Supporting Information

Phosphorothioated DNA Engineered Fusion Liposomes for Ultrasound-Responsive Targeted Intracellular Protein Delivery

Danchu Gong[a], Dajiang Du*[b], Di Li*[a]

a. School of Chemistry and Molecular Engineering, East China Normal University, Shanghai 200241, China, E-mail: dli@chem.ecnu.edu.cn

b. Department of Orthopedic Surgery, Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, 200233, China, E-mail: dudajiang@sjtu.edu.cn

Materials

Chlorpromazine (CPZ), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), cytochrome C (Cyt C), Chlorpromazine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS), iodo-acetic acid sodium salt ($C_2H_2INaO_2$), and Alexa 647-NHS ester were purchased from Sigma-Aldrich (Shanghai, China). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol were purchased from Avanti Polar Lipids. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and sodium iodoacetate were purchased from Aladdin (Shanghai, China). mPEG2000-TK-NH_2 was purchased from Xi'an Ruixi Biological Technology (Xi'an, China). 1,3-Diphenylisobenzofuran (DPBF), Amplex red, Cell Counting Kit-8, Cla I, Rhodamine B isothiocyanate, and Annexin V-FITC/PI cell apoptosis detection kits were purchased from MCE (Shanghai, China). The DNA Gel Extraction Kit, 1,1-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine,4-Chlorobenzenesulfonate (DiD), BCA Protein Concentration Assay Kit, phosphate-buffered saline (PBS) solution, Hoechst 33342, Lyso-Tracker Green, CFDA SE Cell Proliferation Assay and Tracking Kit and Caspase-3 Activity/Mitochondrial Membrane Potential Detection Kit were purchased from Beyotime Biotechnology (Jiangsu, China). Genistein (GEN) and all DNA oligonucleotides were purchased from Sangon Biotech (Shanghai, China), and DNA sequences are listed in the SI Appendix, Table S1.

Cell culture

HepG2, MCF-7, MCF-10A, and HEK293 cells are cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin solution. B16-F10 cells were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with the same supplements. All the cells were incubated in a humidified incubator at 37°C with 5% CO_2. For delivery experiments, cells were plated at a density of 1×10^5 cells/well in 6-well plates. For super-resolution experiments, cells were seeded onto 20 mm × 20

mm coverslips at 5×10^4 cells per coverslip. A cell counter (IN Count, China) was used to count cells.

Synthesis of UR-PD

Incubate the linker-G4 in a solution containing 200 mM KCl, 4 mM $MgCl_2$, and 28 mM Tris-HCl and heated at 95°C for 10 min, followed by slow cooling to room temperature. A circular dichroism (CD) spectrometer (Jasco J-1500, Japan) was employed to investigate the G4 structure before and after ultrasound treatment. EDC was added to a final concentration of 3 mg/mL, and sulfo-NHS to 1 mg/mL, adjusting pH to 6. After activating the carboxyl groups for 20 min, mPEG2000-TK-NH_2 was added to complete conjugation over 24 h.[1] Strand 1, strand 2, and linker-G4-PEG were mixed in a 1:1:1 molar ratio, and hybridized at 37°C for two hours in 1xTAE buffer containing 12.5 mM Mg^{2+}. DNA products were separated using gel electrophoresis for one hour at a voltage of 80 V. The target product band was cut from the gel, and the DNA products were purified using a DNA Gel Extraction Kit. The product concentration was calculated according to the optical absorbance at 260 nm. An equimolar amount of HMME was added to the DNA product, gently mixed by blowing, and ultimately formed UR-PD.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to illustrate the stepwise synthesis of UR-PD and its response to ultrasound. A 1% gel with GelGreen was prepared in 1×TBE buffer. Samples of 10 μL were loaded into each well and run at 90 V for 90 min. The resulting patterns were visualized with a GelDocTM EZ Imager System (BIO-RAD, USA).

The Distribution of HMME

Utilizing the restriction endonuclease Cla I to efficiently cleave G-quadruplexes, HMME bound to G-quadruplexes can be released. The distribution of HMME within the UR-PDEFL can be determined by separating these free HMMEs (Figure S5). To

elaborate, 300 μL of the UR-PDEFL solution was diluted with an equal volume of buffer from the kit, and 30 μL of Cla I solution was added. After incubation at 37°C for 20 min, the first group was directly separated using Amicon centrifugal filters (with a cut-off of 100 kDa, centrifuged at 14,000 rpm for 30 min at 4°C) to isolate free HMME. The second group was further treated with 1% Triton X-100 in the solution, followed by separation using Amicon centrifugal filters (with a cut-off of 100 kDa, centrifuged at 14,000 rpm for 30 min at 4°C) to isolate all HMME. The absorbance of the filtrates from both groups at 394 nm was measured separately, and the proportion of HMME bound to G-quadruplex was calculated using the following formula:

$$HMME\ ratio\ (\%) = \frac{Abs_f \times V_1}{Abs_t \times V_2} \times 100\%$$

Where Abs_f represents the absorbance of the filtrate at 394 nm from the first group, V_1 represents the volume of the filtrate from the first group, Abs_t represents the absorbance of the filtrate from the second group, and V_2 represents the volume of the filtrate from the second group. The calculated HMME ratio was 85%.

Assay for evaluating the catalase-mimic activity of HMME/G4 complex

SOSG was used to detect the presence of singlet oxygen. In the experimental group, 500 μL of H_2O_2 (2%) was mixed with 200 μL of G-quadruplex PBS solution (25 μM), 100 μL of HMME aqueous solution (50 μM), and 200 μL of SOSG solution (5 μM). The control group was replaced with 200 μL of G-quadruplex solution and 200 μL of PBS solution. The mixture was then sonicated (1 MHz, 1 W/cm^2) for 10 min. The SOSG fluorescence at 530 nm (Ex at 470 nm) was measured using a fluorescence spectrophotometer.

Amplex Red was also used to assess the catalase-mimic activity. In the experimental group, 50 μL of H_2O_2 (2%) was mixed with 20 μL of G-quadruplex PBS solution (25 μM), 10 μL of HMME aqueous solution (50 μM), and 20 μL of Amplex Red dissolved in DMSO (25 μM). In the control group, 20 μL of the G-quadruplex

solution was replaced with 20 μL of PBS solution. The mixture was then sonicated (1 MHz, 1 W/cm^2) for 10 min. The fluorescence of catalytically generated resorufin at 585 nm (Ex at 570 nm) was detected using a fluorescence spectrophotometer.

DPBF was also used to detect the presence of singlet oxygen. In the experimental group, 50 μL of 2% H_2O_2 was combined with 20 μL of G-quadruplex PBS solution (25 μM), 10 μL of HMME aqueous solution (50 μM), and 20 μL of DPBF in DMSO (10 mg/mL). In the control group, 20 μL of G-quadruplex solution was replaced with 20 μL of PBS. The mixture was then sonicated (1 MHz, 1 W/cm^2) for 10 min. The absorbance at 410 nm was monitored every minute using a UV-Vis spectrophotometer.

Electron spin resonance (ESR) spectra (Bruker, USA) were used to measure the amount of hydroxyl radicals generated during catalysis. In the experiment group, 50 μL of H_2O_2 (2%) was combined with 20 μL of G-quadruplex PBS solution (25 μM), 10 μL of HMME aqueous solution (50 μM), and 20 μL of DMPO dissolved in H_2O (25 mM). In the control group, 20 μL of the G-quadruplex solution was replaced with 20 μL of PBS solution. The mixture was then sonicated (1 MHz, 1 W/cm^2) for 1 min. The instrument settings were as follows: Center Field 3435G, Sweep Width 200G; Sweep Time, 30 s; and receiver gain, 40 dB.[2]

Preparation of liposomes

Liposomes were prepared using the film hydration method.[3] In a round-bottomed glass flask, DOPC and cholesterol were dissolved in chloroform in a 3:1 mass ratio. The chloroform was then evaporated under a stream of nitrogen at room temperature to form a thin lipid film, which was subsequently hydrated with PBS and sonicated in a water bath for five min. The solution was extruded through a 200 nm polycarbonate membrane using a mini extruder (Avanti, USA) for 20 passes to obtain empty liposomes (10mg/ml). DID-loaded liposomes were prepared by incorporating 1 mg/mL of DID before extrusion. Free DiD was removed from liposomes by a dialysis bag (cut-off 10 kDa 12h at 4°C) and washed twice with fresh PBS.

To obtain Cyt C loaded liposomes (Cyt C@lipo), 1.0 mL of the empty liposomes were mixed with 1.0 mL of a 1.0 mg/mL Cyt C solution in PBS. The mixture was then extruded using the same method to encapsulate the protein. Free Cyt C was removed from liposomes by a dialysis bag (cut-off 30kDa 12h at 4°C) and washed twice with fresh PBS.

The encapsulation efficiency (EE%) of Cyt C@lipo was determined using a UV-Vis spectrophotometer by first plotting a standard curve of Cyt C concentration, then measuring the total Cyt C concentration (Ct) and the concentration of free Cyt C (Cf) that had been dialyzed (Figure S6). The encapsulation efficiency was calculated using the formula:

$$EE\ (\%) = \frac{Ct - Cf}{Cf} \times 100\%$$

The encapsulation efficiency was found to be 39.7%.

UR-PDEFL was prepared by mixing the empty liposomes with UR-PD solution (final concentration: 1 μM) at room temperature for 3h. Free DNA was removed from liposomes by a dialysis bag (cut-off 30kDa 12h at 4°C) and washed twice with fresh PBS. Cyt C@UR-PDEFL was prepared using the same method as for Cyt C@lipo.

Stability test of UR-PDEFL

The stability of UR-PDEFL was investigated in PBS, 10% FBS, and 50% FBS. All samples were stored at 37℃, and the liposome's particle size and polydispersity index (PDI) were measured using dynamic light scattering (DLS) at 0, 2, 4, 8, and 24 hours (Figure S8). In addition, we determined the changes in the liposome's particle size and polydispersity index (PDI) in 50% mouse serum over 24h. (Figure S9) The impact of ultrasound on the stability of the liposomes was also explored, with measurements taken of the particle size and PDI after ultrasound for 0, 1, 2, 5, and 10 min (Figure S10).

Synthesis of fluorescent dye-labeled proteins

The synthesis of AF647-labeled and FITC-labeled Cyt C followed the literature.[4] Cyt C was dissolved in PBS to a concentration of 10 mg/ml, and the dyes were added to maintain a dye-to-protein molar ratio of 1:2, respectively. The mixture was gently vortexed and reacted at room temperature for 3h. Free AF647 or FITC was removed from the protein by dialysis using a dialysis bag with a 10 kDa cut-off for 12h at 4°C, followed by two washes with fresh PBS. Protein concentrations were measured using a BCA assay, employing a pre-established standard curve to determine the concentration of the synthesized fluorescently labeled protein. (Figure S6) The synthesized proteins were stored at -80°C in the dark for future use.

CFSE Staining of HeLa Cells

The Staining begins by collecting 1×10^6 HeLa cells via centrifugation. CFDA SE cell-labeling solution is directly added to the cell pellet and gently mixed, followed by a 10-minute incubation at 37°C, and then washed twice with fresh complete medium. A second 5-minute incubation at 37°C enhances intracellular CFSE retention and removes residual dye. After final centrifugation and supernatant removal, labeled cells are ready for the following application.

Intracellular delivery of liposomes

HepG2 cells were pre-plated on glass-bottomed quadripartite dishes, 100 μL of liposomes (concentration 1 mg/mL) were added to each compartment. The ultrasonic transmitting probe (NSE-UPH, Nasonic) was fixed at 1 cm above the cell culture dish, and the ultrasonic frequency was set at 1 MHz, power density of 1 W/cm^2, and time for 1 min (Figure S11). Subsequently, the cells were incubated at 37°C for 30 min. After washing three times with PBS, the cells were fixed with 4% paraformaldehyde at room temperature for 20 min. Following three more washes with PBS, 200 μL of Hoechst 33342 solution was added to each compartment and incubated for 10 min at room temperature. After three washes with PBS, the cells were imaged using a confocal laser scanning microscope (FV31HSD, Olympus) with a 60× objective lens (NA 1.42). A

405 nm solid-state laser was used to visualize Hoechst 33342, a 488 nm solid-state laser for FITC-labeled Cyt C, and a 640 nm solid-state laser for DID.

Super-resolution imaging

HepG2 cells were pre-plated on 20×20 mm glass slides. After protein delivery with AF647-Cyt C@UR-PDEFL, the cells were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature and washed thrice with PBS. Subsequently, 1 mL of Lyso-Tracker Green at a concentration of 50 nM was added to the cells and incubated for 30 min, followed by three washes with PBS. Before sealing the sample, 30 μL of imaging buffer containing 0.5 mg/mL glucose oxidase, 40 μg/mL catalase, and 140 mM beta-mercaptoethanol (βME) was applied to a 24 × 50 mm microscope slide. Then, the small coverslip on which the cells had been seeded was gently sealed onto the larger one using nail polish.[5]

A STORM microscope (Micro-fields Optics, Guangzhou) with a Nikon 100 × 1.49 NA objective lens was used for dSTORM imaging. The dual-color process involved imaging AF647-Cyt C with a 640 nm laser and lysosomes with a 488nm laser, capturing 2000 frames at 30s exposure per cell. The data were analyzed through the Thunderstorm plugin of Image J. Mander's Overlap Coefficient (MOC) was calculated by ImageJ Coloc2 version 3.0.0.

In vitro apoptosis experiments

The CCK-8 assay was employed to study the cytotoxic effects on Hep G2 and MCF-7 cells following treatment with liposomes containing varying concentrations of Cyt C. HepG2 and MCF-7 cells were pre-plated in 96-well plates and after treatment with liposomes containing 0, 0.1, 0.25, 0.5, and 1 mg/mL of Cyt C, 100 μL of CCK-8 solution was added to each well at 24 and 48h post-treatment. After 2h incubation at 37°C, the absorbance at 450nm was measured using a microplate reader (TECAN, Switzerland). The cell viability was calculated using the formula:

$$Cell\ viability\ (\%) = \frac{CCK_i}{CCK_0} \times 100\%$$

where CCK_i represents the absorbance at 450 nm of each experimental group, and CCK_0 represents the absorbance at 450 nm of the control group.

The Annexin V-FITC/Propidium iodide (PI) assay kit was utilized to investigate the apoptotic status of HepG2 cells following treatments in different groups. HepG2 cells were pre-plated in 12-well plates, and after treatment with liposomes containing 0.25 mg/mL of Cyt C, the cells were collected 24h later. After centrifugation and sedimentation, the cells were mixed with the Annexin V-FITC solution for 15 min, and the samples were then analyzed using a flow cytometer (BD Biosciences, USA).

The Caspase-3 Activity and Mitochondrial Membrane Potential Detection Kit was used to study their changes in HepG2 cells following treatment with Cyt C@UR-PDEFL. HepG2 and MCF-7 cells were pre-plated in glass-bottomed four-well plates, and after treatment with Cyt C@UR-PDEFL containing 0.25 mg/mL of Cyt C, 200μL of detection reagent was added to each well 12h later. After 20 min incubation at room temperature in the dark, the samples were imaged using a confocal microscope, with a 488 nm laser for GreenNuc and a 640 nm laser for Mito-Tracker.

Animal tumor model establishment

Female BALB/c mice, approximately six weeks old, were housed in the Laboratory Animal Centre of East China Normal University (ECNU). The B16-F10 cell xenograft tumor model was established by subcutaneously injecting 5 million B16-F10 cells into the right hind leg of the mice. Subsequent experiments were initiated once the tumor volume reached 100 mm^3.

All experimental animals were treated according to protocols approved by the ECNU Animal Care and Use Committee (protocol ID: m20231201). All sample or data collection procedures used are carried out following the provisions of the Ministry of

Science and Technology of the People's Republic of China on Animal Care Guidelines.

In vivo cycling experiments

Fluorescence imaging technology was utilized to investigate the distribution of liposomes within mice. 100 μL of DiD-labeled UR-PDEFL solution was administered via tail vein injection into three tumor-bearing mice. Fluorescence imaging was performed by the In Vivo imaging system (PerkinElmer, USA) at 0, 2, 4, 6, 8, 10, and 24h post-injection. After the imaging sessions, all mice were sacrificed, and organs (liver, heart, spleen, lung, kidneys) along with the tumors were collected for fluorescence imaging.

In vivo anti-tumor efficacy

Thirty mice with tumors of about 100 mm^3 were randomly assigned to five groups for in vivo anti-tumor experiments. They were treated with either 100 μL of PBS, 100 μL of PBS solution containing Cyt C at a concentration of 0.25 mg/mL, 100 μL of Cyt C@lipo solution at a concentration of 0.25 mg/mL, or 100 μL of Cyt C@UR-PDEFL solution at a concentration of 0.25 mg/mL with or without ultrasound treatment. The treatments were administered twice with a 4-day interval. Tumor volume and mouse body weight were recorded every two days. Tumor volume was calculated using the following formula:

$$V = L \times W \times \frac{W}{2} (mm^3)$$

L and W represent the longest and shortest dimensions of the tumor, respectively.

After 14 days of treatment, all mice were sacrificed. Organs (liver, heart, spleen, lung, kidney) and tumors were collected and fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining. The level of apoptosis in tumor cells was characterized using TdT-mediated dUTP Nick-End Labeling (TUNEL) staining.

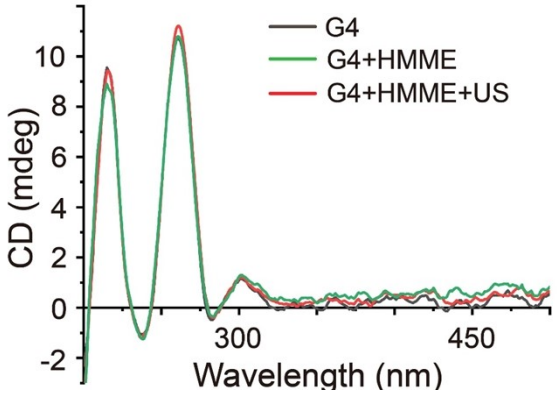

Figure S1. Circular dichroism (CD) spectrum of G4, G4+HMME, and G4+HMME+US.

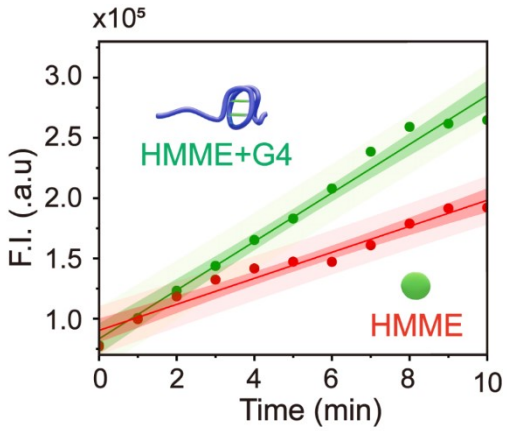

Figure S2. Time-dependent fluorescence changes of Amplex Red catalyzed by HMME or G4/HMME complex in the presence of H_2O_2. The concentration of HMME was 5 μM, and H_2O_2 was 1%. Results are shown as mean ± SD, n=3.

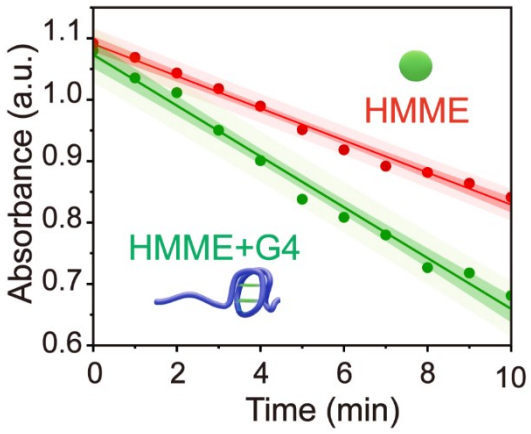

Figure S3. Time-dependent absorbance changes of DPBF catalyzed by HMME or G4/HMME complex in the presence of H_2O_2. The concentration of HMME was 5 μM, and H_2O_2 was 1%. Results are shown as mean ± SD, n=3.

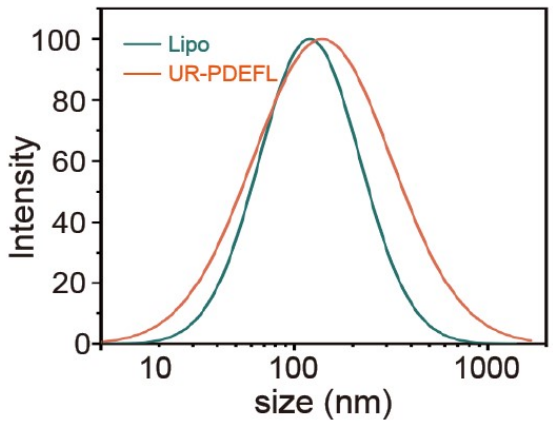

Figure S4. Dynamic light scattering (DLS) measurement of the particle diameter of empty liposomes and UR-PDEFL.

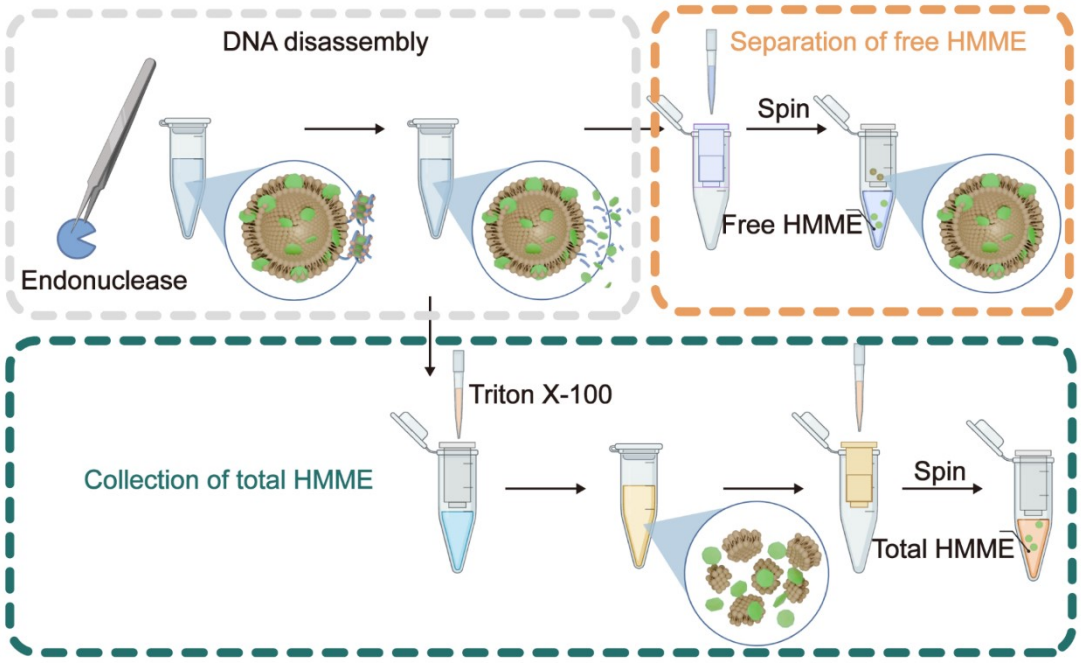

Figure S5. Scheme for exploring the HMME distribution.

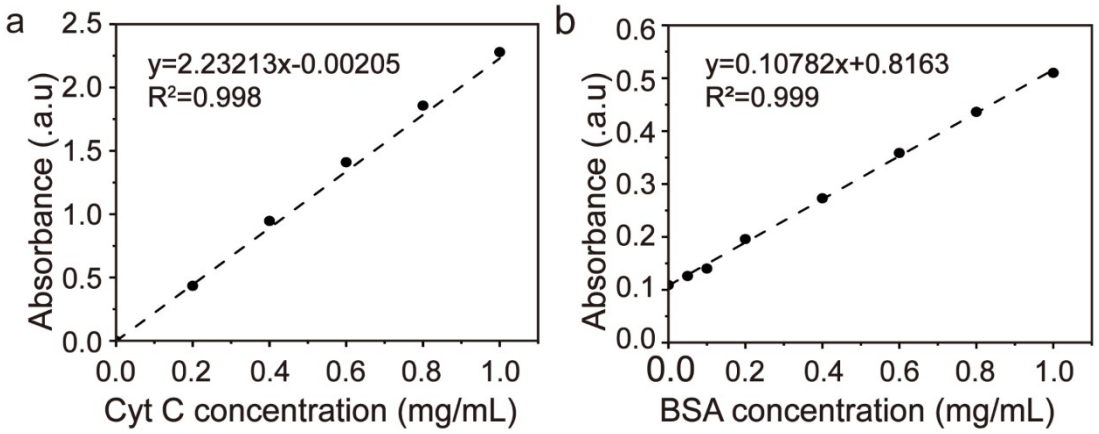

Figure S6. Standard curves of Cyt C (a) and BSA (b) in PBS for calculating liposome loading efficiency.

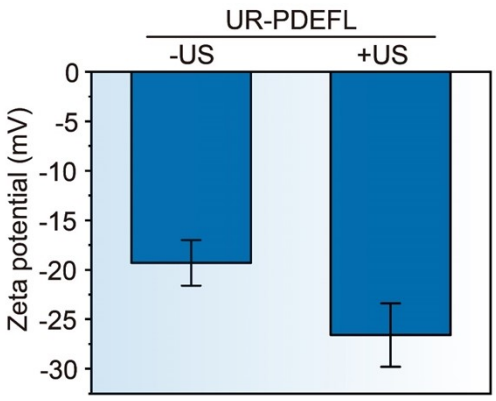

Figure S7. Changes in Zeta potential of UR-PDEFL before and after ultrasound treatment. Results are shown as mean ± SD, n=3.

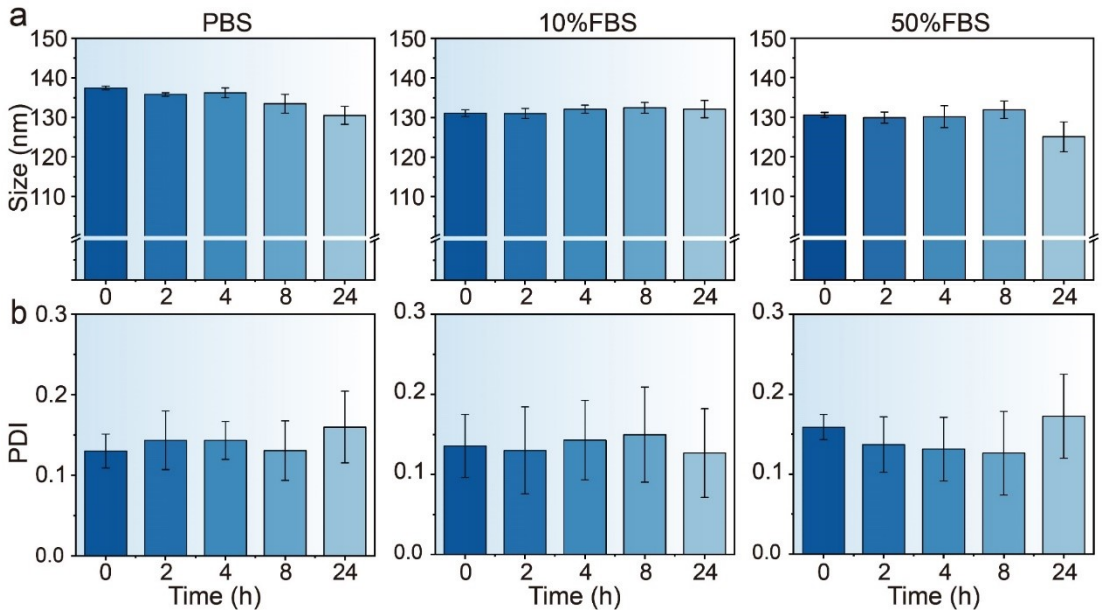

Figure S8. (a) Variation of particle size of UR-PDEFL in PBS solution, 10% FBS and 50% FBS in 24 h. (b) Variation of PDI of UR-PDEFL in PBS solution, 10% FBS and 50% FBS in 24 h. Results are shown as mean ± SD, n=3.

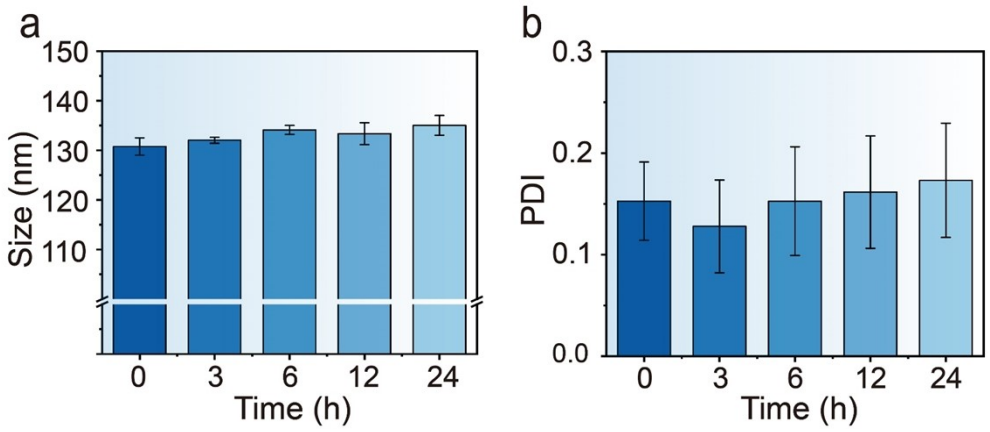

Figure S9. Variation of particle size and PDI of UR-PDEFL within 24h in 50% mouse serum. Results are shown as mean ± SD, n=3.

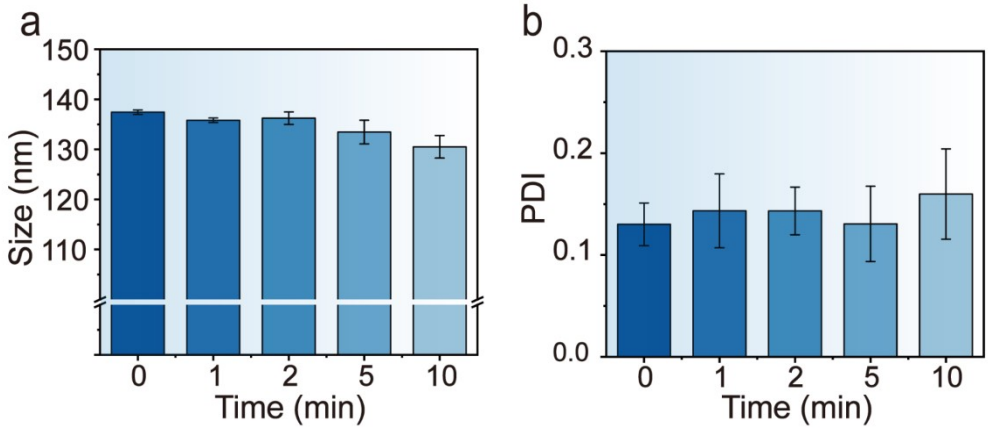

Figure S10. Variations of particle size and PDI of UR-PDEFL within 10 min of ultrasound exposure (1 MHz, 1 W/cm^2). Results are shown as mean ± SD, n=3.

Figure S11. The ultrasonic transmitting probe.

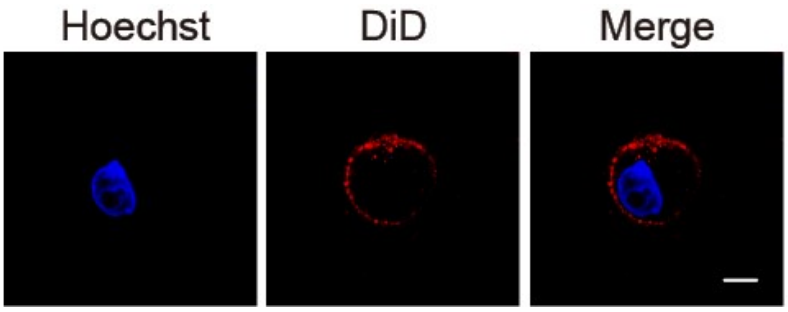

Figure S12. Confocal microscopy images of HepG2 cells after incubating with Cyt C-loaded UR-PDEFL (labeled with DiD) and then subjected to US. Scale bar: 10 μm.

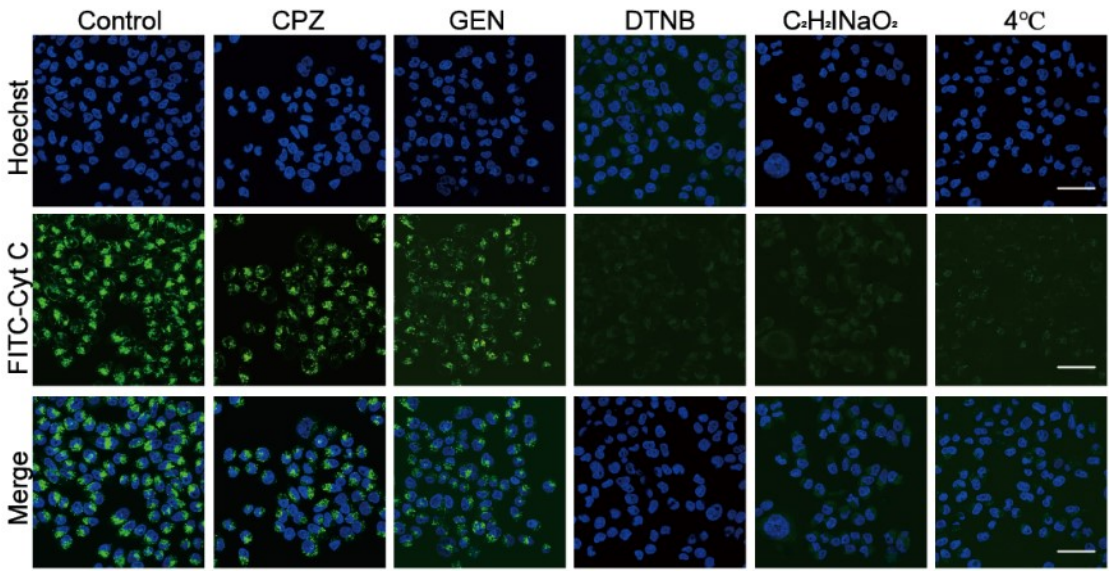

Figure S13. Confocal microscopy images of HepG2 cells treated with different inhibitors or at low temperatures and then incubated with FITC-Cyt C@UR-PDEFL. Scale bar: 50 μm.

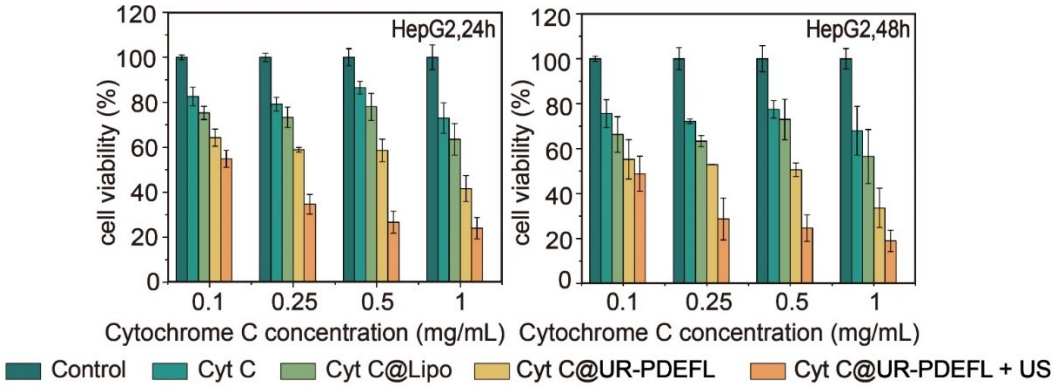

Figure S14. Cell viability of HepG2 cells treated with different concentrations of Cyt C after 24 and 48h in each group.

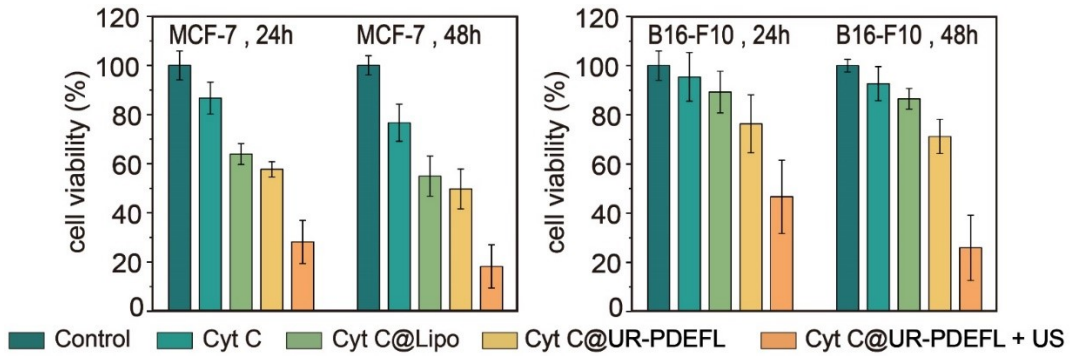

Figure S15. Cell viability of MCF-7 and B16-F10 cells treated with different groups after 24 and 48h.

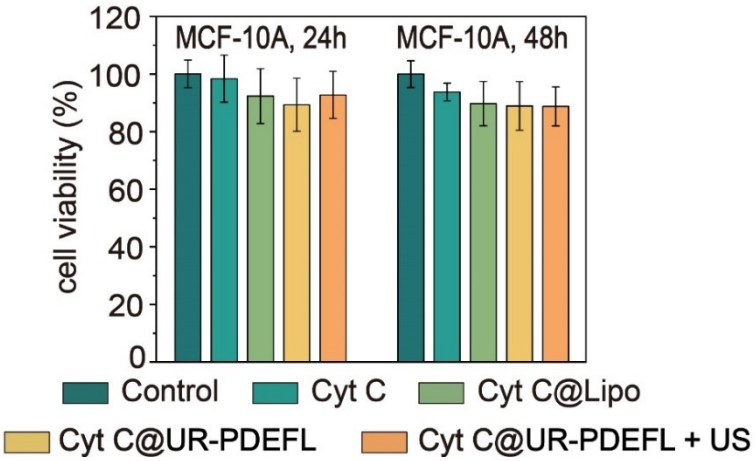

Figure S16. Cell viability of MCF-10A cells treated with different groups after 24 and 48h.

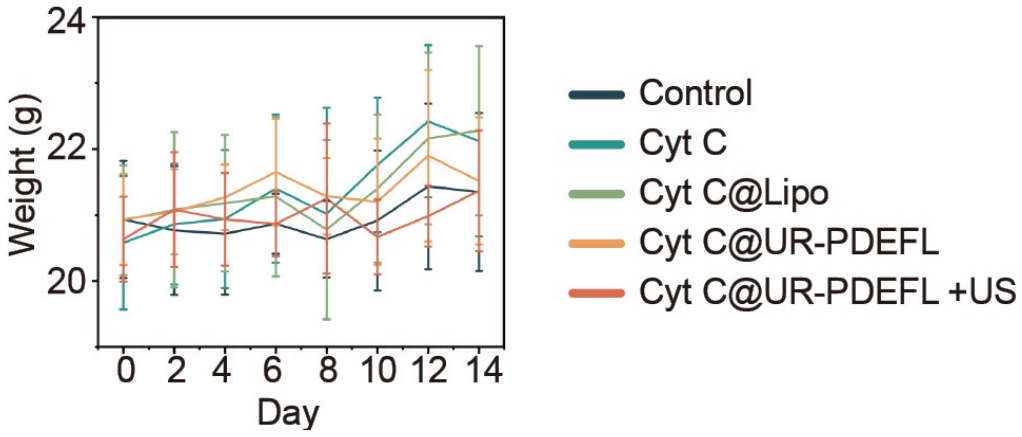

Figure S17. The average body weight of mice in each group was measured over 14 days.

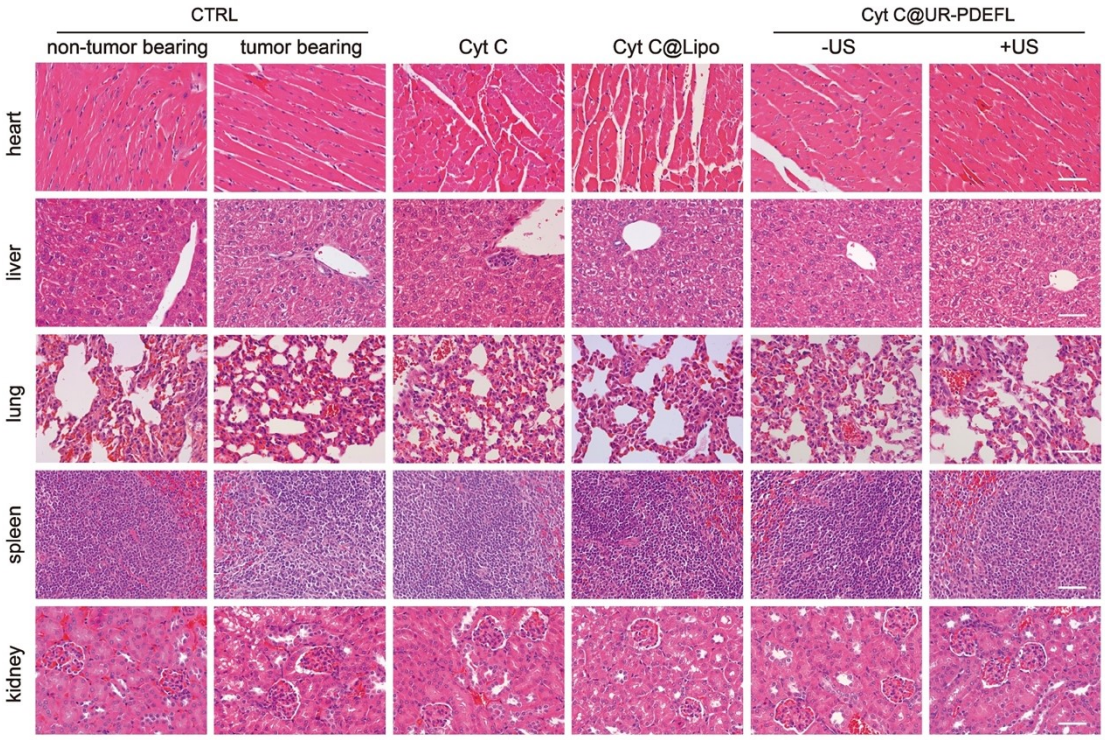

Figure S18. H&E staining of organs in each group after 14 days of treatment. Scale bar: 100 μm.

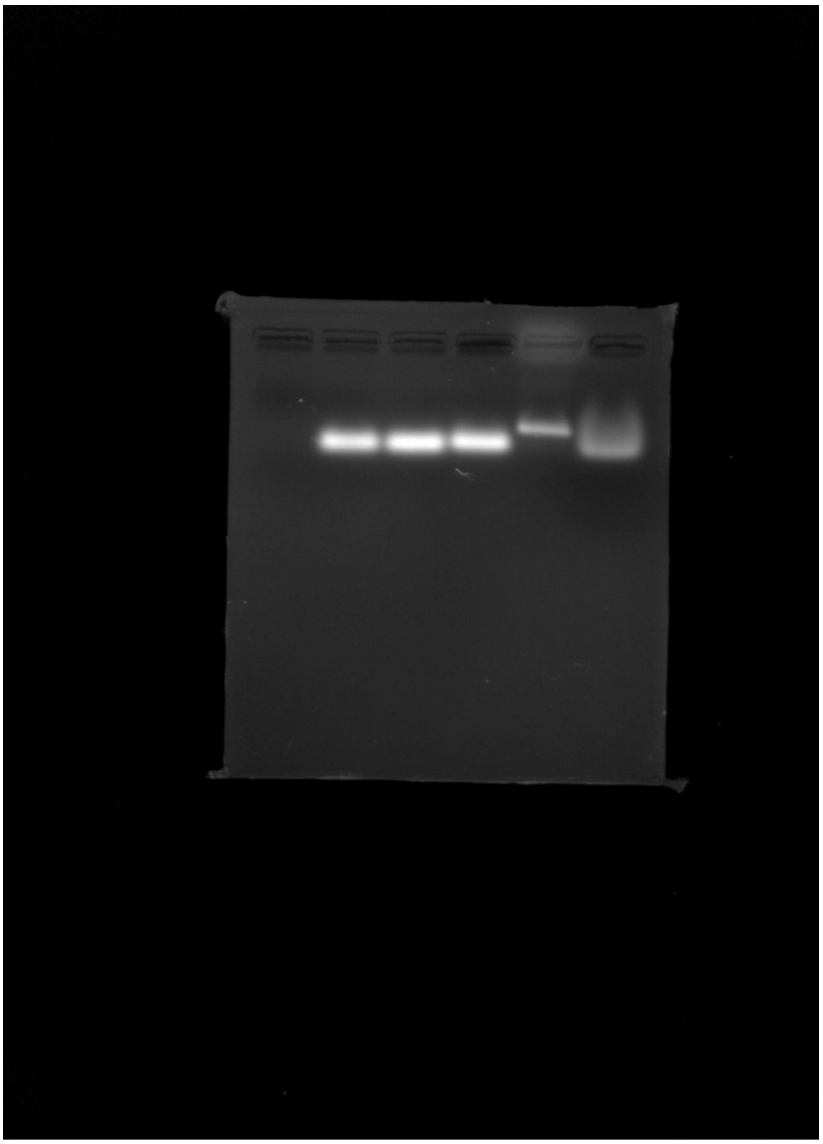

Figure S19. Full-size gel for Figure 1e.

Table. S1. DNA sequence

Name	Sequence (from 5' to 3').
strand1	Cholesteryl- GCG*TTA*G*TCC*T*AAG*A*GT*T*TG*T*G*TG*T*TG*T*T*G*TGGTTT
strand2	CT*CT*TA*G*G*AC*TA*AC*GC-Cholesteryl
linker-G4	COOH-TTTTTGGTGGTGGTGGTTGTGGTGGTGGTGGTTTTTCCACAACAACACAC

*The stars indicate sites of phosphorothioate modification.

Reference:

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