Electronic Supplementary Information

For

Incorporation of 7-dehydrocholesterol into liposomes as a simple, universal and efficient way to enhance anticancer activity by combining PDT and Photoactivated chemotherapy

Youchao Wang,[ab] Nana Tian,[ab] Chao Li,[ab] Yuanjun Hou,[a] Xuesong Wang*[ab] and Qianxiong Zhou*[a]

[a] *Key Laboratory of Photochemical Conversion and Optoelectronic Materials, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing 100190, P. R. China. Fax: +86-10-62564049; Tel: +86-10-82543592;*

[b]*University of Chinese Academy of Sciences, Beijing 100049, P. R. China.*

E-mail:xswang@mail.ipc.ac.cn (X.Wang); zhouqianxiong@mail.ipc.ac.cn (Q. Zhou).

Experimental Section

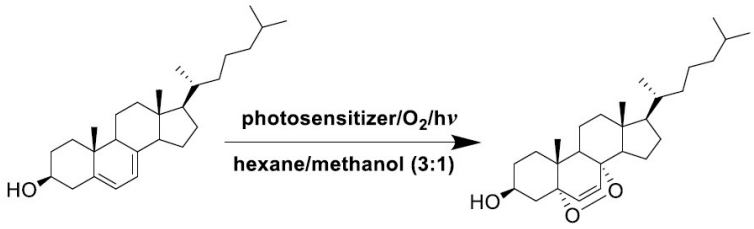
Materials

Cholesterol (CHOL), 7-dehydrocholesterol (7-DHC), meso-tetraphenylporphyrin (TPP) were purchased from Sigma Aldrich. Phosphatidylcholine (PC) was obtained from A.V.T. (Shanghai) Pharmaceutical Co. Ltd. *n*-Hexane, methanol, chloroform of analytical grade were purchased from SCRC (Sino pharm Chemical Reagent Co., Ltd) and used without further treatment. Dulbecco's modification of Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum, and 10 mM PBS (pH = 7.4) were purchased from Corning, apoptosis kit was obtained from Beijing Solarbio Science & Technology Co., Ltd.

Instruments

^{1}H NMR spectra were recorded on a Bruker DMX-400 MHz. High-resolution ESI mass spectra (HR ESI-MS) were determined on a Brucker APEX IV (7.0T) FT-MS. UV-vis absorption spectra were obtained using a Shimadzu UV-1601 spectrophotometer. An LED lamp (420nm) was used as light source for irradiation. The MTT assays were measured on a Thermo MK3 Multiscan microplate reader at 570 nm. Transmission electron microscope (TEM) images were obtained using a JEM-2100. Dynamic light scattering (DLS) was tested by a Dybapro NanoStar.

Synthesis of 7-dehydrocholesterol endoperoxide (CEP)



CEP was synthesized as the reported methods.[1] 0.0012 g (1.9 μmol) of TPP and 0.60 g (1.56 mmol) 7-dehydrocholesterol were added in 20 mL of 3:1 hexane/methanol. The mixture was irradiated for 3 h at 0℃ under magnetic stirring and continuous bubbling with oxygen. A 500 W high-pressure Hg lamp in combination with a 400 nm cut-off glass filter was employed as the light source. Solvent was removed by rotary evaporator. The crude product was purified on silica gel using hexane/ethyl acetate (3: 1) as eluent. Yield: 80%. ^{1}H NMR (400MHz, $CDCl_3$): δ 6.51 (d, J = 8.4 Hz, 1H), 6.24 (d, J = 8.5 Hz, 1H), 3.97 (m, 1H), 2.13-2.08 (dd, J = 13.2, 4.0 Hz, 1H), the signals between 2.05-0.70 ppm cannot be resolved, with total amount of 39 H. HRESI-MS: m/z =439.3166 for $(CEP+Na)^+$, cal: 439.3188.

Fabrication of liposomes

Liposomes with different components were prepared by the film dispersion method.[2] Taking liposome D as an example. PC and 7-DHC with a molar ratio of 9:2 were placed in a round bottom flask and dissolved in chloroform, TPP accounting for 1% of the total weight of starting materials was added to the solvent. After ultrasonication for 30 min, chloroform was removed by rotary evaporation to obtain a lavender liposome membrane. After standing overnight, the membrane was hydrated with 20 mL deionized water. The membrane was fully hydrated by vortexing for 20 min. After another 20 min of sonication, free TPP was removed by centrifugation (10000 rpm, 30 min). The liposomes were obtained by extruded through an ultra-high pressure homogenizer.

The preparation procedure of other liposomes was the same as that of liposome D, just using different components as listed below.

Liposome A: PC/CHOL, with molar ratio of 9:2;

Liposome B: PC/7-DHC, with molar ratio of 9:2;

Liposome C: PC/CHOL/TPP, with PC/CHOL molar ratio of 9:2, and TPP was 1% of the total weight of starting materials;

Liposome E: PC/7-DHC/TPP, with PC/7-DHC molar ratio of 18:1, and TPP was 1% of the total weight of starting materials;

Liposome F: PC/7-DHC/TPP, with PC/7-DHC molar ratio of 9:8, and TPP was 1% of the total weight of starting materials;

Liposome G: PC/7-DHC/TPP, with PC/7-DHC molar ratio of 9:8, and TPP was 3% of the total weight of starting materials;

Liposome H: PC/7-DHC/TPP, with PC/7-DHC molar ratio of 25:1, and TPP was 1% of the total weight of starting materials;

Liposome I: PC/7-DHC/TPP, with PC/7-DHC molar ratio of 36:1, and TPP was 1% of the total weight of starting materials;

Liposome J: PC/CHOL/VER, with PC/CHOL molar ratio of 9:2, and VER was 1% of the total weight of starting materials;

Liposome K: PC/7-DHC/VER, with PC/7-DHC molar ratio of 9:2, and VER was 1% of the total weight of starting materials.

Characterization of liposomes

Drug loading efficiency was defined as the ratio between actual amount of TPP encapsulated in the liposomes versus the amount of liposomes, as described by Equation 1.

$$drug\ loading\ efficiency\% = \frac{actual\ amount\ of\ TPP\ in\ liposomes}{amount\ of\ liposomes} \times 100 \quad (1)$$

The actual amount of TPP in liposome was determined by absorption.

The size distributions of liposomes were measured by dynamic light scattering on a Dybapro NanoStar. The morphology and structure were obtained on a transmission electron microscope (JEM-2100).

Cell Culture

The cells were cultured in DMEM with 10% (v/v) fetal bovine serum, penicillin and streptomycin (100 U/ml) at 37 °C and 5% CO_2.

MTT assay

Cells were seeded in 96-well plates at the density of 5000-8000 cells per well for 24 h, then liposomes with gradient concentrations were added. 4 h later, cell medium was changed for fresh one. Light groups were irradiated for 30 min (420 nm, 22.5 mW/cm^2), and cells were cultivated for another 20 h. Cell medium was changed for fresh one containing MTT (1 mg/ml). After 4 h, MTT solution was removed and DMSO was added. The absorbance at 570 nm was measured using a Thermo MK3 Multiscan microplate reader.

Intracellular ROS detection

SKOV3 cells were seeded onto 35 mm culture dishes and incubated for 24 h. The cells were treated with medium containing liposomes C and D at the same concentration of TPP at 37 °C for 4 h in

the dark. The cells were then washed twice with serum-free medium and incubated with DCFH-DA (10 μM) for 10 min at 37 °C in the dark. The light groups were irradiated with 420 nm laser (22.5 mW/cm^2, 20 min) and washed twice with PBS. The samples were then analyzed immediately by confocal microscopy. Emission was collected at 530 ± 20 nm upon excitation at 488 nm.

Annexin V and PI assay

The annexin V and PI apoptosis detection kit was used to examine the mechanism of cell death. SKOV-3 cells were incubated with different concentrations of liposomes in DMEM without FBS for 4 h. Cell medium was changed for fresh one, and the samples were irradiated for 30 min (420 nm LED lamp) and incubated for another 10 h. Cells were harvested and washed with PBS twice, stained with annexin V and PI, then analyzed by laser scanning confocal microscope or flowcytometry.

Statistical Analysis

All biological experiments were performed at least three times and data were presented as means ± standard deviations (SD).

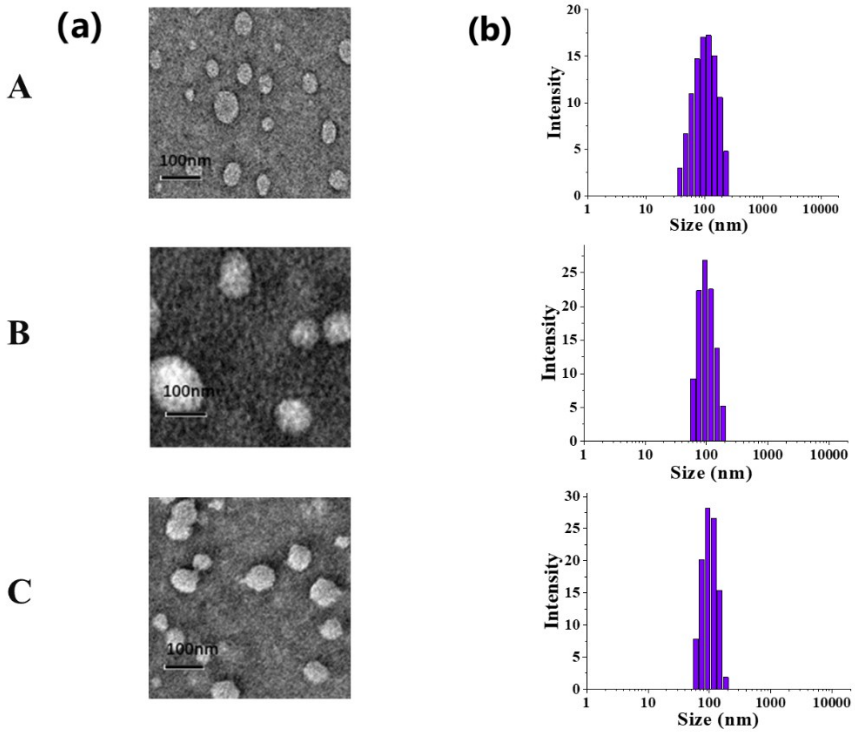


Figure S1. TEM images (a) and DLS analysis (b) of liposomes A-C.

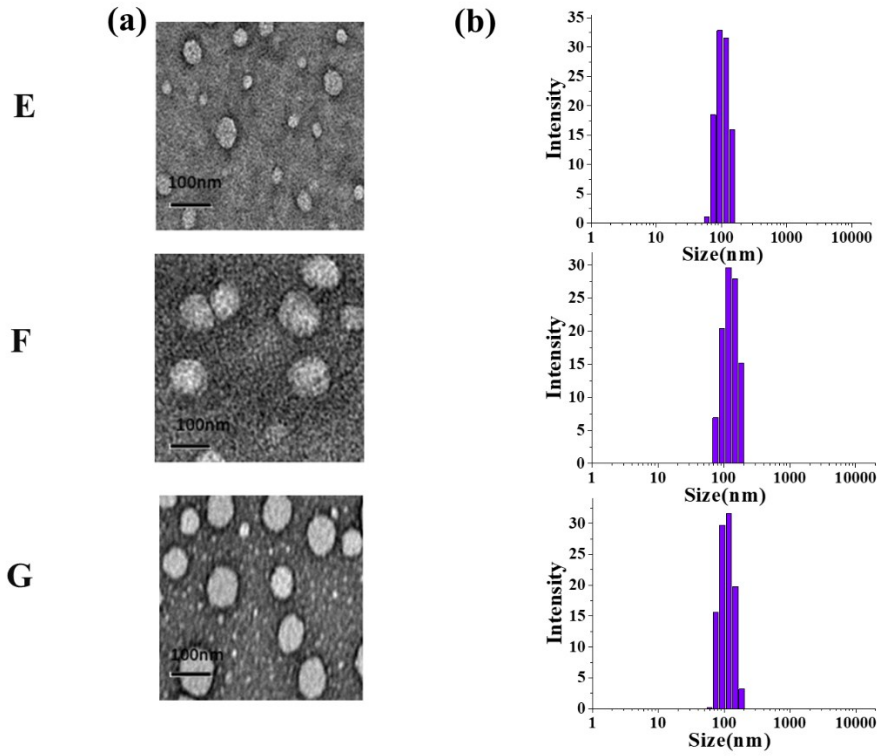


Figure S2. TEM images (a) and DLS analysis (b) of liposomes E-G.

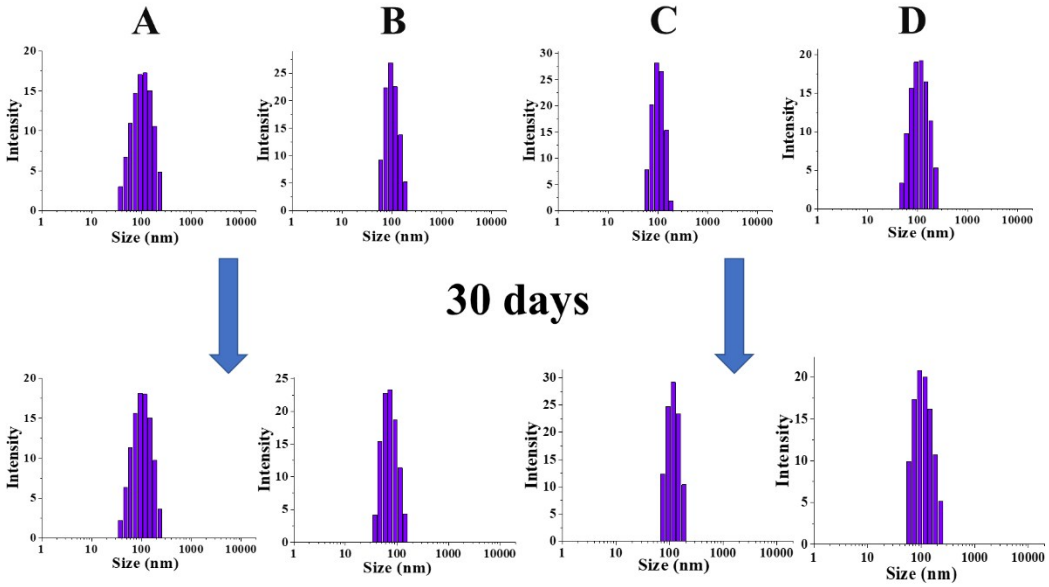


Figure S3. Stability of liposomes A-D examined by DLS.

Table S1. Drug loading efficiencies of liposomes and IC_{50} values expressed by liposome weight and TPP concentration

Liposome	TPP loading efficiency (10^{-2}%)	IC_{50} Liposome (μg/ml)		IC_{50} TPP (μM)
		Light	Dark	Light
A	0	>250	>250	/
B	0	>250	>250	/
C	4.0	236.9 ± 0.2	>250	0.156 ± 0.001
D	4.2	128.5 ± 0.1	>250	0.087 ± 0.001
E	3.4	145.7 ± 0.2	>250	0.080 ± 0.001
F	4.7	103.4 ± 0.5	>250	0.079 ± 0.002
G	6.9	60.3 ± 0.2	>250	0.068 ± 0.001

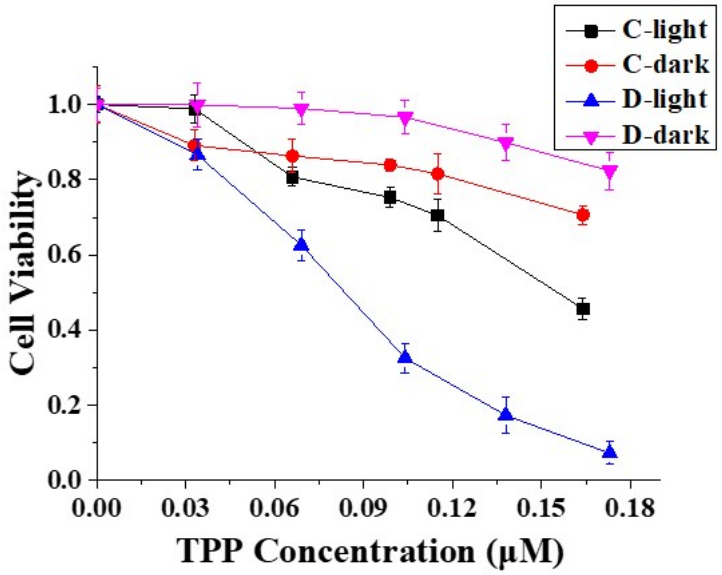


Figure S4. Cytotoxicity expressed by TPP concentrations of liposomes C-D toward SKOV-3 cells in the dark or under light irradiation for 30 min (420 nm LED, 22.5 mW/cm^2).

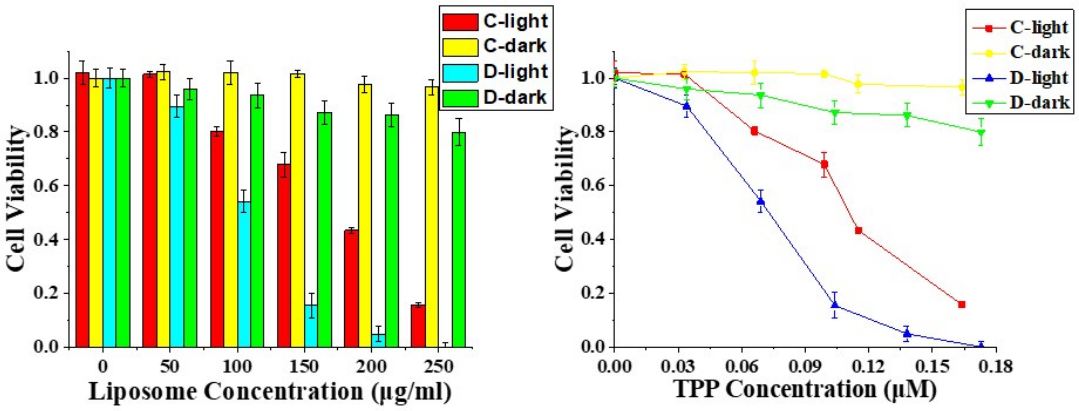


Figure S5. Cytotoxicity expressed by Liposome and TPP concentrations of liposomes C-D toward

A549 cells in the dark or under light irradiation for 30 min (420 nm LED, 22.5 mW/cm^2).

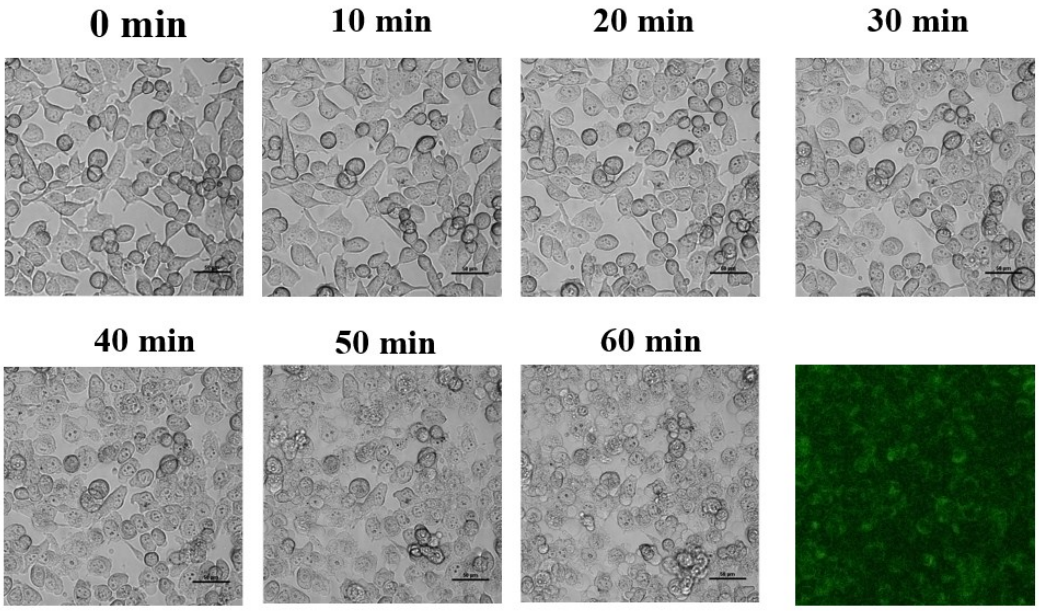
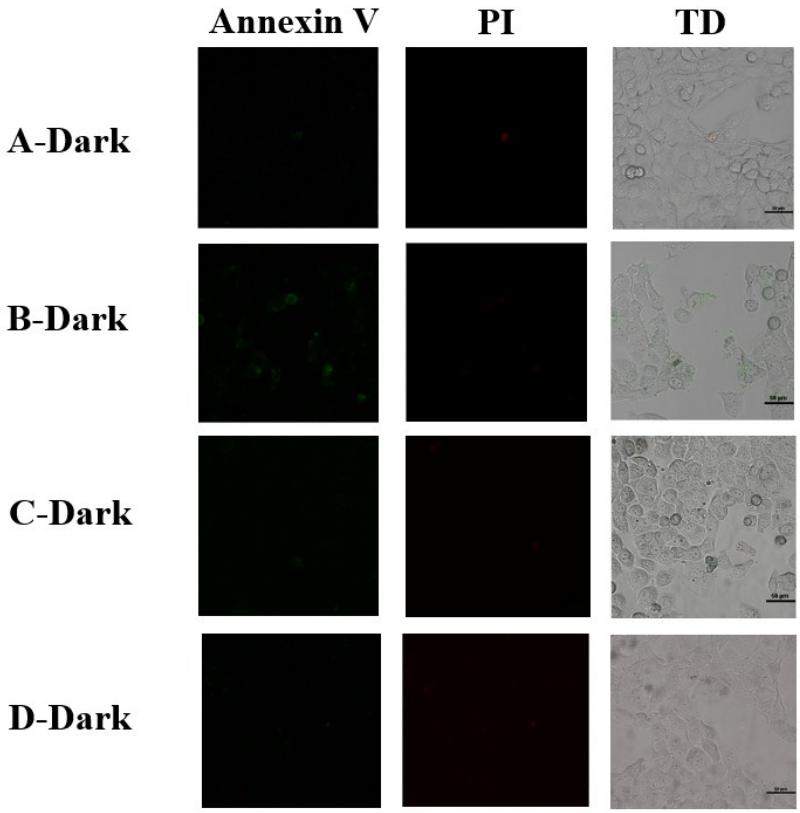


Figure S6. Confocal microscopy images of SKOV-3 cells. The cells were incubated with liposome D for 4 h, then washed with PBS and irradiated with light (420 nm laser of microscope) for different times (0, 10, 20, 30, 40, 50, 60 min). Finally Annexin V was added to image apoptosis cells.



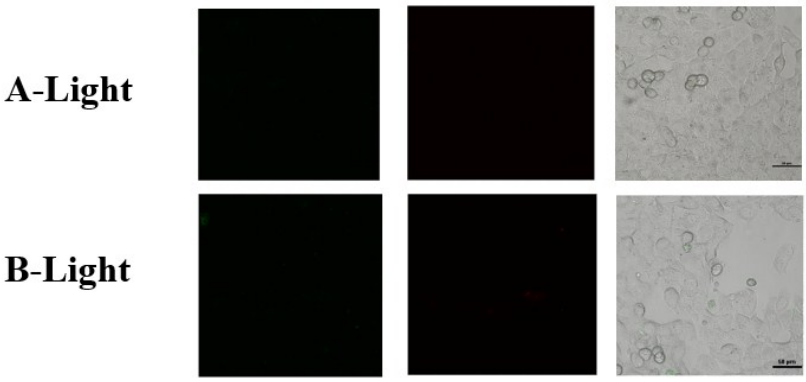


Figure S7. AnnexinV and PI assay of SKOV-3 cells incubated with liposomes A-C (100 μg/ml) and D (95 μg/ml) in the dark or under irradiation for 30 min (420 nm LED, 22.5 mW/cm^2).

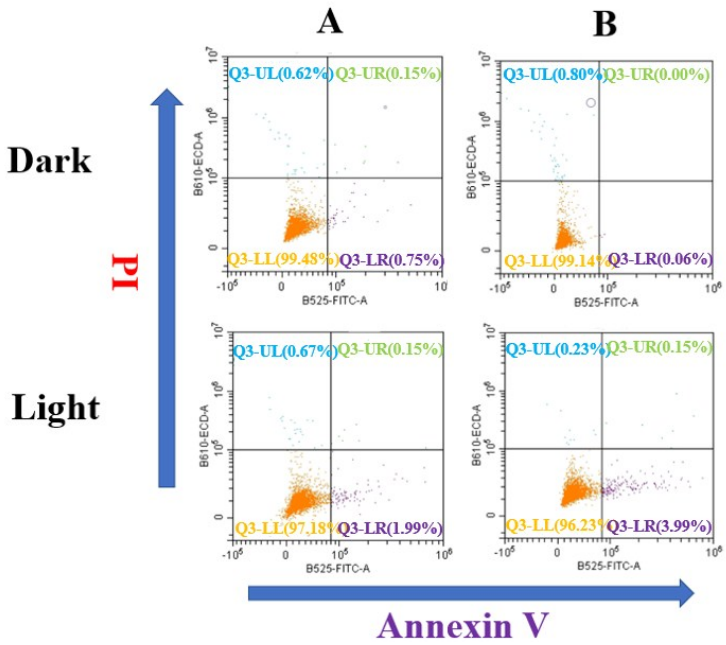


Figure S8. Annexin V and PI assay of SKOV-3 cells incubated with liposomes A-B (100 μg/ml) in the dark or upon irradiation for 30 min (420 nm LED, 22.5 mW/cm^2) analyzed by flow cytometry.

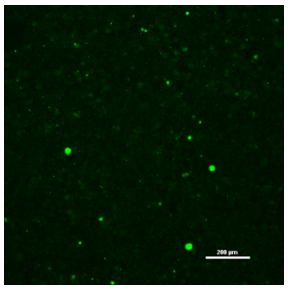


Figure S9. Confocal fluorescence image of SKOV-3 intracellular ROS level of control group (incubated with DCFH-DA alone) upon irradiation by 420 nm LED for 20 min (22.5 mW/cm^2). Scale bar: 200 μm.

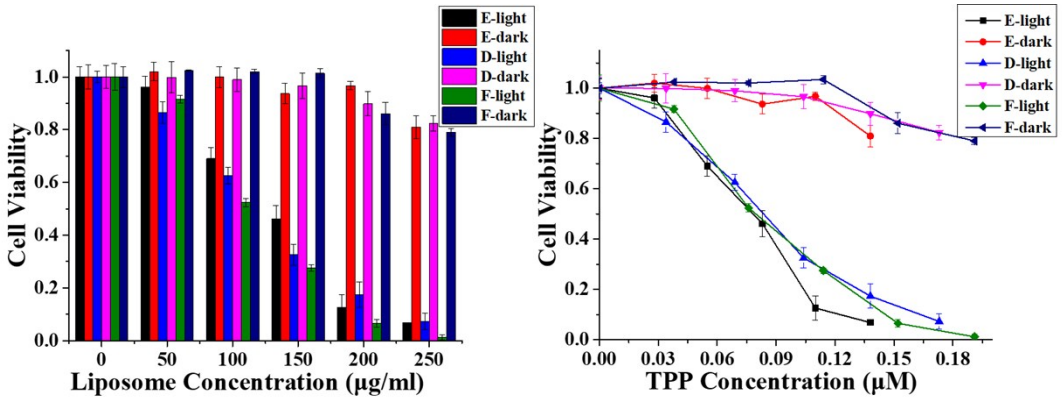


Figure S10. Cytotoxicity of liposomes E, D and F toward SKOV-3 cells in the dark or under light irradiation for 30 min (420 nm LED, 22.5 mW/cm^2).

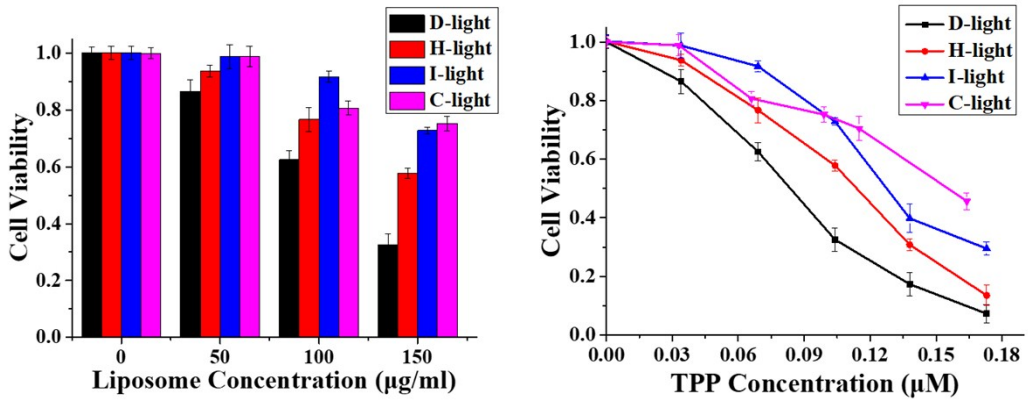


Figure S11. Cytotoxicity of liposomes C-D, H-I toward SKOV-3 cells under light irradiation for 30 min (420 nm LED, 22.5 mW/cm^2).

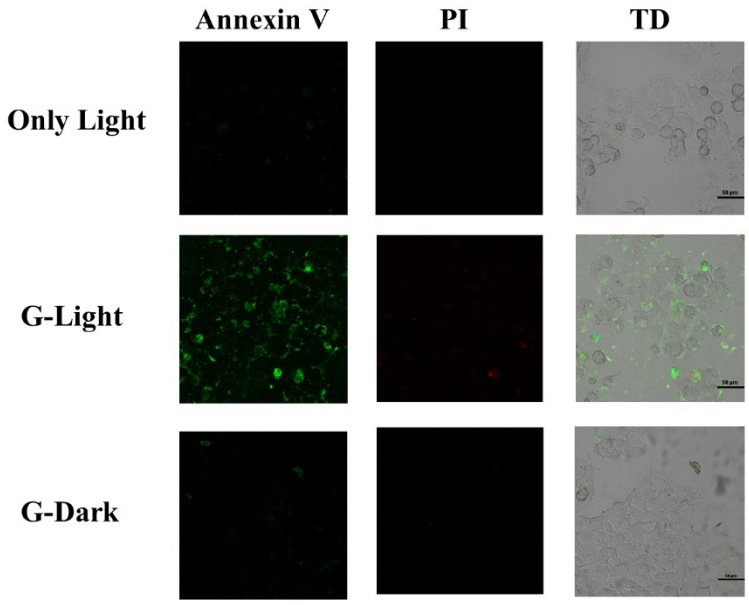


Figure S12. Annexin V and PI assay of SKOV-3 cells incubated with liposome G (100 μg/ml) in

the dark or under irradiation (420 nm LED for 0.5 h, 22.5 mW/cm^2) analyzed by laser scanning confocal microscope.

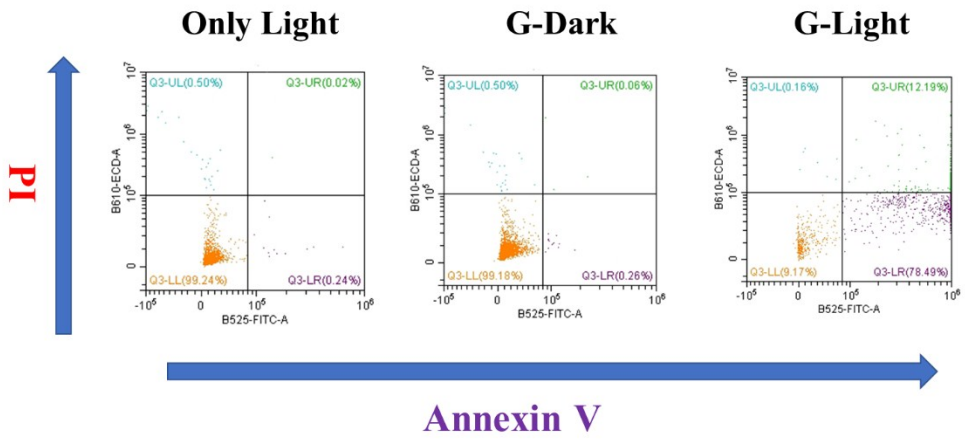


Figure S13. Annexin V and PI assay of SKOV-3 cells incubated with liposome G (100 μg/ml) in the dark or under irradiation (420 nm LED for 0.5 h, 22.5 mW/cm^2) analyzed by flow cytometry.

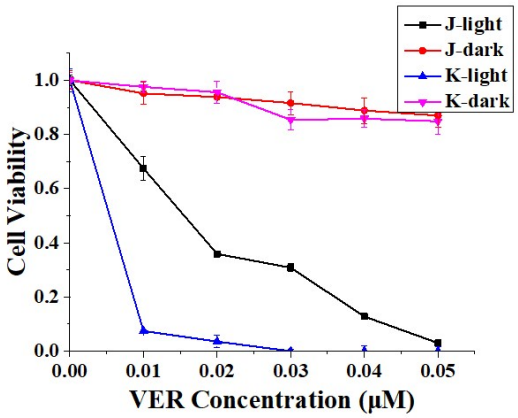


Figure S14. Cytotoxicity of liposomes J-K toward A549 cells in the dark or under light irradiation for 30 min (420 nm LED, 22.5 mW/cm^2).

References

1. N. N. Tian, C. Li, N. Tian, Q. X. Zhou, Y. J. Hou, B. W. Zhang, X. S. Wang, *New J. Chem.*, 2017,**41**, 14843-14846.
2. S. Bonnet, B. Limburg, J. D. Meeldijk, R. J. Gebbink, J. A. Killian, *J. Am. Chem. Soc.*, 2011, **133**, 252-261.