Electronic Supplementary Information (ESI)

A Mitochondria Targeting Artesunate Prodrug Loaded Nanoparticle Exerting Anticancer Activity *via* Iron-Mediated Generation of Reactive Oxygen Species †

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TABLE OF CONTENTS

1. Experimental

1.1. Material

Methoxy-polyethylenelglycol-distearyl phosphatidyl-ethanolamine (mPEG-DSPE) was obtained from Laysan Bio, Inc. Artesunate (ART), dodecylamine, 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl), 1-hydroxypyrrolidine-2,5-dione (NHS), 1 -hydroxybenzotriazole (HOBt), sodium hydroxide (NaOH), chloroform-d ($CDCl_3$), 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS) were purchased from Aladdin, Shanghai. Boc-L-glutamic, ferric chloride were obtained from Energy Chemical. Trifluoroacetic acid (TFA), dichloromethane (CH_2Cl_2), *N,N*-dimethylformamide (DMF) and ethyl alcohol were obtained from Beijing Chemical Works. 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), rhodamine B (RhB), 3,3′-dioctadecyloxacarbocyanine perchlorate (DIO), 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) were purchased from Sigma-Aldrich, Shanghai, China. Annexin-V-FITC Apoptosis Detection Kit and Probe Assay Kit were purchased from Solarbio (Beijing, China).

1.2. Instruments

^{1}H NMR and ^{13}C NMR spectra were acquired on a 300 MHz NMR and 400 MHz NMR spectrometer (Bruker) at 298K. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were obtained by using an Autoflex III (Bruker). Drug quantification

were measured by UV-2550 UV/Vis Spectrophotometer. Reversed phase HPLC experiments were performed on an Agilent HPLC system with a VWD detector using a Agilent 5 TC-C18(2) (4.6×250 mm) column for assay. Eluent A: CH_3OH, Eluent B: H_2O. Particle size and zeta potential measurements were conducted on dynamic light scattering (DLS) *via* Malvern Zetasizer NanoZS90. The transmission electron microscopy (TEM) images were recorded by JEM-1011 electron microscope operated at 100 kV. The growth inhibition was measured by SpectraMax M3. Flow cytometry was determined by using Cytomics FC500 Flow Cytometry (Beckman Coulter Ltd.). Confocal laser scanning microscopy (CLSM) were obtained by using ZEISS LSM880.

1.3. Synthesis of N,N′-bis(dodecyl)-L-glutamic diamide (LGC12)

N,N′-bis(dodecyl)-L-Boc-glutamic diamide (Boc-LGC12) was synthesized through EDC/HOBt condensation reaction. Boc-glutamic acid (2.47 g, 0.01 mol) and dodecylamine (3.71 g, 0.02 mol) were mixed in dichloromethane (200 mL), then 1-ethyl-3-(3-dimethyllaminopropyl)-carbodiimide hydrochloride (EDC·HCl) (4.02 g, 0.022 mol) and 1-hydroxybenzotrizole (HOBt, 2.97 g, 0.022 mol) were added to the mixture. The mixture was stirred at room temperature for 72 h. After completion of the reaction, CH_2Cl_2 was dried under reduced pressure, and the crude product was dissolved in anhydrous ethanol and washed three times by water. MALDI-TOF-MS for $C_{34}H_{67}N_3O_4$: *m*/*z* calcd: 604.51 $[C_{34}H_{67}N_3O_4+Na]^+$, 620.51 $[C_{34}H_{67}N_3O_4+K]^+$; found: 604.1

$[C_{34}H_{67}N_3O_4+Na]^+$, 620.1 $[C_{41}H_{83}N_3O_2+K]^+$.

Then the Boc group was deprotected to free amine *via* trifluoroacetic acid. Boc-LGC12 (2.38 g, 4.10 mmol) and trifluoroacetic acid (TFA; 8 mL) were added to CH_2Cl_2 (50 mL) and stirred at room temperature for 3 h. After the removal of CH_2Cl_2 and excess TFA under reduced pressure, the sample was dissolved in anhydrous ethanol, and washed by saturated $NaHCO_3$ aqueous solution to get crude product. Recrystallization in petroleum ether three times afforded the white pure product. The *N,N′*-bis(dodecyl)-L-glutamic diamide (LGC12) was isolated and yielded 72%. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.38 (s, 1 H), 6.14 (s, 1 H), 3.46 (t, J = 6.8 Hz, 1 H), 3.29-3.14 (m, 4 H), 2.40-2.26 (m, 2 H), 1.95 (d, J = 13.5 Hz, 4 H), 1.58-1.40 (m, 4 H), 1.27 (d, J = 10.5 Hz, 36 H), 0.88 (t, J = 6.8 Hz, 6 H). MALDI-TOF-MS for $C_{29}H_{59}N_3O_2$: *m/z* calcd: 481.46 $[C_{29}H_{59}N_3O_2]$, 504.46 $[C_{29}H_{59}N_3O_2+Na]^+$; found: 481.5 $[C_{41}H_{83}N_3O_2]$, 504.6 $[C_{41}H_{83}N_3O_2+Na]^+$.

1.4. Synthesis of artemisinin-N,N′-bis(dodecyl)-L-glutamic diamide (ART-LGC12)

Artesunate (77 mg, 0.2 mmol) in 30 mL CH_2Cl_2 and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) (76.8 mg, 0.4 mol) and 1-hydroxypyrrolidine-2,5-dione (NHS) (46 mg, 0.4 mol) was stirred and activated at room temperature for 4 h. Then the LGC12 (96.4 mg, 0.2 mmol) were added to the mixture under stirring for 72 h. The obtained mixture was washed for several times with water and dried

under the vacuum to obtain ART-LGC12 (78%) as light-yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.24 (d, J = 7.3 Hz, 1 H), 6.87 (t, J = 5.4 Hz, 1 H), 6.26 (t, J = 5.4 Hz, 1 H), 5.76 (d, J = 9.8 Hz, 1 H), 5.41 (s, 1 H), 4.36 (d, J = 12.0 Hz, 1 H), 3.21 (d, J = 13.2 Hz, 4 H), 2.76 (d, J = 15.0 Hz, 2 H), 2.65-2.44 (m, 2 H), 2.45-2.20 (m, 3 H), 2.06 (d, J = 23.8 Hz, 4 H), 1.94-1.83 (m, 1 H), 1.75 (t, J = 14.0 Hz, 2 H), 1.61 (d, J = 10.8 Hz, 1 H), 1.49 (s, 5 H), 1.42 (s, 3 H), 1.41-1.02 (m, 40 H), 0.97 (d, J = 5.3 Hz, 3 H), 0.87 (d, J = 13.1, 6.4 Hz, 9 H). ^{13}C NMR (75 MHz, $CDCl_3$): δ(ppm) 173.08, 171.92, 171.60, 171.11, 104.49, 92.22, 91.50, 80.07, 52.74, 51.54, 45.19, 39.83, 39.70, 37.30, 36.22, 34.07, 32.81, 31.93, 31.77, 30.68, 29.66, 29.54, 29.37, 28.97, 27.04, 26.99, 25.91, 24.59, 22.70, 22.02, 20.21, 14.13, 12.10. MALDI-TOF-MS for $C_{49}H_{87}N_3O_8$: *m/z* calcd: 868.65 $[C_{49}H_{87}N_3O_8+Na]^+$; found: 870.5 $[C_{49}H_{87}N_3O_8+Na]^+$.

1.5. Synthesis of (3-Carboxypropyl)triphenylphosphonium bromide (TPP) targeting group modification

(3-Carboxypropyl)triphenylphosphonium bromide (21.5 mg, 0.05 mmol) in 10 mL DMF , triethylamine (10 μL), 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) (38.4 mg, 0.2 mol) and 1-hydroxypyrrolidine-2,5-dione (NHS) (23 mg, 0.2 mol) was stirred and activated at room temperature for 4 h. Then the NH_2-PEG-DSPE (400 mg, 0.05 mmol) were added to the mixture under stirring for 72 h. The mixture was dialyzed in a dialysis bag (MWCO: 3500 Da) overnight, lyophilized to obtain white powder and yielded 75%.

1.6. Drug content determination

The amount of artesunate was determined by using a calibration curve at a predetermined wavelength of 290 nm prepared from standard artesunate in 0.1 N NaOH by UV/Vis spectroscopy[1]. The lyophilized powder of nanoparticles was used for drug content and entrapment efficiency. All measurements were in triplicates. The encapsulation efficiency (EE%) was calculated from the equation:

Entrapment efficiency (EE%) =

$$\frac{Calculated\ artemisinin\ weight\ in\ nanoparticles\ (mg)}{Total\ artemisinin\ added\ (mg)} \times 100\%$$

The purity of drugs was determined by C18 column reversed-phase HPLC system, UV/Vis detector at 254 nm, with a mobile phase of acetonitrile and water (70/30, v/v) pumped at a flow rate of 1.0 mL/min, at temperature 25 °C [2].

1.7. Formulation optimization and stability of the nanoparticles

The NPs and NPs-TPP were prepared by a nano-precipitation method. First, ART-LGC12 and mPEG-DSPE were used to prepare nanoparticles in a mass ratio of 1:1 to 1:6 for optimizing the preparation process. Briefly, ART-LGC12 (5 mg) and mPEG-DSPE (20 mg) were dissolved in DMF (2 mL), then added de-ionized water (10 mL) dropwise. The mixture was dialyzed in a dialysis bag (MWCO: 3500 Da) overnight, centrifuged and the supernatant was taken to obtain nanoparticles. Moreover, the time-dependent storage stability in PBS at 4 °C and in the

10% fetal bovine serum (FBS) were evaluated by monitoring the changes in hydrodynamic diameters and polymer dispersity index (PDI) over 7 days.

1.8. Cell culture

A549, A549/DDP (cisplatin resistant), 7404, 7404/DDP (cisplatin resistant), MCF7 and 4T1 cell lines were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin and 1% streptomycin at 37 °C with 5% (v/v) CO_2 atmosphere.

1.9. Cell viability studies

The growth inhibition was measured by the MTT assay in 96-well plates for 72 h at 37 °C with 5% CO_2 atmosphere. Cells were treated with different concentrations of drugs from 0.0125, 0.125, 1.25, 12.5, 25, 50 to 100 μM. The NPs, NPs-TPP as well as ART were freshly prepared and serially diluted to achieve the required dilutions. 10 μL of MTT (5mg/mL) was added to each well, and cells were incubated at 37 °C for 4 h, then 100 μL of 10% SDS was added and incubated for 12 h. Finally, the absorbance was measured at 570/650 nm by a microplate reader (SpectraMax M3). Cell viability was expressed as the ratio of the absorbance of the test wells and control wells, and data were shown as the mean ± standard deviation (S.D.).

1.10. Nanoparticles uptake in the cells

MCF7 cells were seeded in a 6-well plate at a density of 2×10^5 cells/well, and then treated with NPs and NPs-TPP (20 μM) (containing

DIO) for 1 h, 4 h and 7 h. The cells were then washed with PBS (4 °C) for three times and digested with trypsin, collected in tubes and washed twice with PBS. Cellular uptake of nanoparticles was analyzed in the FACS channel on a BD FACSCalibur flow cytometer.

1.11. Intracellular ROS assessments

Cellular ROS were measured by flow cytometry using a 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe. Following drug treatment as section 1.9, the cells were washed with PBS three times. Subsequently the cells were stained with 10 μM DCFH-DA in DMEM without FBS at 37 °C for 30 min in the dark, then washed three times with PBS. The harvested cells were resuspended twice in PBS. Intracellular ROS generation were analyzed on a BD FACSCalibur flow cytometer in the FL1 channel.

To verify the effect of Fe^{2+}, the cells were pre-treated with 10 μM of ferric chloride. After incubation for 4 hours, the cells were washed three times with cold PBS and then followed the above experimental procedure.

1.12. Confocal laser scanning

MCF7 cells were seeded in cell crawling of 6-well plate at a density of 1×10^5 cells/well, then treated with Rhodamine B labeled nanoparticles for 1 h, 4 h and 7 h. Finally, the DMEM culture media were removed followed by washing with cold PBS for three times. In the end, cells were stained with DCFH-DA in serum-free medium for 30 min and washed three times by PBS. Next, the cells were fixed with freshly prepared 4%

paraformaldehyde for 20 min and washed with PBS three times for 15 min. Then, nucleus was stained with 200 μL of propidium iodide (0.1 mg/ml) for 10 min and washed three times by PBS to wipe off excess DAPI. Sealed with sealing liquid containing the anti-fluorescence quencher. Cell images were obtained on CLSM.

1.13. Apoptosis analysis

Apoptosis was evaluated by using the Annexin V-FITC/PI Apoptosis Detection Kit. MCF7 cells were seeded in a 6-well plate at a density of 2×10^5 cells/well, and for 24 h. Then cells were treated with each compound (20 μM) for 24 h. Following drug treatment, cells were collected by trypsin digestion, washed twice with PBS and stained in working solution using an Annexin Vfluorescein isothiocyanate/PI dual staining kit according to the operation of apoptosis kit. Apoptosis was performed after dyeing within 1 h by flow cytometry.

1.14. Cell cycle analysis

Cell cycle distribution was determined by staining DNA with PI. MCF7 cells were seeded in a 6-well plate at a density of 2×10^5 cells/well and allowed to settle for 24 h. After drug treatment (20 μM) for 24 h, cells were trypsinized, collected and washed twice times with cold PBS using 75% ethanol to fix cells overnight at 4 °C. After that, RNAse (100 μg/ml) and propidium iodide (0.1 mg/ml) were added for 30 min at room temperature and then determined by flow cytometry.

1.15. Mitochondrial membrane potential analysis

Mitochondrial membrane potential was estimated in MCF7 cells using the dye JC-10. cells were seeded in a 6-well plate at a density of 2×10^5 cells/well and allowed to settle for 24 h. After drug treatment (40 μM) for 48 h, cells were stained with JC-10 in DMEM without FBS at 37 °C for 30 min in the dark. The positive control group was pretreated with CCCP for 20 min. Then cells were collected and washed twice with JC-10 staining buffer (1×), measured by flow cytometry. JC-10 showed potential-dependent accumulation in the mitochondria, indicated by a fluorescence emission shift from red (approximately 590 nm) to green (approximately 529 nm).

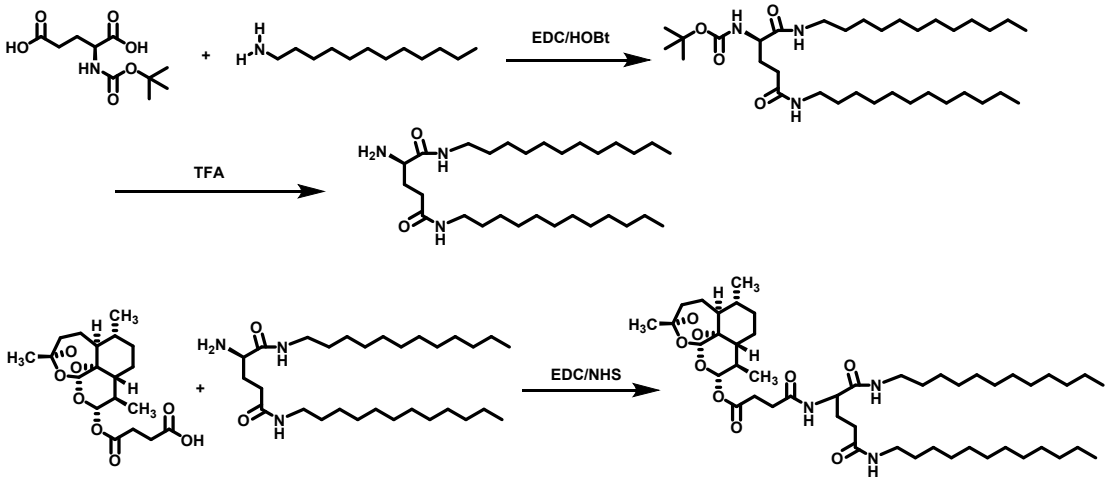
1.16. Mitochondrial targeting validation analysis

MCF7 cells were seeded in cell crawling of 6-well plate at a density of 1×10^5 cells/well, treated with DIO labeled nanoparticles for 7 h. and then the DMEM were removed followed by washing with cold PBS for three times. In the end, cells were stained with Mito Red in serum-free medium for 30 min and washed three times by PBS. Next, the cells were fixed with freshly prepared 4% paraformaldehyde for 20 min and washed with PBS three times for 15 min. Then, nucleus was stained with 200 μL of propidium iodide (2 μg/ml) for 10 min and washed three times by PBS to wipe off excess DAPI. The cells were then sealed with sealing liquid containing the anti-fluorescence quencher. Cell images were obtained on CLSM.

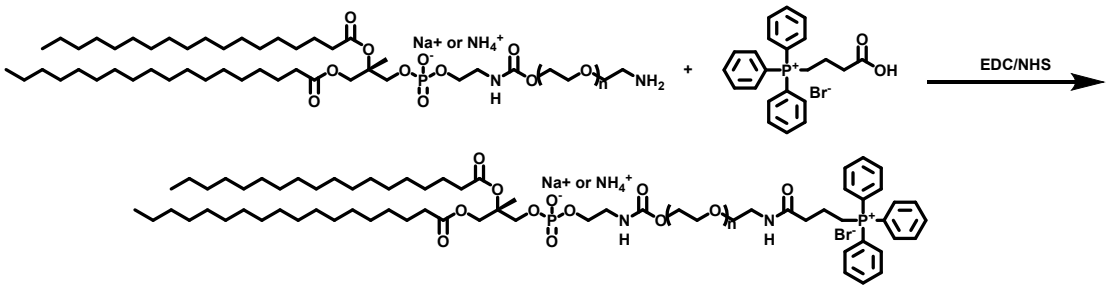
References

1. E. Chinaeke, S. Chime, V. Onyishi, A. Attama and V. Okore, *Drug Delivery*, 2015, **22**, 652-665.
2. M. Ismail, L. Ling, Y. Du, C. Yao and X. Li, *Biomaterials*, 2018, **163**, 76–87.

2. Supplementary figures



Scheme S1 Synthetic routes of *N,N′*-bis(dodecyl)-L-glutamic diamide (LGC12) and artesunate-*N,N′*-bis(dodecyl)-L-glutamic diamide (ART-LGC12) .



Scheme S2 Synthetic routes of (3-Carboxypropyl)triphenylphosphonium bromide (TPP) targeting group modification.

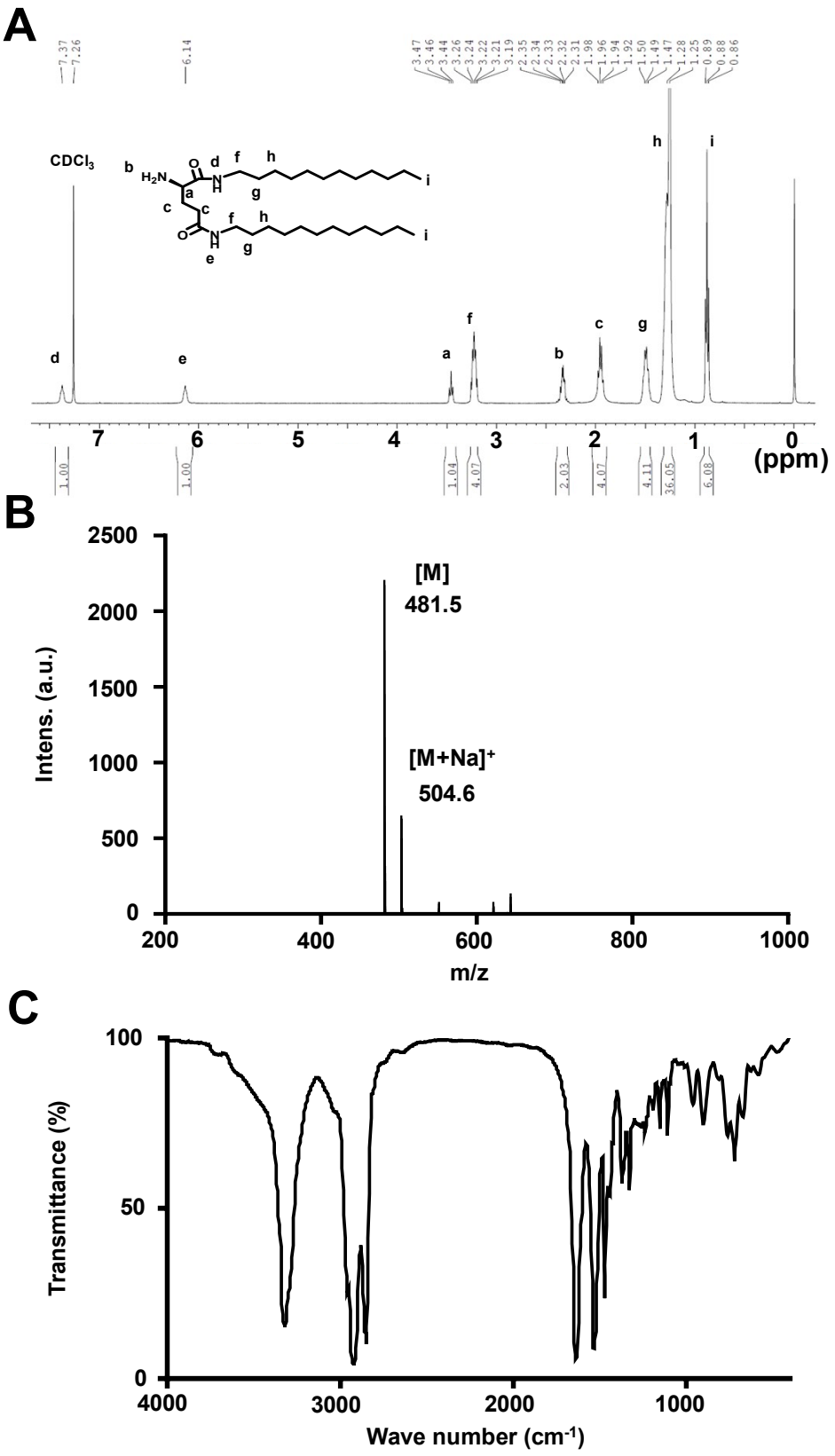
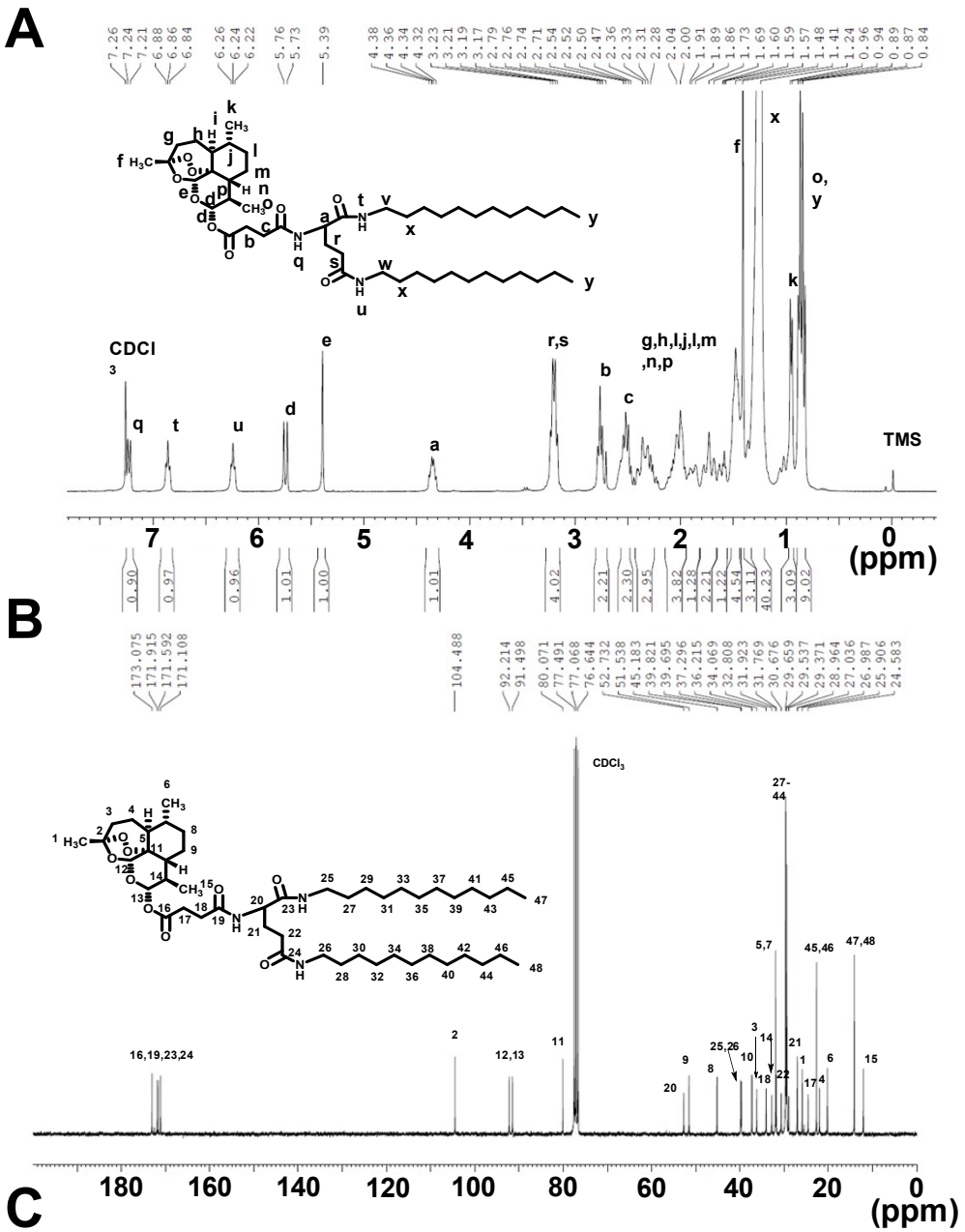


Figure S1 Characterizations of LGC12 by ^{1}H NMR(A), MADLI-TOF (B), and FTIR (C).


A
CDCl3
TMS
(ppm)
B
CDCl3
(ppm)
C
7.585
mAU
Time (min)

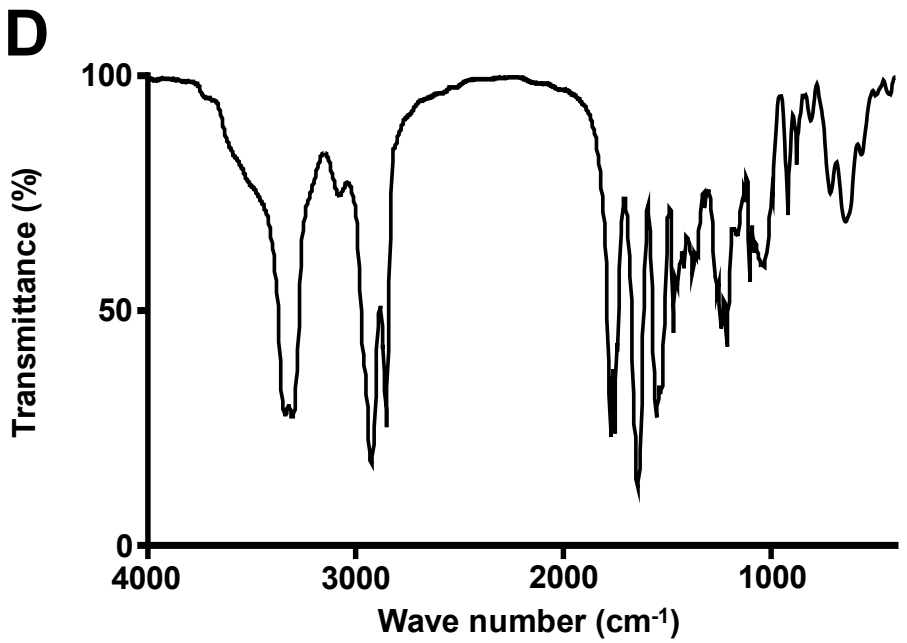


Figure S2 Characterizations of ART-LGC12 by ^{1}H NMR (A), ^{13}C NMR (B), HPLC (C) and FTIR (D).

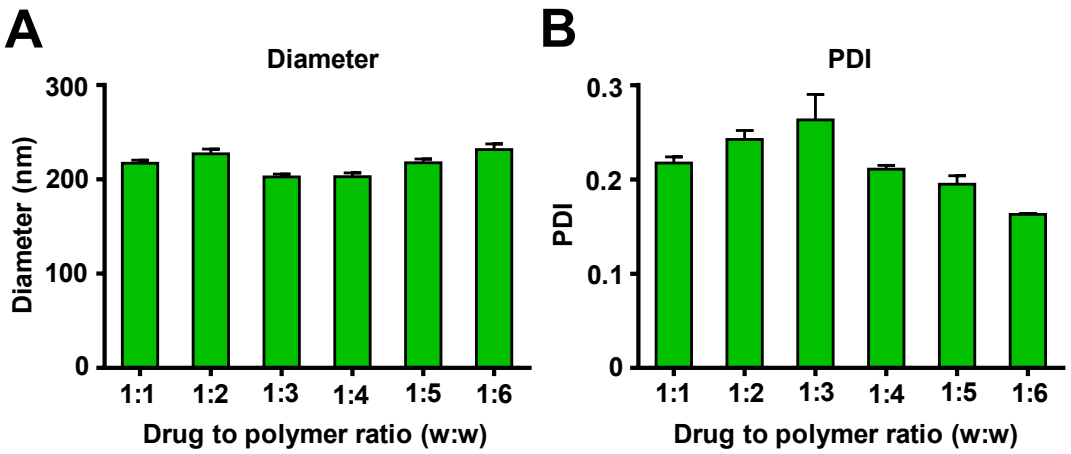


Figure S3 Formulation optimization of the nanoparticles. Diameter (A) and PDI (B).

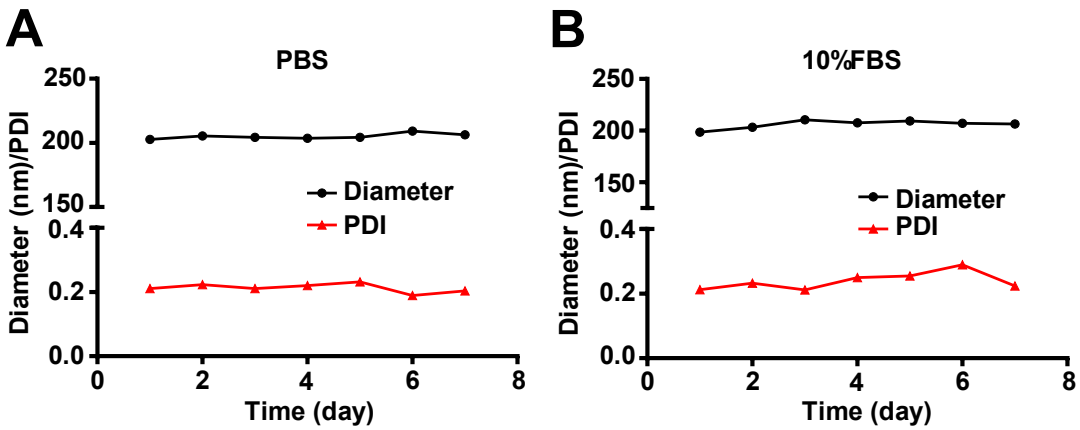


Figure S4 Stability of NPs-TPP. Diameter and PDI of NPs-TPP in PBS (A) and 10% FBS (B).

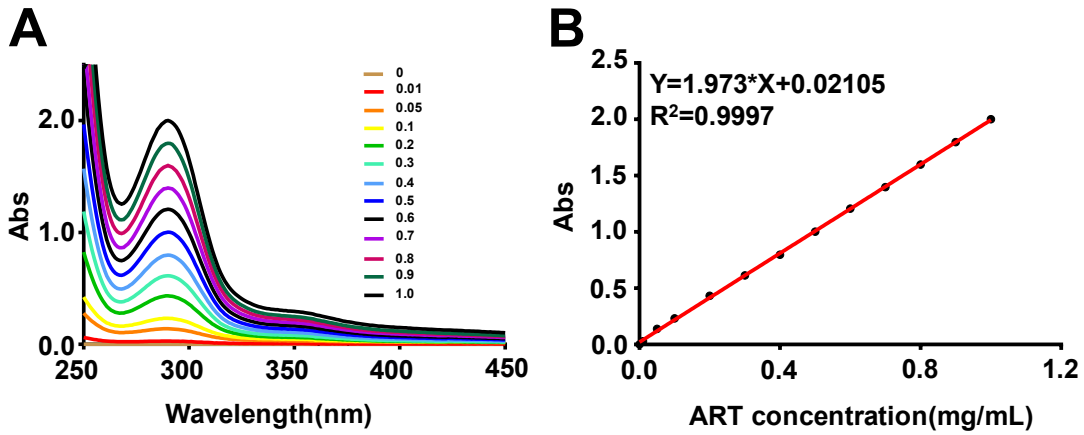


Figure S5 UV absorption diagram of artesunate (A) and standard curve of artesunate at 290 nm.

Table S1 Drug content determination of NPS and NPS-TPP.

Sample	NPs	NPs-TPP
Abs	0.361	0.426
ART concentration (μM)	448.18	533.9
Drug encapsulated rate (%)	27.56	32.85

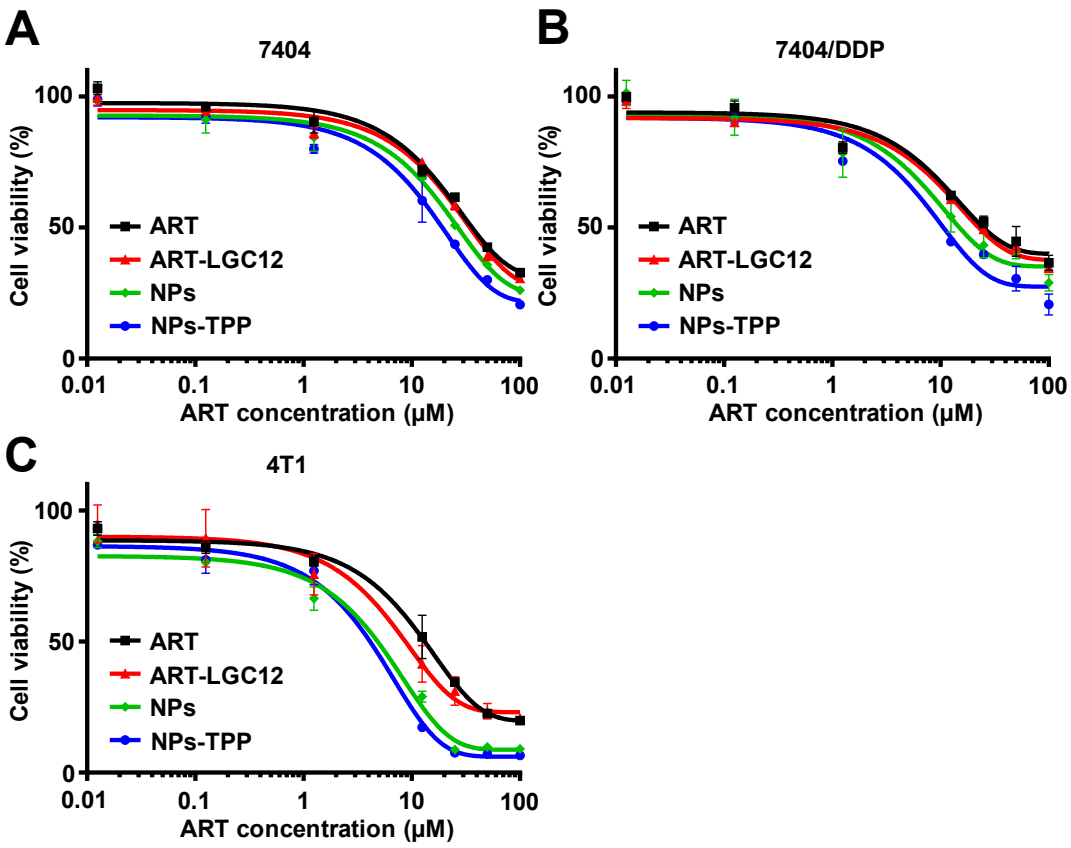


Figure S6 *In vitro* evaluation of nanoparticles. Cytotoxic assay by MTT of ART, ART-LGC12, NPs and NPs-TPP on 7404 (A), 7404/DDP (B) and 4T1(C).

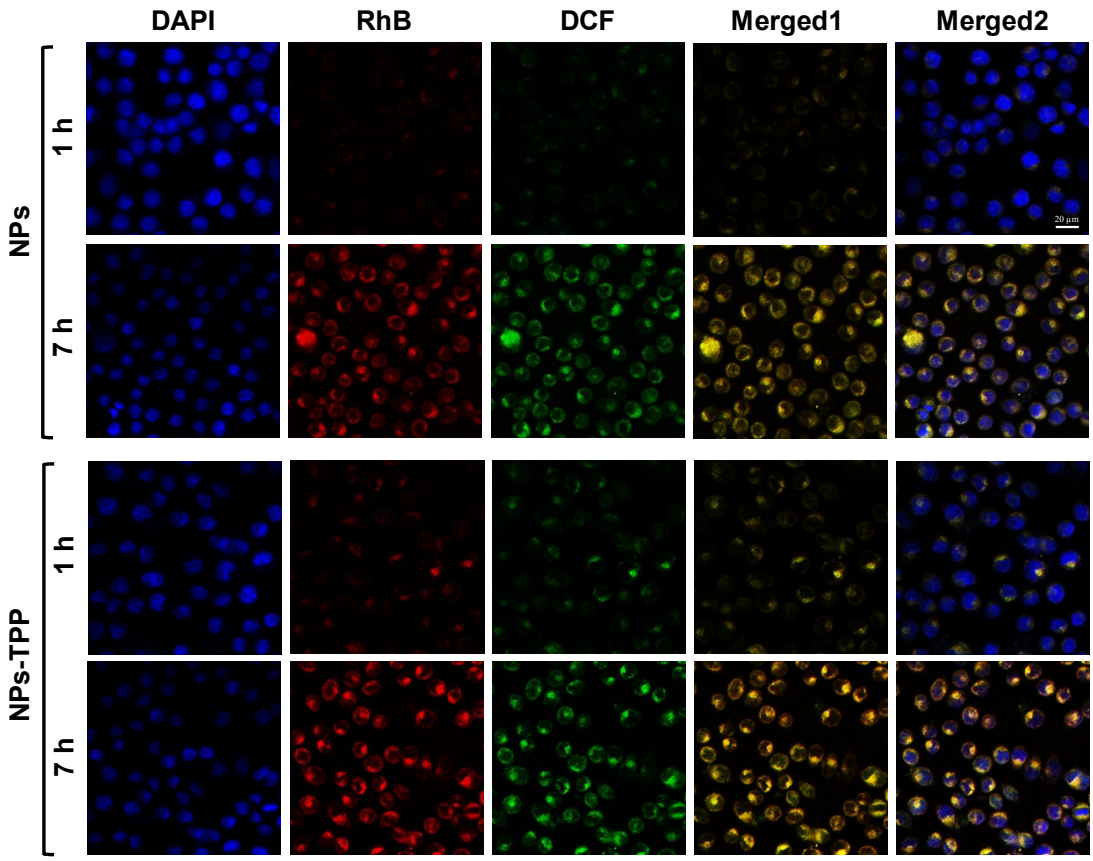


Figure S7 Confocal laser scanning imaging of nanoparticles in the MCF7 cells at 1 h and 7 h. The green fluorescence comes from DCF oxidized by intracellular ROS. The red fluorescence comes from rhodamine B. The blue fluorescence comes from a nuclear dye DAPI (10 μg/mL). Merged 1 : red and green fluorescence merged ; Merged 2 : all fluorescence merged. Scale bar: 20 μm.

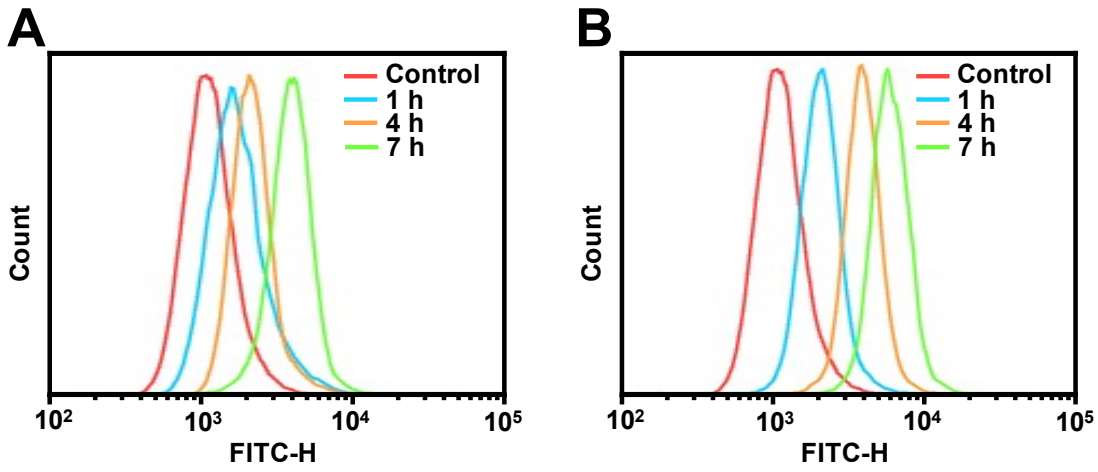


Figure S8 Quantified intracellular uptake of nanoparticles in the MCF7 cells at 1h, 4 h and 7 h by flow cytometry. NPs (A) and NPs-TPP (B).

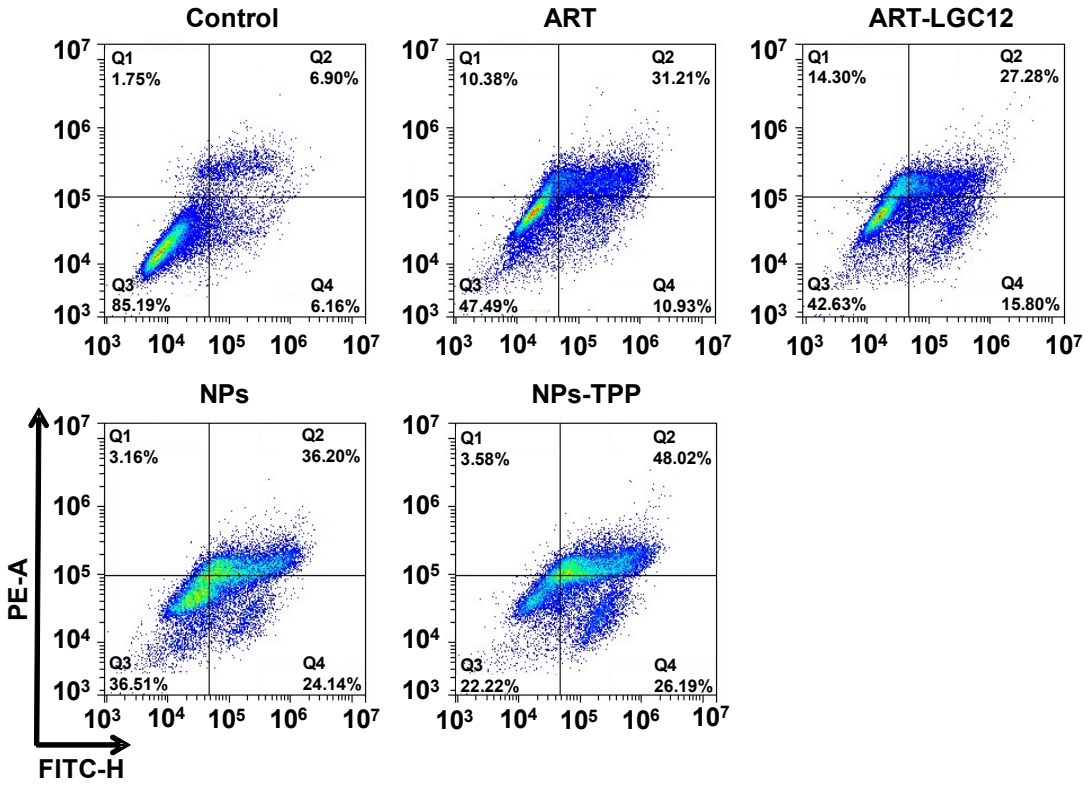


Figure S9 Cell apoptosis of MCF7 cells after 24 h of incubation with PBS, ART, ART-LGC12, NPs and NPs-TPP.

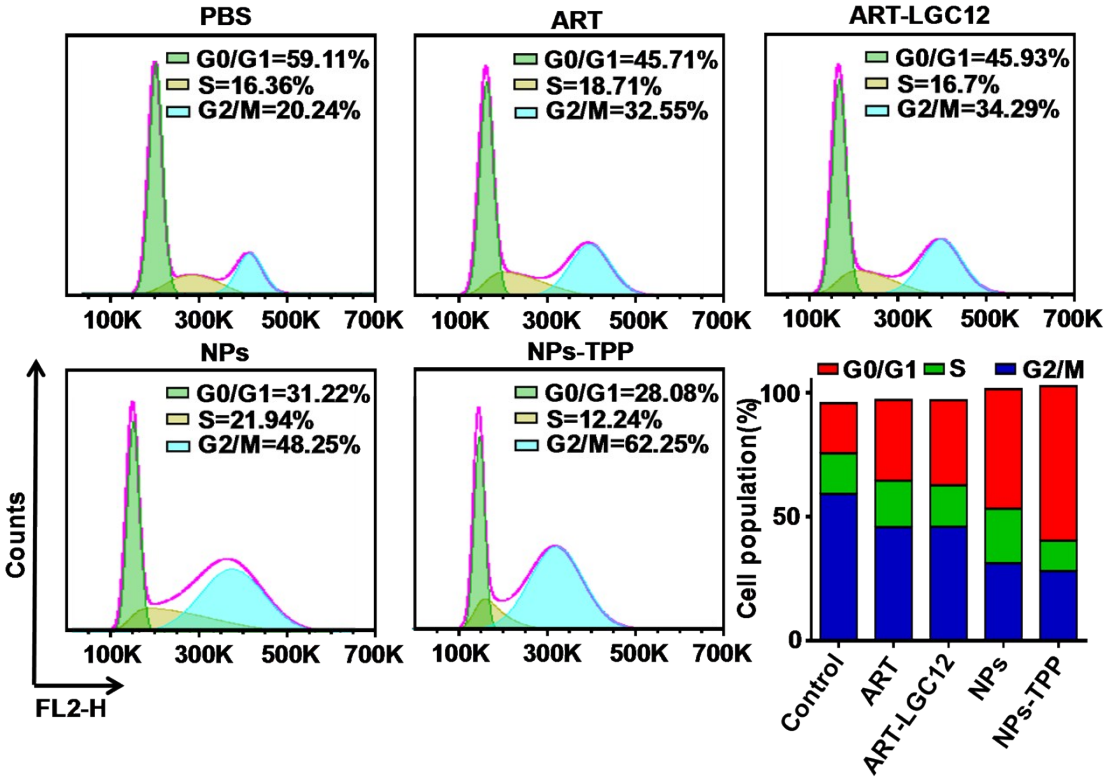


Figure S10 Cell cycle of MCF7 cells of incubation with PBS, ART, ART-LGC12, NPs and NPs-TPP, as well as G0/G1, S and G2/M phase statistics**.**

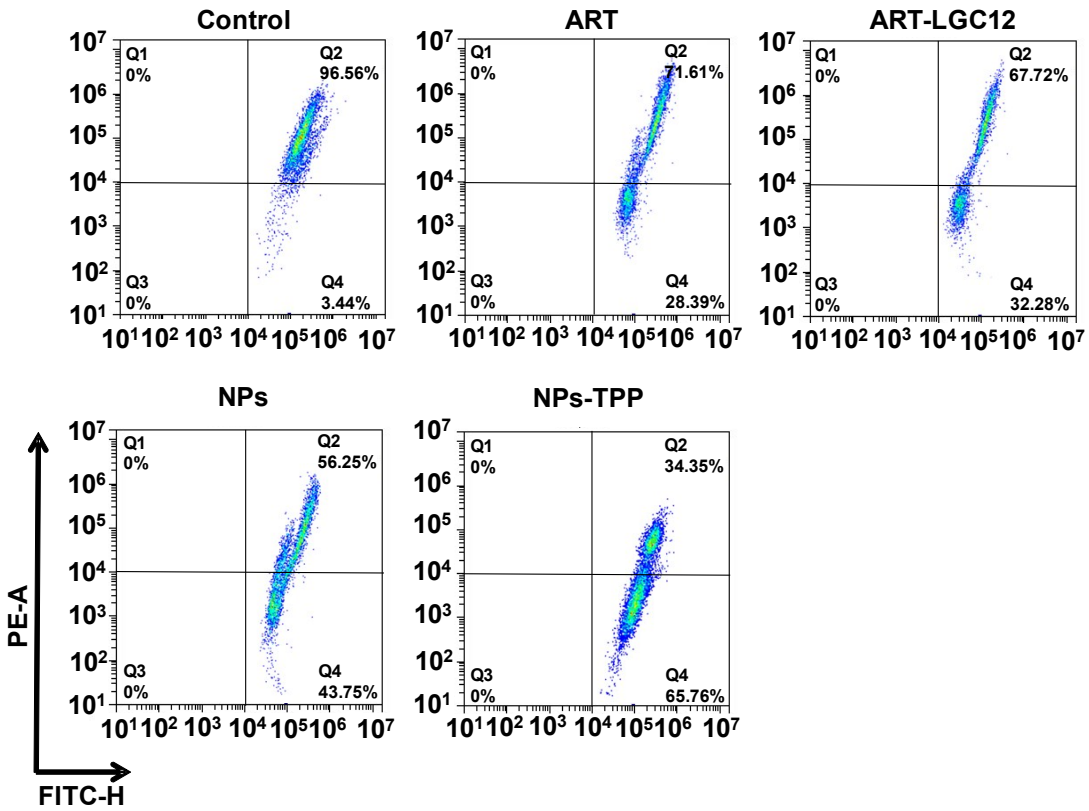


Figure S11 Mitochondrial membrane potential (MMP) changes in MCF7 cells after treatment with PBS, ART, ART-LGC12, NPs and NPs-TPP (40 μM) for 48 h.