Three-in-one Self-assembled Metallo-nanophotosensitizers for Photodynamic/Chemodynamic/Chemo Multimodal Synergistic Cancer Therapy

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Supporting Information

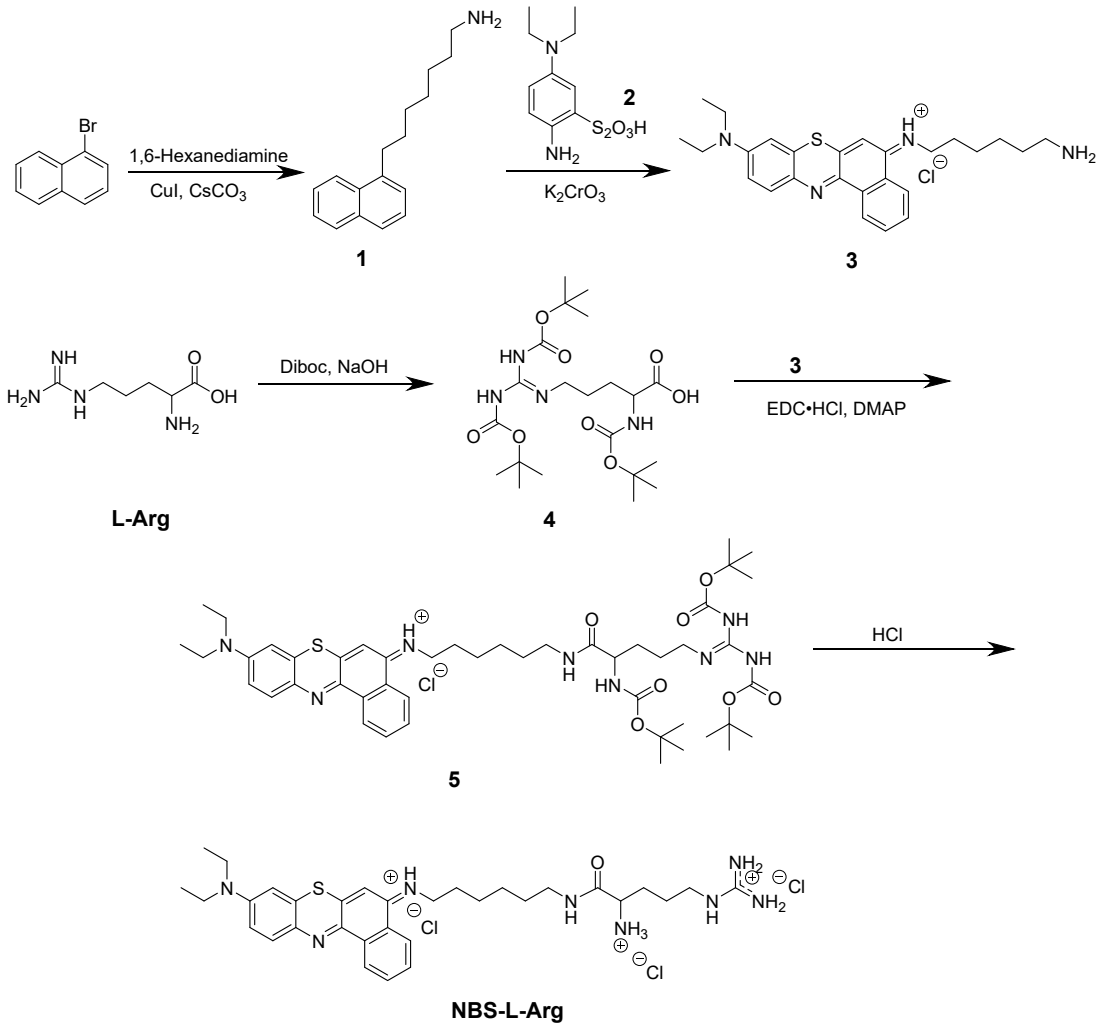
1. General information

All reagents were purchased from commercial suppliers and used without further purification unless specified. Doxorubicin hydrochloride was purchased from Sangon Biotech. ^{1}H NMR spectra were recorded on a Bruker 500 MHz Spectrometer, with working frequencies of 400 MHz for ^{1}H and 101 MHz for ^{13}C nuclei, respectively. SEM image was obtained using a Nano SEM-450 (FEI, U.S.A.) with an accelerating voltage of 10.0 kV. TEM image was obtained by TECNAI G2 SPIRIT BIO (FEI, U.S.A.). UV-vis spectra were recorded with Shimadzu 1750 UV-visible spectrophotometer (Japan) at 298 K. Human liver hepatocellular carcinoma HepG2, and human liver HL7702, were obtained from KeyGEN BioTECH Co. (Nanjing, China). Cell culture was carried out in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C. Dihydrorhodamine 123 (DHR123), Dihydroethidium (DHE), hydroxyphenyl fluorescein (HPF) were purchased from MKbio (China). The green fluorescent probes for labeling endoplasmic reticula, mitochondria, and lysosomes were purchased from Beyotime (China). Annexin V-EGFP/PI apoptosis detection kit was purchased from Solarbio (China).

2. Live subject statement

All experiments were performed in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects of World Health Organization, and approved by the Northwest A&F University Animal Care Committee.

3. Synthesis and characterizations



Scheme S1: Synthesis of the amphiphilic molecule **NBS-L-Arg.**

Synthesis of compound 1. 1-Bromonaphthalene (2.1 g, 10.0 mmol) and 1,6-Hexanediamine (2.35 g, 20.0 mmol) were dissolved in 2-Methoxyethanol (20 mL). CuI (95 mg, 0.5 mmol) and $CsCO_3$ (2.25 g, 7.8 mmol) were added to the above solution, and the ensuing solution was stirred for 12 h under reflux before being filtrated. The resulting filtrate was concentrated by rotary evaporator to obtain the crude product residue. This residue was dissolved in DCM and then washed with H_2O and sat. NaCl (3 × 10 mL), successively. The combined organic phases were dried by Na_2SO_4 and concentrated under reduced pressure to give the crude product. The crude product was purified by column chromatography, using dichloromethane/methanol (DCM/MeOH = 10:1, *v/v*) as eluent to afford a yellow oily liquid (1.37 g, 51 %). The ^{1}H NMR spectrum of compound **1** was shown in Figure S1. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm): δ 7.79 (dd, J = 12.4, 9.2 Hz, 2H), 7.46-7.38

(m, 2H), 7.34 (t, J = 7.9 Hz, 1H), 7.21 (d, J = 8.1 Hz, 1H), 6.58 (d, J = 7.5 Hz, 1H), 3.24 (t, J = 7.0 Hz, 2H), 2.81 (s, 2H), 1.79-1.70 (m, 2H), 1.62-1.55 (m, 2H), 1.53-1.34 (m, 4H).

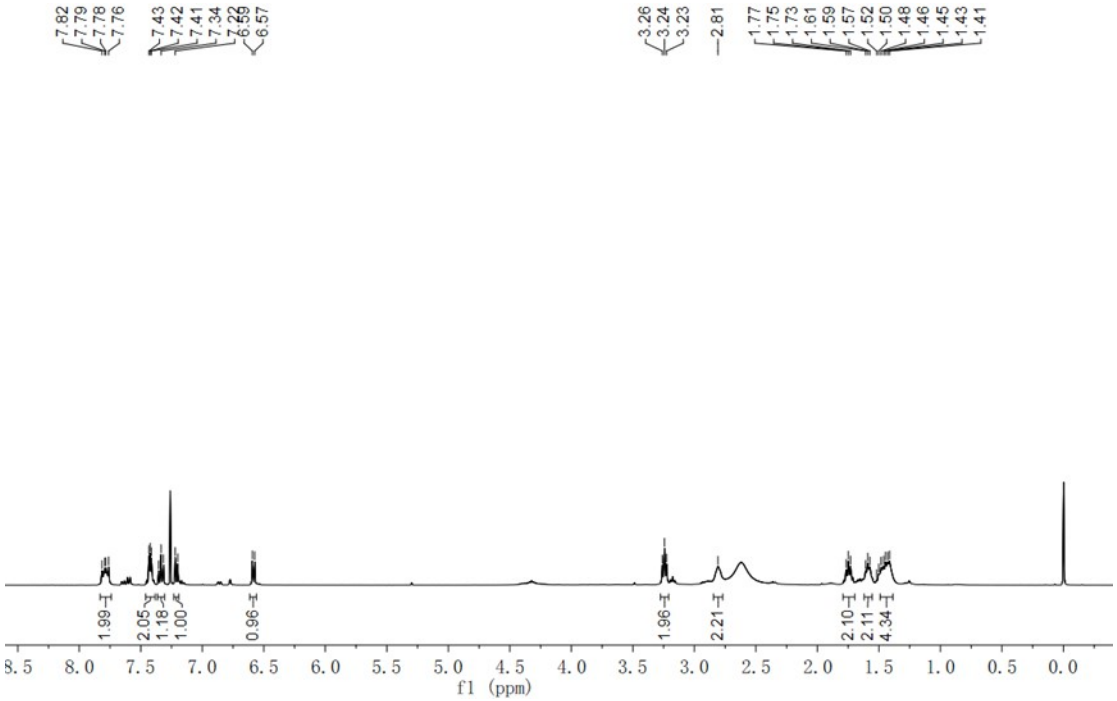


Fig. S1 ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298K) of compound **1**

Synthesis of compound 3. Compound **1** (1.26 g, 5.2 mmol) and compound **2** (1.05 g, 3.8 mmol) (compound **2** was synthesized according to the reported method[S1-S2]) were dissolved in DMSO (20 mL). $K_2Cr_2O_7$ (1.2 g, 4.05 mmol) was added into the above solution. After the solution was stirred for 20 min, MeOH (200 mL) and HCl (2 M) were added. The resulting mixture was concentrated by rotary evaporator and a blue precipitate was obtained by adding sat. NaCl (200 mL), then filtered and dried. The crude product was purified by column chromatography, using dichloromethane/methanol (DCM/MeOH = 8:1, *v/v*) as eluent to afford a blue oily liquid (511 mg, 31 %). ^{1}H NMR (400 MHz, MeOD) δ (ppm): 8.59 (d, J = 8.0 Hz, 1H), 8.18 (d, J = 8.0 Hz, 1H), 7.69 (t, J = 7.5 Hz, 1H), 7.66-7.60 (m, 2H), 7.17 (dd, J = 9.5, 2.4 Hz, 1H), 6.98 (s, 1H), 6.96 (d, J = 2.4 Hz, 1H), 3.66-3.56 (m, 4H), 3.51 (t, J = 7.4 Hz, 2H), 3.01-2.95 (m, 2H), 1.87-1.69 (m, 4H), 1.54 (m, 4H), 1.31 (t, J = 7.0 Hz, 7H).

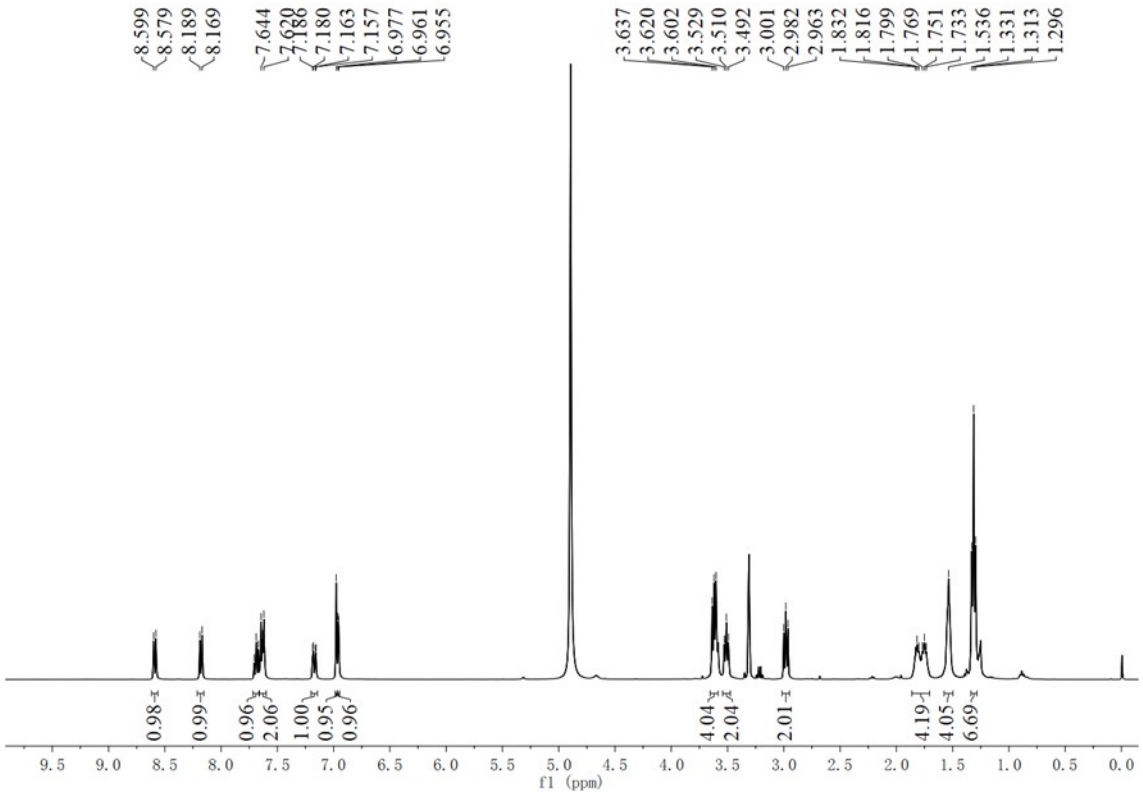


Fig. S2 ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298K) of compound **3**

Synthesis of compound 5. Compound **3** (1 g, 2.0 mmol) and compound **4** (0.96 g, 2.0 mmol) (compound **4** was synthesized according to the reported method[S3]) were dissolved in DMF (20 mL). Then EDC (0.77 g, 4.0 mmol) and DMAP (48.86 mg, 0.4 mmol) were added into above solution. The ensuing solution was stirred for 24 h at room temperature. The reaction solution was diluted with DCM (50 mL) and then washed with deionized water (30 mL×5) to remove DMF. The combined organic phases were dried by Na_2SO_4 and concentrated under reduced pressure to give the crude product. The crude product was purified by column chromatography, using dichloromethane/methanol (DCM/MeOH = 10:1, *v/v*) as eluent to afford compound **5** (0.95 g, 83 %). ^{1}H NMR (400 MHz, MeOD) δ (ppm): 8.68 (d, J = 7.8 Hz, 1H), 8.14 (d, J = 7.9 Hz, 1H), 7.72 (t, J = 8.8 Hz, 2H), 7.66 (t, J = 7.2 Hz, 1H), 7.21 (d, J = 9.3 Hz, 1H), 7.00 (s, 2H), 3.99 (d, J = 6.7 Hz, 1H), 3.62 (q, J = 6.8 Hz, 4H), 3.51 (s, 2H), 3.29-3.23 (m, 1H), 3.18 (m, 1H), 1.77 (m, 2H), 1.62-1.55 (m, 4H), 1.53-1.51 (m, 2H), 1.51-1.37 (m, 33H), 1.32 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, MeOD) δ (ppm) 174.88, 164.50, 157.64, 157.56, 155.97, 154.32, 154.09, 152.50, 141.14, 138.31, 134.98, 134.60, 133.85, 133.02, 132.13, 130.73, 126.20, 125.41, 124.83, 123.56, 118.49, 106.07, 103.24, 85.16, 84.43, 80.49, 80.33, 79.95, 56.09, 49.64, 49.43, 49.21, 49.00, 48.79, 48.57, 48.36, 46.86, 45.37, 41.29, 40.06, 30.68, 30.57, 30.37, 29.82, 29.77, 28.74, 28.62, 28.23, 27.63,

27.58, 27.48, 26.79, 26.40, 13.21. HRMS: m/z calculated for $[M+H]^+$, $C_{47}H_{69}N_8O_7S^+$, m/z 889.5004, found 889.4983.

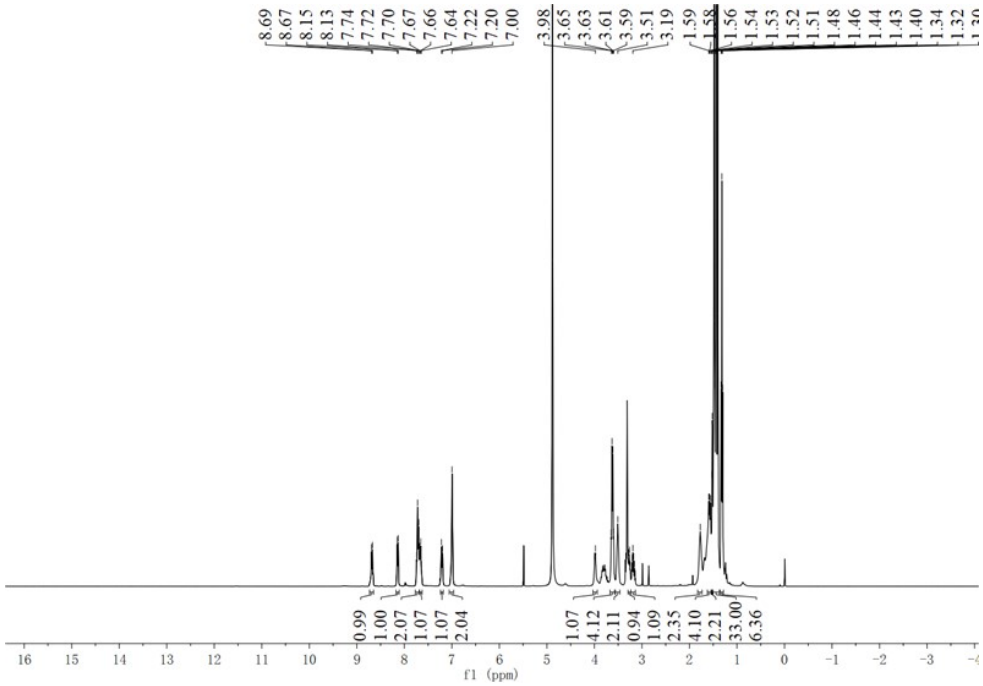


Fig. S3 ^{1}H NMR spectrum (400 MHz, MeOD, 298K) of compound **5**

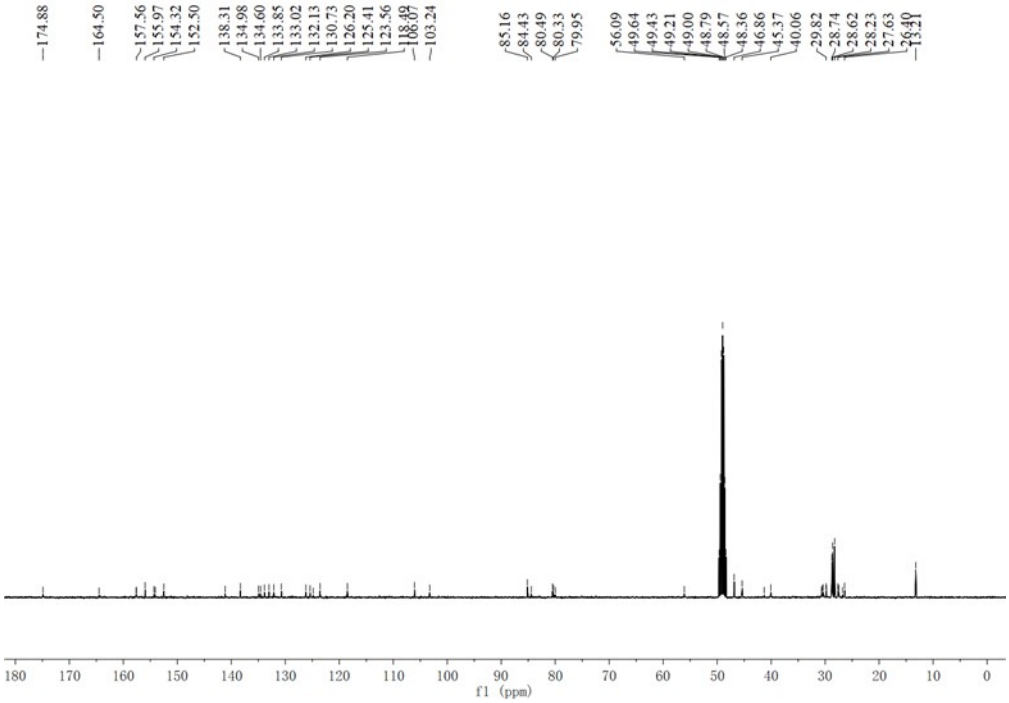


Fig. S4 ^{13}C NMR spectrum (101 MHz, MeOD, 298K) of compound **5**

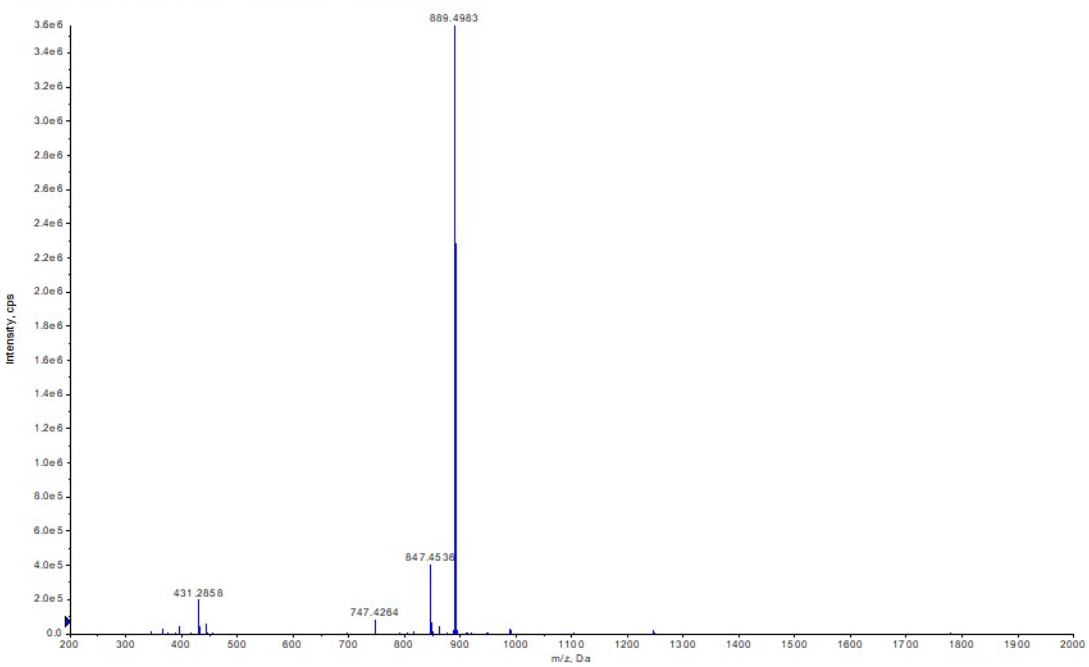


Fig. S5 HRMS spectrum of compound **5**

Synthesis of compound NBS-L-Arg. Compound **5** (88.9 mg, 0.1 mmol) was dissolved in 2 mL DCM. Then 1 M HCl/dioxane solution was added into the mixture. The mixture was stirred for 4 h at room temperature, then concentrated under reduced pressure and washed by diethyl ether to obtain compound **NBS-L-Arg**. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.86 (s, 1H), 8.84 (d, J = 7.6 Hz, 1H), 8.54 (s, 1H), 8.46 (d, J = 7.9 Hz, 1H), 8.24 (s, 3H), 8.11 (s, 1H), 7.86 (t, J = 9.1 Hz, 2H), 7.81-7.74 (m, 1H), 7.40 (s, 2H), 7.29 (d, J = 14.6 Hz, 3H), 3.74 (d, J = 5.4 Hz, 1H), 3.62 (s, 6H), 3.12 (s, 4H), 1.72 (m, 4H), 1.42 (m, 9H), 1.22 (t, J = 6.4 Hz, 8H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 168.15, 157.05, 152.99, 150.76, 139.63, 136.75, 133.49, 133.08, 131.98, 131.21, 131.13, 129.47, 124.65, 124.10, 123.22, 117.24, 113.84, 105.26, 102.94, 51.94, 45.14, 44.06, 28.84, 28.32, 26.19, 24.24, 12.66. HRMS: m/z calculated for $[M+3H]^{3+}$, $C_{32}H_{45}N_8OS^{3+}$, m/z 197.1197, found 197.1194

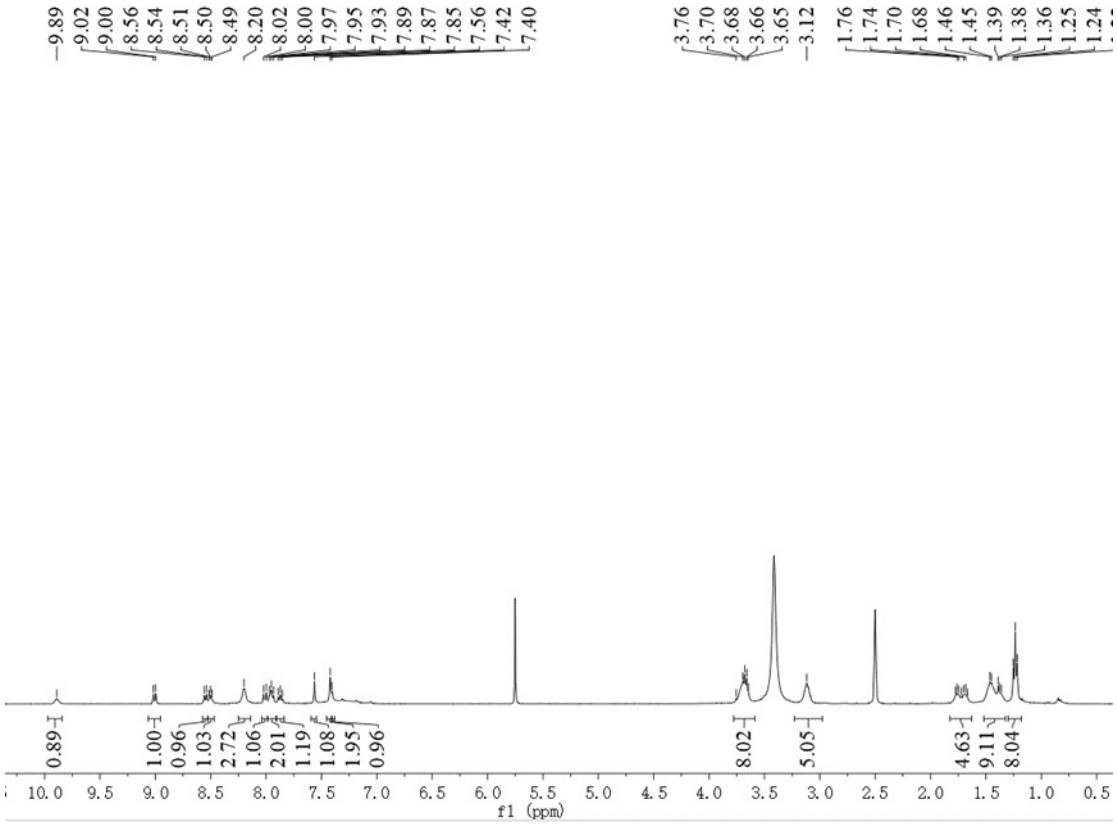


Fig. S6 ^{1}H NMR spectrum (400 MHz, DMSO-d_6, 298K) of compound **NBS-L-Arg**

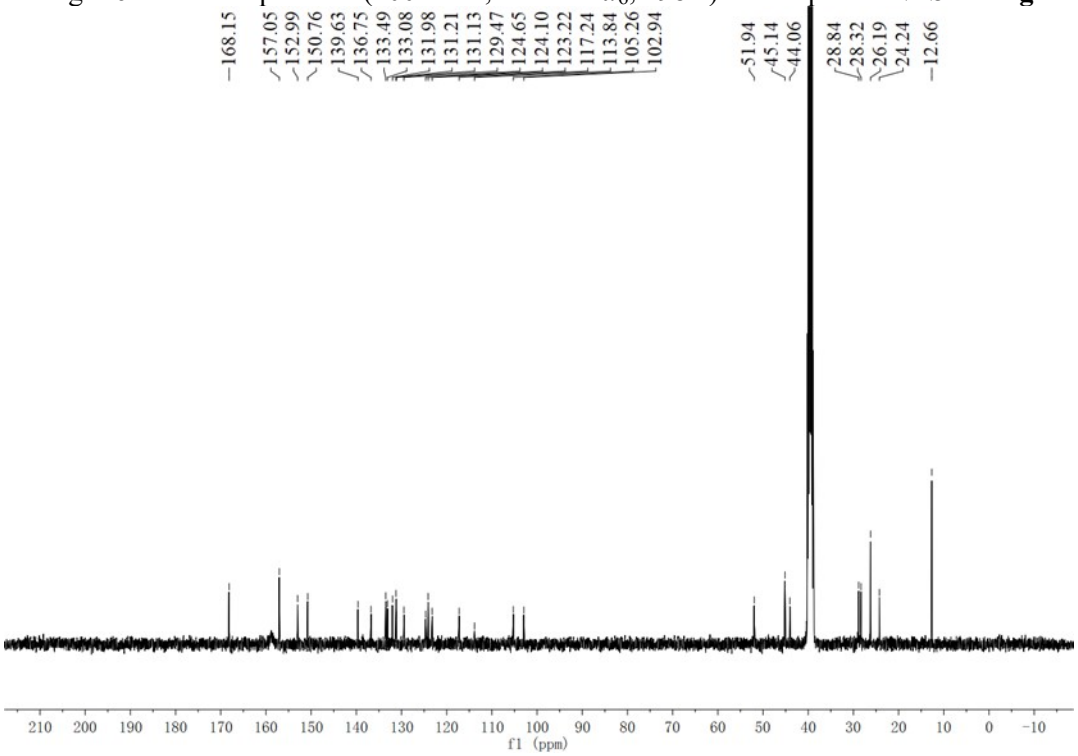


Fig. S7 ^{13}C NMR spectrum (101 MHz, DMSO-d_6, 298K) of compound **NBS-L-Arg**

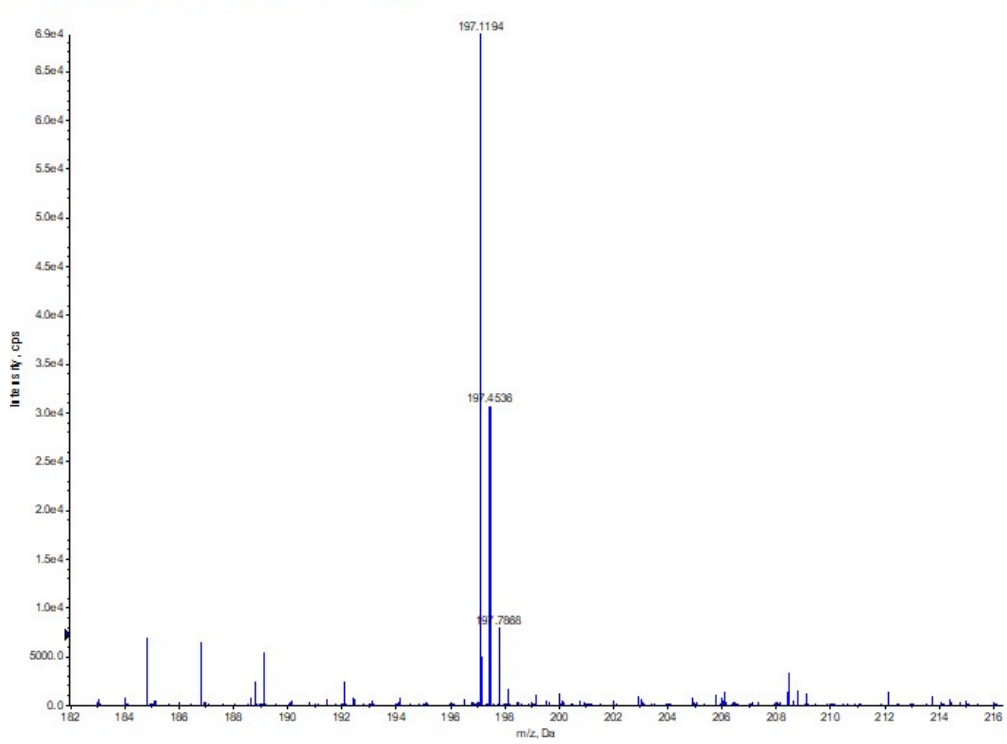


Fig. S8 HRMS spectrum of compound **NBS-L-Arg**

4. The Fluorescence curves of ROS detection

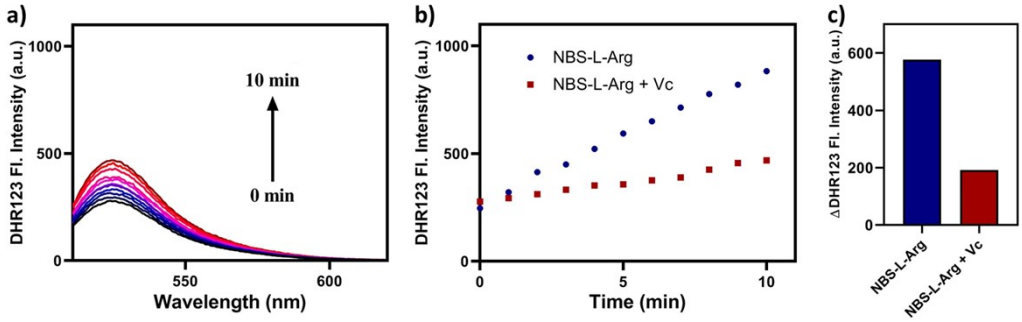


Fig. S9 a) NBS-L-Arg (10 μM) + Vc (100 μM) after 660 nm light irradiation at a power density of 20 mW/cm^2 for different time; b) and c) Fluorescence intensity of DHR123 at 525 nm after 660 nm irradiation for 10 min.

5. Preparation of NLCD

The Cu^{2+}-based coordination nanoparticles NLCD were constructed by the self-assembly of amphiphilic NBS-L-Arg and DOX with different molar ratios in water solutions (NBS-L-Arg/Cu^{2+}/DOX: 1:1:0, 1:1:0.5, 1:1:1). The different molar ratios of NBS-L-Arg, $CuCl_2$ and DOX were dissolved in deionized water and ultrasound for another 3 h at room temperature. After 2 d of dialysis, there is no excess DOX and Cu^{2+} detected in the dialysate.

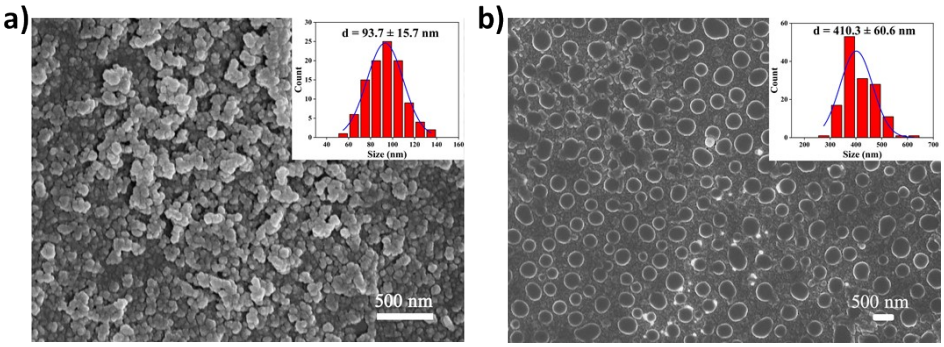


Fig. S10 a) SEM image of NBS-L-Arg/Cu^{2+} (molar ratio of 1:1) NPs; b) SEM image of NBS-L-Arg/Cu^{2+}/DOX (molar ratio of 1:1:1) NPs.

6. Stimuli-responsive behaviour of NLCD

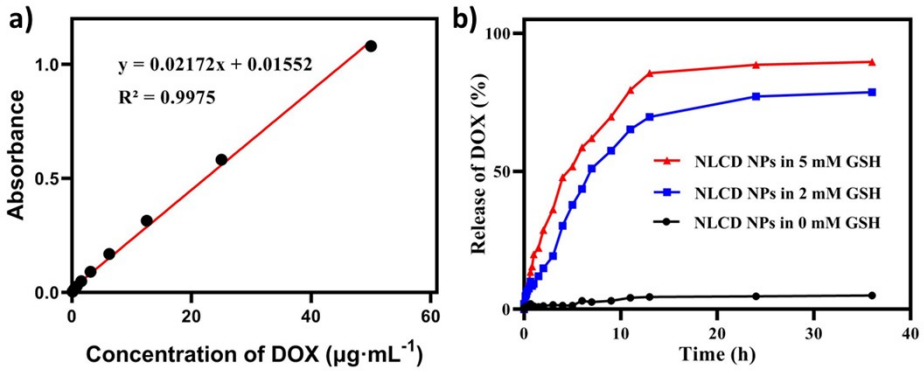


Fig. S11 a) The standard curve of DOX absorption at 480 nm. b) Release rate curve of DOX from NLCD NPs at different concentrations of GSH (0 mM、2 mM、5 mM).

7. Confocal laser scanning microscopy (CLSM) for the cellular uptake of NLCD

Confocal laser scanning microscopy was used for investigating the cellular uptake of NLCD by HepG2 cells. HepG2 cells were plated onto 35 mm confocal dishes and incubated at 37 ℃ under 5% CO_2 for 24 h. HepG2 cells have been incubated with 1.28 μM NLCD for 1 h and then followed by incubation with three different organelle-selective (endoplasmic reticulum, mitochondria, and lysosome) trackers (100 nM) for 30 min, respectively. Afterwards, the cells were washed with PBS (pH 7.4) for imaging by using CLSM. HepG2 cells have been incubated with 1.28 μM NLCD for 1 h and then followed by incubation with 100 nm lysosome trackers. After that, cells were washed with PBS and then irradiated with 660 nm red LED light for 12.5 min at a power density of 20 mW/cm^2 (15 J/cm^2).[S2] The fluorescence was immediately observed using CLSM.

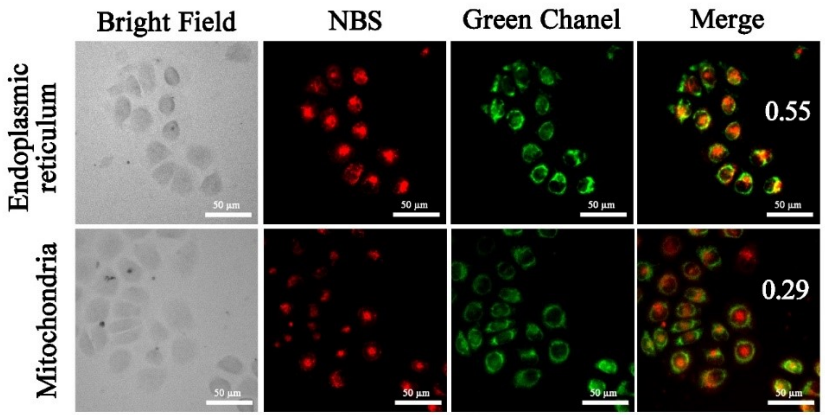


Fig. S12 Colocalization of NLCD NPs with endoplasmic reticulum and mitochondria in HepG2 was detected *via* CLSM.

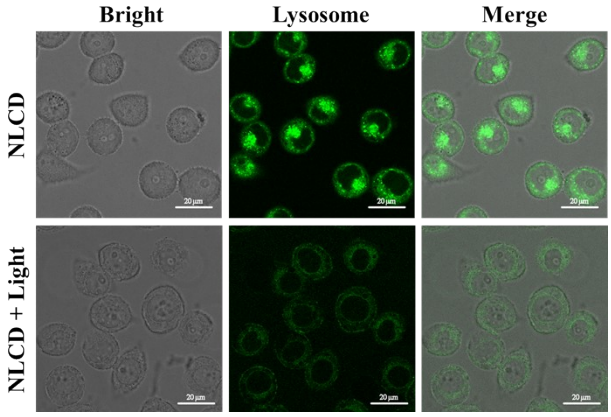


Fig. S13 The morphology of lysosomes in HepG2 before and after irradiation with 660 nm light at a power density of 20 mW/cm^2 for 12.5 min (15 J/cm^2) was detected *via* CLSM.

8. Flow Cytometry

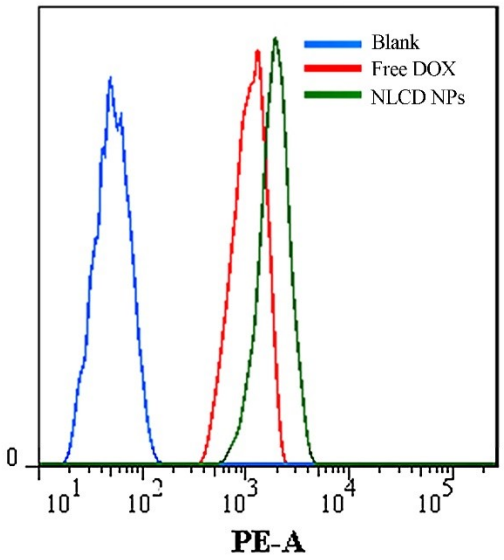


Fig. S14 Flow cytometry analyses of HepG2 cells.

9. CLSM for detecting ROS of NLCD in cells

Generally, HepG2 cells were plated onto 35 mm confocal dishes and incubated at 37 ℃ under 5% CO_2 for 24 h. Liquid paraffin covering method was used to simulate the tumor hypoxic environment. Liquid paraffin was added on the surface of cell culture medium and incubated at 37 ℃ under 5% CO_2 for 24 h. Dihydroethidium (DHE) (λ_{ex} = 535 nm, λ_{em} = 610 nm) and hydroxyphenyl fluorescein (HPF) (λ_{ex} = 490 nm, λ_{em} = 515 nm) as the specific indicators for $O_2^{-\bullet}$ and OH· in cells. The anaerobic indicator ROS-ID was used to prove intracellular hypoxia. In addition, HepG2 cells were incubated with 1.28 μM NLCD for 1 h and followed by incubation with 10 μM DHE for 30 min, 10 μM HPF for 60 min, respectively. After treatments, cells were washed with PBS and then irradiated with 660 nm red LED light for 12.5 min at a power density of 20 mW/cm^2 (15 J/cm^2). The fluorescence was immediately observed using CLSM. The intracellular average fluorescence intensity was calculated by ImageJ.

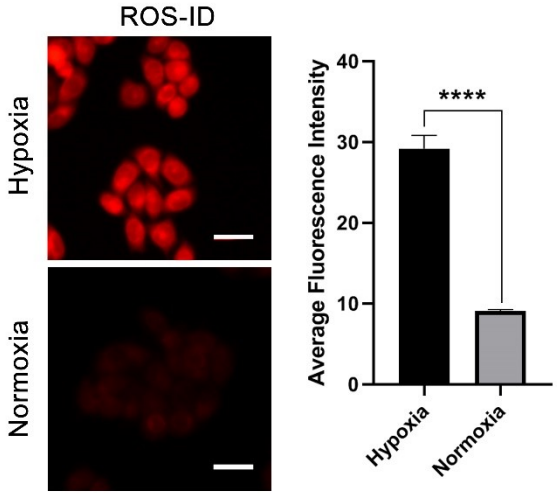


Fig. S15 Intracellular hypoxia imaging using ROS-ID as anaerobic indicator. The scale bar is 20 μm. (****P < 0.001)

10. Cytotoxicity Evaluation

HepG2 and HL7702 cells were cultured in RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin (complete RPMI 1640 medium) in 5% CO_2 at 37 °C. Liquid paraffin covering method was used to simulate the tumor hypoxic environment. The relative cytotoxicity of free DOX, NBS-L-Arg and NLCD were evaluated *in vitro* by MTT assay, respectively. The cells were seeded in 96-well plates. Liquid paraffin was added on the surface of cell culture medium and incubated at 37 ℃ under 5% CO_2 for 24 h to obtain hypoxic state cells. The dark toxicity of NBS-L-Arg was demonstrated on HepG2 and HL7702 cells for 24 h. The anticancer

activity of free DOX, NBS-L-Arg and NLCD were demonstrated on HepG2 cells presence of light irradiation (660 nm, 15 J/cm^2) under hypoxia conditions for 24 h. Subsequently, cells were washed and the fresh medium containing MTT (0.5 mg/mL) was added into each plate. The cells were incubated for another 4 h. After that, the medium containing MTT was removed and dimethyl sulfoxide (100 μL) was added to each well to dissolve the formazan crystals. Finally, the plate was gently shaken for 10 min and the absorbance at 490 nm was recorded with a microplate reader.

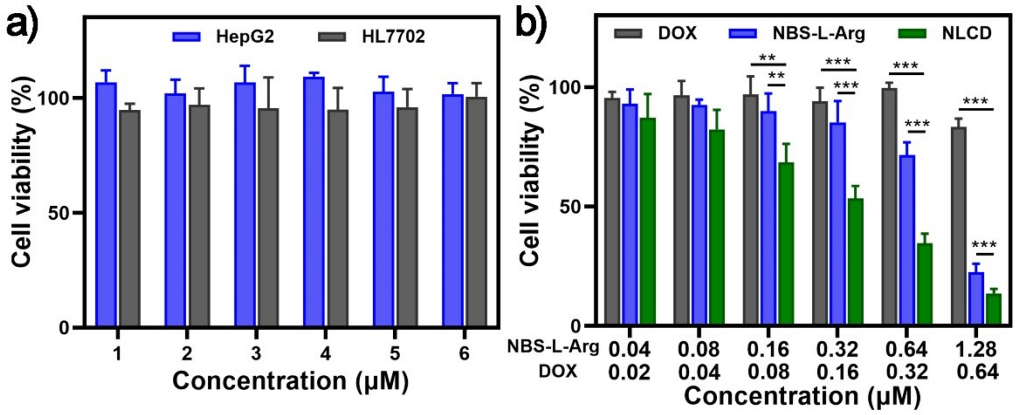


Fig. S16 a) The biocompatibility of NBS-L-Arg NPs on HepG2 cells and HL7702 cells without light irradiation for 24 h; b) The cytotoxicity results of DOX, NBS-L-Arg NPs, and NLCD NPs on HepG2 cells under hypoxia conditions for 24 h after 660 nm light irradiation at a power density of 20 mW/cm^2 for 12.5 min (15 J/cm2). (n = 6, $^{**}P < 0.01$, $^{***}P < 0.001$).

11. Confocal imaging of cell death

Annexin V-FITC and propidium iodide (PI) were used as the indicators to investigate the cell apoptosis and death pathway. HepG2 cells were cultured with 1.28 μM NLCD or NBS-L-Arg for 1 h and then irradiated with 660 nm red LED light for 12.5 min at a power density of 20 mW/cm^2 (15 J/cm^2). After treatments, cells were stained with Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit according to the manufacture instruction. The cell death was visualized by fluorescence microscopy with excitation wavelength of 488 nm, and emission wavelength was collected from 505 to 545 nm (green channel) and from 600 to 700 nm (red channel).

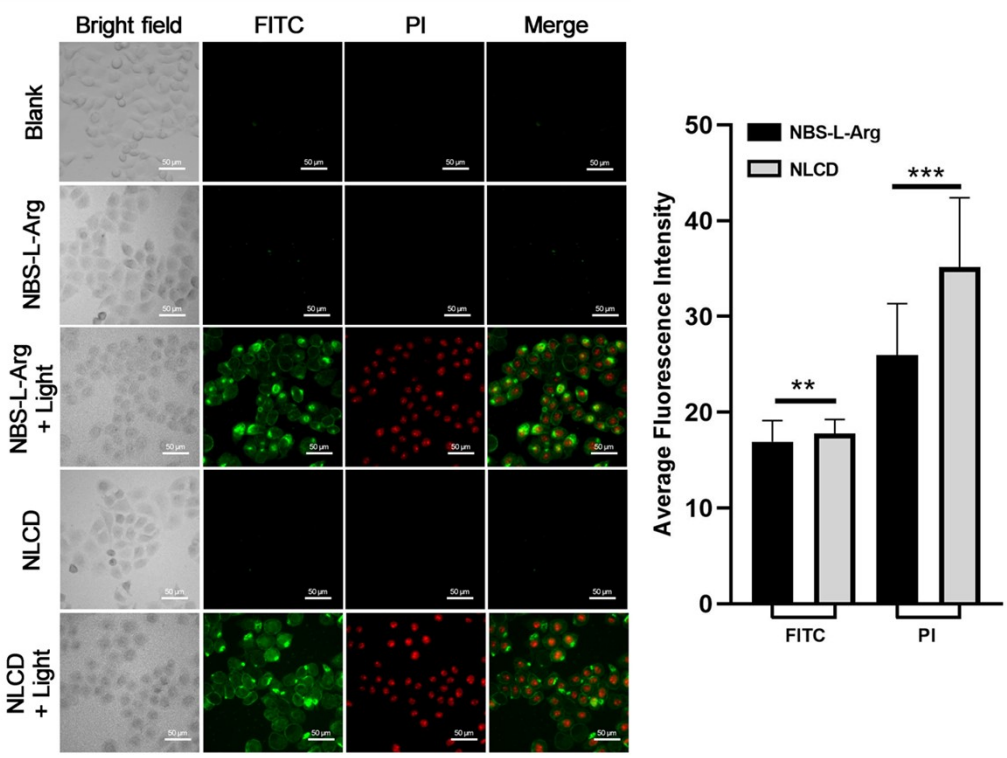


Fig. S17 Apoptosis of cancer cells were measured by Annexin V-FITC/PI Apoptosis Kit after treatment of HepG2 cells with NBS-L-Arg NPs or NLCD NPs and observed by CLSM. (***P < 0.01*, ****P < 0.001*)

12. References

1. M. Li, J. Xia, R. Tian, J. Wang, J. Fan, J. Du, S. Long, X. Song, J. Foley and X. Peng, *J. Am. Chem. Soc.*, 2018, **140**, 14851-14859.
2. S. Chao, Z. Shen, Y. Pei, Y. Lv, X. Chen, J. Ren, K. Yang and Z. Pei, *Chem. Commun.*, 2021, **57**, 7625-7628.
3. S. Meng, Z. Xu, G. Hong, L. Zhao, Z. Zhao, J. Guo, H. Ji and T. Liu, *Eur. J. Med. Chem.*, 2015, **92**, 35-48.