Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Biomimetic Nanothylakoids for Efficient Imaging-Guided Photodynamic Therapy of Cancer

Jiang Ouyang, Liqiang Wang, Wansong Chen, Ke Zeng, Yajing Han, Yan Xu, Qunfang Xu, Liu Deng, and You-Nian Liu**

Table of Contents

1. Experimental Procedures

1.1. Materials. 4T1, HeLa and L929 cells were obtained from Xiangya Hospital of Central South University (Changsha, China). Fetal bovine serum (FBS), DMEM culture medium, penicillin-streptomycin and trypsin were purchased from HyClone (Beijing, China). Hoechst 33342, calcein-AM, propidium iodide (PI), catalase assay kit, CCK-8 kit, and JC-1 staining kit were provided by Beyotime Inst. Biotech. (Haimen, China). Amplex red, zinc phthalocyanine (ZnPc), singlet oxygen sensor green (SOSG) and deferoxamine were purchased from Sigma-Aldrich (Shanghai, China). Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride ($[Ru(dpp)_3]^{2+}Cl_2$) was purchased from HWRK (Beijing, China). Egg phosphatidylcholine (EPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG_{2000}) were bought from Ruixi Biotech. (Xi'an, China). Other reagents and solvents were of analytical purity and used without further purification. Ultrapure water was obtained from a Milli-Q ultrapure (18.2 MΩ cm) system and used in all experiments.

1.2. Preparation of nanothylakoids. Thylakoid membrane was isolated from fresh spinach following the previously reported method.[1] In detail, 100 g of spinach leaves were washed and then kept at 4 °C overnight. Subsequently, the spinach leaves were homogenized in a precooled mortar, together with 300 mL of homogenization buffer (20 mM Tricine, 0.4 M sucrose, 2 mM ascorbic acid, 2 mM $MgCl_2$, 40 mM NaCl, 0.2% BSA, pH 7.8). Then, the leaf fragments were filtered through ten layers of cotton gauze and the filtrate was collected. Afterwards, the filtrate was centrifuged at 8000 rpm for 10 min to collect intact chloroplasts. The obtained chloroplasts were re-suspended in hypotonic lysis buffer (10 mM HEPES, pH 8.0) for 2 h, followed by centrifugation at 8000 rpm for 10 min to remove the stroma extract in the supernatant. The pellet containing thylakoids was then collected and washed with 10 mM HEPES buffer twice. Finally, thylakoids were suspended in water and fractioned under sonication at 4 °C for 5 min. The lumen extract was removed by centrifugation at 16000 rpm for 15 min, and the thylakoid membrane was obtained as dark green pellets.

To prepare nanothylakoids, the thylakoid membrane was dispersed in water, and the pH of the solution was adjusted to 7.4 by addition of dilute NaOH solution. After sonication for 5 min, the mixture solution gradually turned clear and then extruded through 100 nm porous polycarbonate membrane to obtain nanothylakoids.

Chlorophyll concentration was calculated according to the following equations:[2]

$$\text{chlorophyll a } (\mu g\ mL^{-1}) = 13.95 \times A_{665} - 6.88 \times A_{649} \quad (1)$$

chlorophyll b (μg mL^{-1}) = 24.96 × A_{649} – 7.32 × A_{665} (2)

Total chlorophyll (μg mL^{-1}) = chlorophyll a + chlorophyll b (3)

Where, A_{649} and A_{665} represent the absorption of nanothylakoids at 649 and 665 nm, respectively.

1.3. Preparation of chlorophylls loaded liposomes. Fresh spinach was homogenized in water and filtered through ten layers of cotton gauze. The filtrate was collected and mixed with ethyl acetate to extract chlorophylls. The crude chlorophylls were then purified through flash column chromatography. To prepare chlorophylls loaded liposomes, chlorophylls in ethyl acetate were added into the mixture solution of EPC and DSPE-PEG_{2000} in methanol with weight ratio at 9.1:85.9:5 (chlorophyll: EPC: DSPE-PEG_{2000}). After remove of the solvents under vacuum, the lipid mixture was hydrated with water, sonicated for 3 min, and then extruded through 100 nm porous polycarbonate membrane to obtain chlorophylls loaded liposomes.

1.4. Characterization. The morphology of nanothylakoids was observed by transmission electron microscopy (TEM, Tecnai G2, FEI, USA). Dynamic light scattering (DLS) was performed on the Malvern Zetasizer Nano-ZS instrument (ZEN3600, Malvern Instruments, UK). UV-vis absorbance spectrum was measured on a UV-2450 spectrophotometer (Shimadzu, Japan). The fluorescence spectra were recorded on a Hitachi F-4600 spectrophotometer (Hitachi, Japan). For membrane protein analysis, thylakoids or nanothylakoids were dissolved in 5% Triton aqueous solution, and then sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed.

1.5. Catalytic decomposition of H_2O_2 by nanothylakoids. To detect the catalase activity of nanothylakoids, 10 μL of H_2O_2 (10 mM) was mixed with 1 mL of nanothylakoids (0.1 mg mL^{-1}) at room temperature for 2 min. Then the H_2O_2 concentration was quantified by Amplex red (200 μM) with horseradish peroxidase (0.2 U mL^{-1}) as catalyst.[3] The absorbance at 585 nm was measured and the H_2O_2 content was calculated according to the standard curve. H_2O_2 was repeatedly added for four times to study the catalytic stability. The dissolved O_2 content in the solution was monitored by the pen type dissolved O_2 meter (AMT08, USA). To study the inhibition of 3-AT to the catalase activity of nanothylakoids, 3-AT (10 mM) was pre-incubated with nanothylakoids for 30 min at room temperature. Afterwards, both H_2O_2 decomposition and O_2 generation were measured following to the above-mentioned procedures.

1.6. Determination of catalase activity. Catalase activity was determined using catalase assay kit and calculated according equation (4).

$$Catalase\ activity\ (U \cdot g^{-1}) = \frac{\Delta H_2O_2}{t \times m} \quad (4)$$

where, ΔH_2O_2 : the change of H_2O_2 concentration [μM]; t : reaction time [min]; m : total weight of the nanothylakoids [g].

1.7. Measurements of $^1O_2$ generation. SOSG was employed as a fluorescent probe to detect $^1O_2$ generation from nanothylakoids. In detail, 1 μL of SOSG (2.5 mM) in methanol was added into 1 mL of nanothylakoids (0.1 mg mL^{-1}) or chlorophylls loaded liposomes (0.1 mg mL^{-1}) in HEPES buffer (10 mM, pH 6.8). Then the mixture solution was irradiated under 660 nm laser (1 W cm^{-2}) for 5 min. The fluorescence spectra of the solution at different irradiation time were measured with excitation wavelength at 488 nm. To study the $^1O_2$ generation in artificially hypoxic environments, the measurements were repeated in the presence of 100 μM H_2O_2 under 1% O_2 atmosphere. To study the inhibition of NaN_3 to $^1O_2$ generation, NaN_3 was added prior to laser irradiation with the final concentration at 1mg mL^{-1}.

1.8. Measurements $^1O_2$ quantum yield of chlorophylls

The $^1O_2$ quantum yield of chlorophylls was measured using DPBF as probe according to previsouly reported method.[4] ZnPc, a typical photosensitizer, was employed as reference. In detail, 30 μL of ZnPc (1 mM) or chlorophylls (1 mM) in *N*,*N*-dimethylformamide (DMF) was mixed with 3 mL of DPBF (50 μM) solution in DMF. Then the solution was exposed to 660 nm light irradiation (15 mW cm^{-2}) for 5 min, and the absorption change at 410 nm (ΔOD_{410nm}) can be measured using UV-Vis absorption spectrometer. The $^1O_2$ quantum yield of ZnPc in DMF is 0.58,[5] and the $^1O_2$ quantum yield of chlorophylls can be calculated according to the following equation.

$$\phi^{\text{pro}} = \phi^{\text{ref}} \frac{m^{\text{pro}}}{m^{\text{ref}}} \frac{F^{\text{ref}}}{F^{\text{pro}}} \qquad (5)$$

where, ϕ represents $^1O_2$ quantum yield, superscript "ref" and "pro" represent ZnPc and chlorophylls, m is the slope of a curve of ΔOD_{410nm} vs. irradiation time, F is the absorption correction factor ($F = 1 - 10^{-OD_{660\,nm}}$).

1.9. Detection of cellular hypoxia. 4T1 cells were seeded in a 96-well plate at the density of 1×10^4 cells per well and cultured overnight. Then fresh medium containing nanothylakoids (0.1 mg mL^{-1}) or chlorophylls loaded liposomes (0.1 mg mL^{-1}) was added into each well. To induce the cellular hypoxia, deferoxamine (3×10^{-5} M) was also added, and the wells were sealed with 100 μL of paroline. After 4 h of incubation, 10 μL of $[Ru(dpp)_3]^{2+}Cl_2$ was carefully added with final concentration at 30 μM. Thirty minutes later, cells were washed with PBS for three times and imaged under an inverted fluorescent microscope (IX

83, Olympus, Japan). The intracellular fluorescence intensity was analyzed through CellSens software (Olympus, Japan).

1.10. Imaging of intracellular ROS. 4T1 cells were seeded into a 96-well plate at density of 1×10^4 cells per well and cultured overnight. Then, the medium was replaced by cell culture medium including nanothylakoids (0.1 mg mL^{-1}) or chlorophylls loaded liposomes (0.1 mg mL^{-1}). Deferoxamine (3×10^{-5} M) was added to induce cellular hypoxia, and paroline was also used to form liquid seal. After 4 h of incubation, SOSG was added into each well with the final concentration at 20 μM. Thirty minutes later, cells were irradiated under 660 nm laser (1 W cm^{-2}) for 5 min. After that, cells were washed with PBS, and the nucleus was stained by Hoechst 33342 for 10 min. The fluorescence images were taken under the inverted fluorescent microscope.

1.11. In vitro antitumor study. 4T1 cells were seeded into 96-well plate and treated with deferoxamine following the above mentioned procedures. Then cells were cultured with nanothylakoids (0.1 mg mL^{-1}) or chlorophylls loaded liposomes (0.1 mg mL^{-1}) for another 4 h. Afterwards, the cells were irradiated under 660 nm NIR light (1 W cm^{-2}) for 5 min. The cell viability was determined using CCK-8 assay kit following the manufacturer's protocol.

1.12. Mitochondrial integrity study. 4T1 cells were cultured in culture medium containing nanothylakoids (0.1 mg mL^{-1}) for 4 h. Then the cells were irradiated by 660 nm NIR light (1 W cm^{-2}) for 5 min. Meanwhile, cells treated with nanothylakoids in the dark or NIR light only were set as control. After 1 h of incubation, the culture medium was replaced with JC-1 staining solution according to the manufacturer's protocol. Then cells were washed twice with precooled PBS and the nucleus was stained by Hoechst 33342 for 10 min. Subsequently, the cells were imaged under inverted fluorescence microscope.

1.13. In vivo hypoxia modulation. Male BALB/c mice (6 weeks old) were provided by Hunan Silaike Experimental Animal Co. Ltd (Changsha, China). All the animal experiments were performed under regulation approved by the Laboratory Animal Center of the Xiangya School of Medicine, Central South University (Changsha, China). 4T1 cells were subcutaneously injected into mice to establish tumor-bearing mouse model. When tumor size reaches ~100 mm^3, mice were randomly divided into three groups (n = 4): (1) saline (control); (2) chlorophylls loaded liposomes; (3) nanothylakoids. For group (2) and (3), mice were received intravenously injection of chlorophylls loaded liposomes (50 mg kg^{-1}) or nanothylakoids (50 mg kg^{-1}), respectively. At 24 h of postinjection, all the mice were sacrificed and the tumors were collected and

fixed in formalin. The tumor slices were immunostained with rabbit anti-mouse HIF-α primary antibody (AP7759c, Abgent, USA) and Alexa Fluor 488-labeled Goat Anti-Rabbit secondary antibody (A0423, Beyotime Inst. Biotech., China). Nucleus was stained with Hoechst 33342, and the images were obtained under the inverted fluorescence microscope.

1.14. In vivo fluorescence imaging. Tumor bearing mice were received intravenously injection of nanothylakoids (50 mg kg^{-1}). The fluorescence images were taken using a Caliper VIS Lumina XR small animal optical in vivo imaging system. The fluorescence intensity (*FI*) in main organs (heart, liver, spleen, lung and kidneys) and tumor were calculated according the following equation:

$$FI = I \times m \quad (6)$$

where, *I* and *m* represent the average fluorescence intensity and weight of organ or tumor, respectively. Accordingly, the *FI* ratios in main organs (including heart, liver, spleen, lung, kidneys) or tumor can be calculated, without consideration the fluorescence distributions in other organs (such as skin and muscle).

1.15. In vivo $^1O_2$ generation. Tumor-bearing mice with tumor size around 100 mm^3 were randomly divided into six groups (n = 4): (1) saline (blank control); (2) NIR; (3) chlorophylls loaded liposomes; (4) nanothylakoids; (5) chlorophylls loaded liposomes with NIR irradiation; and (6) nanothylakoids with NIR irradiation. Afterwards, the mice in group (3) – (6) were received an intravenously injection of chlorophylls loaded liposomes (50 mg kg^{-1}) or nanothylakoids (50 mg kg^{-1}). At 24 h post injection, 20 μL of SOSG (100 μM) in saline was intratumorally injected to monitor the *in vivo* $^1O_2$ generation. After another 1 h, the mice in group (2), (5) and (6) were irradiated by 660 nm laser (1 W cm^{-2}) for 10 min. Then tumors were harvested and homogenized to obtain cell suspensions. The intracellular SOSG fluorescence was analyzed through flow cytometry (FACS-Calibur, Becton Dickinson, USA).

1.16. In vivo PDT efficacy. Tumor-bearing mice with tumor size around 100 mm^3 were randomly divided into six groups (n = 4): (1) saline (blank control); (2) NIR; (3) chlorophylls loaded liposomes; (4) nanothylakoids; (5) chlorophylls loaded liposomes with NIR irradiation; an. d (6) nanothylakoids with NIR irradiation. Afterwards, chlorophylls loaded liposomes (50 mg kg^{-1}) or nanothylakoids (50 mg kg^{-1}) intravenously injected into mice. After 24 h, the mice in group (2), (5) and (6) were irradiated by 660 nm laser (1 W cm^{-2}) for 10 min. All the treatments were repeated on day 4 and day 7. The tumor size and body weight of mice were recorded every other day. The tumor volume was calculated according to the formula:

$$V = L \times D^2/2 \quad (7)$$

Where, *L* and *D* represent the length and width of the tumor, respectively.

On day 15, the mice were sacrificed and the main organs (heart, liver, spleen, lung and kidney) as well as tumors were harvested, fixed in 4% formalin solution and sectioned into slices for H&E or TUNEL staining.

1.17. Biocompatibility and biosafety studies. To evaluate the cytotoxicity of nanothylakoids, 4T1, L929 and Hela cells were seeded into a 96-well plate at a density of 1×10^5 cells per milliliter, respectively. After 12 h of incubation, the complete DMEM medium was replaced with fresh DMEM medium containing various concentrations of nanothylakoids (0, 0.1, 0.2, 0.5, 1.0 mg mL^{-1}). After cultured for 24 h, the cell viability was measured using CCK-8 assay kit following the manufacturer's protocol.

To study the hemolysis rate, red blood cells were separated from the whole blood of mouse by centrifugation (6000 rpm, 5 min) and washed with PBS solution for three times. Subsequently, 0.15 mL of 4% red blood cell (*v*/*v*) was mixed with 0.15 mL of nanothylakoids solution at various concentrations (0, 0.1, 0.2, 0.5, 1.0 mg mL^{-1}) and incubated at 37 ℃ for 8 h. The mixture was centrifuged and the absorbance of the supernatants at 540 nm was recorded by UV-Vis spectrophotometer. The hemolysis percentage was calculated by following equation.

$$\text{Hemolysis (\%)} = (I/I_0) \times 100\% \quad (8)$$

Where, I represents the absorbance of supernatant for red blood cell with different concentrations of nanothylakoids, and I_0 is the absorbance of complete hemolysis in pure water.

To study the *in vivo* biosafety of nanothylakoids, male BALB/c mice (6 weeks old) were receive intravenously injection of the nanothylakoids at a dosage of 50 mg kg^{-1}. After 7 days, mouse blood was collected and analyzed on a blood chemistry analyzer (Pointcare V2, MNCHIP, Tianjin, China).

2. Supplementary Figures and Table

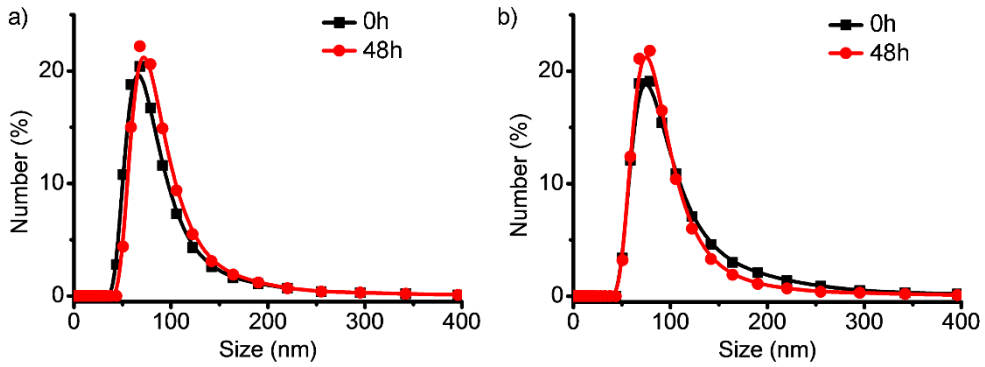

Fig. S1 Size distributions of nanothylakoids in a) water or b) cell culture medium within 48 h.

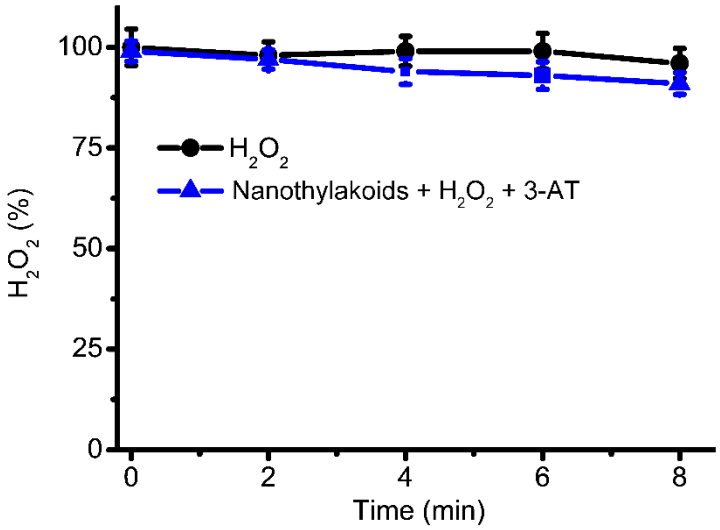

Fig. S2 3-AT inhibits H_2O_2 decomposition by the nanothylakoids.

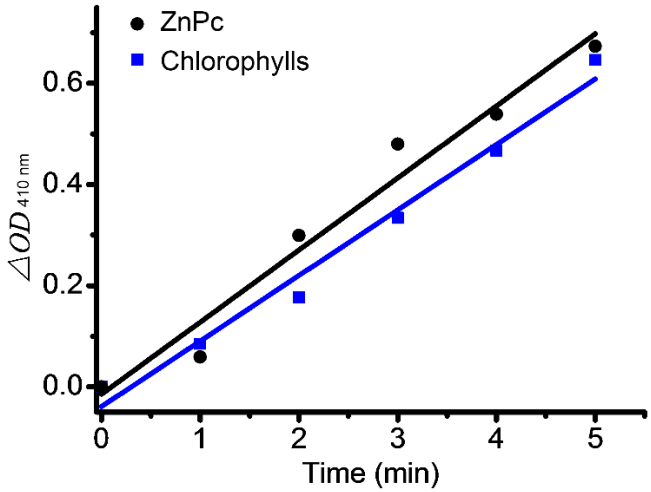

Fig. S3 Decrease of DPBF absorption at 410 nm vs. irradiation time (λ = 660 nm) in the presence of chlorophylls or ZnPc.

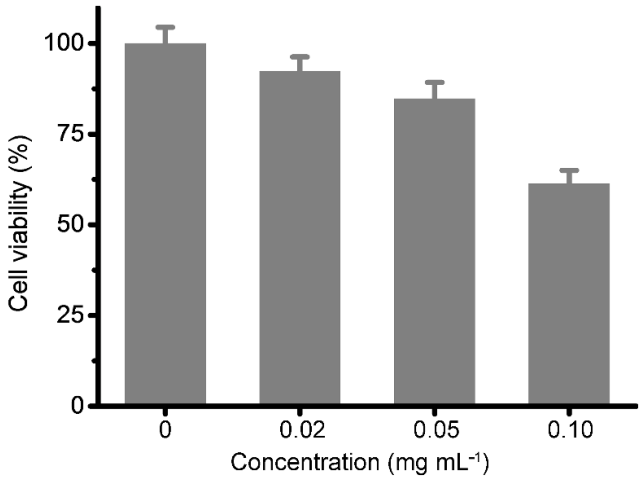

Fig. S4 The cytotoxicity of chlorophylls loaded liposomes under 660 nm NIR light irradiation (1 W cm^{-2}, 5 min).

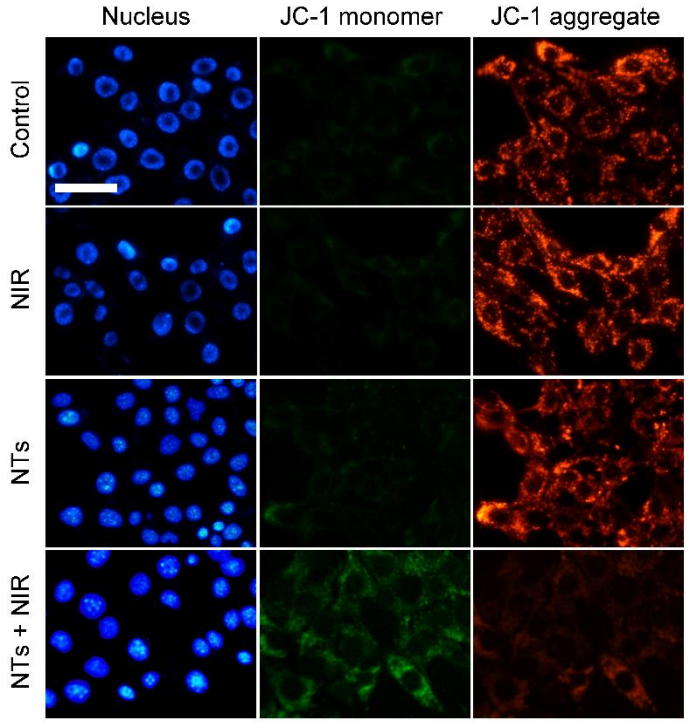

Fig. S5 The integrity of mitochondria after different treatments was imaged by JC-1 staining. All scale bars are 25 μm.

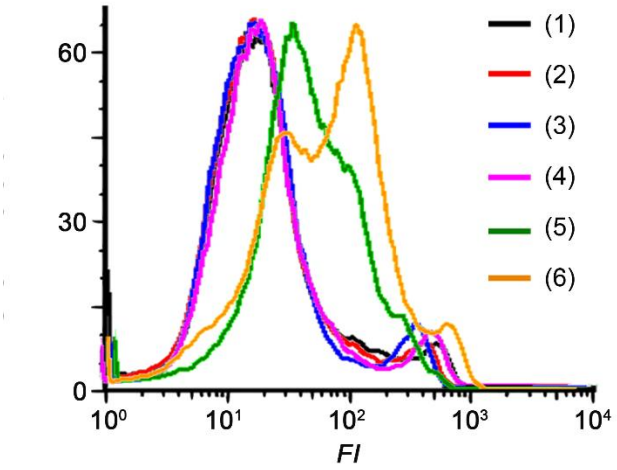

Fig. S6 Intracellular $^1O_2$ contents after different treatments were analyzed through flow cytometry: (1) saline; (2) NIR; (3) chlorophylls loaded liposomes (LPs); (4) nanothylakoids (NTs); (5) LPs + NIR; and (6) NTs + NIR.

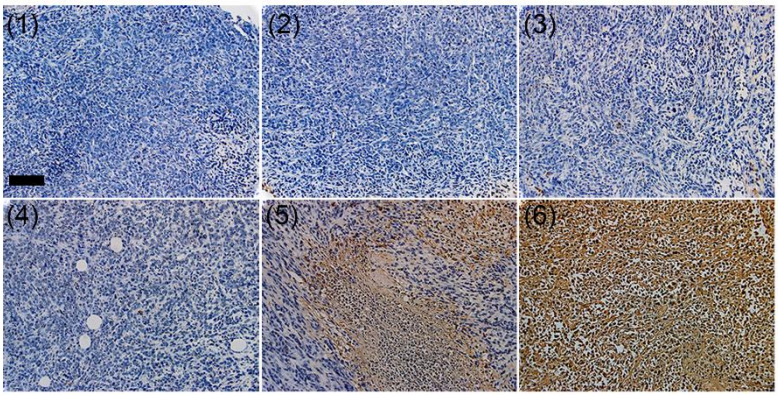

Fig. S7 Cell apoptosis was studied through TUNEL staining of tumor slices after different treatments: (1) blank; (2) NIR; (3) LPs; (4) NTs; (5) LPs + NIR; (6) NTs + NIR (scale bar = 100 μm).

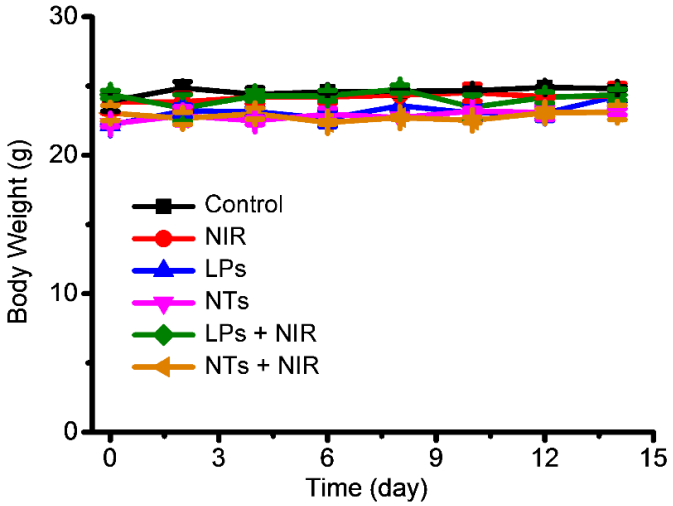

Fig. S8 Change of mouse body weights during various treatments: (1) blank contro; (2) NIR; (3) chlorophylls loaded liposomes (LPs); (4) nanothylakoids (NTs); (5) LPs + NIR; and (6) NTs + NIR.

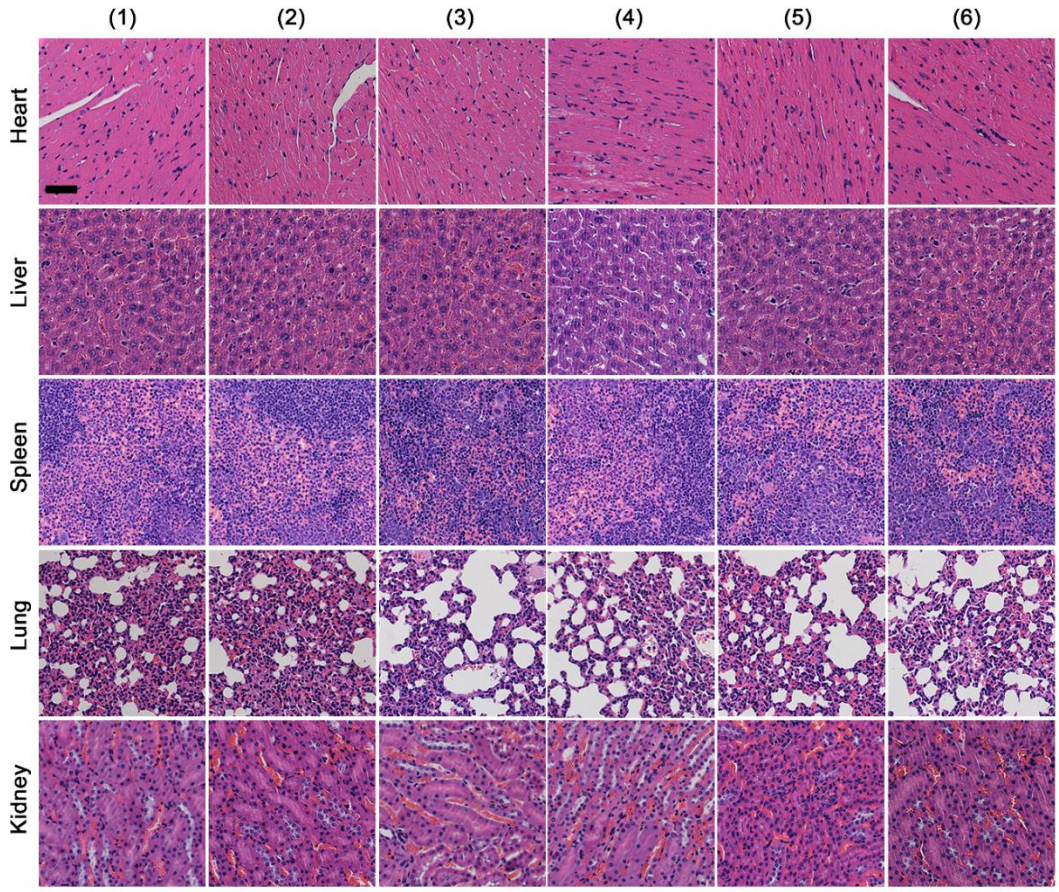

Fig. S9 H&E staining of tissue slices from main organs after different treatments: (1) blank control; (2) NIR; (3) chlorophylls loaded liposomes; (4) nanothylakoids; (5) chlorophylls loaded liposomes with NIR irradiation; and (6) nanothylakoids with NIR irradiation (scale bar = 50 μm).

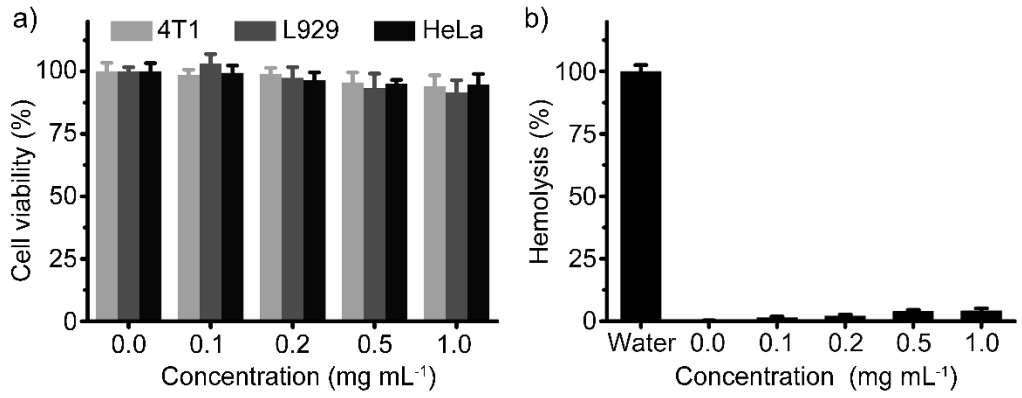

Fig. S10 a) Cytotoxicity of nanothylakoids to three different cell lines (4T1, L929 and HeLa). b) Hemolysis rates of red blood cells under different concentration of nanothylakoids.

Table S1 Blood biochemistry analysis of mice injected with nanothylakoids.

	Normal Range [a]	Control	Nanothylakoids
RBC ($\times 10^{12}$)	6.93 − 12.24	10.99 ± 0.37	11.16 ± 1.01
WBC ($\times 10^{9}$)	8.62 − 14.03	10.6 ± 1.83	10.5 ± 0.57
PLT ($\times 10^{12}$)	0.42 − 1.70	0.93 ± 0.14	1.07 ± 0.10
HGB (g L^{-1})	126 − 205	174.5 ± 12.9	169.0 ± 15.2
BUN (mmol L^{-1})	2.50 − 9.28	6.05 ± 0.35	6.45 ± 0.52
Cr (mmol L^{-1})	17.68 − 35.36	31.25 ± 2.87	32.71 ± 2.38
AST (IU L^{-1})	55 − 352	68.24 ± 7.39	70.51 ± 10.16
T-Bil (mmol L^{-1})	3.42 − 8.55	8.26 ± 0.31	7.84 ± 0.59

[a] Normal ranges of healthy male Balb/c mice were referred to the Jackson Laboratory (https://www.jax.org/) and Charles River Laboratories (http://www. criver.com/).

References

[1]. a) H. Itzhaki, L. Naveh, M. Lindahl, M. Cook and Z. Adam, *J. Biol. Chem.*, **1998**, 273, 7094-7098; b) D. J. Chapman, J. De-Felice and J. Barber, *Plant Physiol.*, **1983**, 72, 225.

[2]. E. J. Clerkx, H. Blankestijn-De Vries, G. J. Ruys, S. P. Groot and M. Koornneef, *Plant Physiol.*, **2003**, 132, 1077-1084.

[3]. W. Chen, J. Ouyang, X. Yi, Y. Xu, C. Niu, W. Zhang, L. Wang, J. Sheng, L. Deng, Y. N. Liu and S. Guo, *Adv. Mater.*, **2018**, 30, 1703458.

[4]. A. P. Thomas, P. Saneesh Babu, S. Asha Nair, S. Ramakrishnan, D. Ramaiah, T. K. Chandrashekar, A. Srinivasan and M. Radhakrishna Pillai, *J. Med. Chem.*, 2012, **55**, 5110-5120.

[5]. H. Shinohara, O. Tsaryova, G. Schnurpfeil and D. Wöhrle, *J. Photochem. Photobiol., A*, 2006, **184**, 50-57.