Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary Information

Transporting mitochondrion-targeting photosensitizers into cancer cells by low-density lipoproteins for fluorescence-feedback photodynamic therapy

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Experimental Section

Chemicals and Materials. Low-density lipoprotein (LDL) particles isolated from human plasma were purchased from LEE Biosolutions. Lauric acid, stearic acid, and potato starch were obtained from TCI, Maryer, and Solarbio, respectively. 4-Fluorobenzophenone, diphenylamine, 4-benzoylpyridine, titanium(IV) chloride ($TiCl_4$), iodomethane (CH_3I), rose bengal (RB), dry tetrahydrofuran (THF), dry *N*,*N*-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were ordered from Adamas. Petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), heptane, toluene, dioxane, ethyl ether (Et_2O), potassium *tert*-butoxide (t-BuOK), potassium hexafluorophosphate (KPF_6), and methanol were purchased from Tianjin Bohai Chemical Industry Group Co., Ltd. Zinc dust was obtained from Energy chemical. 2',7'-Dichlorofluorescin diacetate (DCFH-DA) and 9,10-anthracenediyl-*bis*(methylene)-dimalonic acid (ABDA) were purchased from Sigma-Aldrich. Roswell Park Memorial Institute 1640 (RPMI 1640), penicillin-streptomycin solution (100×), and phosphate buffered saline (PBS, 10 mM, pH = 7.4) were ordered from Thermal Fisher Scientific. Fetal bovine serum (FBS) was obtained from Zhejiang Tianhang Biotechnology co., Ltd. Hoechst 33342, Lyso-Tracker Green, Mito-Tracker Green, live/dead cell viability assay kit, enhanced bicinchoninic acid (BCA) protein assay kit, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Beyotime Biotechnology. All chemicals were used as received without further purification. The water used in all experiments was obtained by filtering through a set of HEAL FORCE cartridges (Smart-N15VF).

Synthesis of TPA-DPPy. Compound **1** was prepared by referring to a previously published paper.[1] Zinc dust (2.86 g, 44 mmol) was placed in a 250-mL three-necked round-bottomed flask equipped with a reflux condenser. The flask was evacuated under vacuum and purged with dry argon three times, followed by the addition of dry THF (33 mL). The system was cooled to −50 °C, and then $TiCl_4$ (2.42 mL, 22 mmol) was added. The mixture was recovered to room temperature and then heated to reflux for 2 h. After cooling to room temperature, compound **1** (1.43 g, 4.1 mmol) and

4-benzoylpyridine (0.92 g, 4.92 mmol) were added into the reaction system, followed by refluxing for another 5 h. The reaction was then quenched with aqueous K_2CO_3 (10 wt.%). After filtration, the residue was extracted by DCM three times. The combined organic layers were washed with brine twice and dried over anhydrous Na_2SO_4. The crude product was purified on a silica-gel column (DCM/PE=1/10) to afford an orange solid (compound **2**) in 21% yield. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.45–8.23 (m, 2H), 7.25–7.18 (m, 4H), 7.18–6.94 (m, 17H), 6.90 (d, 1H), 6.87–6.74 (m, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 152.25, 151.98, 149.29, 147.51, 147.44, 147.01, 146.68, 143.55, 142.82, 142.61, 142.54, 137.64, 136.62, 136.24, 132.12, 131.38, 131.31, 129.36, 129.27, 128.10, 128.02, 127.82, 127.37, 127.10, 126.98, 126.91, 126.17, 124.74, 124.58, 123.23, 123.07, 122.30. HRMS (ESI, m/z, $C_{37}H_{28}N_2$, $[M + H^+]$): calcd, 501.2325; found, 501.2332.

Compound **2** (0.1488 g, 0.3 mmol) was dissolved in toluene (3 mL) followed by the addition of CH_3I (0.3 mL). The reaction mixture was stirred at 90 °C for 3 h. After cooling to room temperature, the precipitates were filtered and washed with cold toluene three times. The obtained solid was dissolved in 9 mL acetone, which was subjected to ion-exchange with KPF_6 (93.7 mg) for 12 h. After solvent removal, the resultant solid was washed with water three times. The crude product was purified on a silica-gel column (DCM/methanol=50/1) to afford a red solid (TPA-DPPy) in 73% yield. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.60 (d, 2H), 7.46 (d, 2H), 7.31 (dd, 4H), 7.19–7.11 (m, 10H), 7.10 –7.01 (m, 5H), 6.98 (dd, 2H), 6.84 (s, 4H), 4.41 (s, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 161.91, 150.94, 148.97, 146.96, 146.70, 143.90, 143.69, 141.68, 141.00, 140.88, 134.06, 132.85, 132.65, 132.39, 131.62, 131.39, 129.66, 129.56, 129.41, 128.85, 128.42, 127.98, 125.76, 125.23, 124.25, 123.81, 120.89, 47.96. HRMS (ESI, m/z, $C_{38}H_{31}N_2$, $[M]^+$): calcd, 515.2482; found, 515.2488.

Theoretical Calculation. Theoretical calculation was carried out using the Gaussian 09 software. Geometry optimization was performed using density functional theory (DFT) at B3LYP/6-31G* level of theory. The electronic distribution of the frontier molecular orbitals (FMOs) were drawn using Gaussview 5.0.9.

Preparation and characterization of reconstituted LDL particles. The reconstituted LDL (rLDL) particles were fabricated according to our previous report.[2] Briefly, 1 mg of native LDL particles in 200 μL of water was vortexed with 12.5 mg of potato starch, followed by freeze-drying. After extraction of endogenous lipids with heptane three times, 100 μL of permixed payloads in toluene (TPA-DPPy = 0.5 mg mL^{-1}, lauric acid = 4 mg mL^{-1}, stearic acid = 1 mg mL^{-1}) was added to the LDL-starch mixture. As a control, a mixture of fatty acids at the identical concentration and volume were also prepared for comparative experiments. After incubation at –20 °C for 20 min, toluene was removed under argon in an ice bath. The mixture was then dispersed in 300 μL of tricine buffer (10 mM, pH=8.4) at 4 °C for 18 h, following by centrifugation at 2000 rpm for 10 min. The supernatant was subjected to additional two rounds of centrifugation at 10000 rpm for 10 min to further purify the rLDL particles. After passing through a 0.22-μm sterile filter (Millipore), the resultant rLDL particles were stored at 4 °C for subsequent use. The rLDL particles with and without TPA-DPPy were denoted as rLDL(+) and rLDL(–) particles, respectively. To quantify the concentration of the encapsulated TPA-DPPy, rLDL(+) particles were disassembled and extracted with methanol under ultrasonication, followed by centrifugation at 10000 rpm for 5 min. The supernatant was then measured by a UV/vis spectrometer (Shimadzu, UV-2600). The concentration of TPA-DPPy was determined by referring to the calibration curve of TPA-DPPy in methanol. The concentration of apolipoprotein B-100 (ApoB-100) protein in rLDL(+) particles solution was quantified using an enhanced BCA protein assay kit according to the manufacturer's instruction. The molar ratio of TPA-DPPy and ApoB-100 was calculated using the molecular mass of ApoB-100 of 550 kDa in each LDL particle. The morphology of all rLDL particles was characterized by transmission electron microscopy (TEM, Tecnai, G2 F20). Prior to TEM characterization, the samples were negatively stained with 2 wt.% uranyl acetate.

Reactive oxygen species (ROS) measurements. Prior to ROS measurements, the chemical probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) was chemically activated to its deacetylated form

2',7'-dichlorofluorescin (DCFH) according to a previous study with the final concentration of DCFH at 40 μM.[3] To 1 mL of DCFH solution was added TPA-DPPy with a final concentration of 10 μM. The fluorescence spectra were recorded at 30-second intervals on a fluorometer (HITACHI, F-4700, Ex = 488 nm) under white-light irradiation (380−800 nm) at a power density of 30 mW cm^{-2}.

Singlet oxygen quantum yield measurements. 9,10-Anthracenediyl-*bis*(methylene)-dimalonic acid (ABDA) was used as the indicator of singlet oxygen, where rose bengal (RB) was employed as the reference photosensitizer. To eliminate the inner-filter effect, the absorption maxima were controlled at *ca.* 0.2. The measurements were carried out under white-light irradiation at a power density of 50 mW cm^{-2} in the mixture of DMSO/water (v/v = 1/99). Briefly, 15 μL of ABDA stock solution (10 mM) was added to 2 mL of TPA-DPPy (20 μM) or RB (2.5 μM) in PBS. The absorbance of ABDA at 378 nm was then recorded at 1-min intervals to obtain the decay rate of ABDA. The singlet oxygen quantum yield of TPA-DPPy was calculated by the following equation:

$$\Phi_{\text{TPA-DPPy}} = \Phi_{\text{RB}} (k_{\text{TPA-DPPy}}/k_{\text{RB}})/(A_{\text{TPA-DPPy}}/A_{\text{RB}})$$

k_{AIE} and k_{RB} are the decomposition rate constants of ABDA with TPA-DPPy and RB, respectively, which were the slopes of the plots of $\ln(A_0/A)$ against irradiation time. $A_{\text{TPA-DPPy}}$ and A_{RB} represent the light energy absorbed by TPA-DPPy and RB, respectively, which were determined by integrating the areas under the absorption bands in the wavelength range of 380−800 nm. Φ_{RB} is the singlet oxygen quantum yield of RB (0.75 in water).

Cell culture. A549 cells, a human lung adenocarcinoma epithelial cell line, were cultured in RPMI-1640 containing 10% FBS and 1% antibiotics (100 units/mL penicillin and 100 g/mL streptomycin) in a 5% CO_2 humidity incubator at 37 °C. The culture medium was changed every other day.

Cellular uptake of rLDL particles. A549 cells were seeded in 35-mm petri dishes at a density of 3–4×10^5 cells and cultured in a 5% CO_2 humidity incubator at 37 °C overnight. The cells were incubated with rLDL(+) particles ([TPA-DPPy] = 20 μM) at 37 °C for 0.5, 1, 2, 4, and 6 h, respectively. After washing with PBS, the cells were stained with Hoechst 33342 (10 μg mL^{-1}) in RPMI-1640 at 37 °C for 20 min. After washing with PBS, the cells were supplemented with fresh culture medium for confocal laser scanning microscopy (CLSM) characterization (NIKON, A+). To study the endocytic pathway, the cells were incubated with rLDL(+) particles ([TPA-DPPy] = 20 μM) at 37 °C for 6 h. After washing with PBS, the cells were stained with Hoechst 33342 (10 μg mL^{-1}) in RPMI-1640 and Lyso-Tracker Green (75 nM) or Mito-Tracker (200 nM) in RPMI-1640 at 37 °C for 20 min. After washing with PBS, the cells were supplemented with fresh culture medium for CLSM characterization. To demonstrate the LDL-receptor (LDLR) mediated endocytosis, the cells were incubated with rLDL(+) particles ([TPA-DPPy] = 20 μM) and a mixture of 20-fold excess of native LDL particles and rLDL(+) particles ([TPA-DPPy] = 20 μM) at 37 °C for 6 h. After washing with PBS, the cells were stained with Hoechst 33342 (10 μg mL^{-1}) in RPMI-1640 at 37 °C for 20 min. After washing with PBS, the cells were supplemented with fresh culture medium for CLSM characterization.

Fluorescence monitoring of cell apoptosis. A549 cells were seeded in 35-mm petri dishes at a density of 3–4×10^5 cells and cultured in a 5% CO_2 humidity incubator at 37 °C overnight. The cells were incubated with rLDL(+) particles ([TPA-DPPy] = 20 μM) at 37 °C for 6 h. After washing with PBS, the cells were subjected to *in situ* irradiation with a 405-nm light and examined in a real-time fashion under a fluorescence microscope (OLYMPUS, CKX53).

Intracellular ROS generation. DCFH-DA was used as the ROS-responsive fluorescent probe to indicate the intracellular generation of ROS. A549 cells were incubated with rLDL(+) particles ([TPA-DPPy] = 20 μM) at 37 °C for 6 h. Both fresh culture medium and rLDL(−) particles were

used as the control groups, where the dilution factor for rLDL(−) particles was identical to that of rLDL(+) particles. After incubation with DCFH-DA (10 μM) in PBS at 37 °C for 20 min, the cells were placed in the dark or irradiated with a 450-nm lamp at a power density of 100 mW cm^{-2} for 15 min. After washing with PBS, the cells were supplemented with fresh culture medium for CLSM characterization.

Live/dead staining with calcein-AM and PI. Calcein-AM and propidium iodide (PI) were used to distinguish live (green) and dead (red) cells, respectively. Briefly, A549 cells were incubated with fresh medium, rLDL(−) particles, and rLDL(+) particles ([TPA-DPPy] = 20 μM) at 37 °C for 6 h. After washing with PBS, the cells were placed in the dark or irradiated with a 450-nm lamp at a power density of 100 mW cm^{-2} for 15 min. The cells were replenished with fresh culture medium and incubated for another 12 h. The working solutions of calcein-AM (1000-fold dilution) and PI (2000-fold dilution) were then added, followed by incubation for 20 min at 37 °C. After washing with PBS, the cells were supplemented with fresh culture medium for CLSM characterization.

Cell viability and competitive assay of LDL receptors. A549 cells were seeded in a 96-well plate at a density of 8000 cells per well and cultured overnight. The rLDL(−) and rLDL(+) particles with varying concentrations (n = 3 per group) were added into the wells, followed by incubation at 37 °C for 6 h. After washing with PBS, the cells were placed in the dark or irradiated with a 450-nm lamp at a power density of 100 mW cm^{-2} for 15 min. The cells were replenished with fresh culture medium and incubated for another 12 h. MTT (0.5 mg mL^{-1} in medium, 100 μL per well) was added to the wells followed by incubation at 37 °C for 4 h. After removing the supernatant, DMSO (100 μL per well) was then added to dissolve the produced formazan, and the absorbance values at 490 nm was recorded by a microplate reader (Tecan, Infinite M Nano). The cells without light irradiation was used as the blank control. For competitive binding assay, 20-fold excess of native LDL particles, rLDL(+) particles ([TPA-DPPy] = 20 μM), and the mixture of 20-fold excess of native LDL particles and rLDL(+) particles ([TPA-DPPy] = 20 μM) (n = 3 for each group) were

added in the wells, respectively, while keeping other conditions unchanged. The cell viability was defined as the percentage of surviving cells *versus* untreated cells. The results were presented as mean ± standard deviation, with "*n*" indicating the number of samples per group.

References

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2 C. Zhu, P. Pradhan, D. Huo, J. Xue, S. Shen, K. Roy and Y. Xia, *Angew. Chem. Int. Ed.*, 2017, **56**, 10399−10402.

3 L. Bourré, S. Thibaut, A. Briffaud, N. Rousset, S. Eléouet, Y. Lajat and T. Patrice, *J. Photochem. Photobiol. B*, 2002, **67**, 23−31.

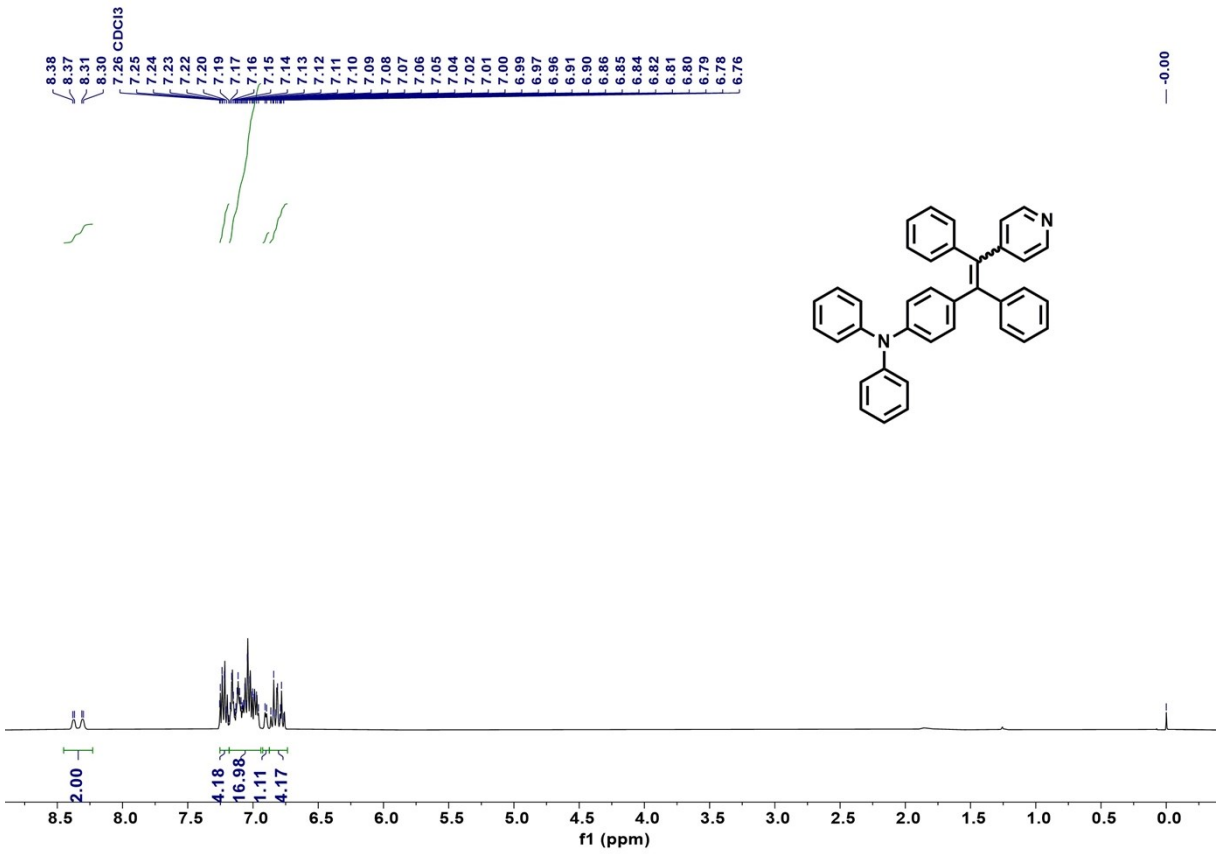

Fig. S1 ^{1}H-NMR spectrum of compound **2** in $CDCl_3$.

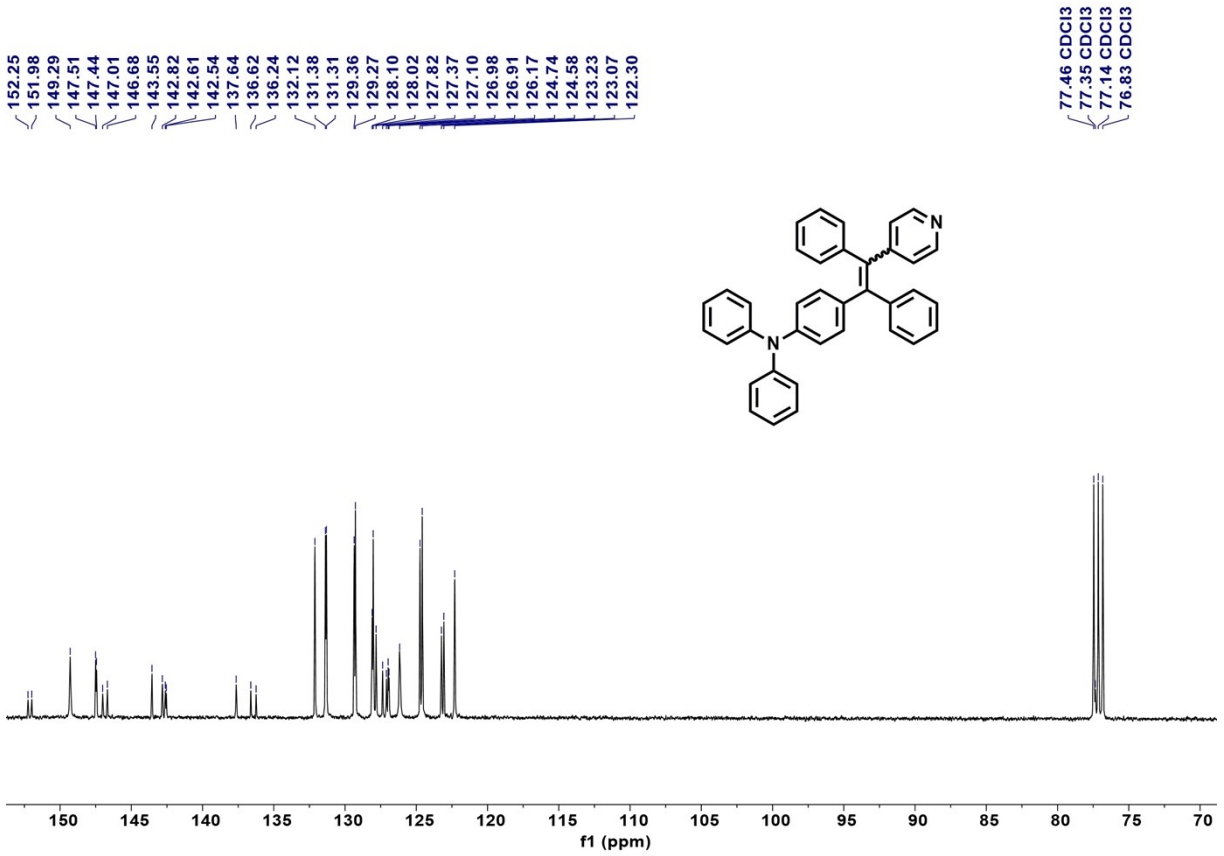

Fig. S2 ^{13}C-NMR spectrum of compound **2** in $CDCl_3$.

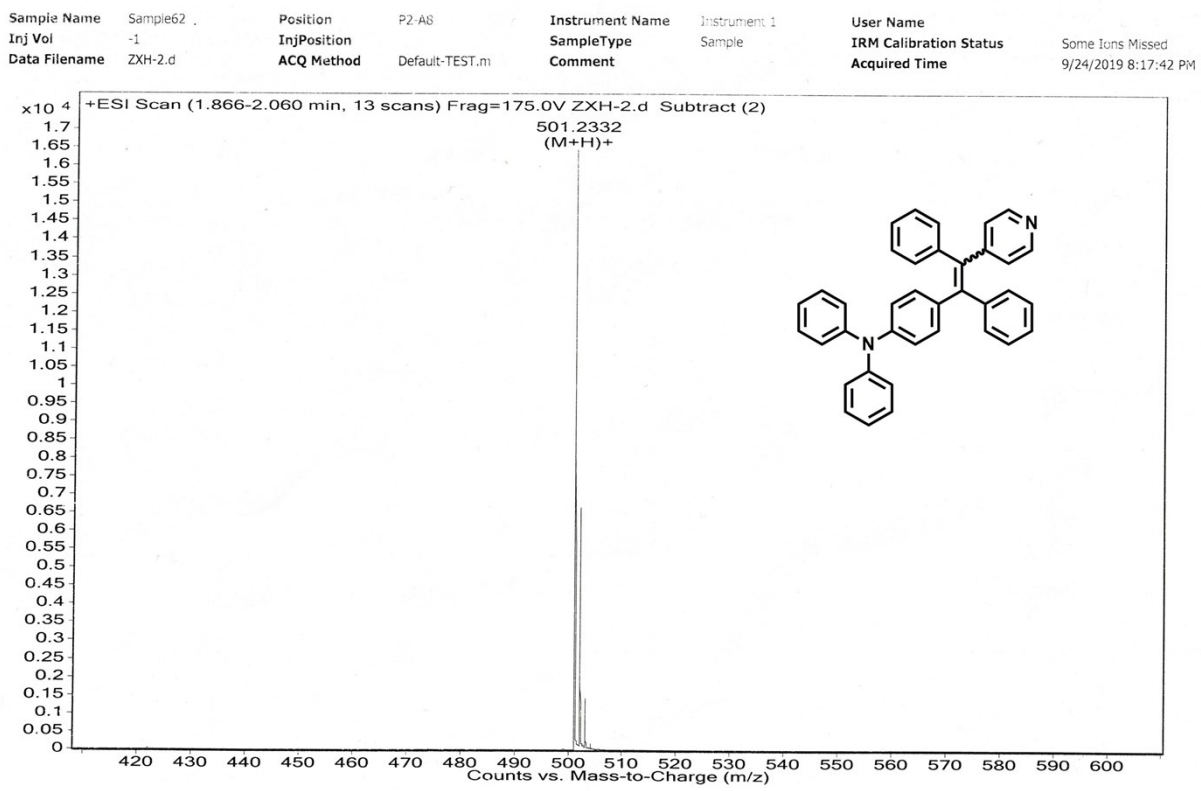

Fig. S3 HRMS spectrum of compound **2**.

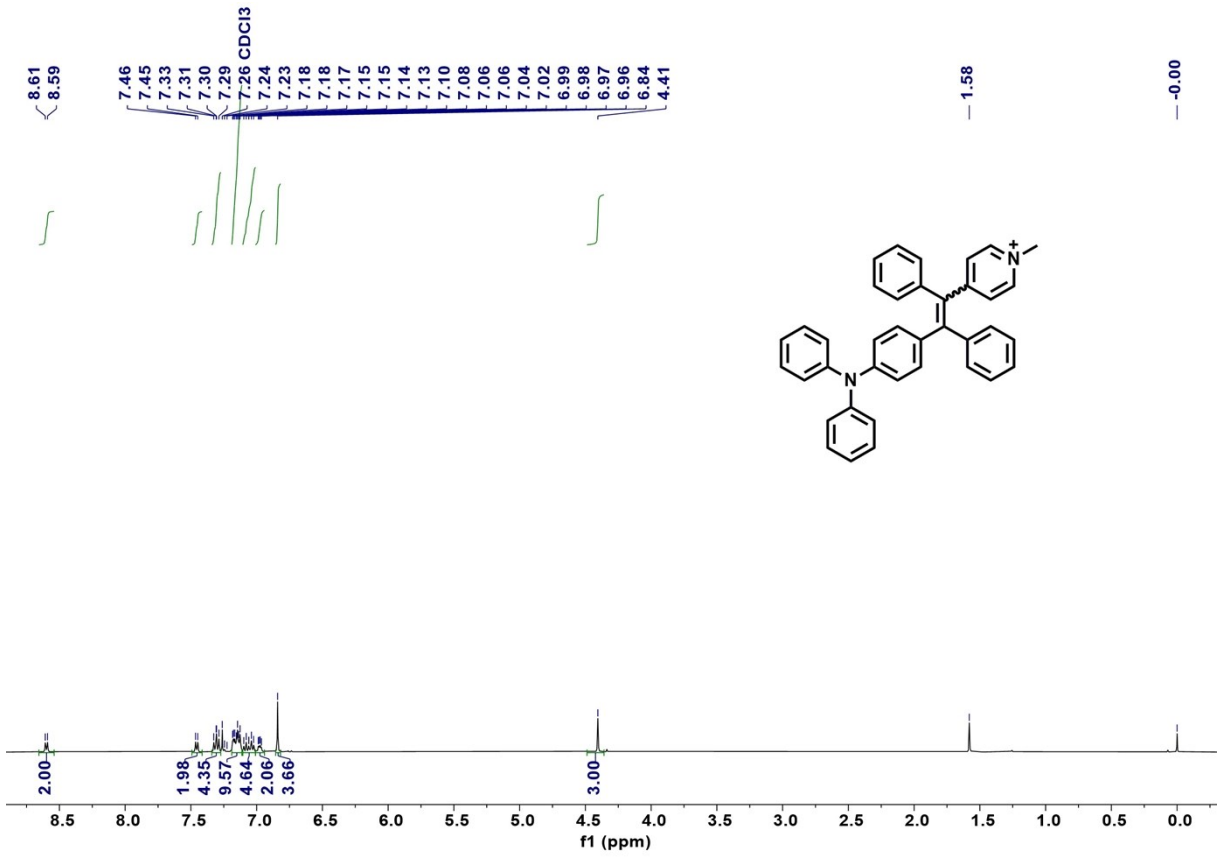

Fig. S4 ^{1}H-NMR spectrum of TPA-DPPy in $CDCl_3$.

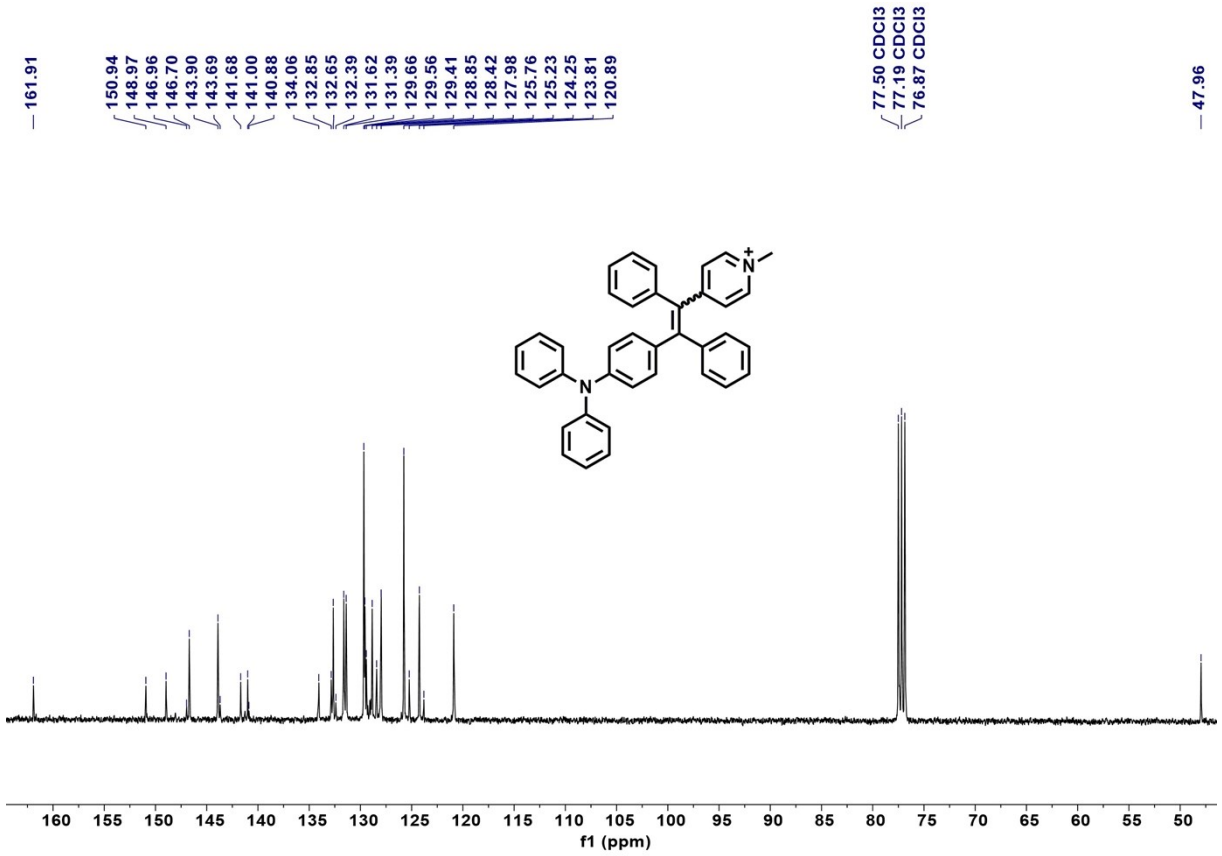

Fig. S5 ^{13}C-NMR spectrum of TPA-DPPy in $CDCl_3$.

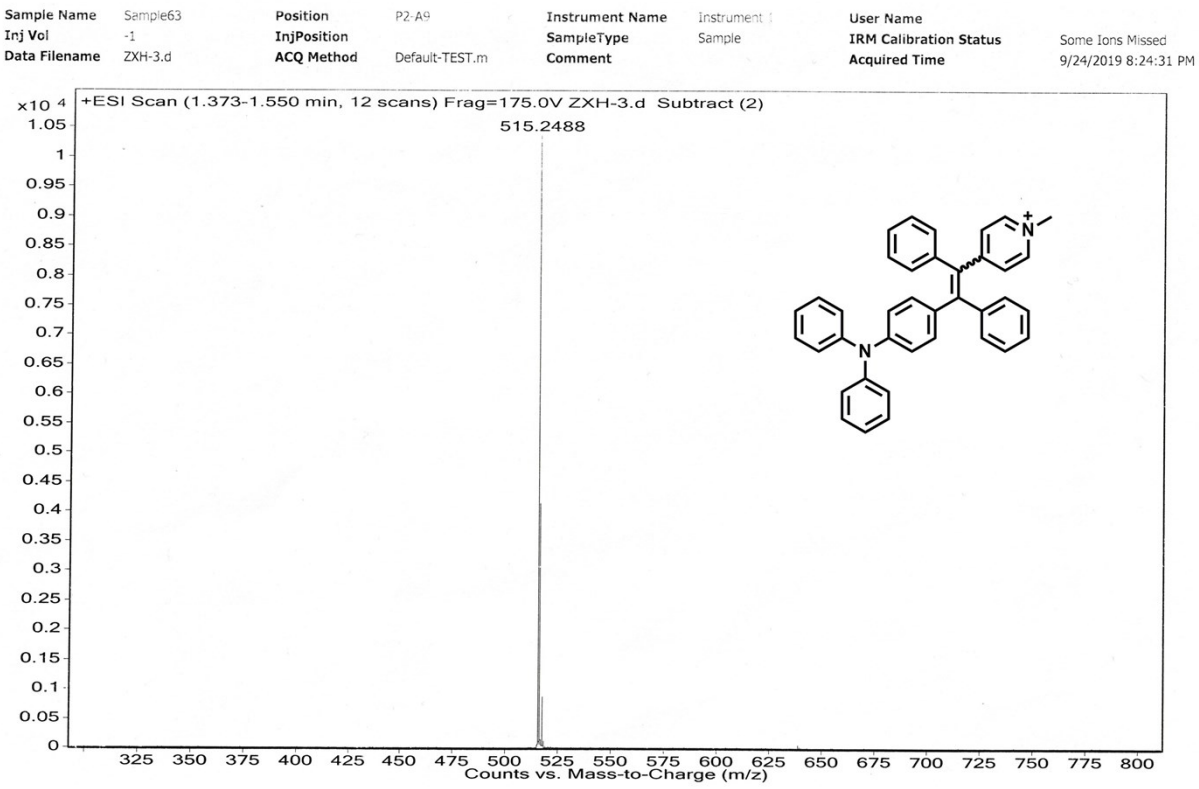

Fig. S6 HRMS spectrum of TPA-DPPy.

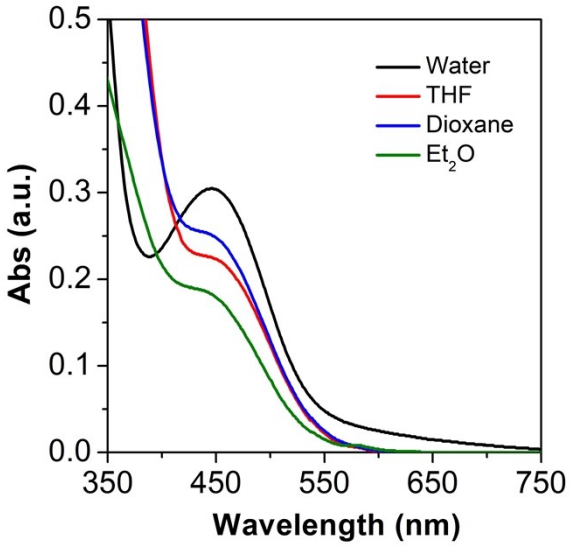

Fig. S7 UV-vis spectra of TPA-DPPy in H_2O and a series of organic solvents. [TPA-DPPy] = 50 μM.

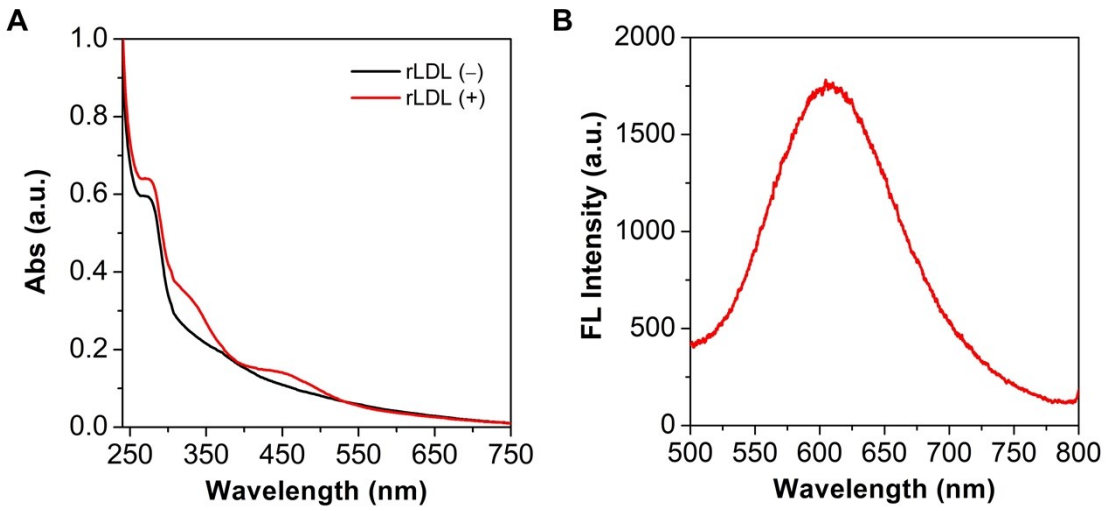

Fig. S8 Absorption and emission spectra of rLDL(+) particles. (A) UV/vis spectra of rLDL(–) and rLDL(+) particles, showing the characteristic absorption of apolipoprotein B-100 at *ca.* 280 nm and TPA-DPPy at *ca.* 450 nm. (B) Fluorescence (FL) spectrum of rLDL(+) in tricine buffer (Ex = 440 nm).

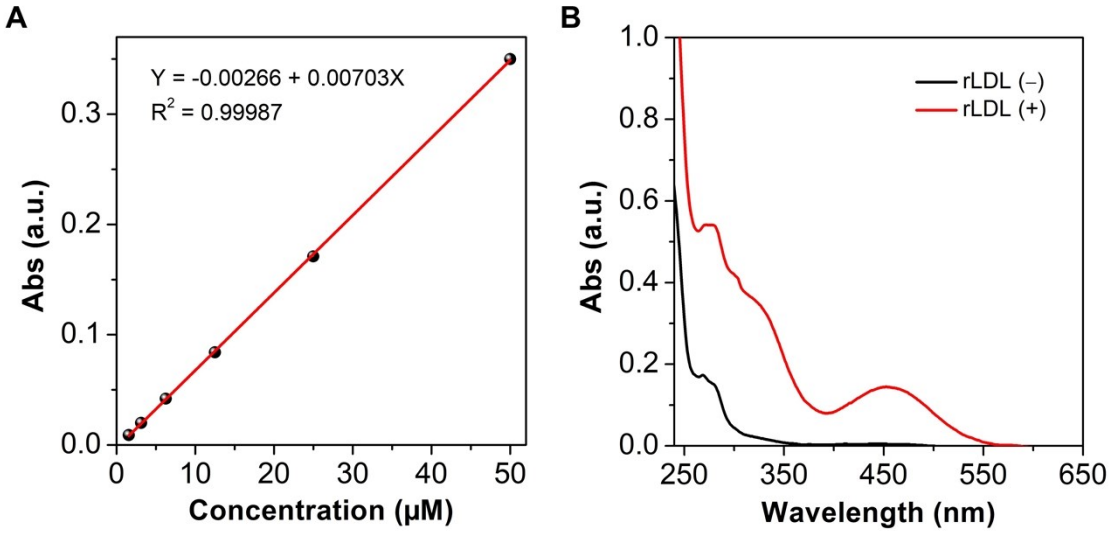

Fig. S9 Quantification of TPA-DPPy in rLDL(+) particles. (A) Calibration curve for TPA-DPPy in methanol. (B) UV-vis spectra of methanol extracts from rLDL(–) and rLDL(+) particles.

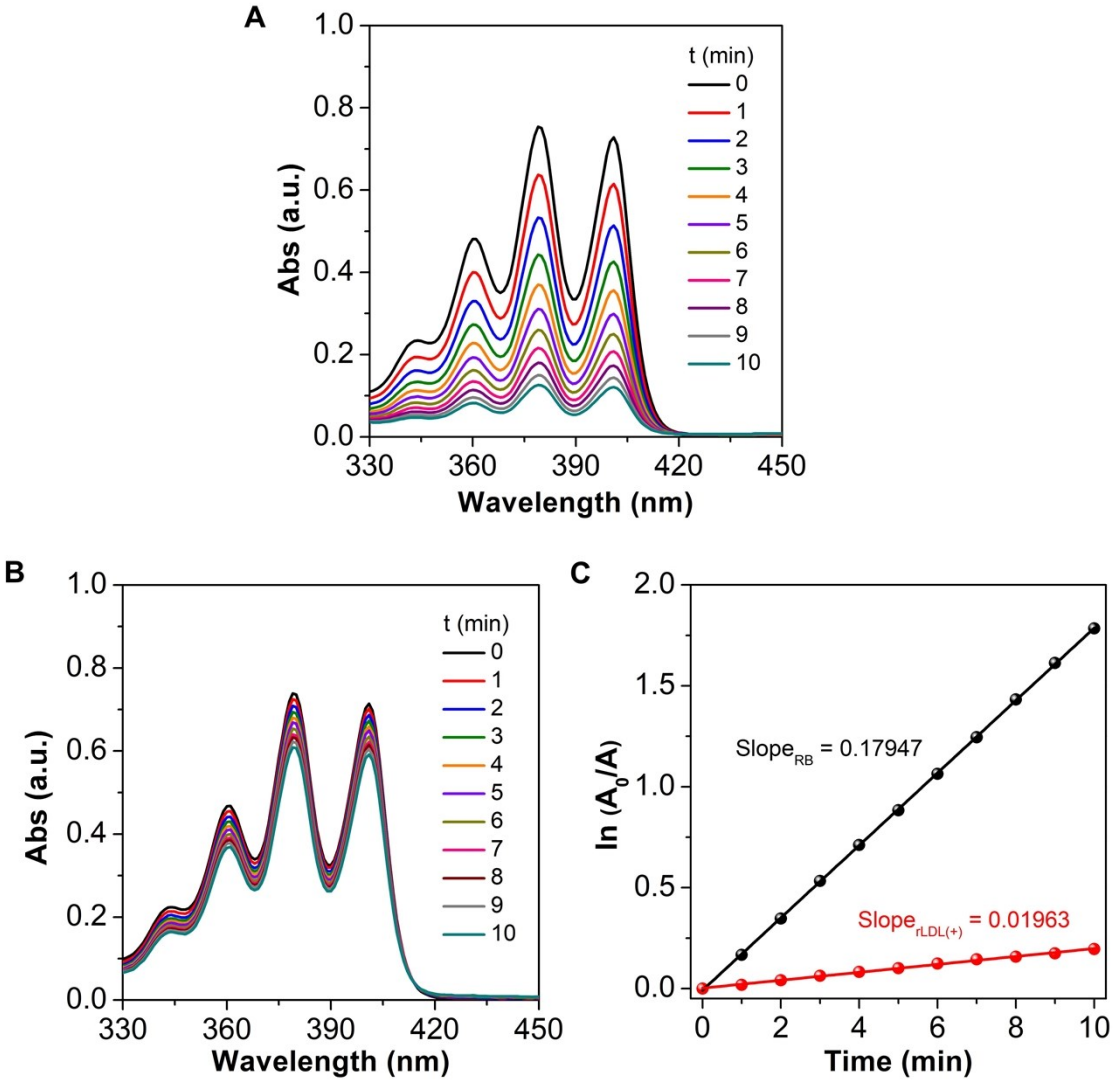

Fig. S10 Singlet oxygen measurements. (A and B) Absorption spectra of ABDA for (A) RB and (B) rLDL(+) particles under white-light irradiation. (C) Decomposition rates of ABDA for RB and rLDL(+) particles, where A_0 and A represent the absorbance at 378 nm before and after light irradiation, respectively.

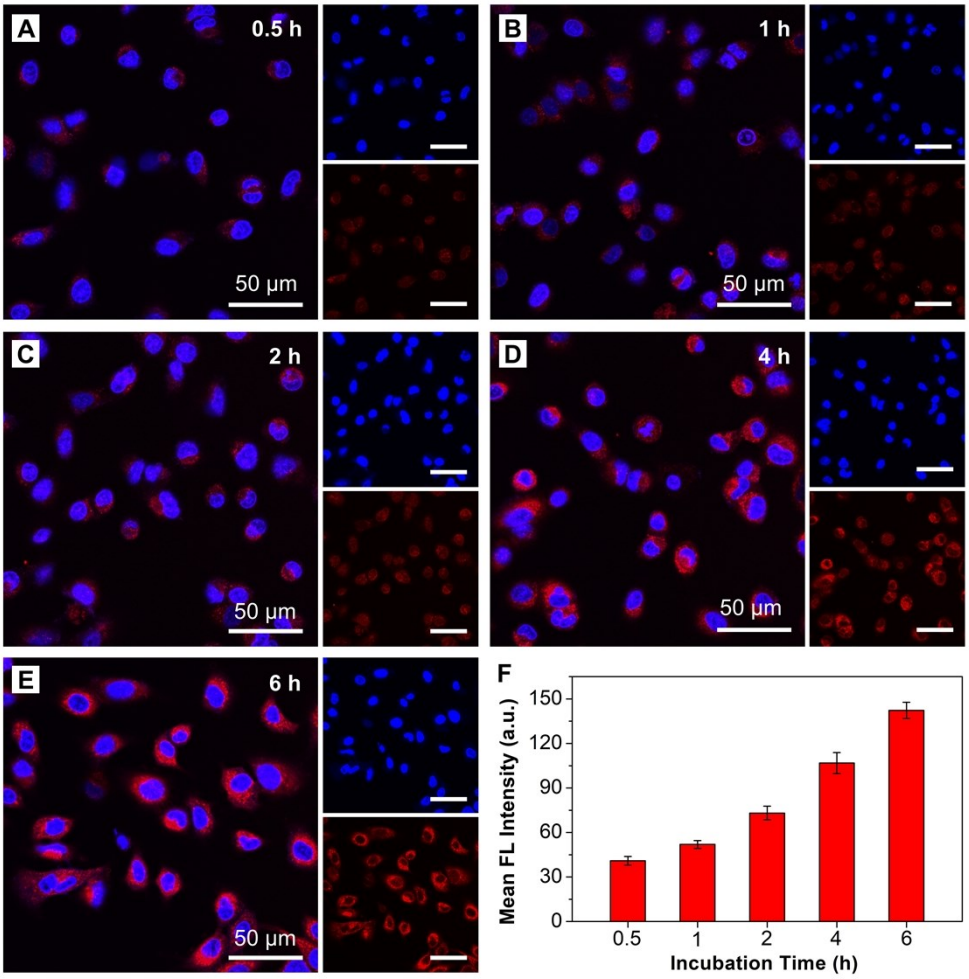

Fig. S11 Uptake dynamics of rLDL(+) particles by A549 cells. (A–E) A549 cells incubated with rLDL(+) particles for 0.5, 1, 2, 4, and 6 h, respectively. (F) Mean fluorescence (FL) intensity of TPA-DPPy extracted from (A–E) using the ImageJ software.

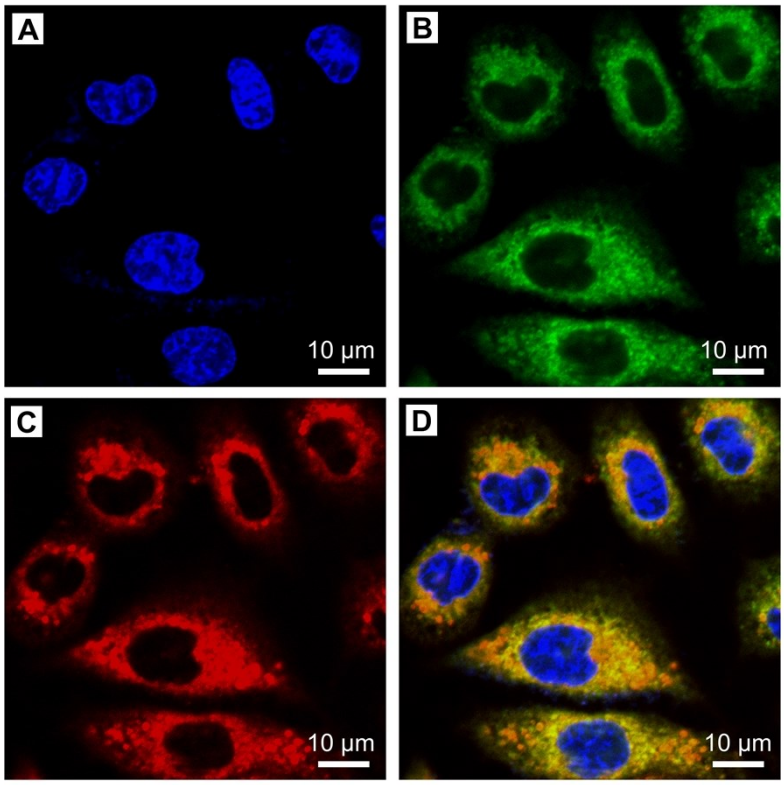

Fig. S12 Co-localization of TPA-DPPy with mitochondria in A549 cells. (A) Hoechst 33342 (blue). (B) Mito-Tracker Green (green). (C) TPA-DPPy (red). (D) Merged image.

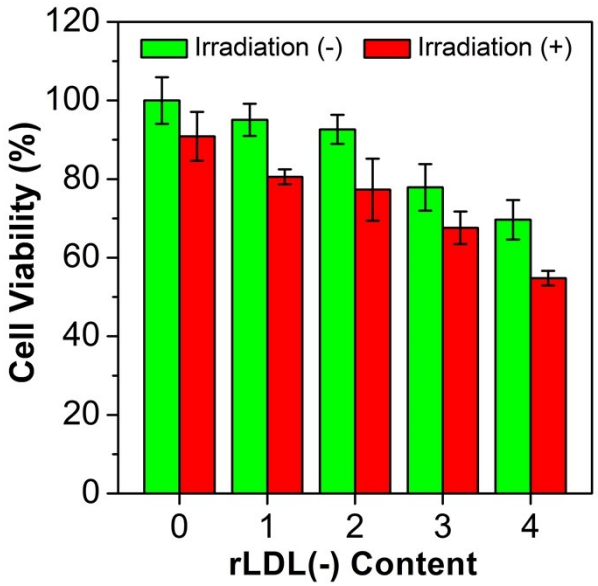

Fig. S13 Cell viability of A549 cells incubated with rLDL(–) particles in the absence and presence of light irradiation, where "rLDL(–) content" corresponds to rLDL(–) particles with identical dilution ratio as rLDL(+) particles as shown in Fig. 7B.