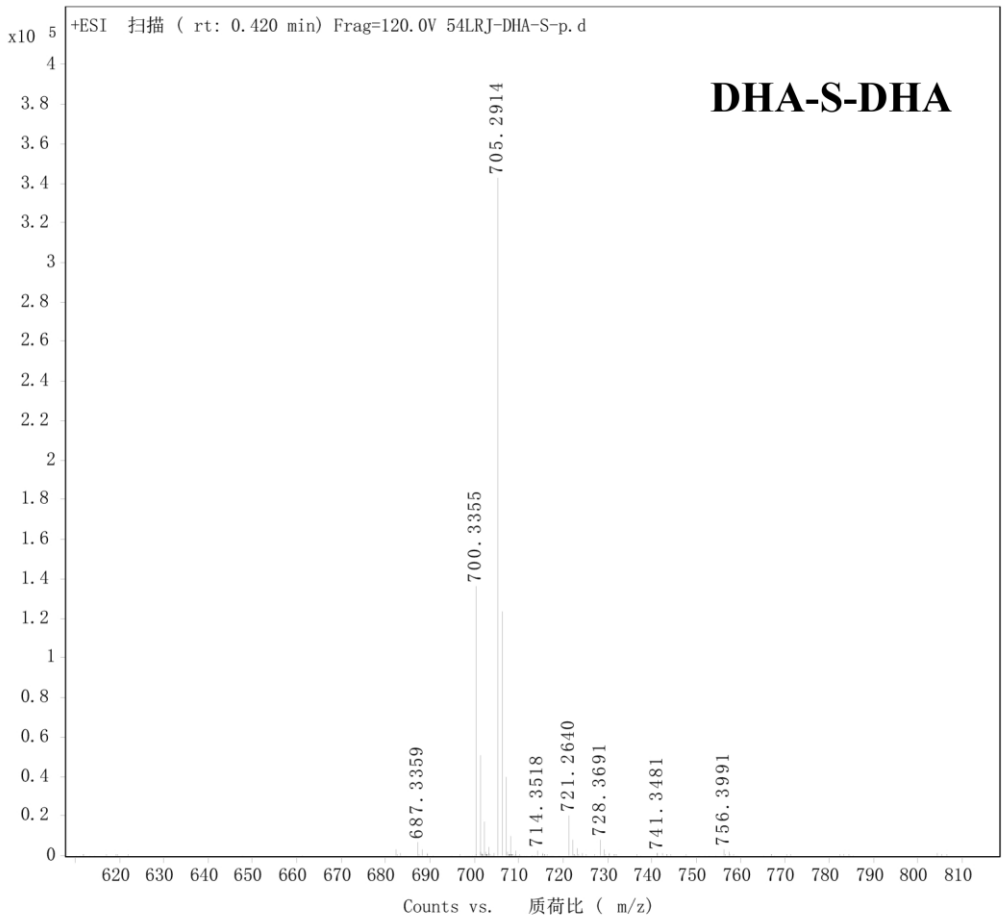

Figure S2. ESI-MS spectrum of DHA-S-DHA.

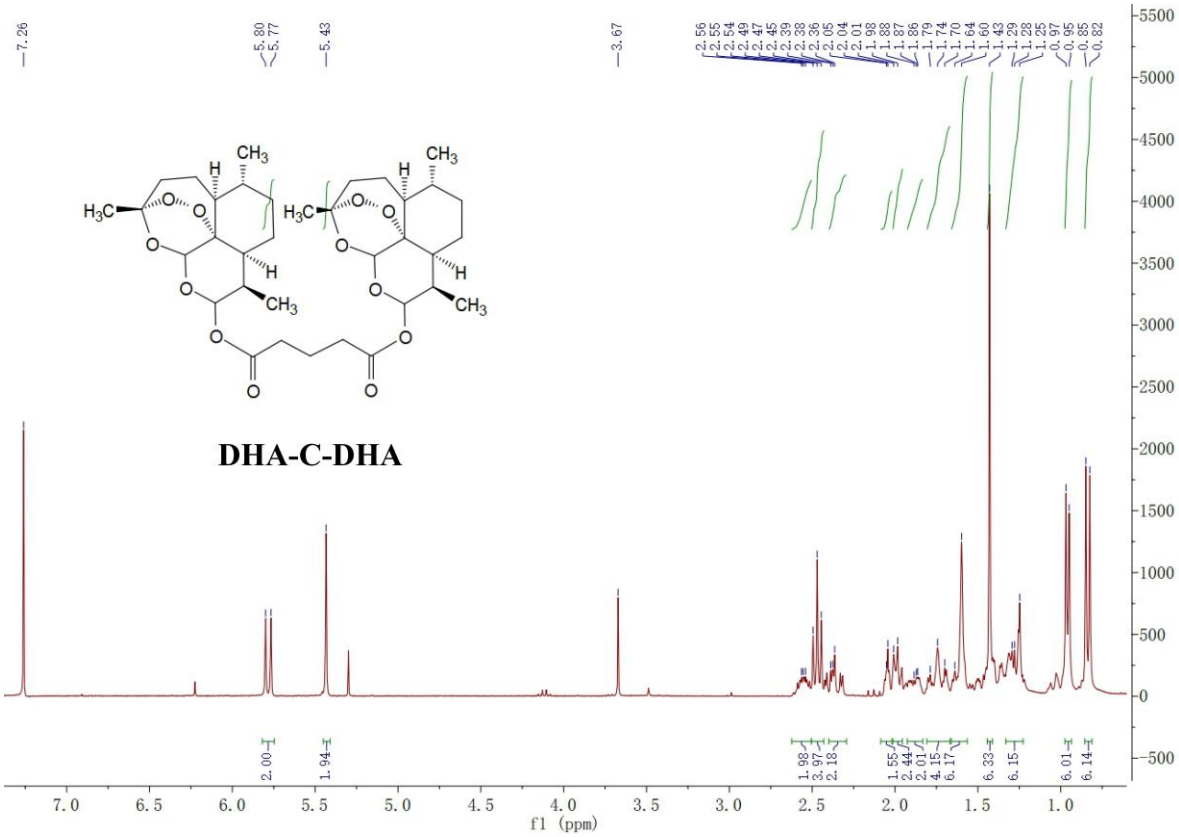

Figure S3. ^{1}H NMR spectrum of DHA-C-DHA.

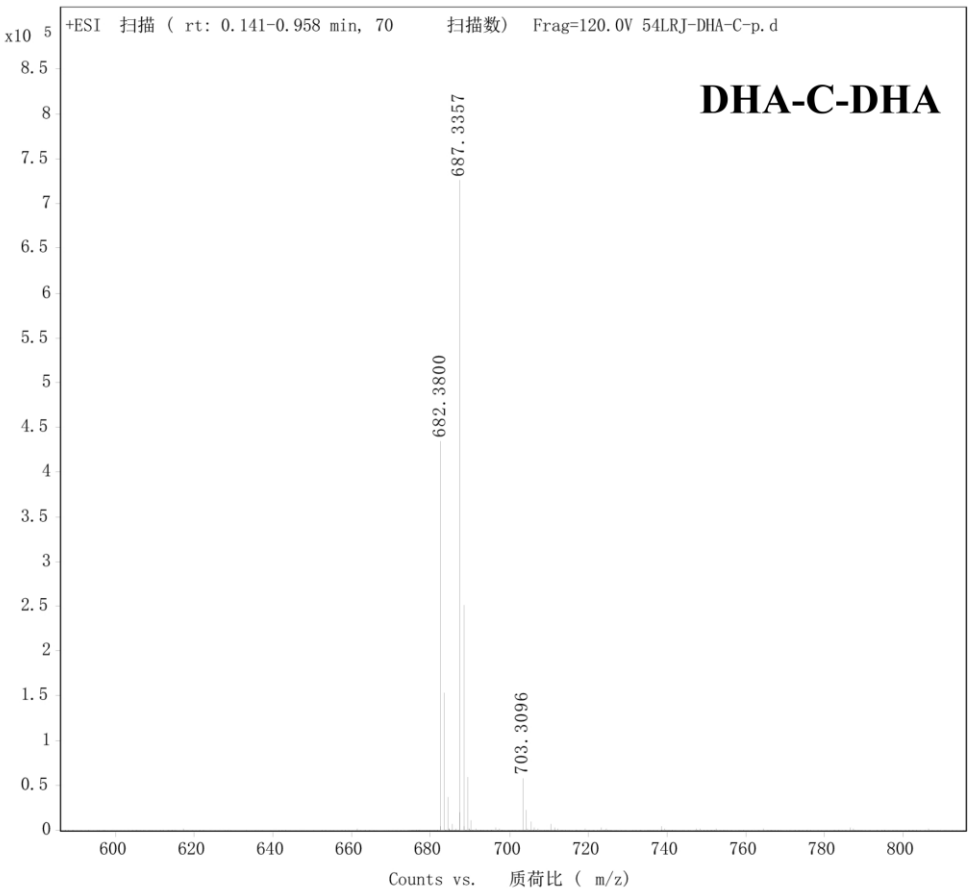

Figure S4. ESI-MS spectrum of DHA-C-DHA.

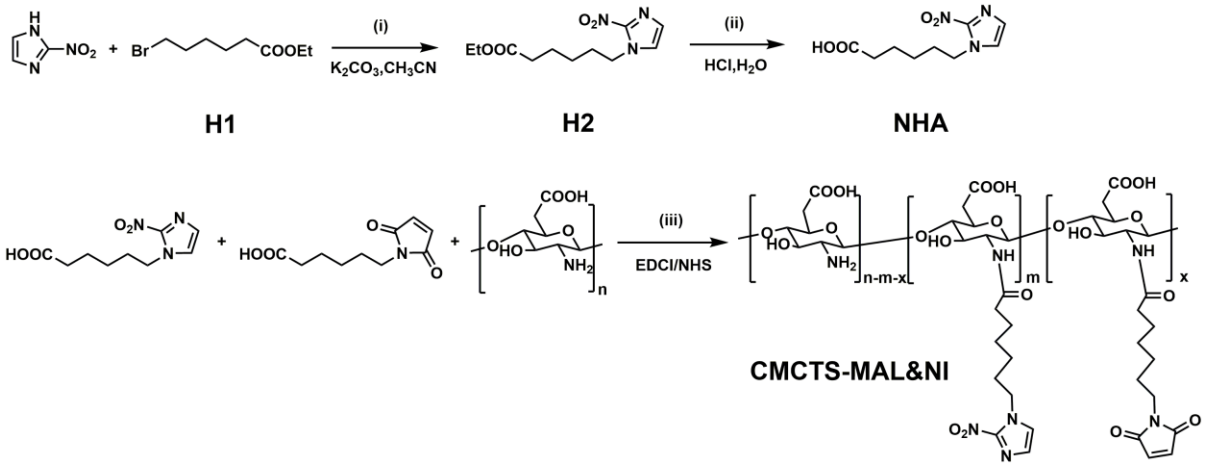

Figure S5. The synthesis scheme of CMN.

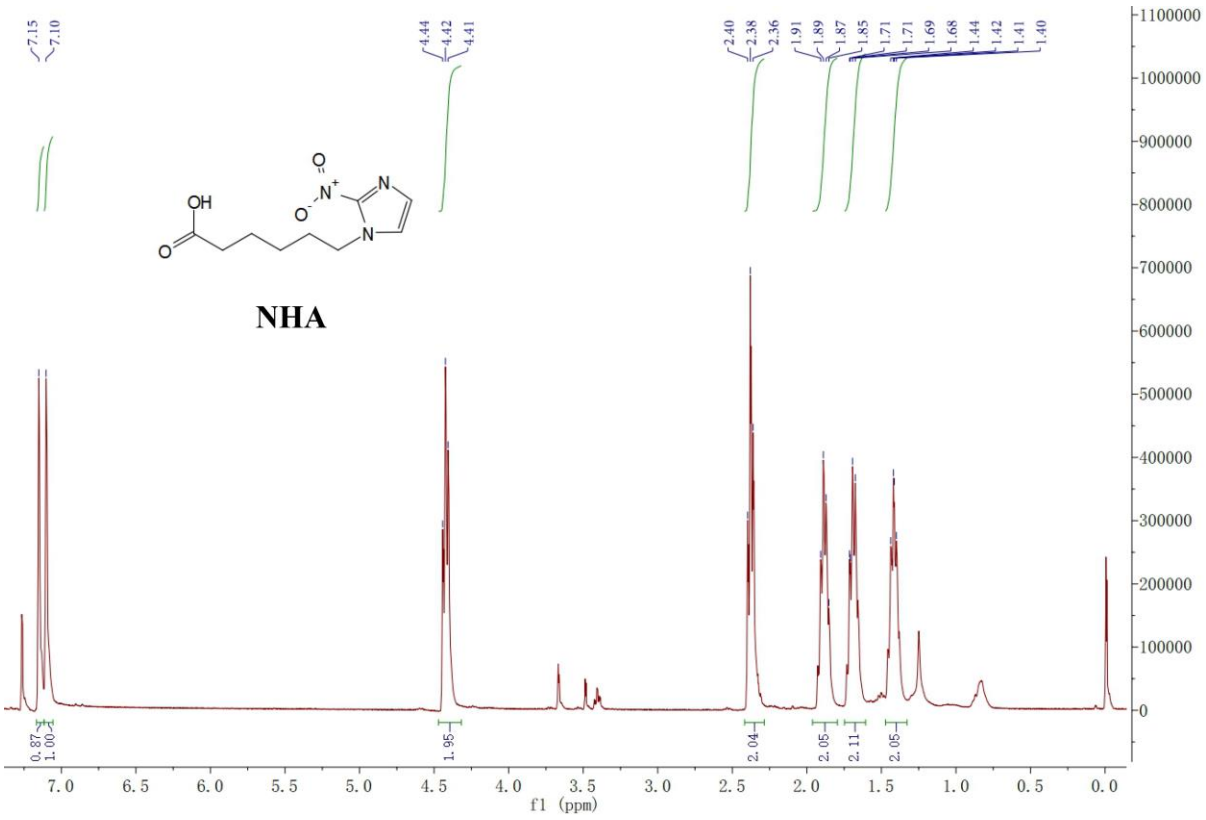

Figure S6. ^{1}H NMR spectrum of NHA.

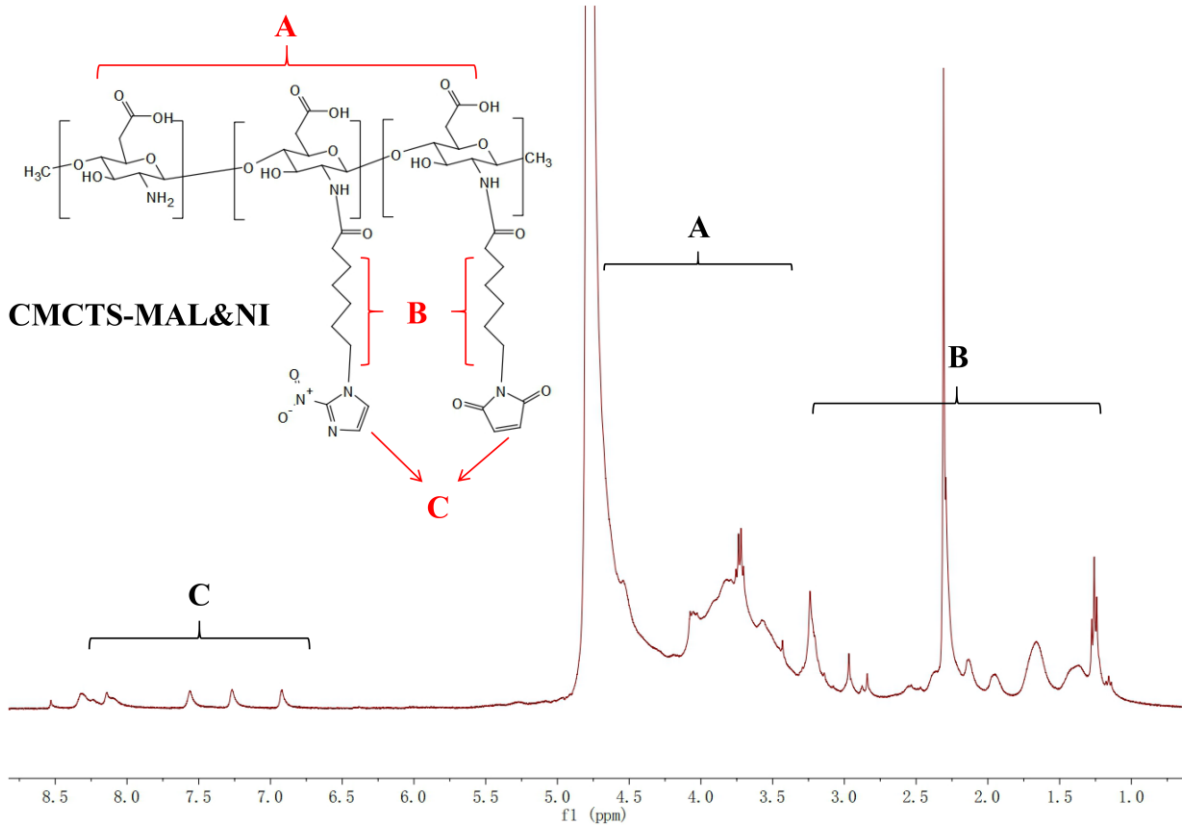

Figure S7. ^{1}H NMR spectrum of CMN.

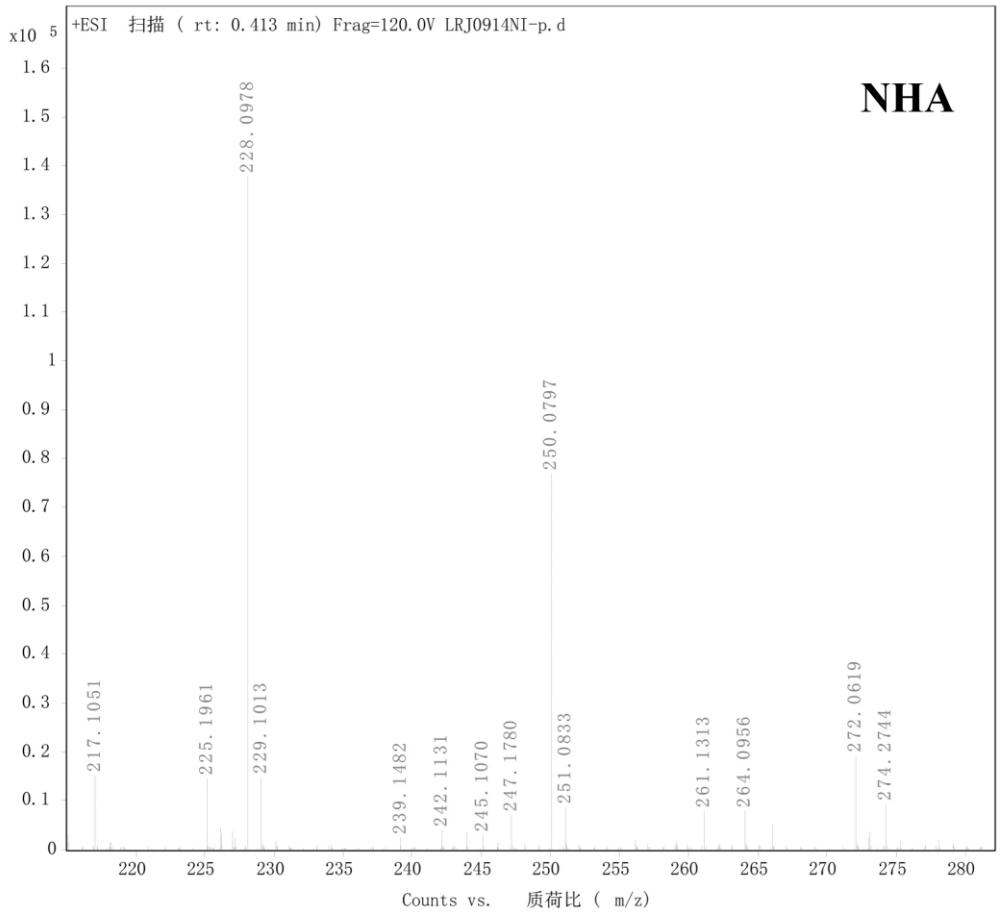

Figure S8. ESI-MS spectrum of NHA.

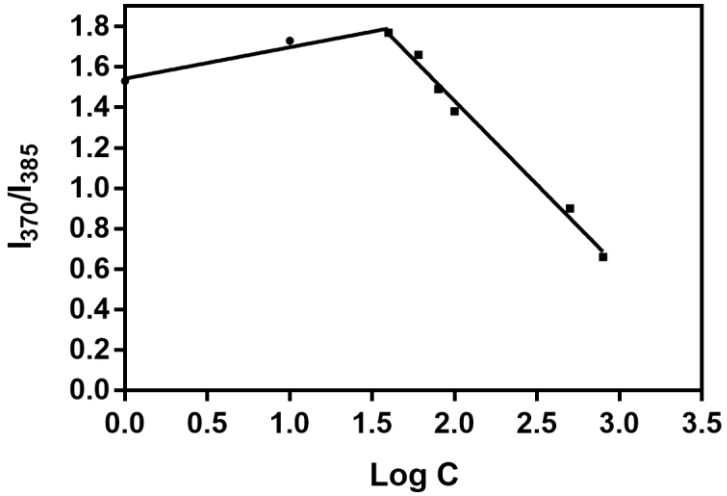

Figure S9. Critical micelle concentration measurement of CMN using fluorescence excitation spectra at a wavelength of 337 nm.

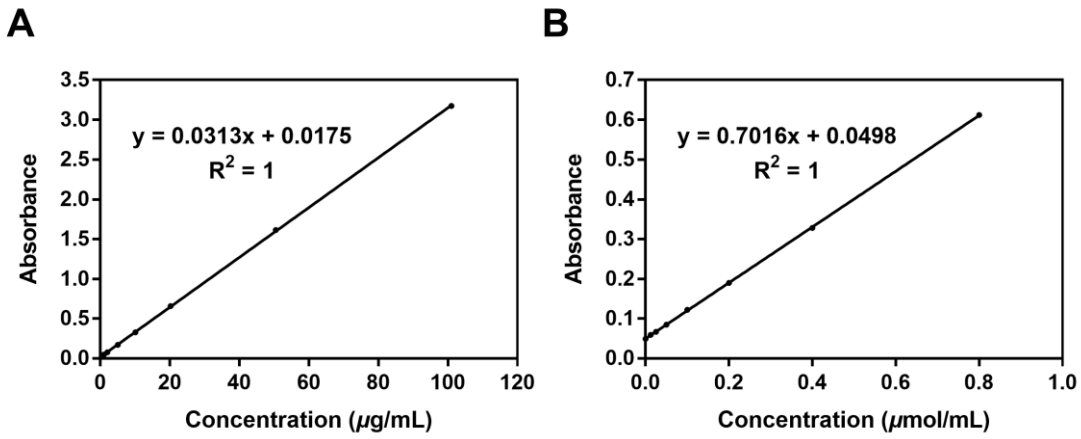

Figure S10. The standard curves of 2-nitroimidazole (A) and L-cysteine hydrochloride monohydrate (B).

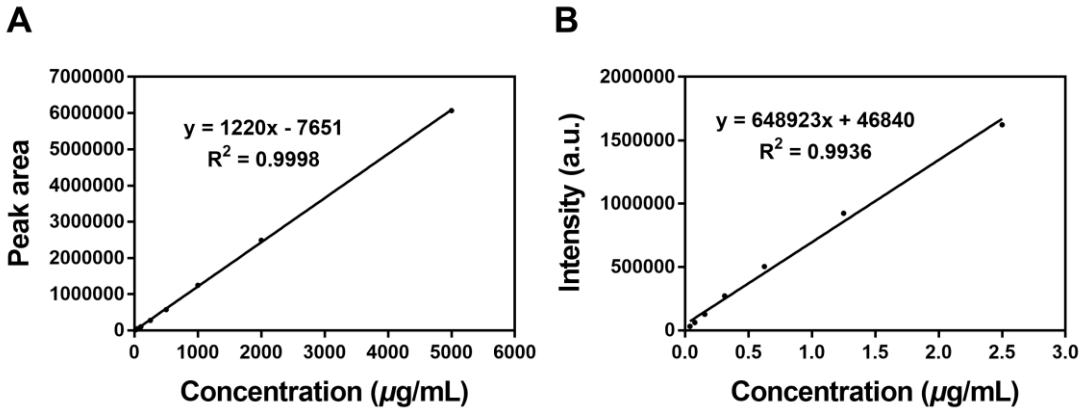

Figure S11. The standard curves of DHA-S-DHA and Ce6.

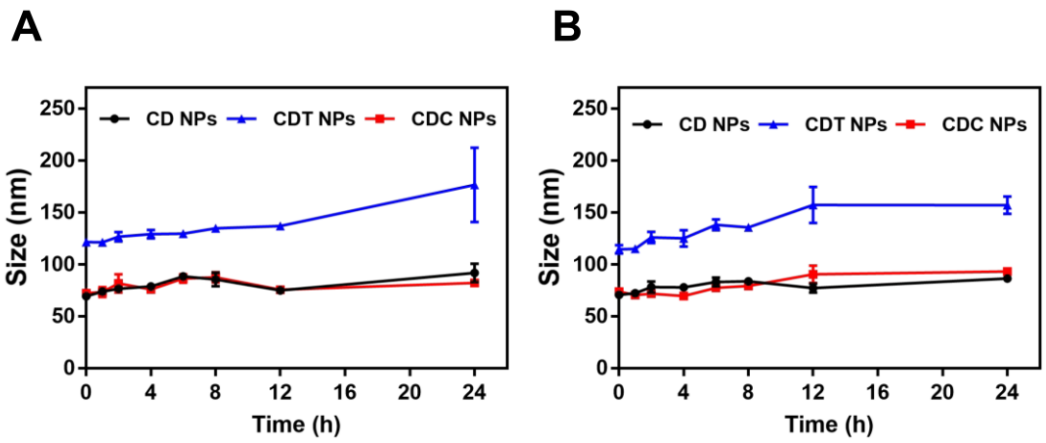

Figure S12. Stability studies. Size of CD NPs, CDT NPs, CDC NPs in PBS solutions (A) or PBS solutions containing 4 mg/mL of BSA (B) (n = 3).

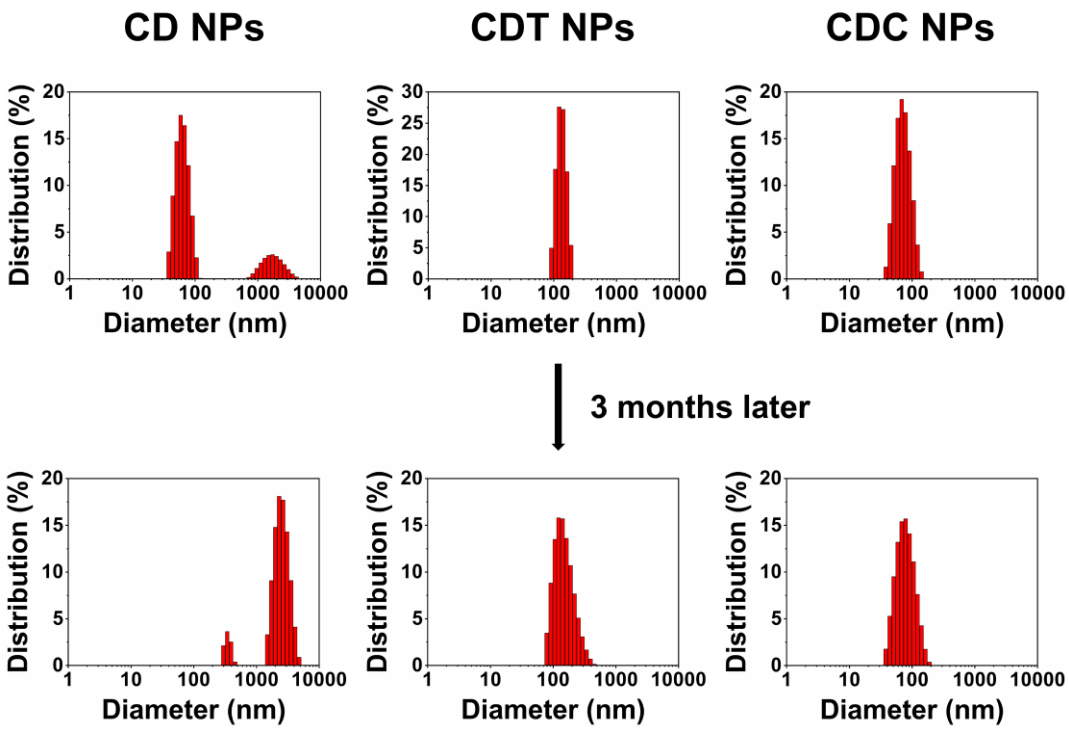

Figure S13. Long-term stability studies. The change of size distribution of three formations dispersed in PBS solution after 3 months.

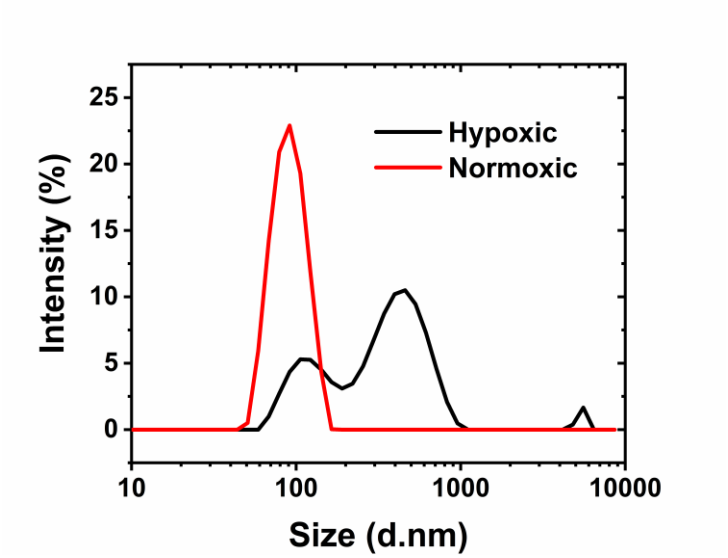

Figure S14. The size distribution of CDC NPs incubated with 100 μM NADPH under normoxia or hypoxia condition (degassed with N_2) for 4 h.

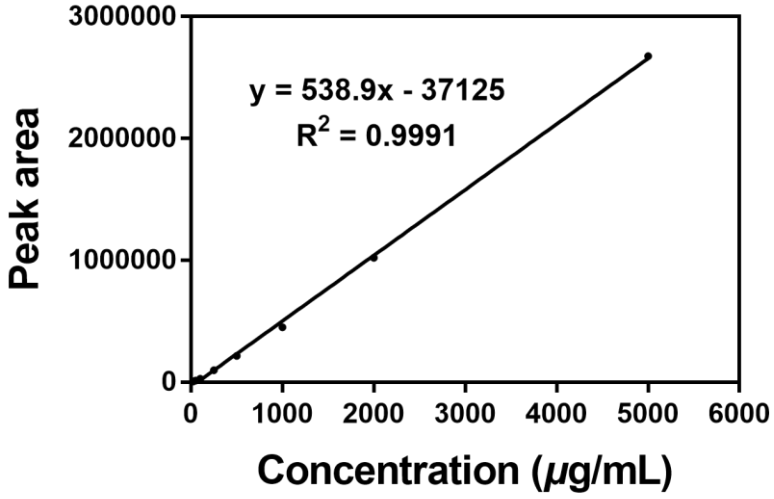

Figure S15. The standard curves of DHA-C-DHA.

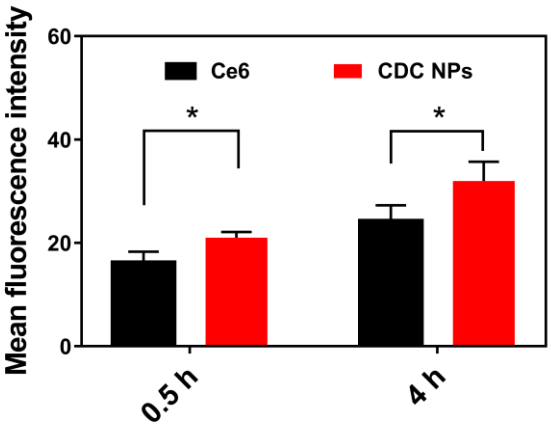

Figure S16. Quantitative result for the mean fluorescent intensity in CLSM images of cellular uptake (n = 3). *$P < 0.05$.

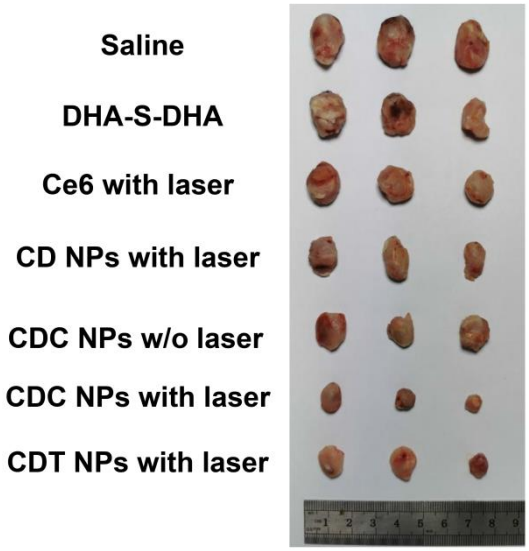

Figure S17. Photographs of ex-tumor images of each group.

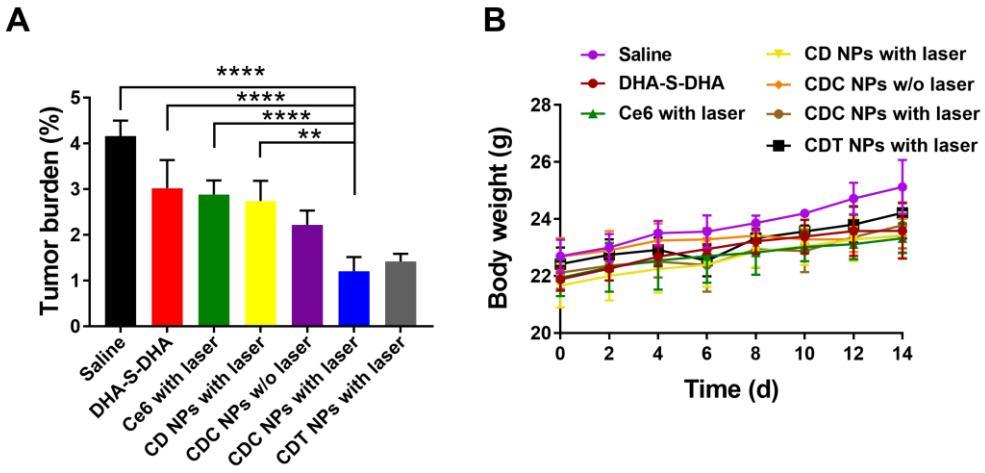

Figure S18. (A) Tumor burden measured at days 15 (n = 5). $^{**}P < 0.01$, $^{****}P < 0.0001$. (B) The change of body weight during the pharmacodynamic study (n = 5).

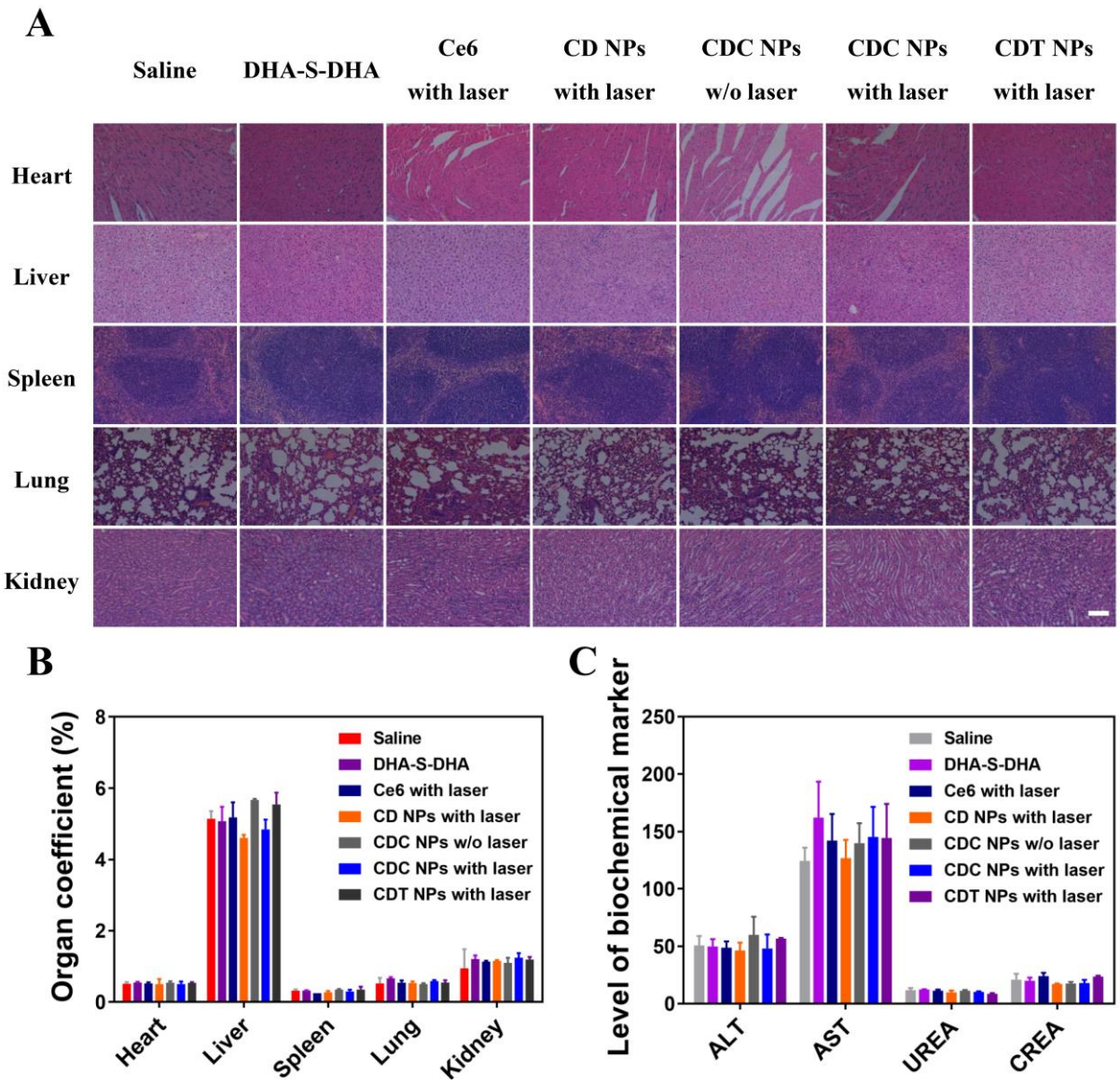

Figure S19. Safety evaluation. (A) H&E staining of mice major organs (hearts, livers, spleens, lungs, and kidneys) in the experiment of safety evaluation (scale bar = 100 μm). (B) Organ coefficients of mice after various treatments on day 15 (n = 3). (C) Level of biochemical markers of renal and liver function (n = 3).

Table S1. 50% inhibitory concentration (IC50) values of CDC NPs on Lewis Lung Carcinoma (LLC) cells under normoxic or hypoxic condition.

Incubation condition	IC50 of CDC NPs (μg/mL)
Normoxic	6.732
Hypoxic	2.749

Table S2. *In vivo* pharmacokinetic parameters of DHA after intravenous administration of different drugs or nanoparticles in rats (5 mg/kg of Ce6 and 10 mg/kg of DHA-S-DHA equivalent, mean ± SD, n = 3).

Preparations	$t_{1/2}$ (h)	$AUC_{(0-t)}$ (μg/mL*h)	$MRT_{(0-\infty)}$ (h)	Cl ((mg/kg)/(μg/mL)/h)
DHA-S-DHA	1.28 ± 0.19	1160.29 ± 225.49	0.50 ± 0.19	0.0073 ± 0.0014
CD NPs	1.15 ± 0.61	1435.05 ± 209.97	0.54 ± 0.09	0.0059 ± 0.0009
CDT NPs	6.48 ± 0.85	13895.69 ± 844.97	9.69 ± 1.17	0.0005 ± 0.0000
CDC NPs	8.46 ± 3.44	18017.84 ± 1475.94	9.21 ± 2.17	0.0004 ± 0.0001
CDT NPs (Second injection)	2.56 ± 0.58	4654.15 ± 505.75	2.49 ± 0.13	0.0018 ± 0.0002
CDC NPs (Second injection)	5.18 ± 0.94	17491.11 ± 3487.17	7.57 ± 1.33	0.0005 ± 0.0001

Table S3. Routine blood test results of C57BL/6 mice after different treatments (data are presented as mean ± SD, n = 3).

Item \ Group	Saline	DHA-S-DHA	Ce6 with laser	CD NPs with laser	CDC NPs w/o laser	CDC NPs with laser	CDT NPs with laser
WBC ($10^3/\mu L$)	4.40 ± 0.35	6.47 ± 0.99	6.23 ± 0.87	5.87 ± 0.51	6.30 ± 0.61	5.63 ± 0.76	6.00 ± 1.65
Lymphocyte ($10^3/\mu L$)	2.83 ± 0.21	4.50 ± 0.78	4.37 ± 0.47	4.37 ± 0.40	4.20 ± 0.56	4.03 ± 0.74	3.97 ± 1.63
Monocyte ($10^3/\mu L$)	0.13 ± 0.06	0.23 ± 0.06	0.23 ± 0.06	0.17 ± 0.06	0.20 ± 0.10	0.10 ± 0.00	0.17 ± 0.06
Neutrophil ($10^3/\mu L$)	1.43 ± 0.25	1.73 ± 0.21	1.63 ± 0.35	1.33 ± 0.15	1.90 ± 0.10	1.50 ± 0.40	1.87 ± 1.06
Lymph (%)	64.50 ± 4.14	69.80 ± 2.95	70.40 ± 2.33	73.87 ± 2.30	66.33 ± 2.97	71.13 ± 6.13	65.70 ± 16.74
Mon (%)	3.43 ± 0.42	3.60 ± 0.44	3.63 ± 0.32	2.97 ± 0.46	3.90 ± 1.04	2.23 ± 0.25	3.17 ± 0.06
Gran (%)	32.07 ± 4.12	26.60 ± 2.72	25.97 ± 2.22	23.17 ± 1.92	29.77 ± 2.06	26.63 ± 6.37	31.13 ± 16.71
RBC ($10^6/\mu L$)	8.88 ± 0.18	9.18 ± 0.31	8.91 ± 0.34	9.47 ± 0.50	8.90 ± 0.21	8.08 ± 0.35	7.60 ± 1.46
Hemoglobin (g/L)	139.00 ± 2.65	141.00 ± 5.29	138.33 ± 5.13	146.67 ± 7.51	139.00 ± 4.58	126.33 ± 5.03	119.67 ± 25.01
HCT (%)	41.07 ± 0.76	42.93 ± 1.97	41.77 ± 1.03	43.80 ± 2.26	40.73 ± 1.04	37.93 ± 1.69	35.33 ± 7.13
MCV (fl)	46.33 ± 0.23	46.80 ± 0.96	46.93 ± 0.75	46.33 ± 0.21	45.80 ± 0.62	47.00 ± 0.20	46.53 ± 0.85

MCH (pg)	15.60 ± 0.26	15.30 ± 0.26	15.47 ± 0.15	15.43 ± 0.12	15.57 ± 0.32	15.60 ± 0.26	15.63 ± 0.29
MCHC (g/L)	338.00 ± 7.21	328.00 ± 2.65	330.67 ± 4.04	334.33 ± 1.15	340.67 ± 2.52	332.67 ± 7.57	337.67 ± 7.23
RDW (%)	11.87 ± 0.58	12.97 ± 0.75	12.63 ± 0.40	12.43 ± 0.57	12.47 ± 0.91	12.60 ± 0.00	12.47 ± 0.61
PLT ($10^3/\mu$L)	461.33 ± 124.80	460.00 ± 54.84	426.00 ± 63.00	383.00 ± 97.75	521.33 ± 56.05	538.67 ± 63.52	477.67 ± 279.35
MPV (fl)	5.43 ± 0.15	5.30 ± 0.36	5.40 ± 0.10	5.47 ± 0.06	5.47 ± 0.32	5.33 ± 0.15	5.40 ± 0.17
PDW	16.90 ± 0.10	16.80 ± 0.26	16.77 ± 0.12	16.90 ± 0.17	16.93 ± 0.32	16.90 ± 0.10	17.13 ± 0.23
PCT (%)	0.25 ± 0.06	0.24 ± 0.03	0.23 ± 0.04	0.21 ± 0.05	0.28 ± 0.02	0.29 ± 0.03	0.25 ± 0.14

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