Electronic Supplementary Information

Amphiphilic Phosphorous Dendron Micelles Co-deliver MicroRNA Inhibitor and Doxorubicin for Augmented Triple Negative Breast Cancer Therapy†

Liang Chen[a,b,c], Mengsi Zhan[a], Jin Li[a], Liu Cao[a], Huxiao Sun[a], Régis Laurent[b,c], Serge Mignani[d,e], Anne-Marie Caminade[b,c]*, Jean-Pierre Majoral[b,c]*, Xiangyang Shi[a,e]*

[a] State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, Shanghai Engineering Research Center of Nano-biomaterials and Regenerative Medicine, College of Biological Science and Medical Engineering, Donghua University, Shanghai 201620, People's Republic of China

[b] Laboratoire de Chimie de Coordination du CNRS, 205 Route de Narbonne, BP 44099, 31077 Toulouse CEDEX 4, France

[c] Université de Toulouse, UPS, INPT, 31077 Toulouse CEDEX 4, France

[d] Université Paris Descartes, PRES Sorbonne Paris Cité, CNRS UMR 860, Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologique, 45, rue des Saints Pères, 75006 Paris, France

[e] CQM - Centro de Química da Madeira, Universidade da Madeira, Campus da Penteada, 9020-105 Funchal, Portugal

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* Corresponding author. E-mail: anne-marie.caminade@lcc-toulouse.fr (A. M. Caminade), jean-pierre.majoral@lcc-toulouse.fr (J. P. Majoral), and xshi@dhu.edu.cn (X. Shi)

More Experimental Details

Materials

All reactions were carried out in organic solvents using standard high-vacuum and dry-argon techniques. All chemicals were purchased from Acros, Aldrich, or Fluka. All solvents were freshly dried and distilled according to routine procedures before use. MDA-MB-231 cells (a human triple-negative breast cancer cell line) and NIH-3T3 cells (a mouse embryonic fibroblast cell line) were supplied from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute Medium (RPMI-1640), Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Carlsbad, CA). Penicillin and streptomycin were from Gino Biomedical Technology Co., Ltd. (Hangzhou, China). Cell Counting Kit-8 (CCK-8) was from 7 Sea Biotech Co., Ltd. (Shanghai, China). 4',6-Diamidino-2-phenylindole (DAPI) stain solution and F-Actin Labelling Kit *Green Fluorescence* were form Sangon Biotech (Shanghai, China). Annexin V-FITC (Fluorescein Isothiocyanate)/PI (propidium iodide) Apoptosis Detection Kit and PI Cell Cycle Analysis Kit were acquired from BestBio Biotechnology Co., Ltd. (Shanghai, China). Western blot kit and horse radish peroxidase-labeled Goat Anti-Mouse IgG(H+L) were from Beyotime Biotechnology Co., Ltd. (Shanghai, China). The Bicinchoninic Acid Protein Quantitation Kit was from Shanghai Yeason Biotechnology Co., Ltd. (Shanghai, China). Some antibodies including p53, PTEN, BAX and GAPDH were obtained from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China). Water used in all experiments was purified using a PURIST UV Ultrapure Water System (RephiLe Bioscience, Ltd., Shanghai, China) with a resistivity higher than 18.2 MΩ·cm.

NMR Characterizations

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded with Bruker AV300, DPX300, or AV400 spectrometers. All ^{13}C NMR and ^{31}P NMR spectra were generally recorded by avoiding the disturbance of $\{^{1}H\}$. The signal of the non-deuterated solvent served as internal standard.

Compound 3: This compound was prepared and characterized according to the literature.[1] The solution of hexachlorocyclotriphosphazene (4.52 mmol, 1.57 g, 30 mL tetrahydrofuran (THF)) was added into the mixture of 4-hydroxybenzaldehyde (22.60 mmol, 2.75 g) and cesium carbonate (45.20 mmol, 14.72 g) in 50 mL THF at 0 ^{o}C. The reaction mixture was stirred overnight at room temperature. Salts were then removed by centrifugation and the supernatant was concentrated under

reduced pressure. The residue was then purified by silica column chromatography (hexane/ethyl acetate, 8/2 to 6/4, v/v) to afford compound 3 as a colorless oil in a 76% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ= 7.24 (m, 10 H, C2 0-H), 7.82 (m, 10 H, C3 0-H), 9.98 (5H, CHO) ppm. ^{31}P{^{1}H} NMR (121 MHz, $CDCl_3$): δ= 5.19 (d, $^{2}J_{(p\text{-}p)}$ =84 Hz, P_{01}, P_{02}), 20.73 (dd, $^{2}J_{P\text{-}P}$ = 88 Hz, $^{2}J_{P\text{-}P}$ = 84 Hz, P_{03}) ppm. ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ= 121.40 (m, C2 0), 131.44 (s, C3 0), 133.77 (m, C4 0), 154.29 (m, C1 0), 190.36 (s, CHO), 190.48 (s, CHO) ppm.

Compound 4: To a solution of the tyramine (3.64 mmol) in 5 mL of methanol (MeOH) was added a solution of 4-dodecyloxybenzoic acid (3.64 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 3.64 mmol) in $CHCl_3$ (5 mL). This mixture was refluxed overnight. The solution was concentrated under reduced pressure. The resulting product was then purified by silica column chromatography (MeOH/dichloromethane (DCM), 1/19, v/v) to afford compound 4 as a pale yellow powder in an 85% yield. ^{1}H NMR (400 MHz, DMSO): δ= 0.85 (t, $^{3}J_{(H\text{-}H)}$ = 8 Hz, 3 H, Co ‘0-H), 1.24 (m, 16 H, Cn ‘0-H, Cm ‘0-H, Cl ‘0-H, Ck ‘0-H), 1.40 (m, 2 H, Cj ‘0-H), 1.71 (t, $^{3}J_{(H\text{-}H)}$ = 8 Hz, 2 H, Ci ‘0-H), 2.70 (t, $^{3}J_{(H\text{-}H)}$ = 6 Hz, 2 H, Ca ‘0-H), 3.39 (m, 2 H, Cb ‘0-H), 4.00 (t, $^{3}J_{(H\text{-}H)}$ = 6 Hz, 2 H, Ch ‘0-H), 6.68 (d, $^{3}J_{(H\text{-}H)}$ = 8 Hz, 2 H, C2 ‘0-H), 6.96 (d, $^{3}J_{(H\text{-}H)}$ = 8 Hz, 2 H, C3 ‘0-H), 7.02 (d, $^{3}J_{(H\text{-}H)}$ = 12 Hz, 2 H, Cf ‘0-H), 7.79 (d, $^{3}J_{(H\text{-}H)}$ = 12 Hz, 2 H, Ce ‘0-H), 8.35 (t, $^{3}J_{(H\text{-}H)}$ = 6 Hz, 1 H, NH-Cc ‘0), 9.16 (s, 1 H, OH) ppm. ^{13}C {^{1}H} NMR (100 MHz, DMSO): δ= 14.41 (s, Co ‘0), 22.56 (s, Cn ‘0), 25.92 (s, Cj ‘0), 29.05 (s, Cl ‘0), 29.34 (m, Ck ‘0, Ci ‘0), 31.76 (s, Cm ‘0), 34.91 (s, Ca ‘0), 41.66 (s, Cb ‘0), 68.05 (s, Ch ‘0), 114.28 (s, Cf ‘0), 115.54 (s, C2 ‘0), 127.15 (s, Cd ‘0), 129.32 (s, C3 ‘0), 129.91 (s, Ce ‘0), 130.08 (s, C4 ‘0), 156.05 (s, C1 ‘0), 161.31 (s, Cg ‘0), 165.99 (s, Cc ‘0) ppm.

Compound 5: Compound 4 was added at 0 °C to a solution of cesium carbonate and compound 3 in anhydrous THF (20 mL). The reaction mixture was stirred overnight at room temperature and then centrifuged. The supernatant was then concentrated under reduced pressure. The resulting product was purified by silica column chromatography (pentane/ethyl acetate, 8/2 to 6/4, v/v) to afford compound 5 as a white powder in an 80% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ= 0.89 (t, $^{3}J_{(H\text{-}H)}$ = 8 Hz , 3 H, Co ‘0-H), 1.27 (m, 16 H, Cn ‘0-H, Cm ‘0-H, Cl ‘0-H and Ck ‘0-H), 1.44 (m, 2 H, Cj ‘0-H), 1.79 (t, $^{3}J_{(H\text{-}H)}$ = 8 Hz, 2 H, Ci ‘0-H), 2.91 (t, $^{3}J_{(H\text{-}H)}$ = 8 Hz, 2 H, Ca ‘0-H), 3.67 (m, 2 H, Cb ‘0-H), 3.98 (t, $^{3}J_{(H\text{-}H)}$ = 8 Hz, 2 H, Ch ‘0-H), 6.22 (t, $^{3}J_{(H\text{-}H)}$ = 6 Hz, 1 H, NH-Cc ‘0), 6.89 (d, $^{3}J_{(H\text{-}H)}$ = 8 Hz, 2 H, Cf ‘0-H), 6.94 (d, $^{3}J_{(H\text{-}H)}$ = 8 Hz, 2 H, Ce ‘0-H), 7.13 (m, 12 H, C2 ‘0-H and C2 0-H), 7.71 (m, 12 H, C3 ‘0-H and C3 0-H), 9.94 (s, 5 H, CHO) ppm. ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$):

δ= 7.40 (m, P_0) ppm. $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$): δ= 14.12 (s, Co ‘0), 22.68 (s, Cn ‘0), 25.98 (s, Cj ‘0), 29.13 (s, Cl ‘0-H), 29.49 (m, Ck ‘0 and Ci ‘0), 31.90 (s, Cm ‘0), 35.10 (s, Ca ‘0), 40.99 (s, Cb ‘0), 68.21 (s, Ch ‘0), 77.03 ($CDCl_3$), 114.24 (s, Cf ‘0), 120.74 (br s, C2 ‘0), 121.25 (s, C2 0), 126.36 (s, Cd ‘0), 128.55 (s, C4 0), 129.91 (s, C3 ‘0), 131.34 (s, C3 0), 133.67 (s, Ce ‘0), 136.71 (s, C4 ‘0), 148.53 (br s , C1 ‘0), 154.60 (s, C1 0), 161.82(s, Cg ‘0), 167.03 (s, Cc ‘0), 190.39 (s, CHO), 190.43 (s, CHO), 190.54 (s, CHO) ppm.

Compound 7: A freshly compound 6 (2.1 mmol) synthesized according to the literature[2] was added at 0 °C to a solution of compound 5 (4.2 mmol) in anhydrous DCM (50 mL). The solution was stirred for 6 h at room temperature and then concentrated under reduced pressure (10 mL). Then, 100 mL of pentane was added to the residue and the resulting precipitate was filtered off and dried under reduced pressure to afford compound 7 as a white powder in a 92% yield. ^1H NMR (400 MHz, $CDCl_3$): δ= 0.90 (t, $^3J_{(H-H)}$ =8 Hz ,3 H, Co ‘0-H), 1.28 (m, 16 H, Cn ‘0-H, Cm ‘0-H, Cl ‘0-H and Ck ‘0-H), 1.46 (m, 2 H, Cj ‘0-H), 1.80 (t, $^3J_{(H-H)}$ = 8 Hz, 2 H, Ci ‘0-H), 2.88 (t, $^3J_{(H-H)}$ = 8 Hz, 2 H, Ca ‘0-H), 3.50 (d, $^3J_{(H-P1)}$ = 16 Hz, 15 H, CH_3-N-P_1), 3.61 (m, 2 H, Cb ‘0-H), 3.99 (t, $^3J_{(H-H)}$ = 8 Hz, 2 H, Ch ‘0-H), 6.04 (t, $^3J_{(H-H)}$ = 6 Hz, 1 H, NH-Cc ‘0), 6.92 (m, 4 H, Cf ‘0-H and Ce ‘0-H), 7.03 (m, 12 H, C2 ‘0-H and C2 0-H), 7.64 (m, 17 H, C3 ‘0-H, C3 0-H and CH=N) ppm. $^{31}P\{^1H\}$ NMR (162 MHz, $CDCl_3$): δ = 8.36 (m, P_0), 62.41, 62.44 (s, P_1) ppm. $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$): δ= 14.13 (s, Co ‘0), 22.69 (s, Cn ‘0), 26.00 (s, Cj ‘0), 29.14 (s, Cl ‘0), 29.50 (m, Ck ‘0 and Cj ‘0), 31.89 (s, Cm ‘0), 31.97 (d, $^2J_{(C-P1)}$ = 11 Hz, CH_3-N-P_1), 35.23 (s, Ca ‘0), 41.21 (s, Cb ‘0), 68.24 (s, Ch ‘0), 114.31 (s, Cf ‘0), 121.12 (br s, C2 ‘0), 121.38 (s, C2 0), 126.24 (s, Cd ‘0), 128.60 (s, C4 0), 129.76 (s, C3 ‘0), 131.27 (m, C3 0), 131.16 (s, Ce ‘0), 136.02 (s, C4 ‘0), 140.68 (m, CH=N), 148.77 (br s , C1 ‘0), 151.66 (s, C1 0), 161.87(s, Cg ‘0), 166.95 (s, Cc ‘0) ppm.

Compound C12G1NC4: To a solution of compound 7 (2.26 mmol) in THF (40 mL) was added at 0 °C compound 8 (22.6 mmol) and N,N-diisopropylethylamine (27.12 mmol). The reaction mixture was stirred overnight at room temperature and then concentrated under reduced pressure. After that, 100 mL of pentane was added to the residue and the resulting precipitate was filtered off and dried under reduced pressure to afford C12G1NC4 as a white powder in a 95% yield. ^1H NMR (400 MHz, $CDCl_3$): δ= 0.89 (t, $^3J_{(H-H)}$ = 8 Hz ,3 H, Co ‘0-H), 1.28 (m, 16 H, Cn ‘0-H, Cm ‘0-H, Cl ‘0-H and Ck ‘0-H), 1.45 (m, 2 H, Cj ‘0-H), 1.76 (br s, 40 H, C4 1-H), 1.80 (m, 2 H, Ci ‘0-H), 2.58 (br s, 40 H, C3 1-H), 2.67 (m, 20 H, C2 1-H), 2.83 (m, 2 H, Ca ‘0-H), 3.09 (m, 20 H, C1 1-H), 3.18 (d, $^3J_{(H-P1)}$ = 8 Hz, 15 H, CH_3-N-P_1), 3.51 (m, 2 H, Cb ‘0-H), 3.99 (t, $^3J_{(H-H)}$ = 8 Hz, 2 H, Ch ‘0-H),

4.20 (m, 10 H, NH-P_1), 6.92 (d, $^3J_{(H-H)}$ = 8 Hz , 2 H, Ce ‘0-H), 6.97 (m, 14H, Cf ‘0-H, C2 ‘0-H and C2 0-H), 7.64 (m, 17 H, C3 ‘0-H, C3 0-H and CH=N) ppm. $^{31}P\{^1H\}$ NMR (162 MHz, $CDCl_3$): δ= 8.56 (m, P_0), 68.27, 68.49 (s, P_1) ppm. $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$): δ= 14.12 (s, Co ‘0), 22.68 (s, Cn ‘0), 23.51 (s, C4 1), 26.02 (s, Cj ‘0), 29.18 (s, Cl ‘0), 29.50 (m, Ck ‘0 and Cj ‘0), 30.90 (d, $^2J_{(C-P1)}$ = 7 Hz, CH_3-N-P_1), 31.90 (s, Cm ‘0), 35.36 (s, Ca ‘0), 39.63 (s, C2 1), 41.39 (s, Cb ‘0), 53.84 (s, C3 1), 51.23 (d, $^2J_{(C-P1)}$ = 8 Hz, C1 1), 68.17 (s, Ch ‘0), 114.11 (s, Cf ‘0), 120.77 (br s, C2 ‘0), 121.12 (s, C2 0), 126.42 (s, Cd ‘0), 127.49 (s, C3 0), 128.91 (s, C4 0), 129.56(s, C3 ‘0), 132.79 (s, Ce ‘0), 132.86 (s, C4 ‘0), 135.98 (m, CH=N), 150.52 (br s , C1 ‘0 and C1 0), 161.69(s, Cg ‘0), 166.90 (s, Cc ‘0) ppm.

Critical Micelle Concentration Determination

The critical micelle concentration (CMC) of 1-C12G1 was assessed using pyrene as a fluorescence probe. Water solutions of 1-C12G1 with concentrations ranging from 2.4 × 10^{-6} to 4.9 × 10^{-4} M were prepared. Then, each solution was added to a flask containing pyrene at a final concentration of 6.0 × 10^{-7} M. The solutions were then sonicated for 30 min and kept overnight at room temperature to finalize the micelle formation. Next, the fluorescence spectra were recorded at the excitation wavelength of 333 nm by a fluorescence spectrophotometer (Protein Technologies, Inc., Tucson, MA). Both the excitation and emission bandwidths were set at 5 nm. The pyrene fluorescence intensity ratio of I_{373}/I_{393} was analyzed as a function of logarithm 1-C12G1 concentration.

Preparation and Characterization of Doxorubicin (DOX)-Loaded Micelles

Doxorubicin hydrochloride (1.2 to 3.2 mg) was dissolved in 100 μL methanol, and added with triethylamine (molar ratio of DOX/triethylamine = 1: 1) to obtain hydrophobic DOX, which was then mixed with 1-C12G1 (0.5 mg) in 3.0 mL water at different molar ratios (1-C12G1/DOX = 1: 15, 1: 20, 1: 25, 1: 30 or 1: 35). Each mixture solution was stirred overnight at room temperature to allow the evaporation of methanol, and centrifuged (7000 rpm for 10 min) to remove the precipitate related to the non-encapsulated DOX. The precipitate was collected and dissolved into 1 mL methanol for UV-vis spectral analysis using a Lambda 25 UV-vis spectrophotometer (Perkin Elmer, Waltham, MA). The supernatant was lyophilized for 3 days to obtain the 1-C12G1@DOX complex. The drug loading content and drug encapsulation efficiency were calculated according to the following equations:

$$\text{Loading content (\%)} = W_t / W_s \times 100\% \quad (1)$$

$$\text{Encapsulation efficiency (\%)} = W_t / W_0 \times 100\% \quad (2)$$

where W_t represents the mass of loaded DOX within the micelles, W_0 the initial mass of DOX, and W_s the mass of DOX-loaded micelles.

The 1-C12G1@DOX micelles were characterized by fluorescence spectroscopy. Free DOX with the same DOX concentrations was also recorded. The fluorescence emission spectra of the solutions were measured at an excitation wavelength of 365 nm, while the fluorescence excitation spectra of the solutions were collected at an emission wavelength of 595 nm. The micelles before and after DOX loading were also examined using zeta-potential and dynamic light scattering (DLS), which were performed using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633-nm laser. Transmission electron microscopy (TEM) imaging was executed using the JEOL 2010 analytical electron microscope (JEOL, Tokyo, Japan) at an operating voltage of 200 kV. Each sample was prepared by dropping its aqueous solution onto a carbon-coated copper grid and air dried before measurements.

Preparation and Characterization of 1-C12G1@DOX/miR-21i Polyplexes

The 1-C12G1@DOX/miR-21i (microRNA-21 inhibitor) polyplexes were formed by incubating 1 μg miR-21i and appropriate amount of 1-C12G1@DOX under different N/P ratios in diethyl procarbonate (DEPC) water, and incubated for 30 min at room temperature. The final volume of the polyplexes was 20 μL for gel retardation assay. The gel was prepared by dissolving 1% (w/v) agarose gel and 4S Green Plus Nucleic Acid Stain in Tris-acetate-EDTA buffer, and melted using a microwave. Then, the 1-C12G1@DOX/miR-21i polyplexes with different N/P ratios were loaded into the respective wells of the gel, and electrophoresis was carried out at 85 V for 30 min. The retardation of the miR-21i was visualized using a UV transilluminator (Shanghai FURI Science & Technology, Shanghai, China).

For hydrodynamic size and zeta-potential measurements, the 1-C12G1@DOX micelles were dispersed in DEPC water, and then complexed with miR-21i under different N/P ratios. In this case, 5 μg of miR-21i dissolved in DEPC water was complexed with the 1-C12G1@DOX to reach a final volume of 1 mL. After incubation for 30 min, the prepared polyplexes were subjected to DLS and zeta potential measurements. All measurements were performed at room temperature.

Drug Release Kinetics

The DOX release from the 1-C12G1@DOX/miR-21i polyplex was determined by dialysis using a membrane with a molecular weight cut-off (MWCO) of 8,000-12,000. In brief, 200 μL of the 1-C12G1@DOX/miR-21i polyplex (1 mg/mL) was dialyzed against phosphate buffer at two different

pHs (pH 7.4 and pH 5.0) at 37 °C according to the literature.[3] At a predetermined time interval, 1 mL of aliquot of the outer phase medium was withdrawn, and replenished with 1 mL of fresh corresponding medium. The DOX concentration was determined by UV-vis spectrophotometer to calculate the accumulative release of DOX from the 1-C12G1@DOX/miR-21i polyplex.

Cell Culture

NIH-3T3 and MDA-MB-231 cells were regularly cultured and passaged using DMEM and RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin, respectively. The cells were incubated at 37 °C in a Thermo Scientific cell incubator (Waltham, MA) with 5% CO_2.

Cytotoxicity Assay

The cytotoxicity of 1-C12G1, free DOX, 1-C12G1/miR-21i, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i was quantitatively evaluated by CCK-8 cell viability assay. Cells were cultured in a 96-well plate at a density of 1×10^4 cells per well in 100 μL medium overnight to bring the cell adherence to the well. Then, the medium of each well was discarded and added with fresh medium containing different materials with varying concentrations. After 24 h, the medium in each well was replaced with 100 μL serum-free medium containing 10% CCK-8 solution and then the cells were incubated for another 2 h under regular cell culture conditions. After that, the cells in each well were analyzed using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA) at a wavelength of 450 nm. Each sample was tested in sextuplicate.

Cellular Uptake Assay

Cellular uptake of free DOX, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i was investigated by qualitative confocal/fluorescence microscopic imaging and quantitative flow cytometry analysis. For confocal microscopic imaging, MDA-MB-231 cells were seeded into 35-mm confocal dishes (1.5×10^5 cells per dish, 1 mL medium), incubated at 37 °C overnight, and then treated with medium containing free DOX, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i at the equivalent DOX concentration of 1 μM for 4 h. Cells were then washed with phosphate buffered saline (PBS), stained with F-Actin Labeling Kit *Green Fluorescence* for 30 min, washed with PBS, and stained with DAPI for 5 min. Then, the cells were observed by ZEISS laser scanning confocal microscope (LSM-700, Jena, Germany) at an excitation wavelength of 488 nm. For fluorescence microscopic imaging, cells were seeded into 24-well plates at a density of 1×10^5 cells per well, then treated with the polyplexes as described above. The cells were observed by an Axio Vert.A1 Carl Zeiss fluorescence microscope (Jena, Germany) to validate the DOX uptake. For flow cytometry assay, MDA-MB-231

cells or NIH-3T3 cells were seeded into 6-well plates at a density of 3 × 10^5 cells per well, then treated with free DOX, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i as described above. Cells were then harvested, treated with Trypsin-EDTA, and analyzed using a Becton Dickinson Facscan analyzer (Franklin Lakes, NJ).

Cell Apoptosis Assay

Annexin V-FITC/PI apoptosis detection kit was used for cell apoptosis assessments. In brief, 3× 10^5 MDA-MB-231 cells per well were seeded into 6-well plates, then incubated with 1-C12G1, free DOX, 1-C12G1/miR-21i, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i at the equivalent DOX (or 1-C12G1) concentration ([DOX] = 1 μM, or [1-C12G1] = 0.04 μM) for 4 h. Then, cells were washed with PBS, and the medium was replaced with fresh medium containing 10% FBS and 1% penicillin-streptomycin. After 24 h, cells were harvested and processed using Annexin V-FITC/PI apoptosis detection kit in compliance with the manufacturer's protocol, and measured by a Becton Dickinson Facscan analyzer. Each sample was measured in triplicate.

Western Blot Assay *in Vitro*

To elucidate the molecular mechanism of the cell apoptosis, the cell apoptosis-related proteins were inspected *via* Western blotting *in vitro*. MDA-MB-231 cells were treated as described above, and then exposed to cell lysate buffer to extract proteins. The lysates were centrifuged at 12,000 rpm for 5 min at 4 °C. Thereafter, the supernatants were collected for Western blot assay according to the manufacturer's instructions. Protein concentration was determined with a commercial Bradford Protein Assay Kit. The whole cell protein lysates were electrophoresed on 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), followed by blocking in Tris-buffered saline with 5% non-fat milk, and then incubated with primary antibodies (Cell Signaling Technology, Beverly, MA) at 4 °C overnight. Membranes were then incubated with secondary antibody for 2 h at room temperature. The membranes were detected with an enhanced chemiluminescent reagent (Merck Millipore, Burlington, MA) and exposed to Kodak X-Omat films (Kodak, Xiamen, China). Finally, the films were developed and the intensity of immunoreactivity was measured by densitometry using Image J 1.40 G software (http://rsb.info.nih.gov/ij/download.html).

***In Vivo* Antitumor Activity Using an Orthotopic TNBC Tumor Model**

All animal experiments were carried out after approval by the ethical committee for animal care of Donghua University and also in accordance with the policy of the National Ministry of Health

(China). To build up an orthotopic triple negative breast cancer (TNBC) tumor model, each BALB/c nude mouse (4-weeks-old, Shanghai Laboratory Animal Center, Shanghai, China) was injected in the right axilla with 5×10^6 MDA-MB-231 cells suspended in 100 μL of 0.9% normal saline (NS). At about 2 weeks post-injection, the tumor reached about a volume of 100 mm^3. To investigate the antitumor activity *in vivo*, the tumor-bearing nude mice were divided into 6 groups, and the mice were intravenously injected with **NS** (Group 1), 1-C12G1 (Group 2), free DOX (Group 3), 1-C12G1/miR-21i (Group 4), 1-C12G1@DOX (Group 5), and 1-C12G1@DOX/miR-21i (Group 6), respectively every 3 days for 5 times. The dose of 5 mg DOX/kg or 1.2 mg 1-C12G1/kg was used for each mouse. The tumor volumes and body weights were recorded every two days. The tumor volume (V) was calculated according to a formula of $V = W^2 \times L/2$, where W and L represent the width and length of tumor, respectively. The relative tumor volume and body weight was calculated based on the tumor volume on the first day. On the 16th day, the main organs (heart, liver, spleen, lung, and kidney) and the tumor tissues were removed from sacrificed mice for hematoxylin-eosin (H&E) and TdT-mediated dUTP Nick-End Labeling (TUNEL) staining according to standard protocols. The TUNEL staining images were also applied to detect the apoptosis rate of tumor cells in different treatment groups. Western blot assay was also applied to detect the relative expression level of apoptosis-related proteins in tumor cells.

Statistical Analysis

Data were presented as the mean ± standard deviation ($n \geq 3$). One-way analysis of variance statistical method was adopted to analyze the experimental results. A p value of 0.05 was selected as the significance level, and the data were marked with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Polydispersity index (PDI) of 1-C12G1, 1-C12G1@DOX and 1-C12G1@DOX/miR-21i.

Samples	Low concentration (30.2 μM)	High concentration (151 μM)
1-C12G1	0.314	0.798
1-C12G1@DOX	0.290	0.286
1-C12G1@DOX/miR-21i	0.355	0.144

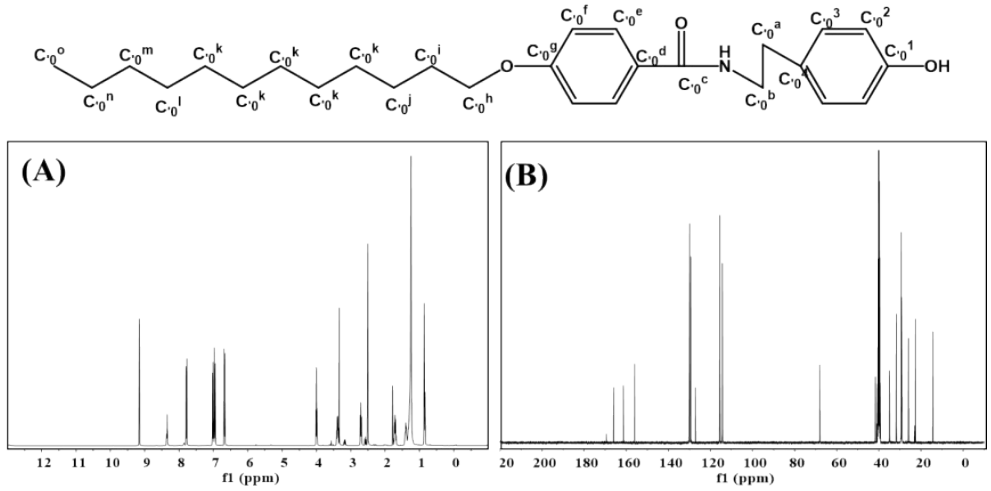

Figure S1. ^{1}H NMR (A) and $^{13}C\{^{1}H\}$ NMR (B) spectra of compound 4.

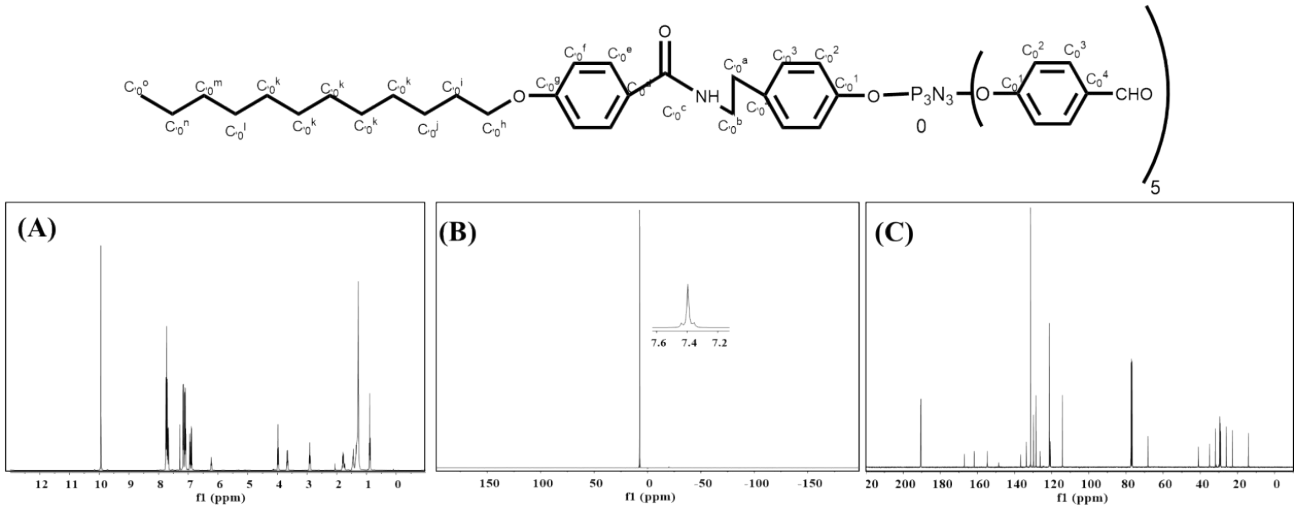

Figure S2. ^{1}H NMR (A), $^{31}P\{^{1}H\}$ NMR (B) and $^{13}C\{^{1}H\}$ NMR (C) spectra of compound 5.

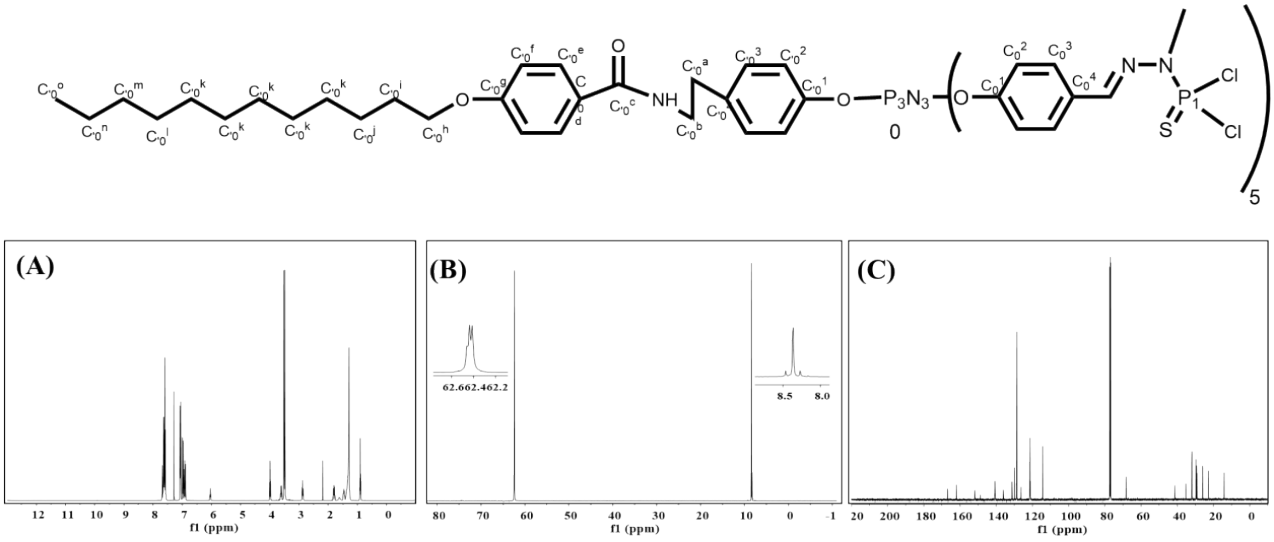

Figure S3. ^{1}H NMR (A), $^{31}P\{^{1}H\}$ NMR (B) and $^{13}C\{^{1}H\}$ NMR (C) spectra of compound 7.

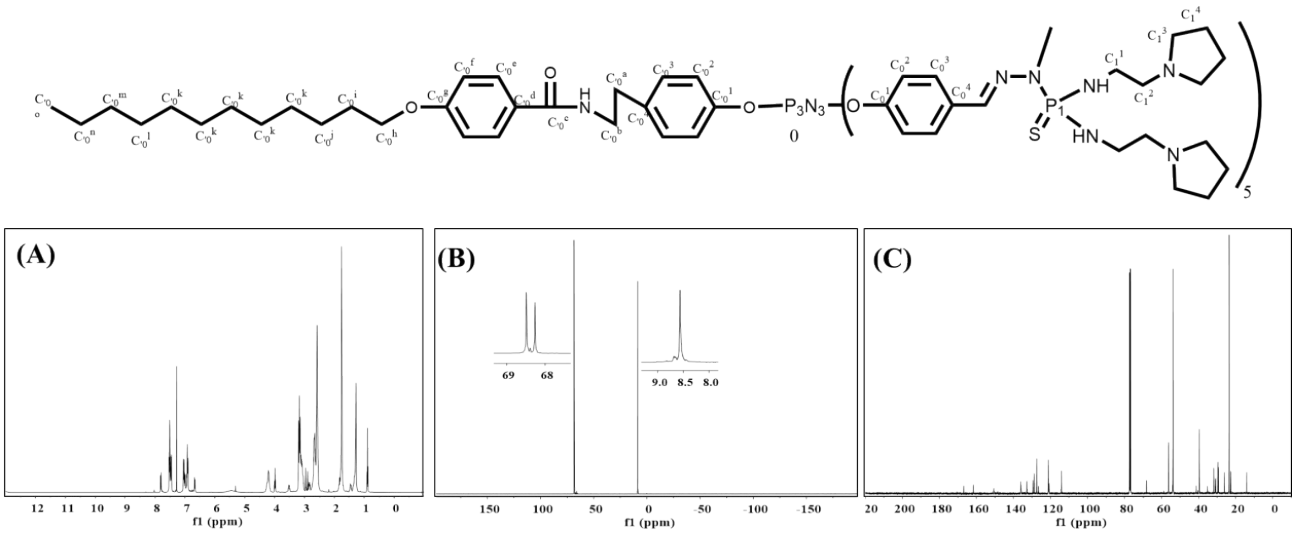

Figure S4. ^{1}H NMR (A), $^{31}P\{^{1}H\}$ NMR (B) and $^{13}C\{^{1}H\}$ NMR (C) spectra of C12G1NC4.

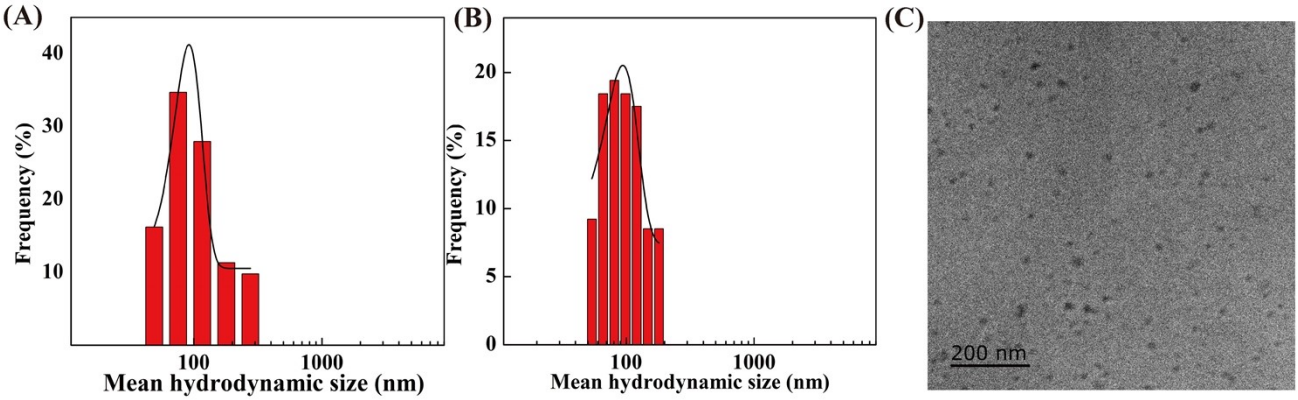

Figure S5. Hydrodynamic size distribution of 1-C12G1 micelles dispersed in water at a concentration of (A) 300 μM and (B) 30 μM. (C) TEM image of 1-C12G1 micelles at the dendron concentration of 300 μM.

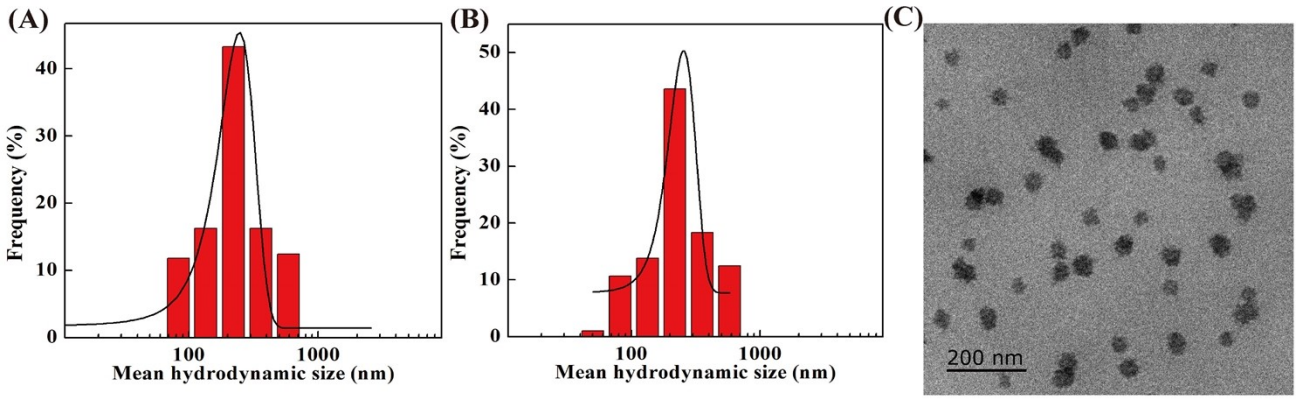

Figure S6. Hydrodynamic size distribution of 1-C12G1@DOX micelles dispersed in water at the equivalent DOX concentration of (A) 79.6 μM and (B) 7.96 μM. (C) TEM image of 1-C12G1@DOX micelles at the equivalent DOX concentration of 79.6 μM.

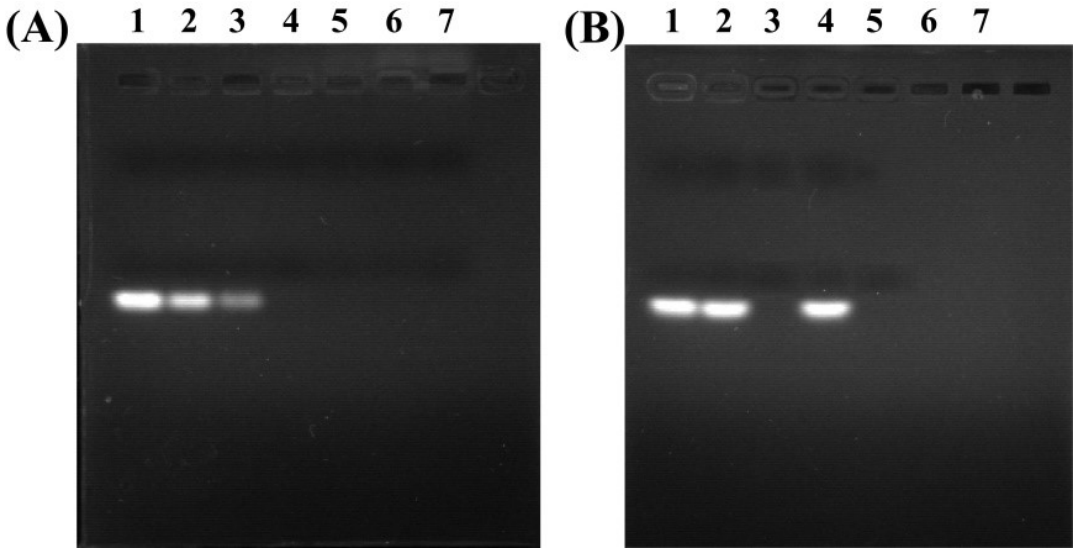

Figure S7. (A) Gel retardation assay of miR-21i complexed with 1-C12G1@DOX at various N/P ratios. Lane 1: miR-21, lane 2: N/P = 0.25: 1, lane 3: N/P = 0.5:1, lane 4: N/P = 1: 1, lane 5: N/P = 2: 1, lane 6: N/P = 3:1, and lane 7: N/P = 4: 1. (B) The stability assessment of free miR-21i and miR-21i complexed with the micelles monitored under normal saline at 37 ^{o}C (Lane 1: free miR-21i for 0 h, lane 2: 1-C12G1@DOX/miR-21i for 12 h, lane 3: free miR-21i for 12 h, and lane 4: 1-C12G1@DOX/miR-21i for 24 h, and lane 5: free miR-21i for 24 h).

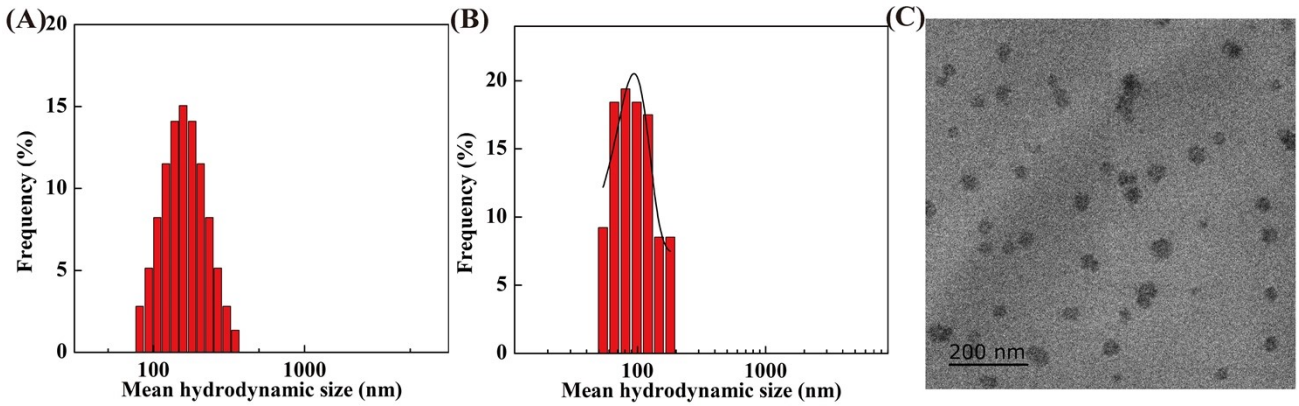

Figure S8. Hydrodynamic size distribution of 1-C12G1@DOX/miR-21i dispersed in water at the equivalent DOX concentration of (A) 79.6 μM and (B) 7.96 μM. (C) TEM image of 1-C12G1@DOX/miR-21i micelles at the equivalent DOX concentration of 79.6 μM.

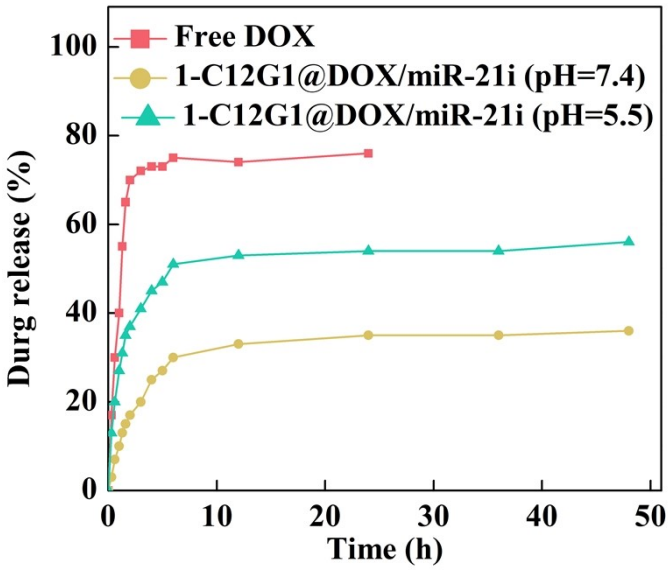

Figure S9. *In vitro* DOX release from 1-C12G1@DOX/miR-21i micelles in phosphate buffer at pH 5.5 or pH 7.4 at 37 °C. Free DOX was also tested for comparison.

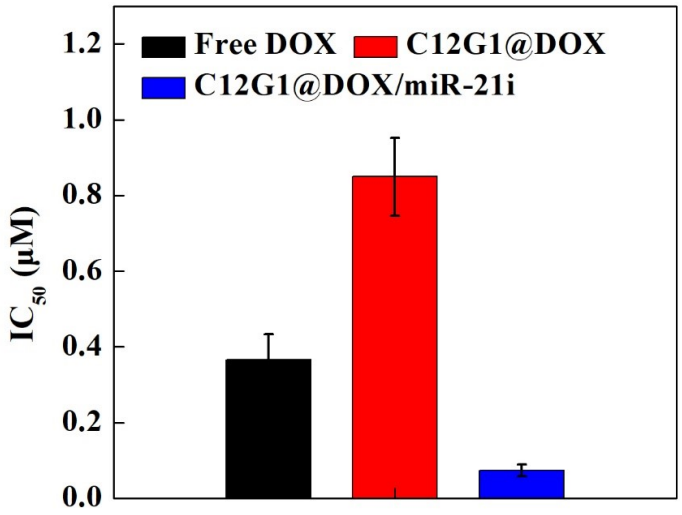

Figure S10. IC_{50}s of free DOX and complexes. Data are shown as the mean of triplicate measurements with standard deviation (n = 3).

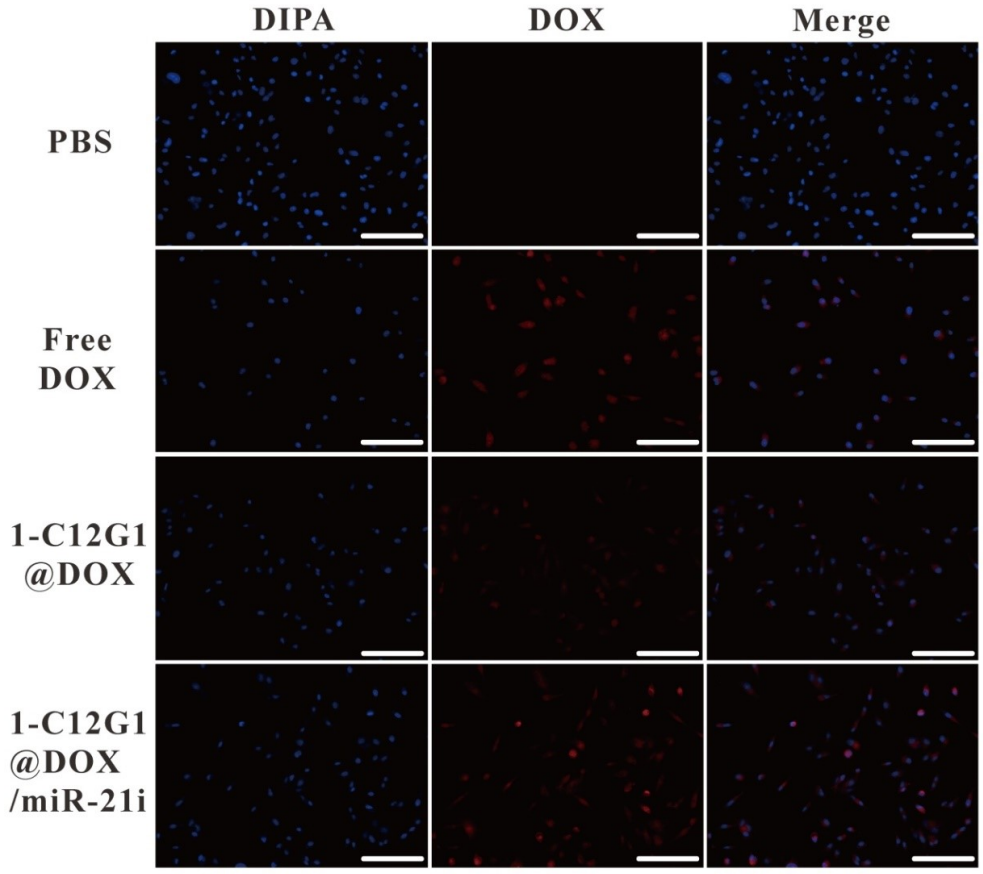

Figure S11. Fluorescence microscopic images of MDA-MB-231 cells treated with free DOX, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i for 4 h (the equivalent concentration of DOX was 1 μM). Scale bar in each panel represents 100 μm.

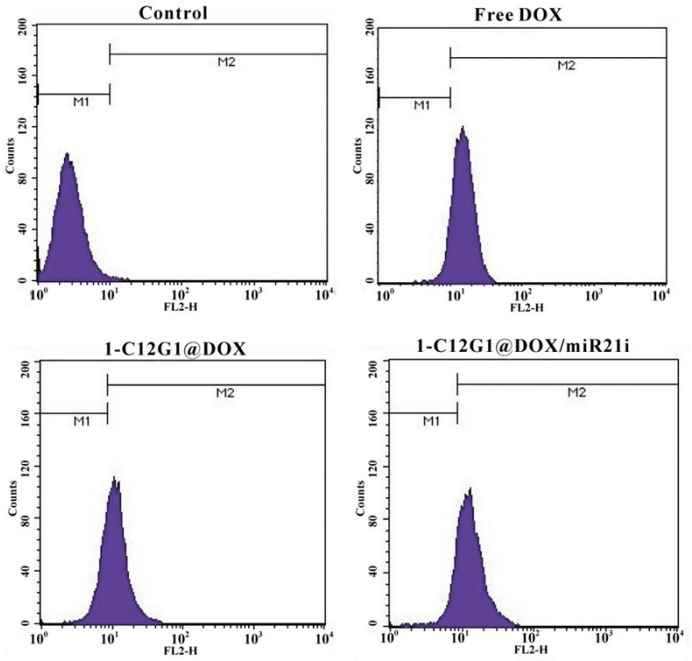

Figure S12. Flow cytometry analysis of MDA-MB-231 cells after treatment with free DOX, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i for 4 h (the equivalent concentration of DOX was 1 μM).

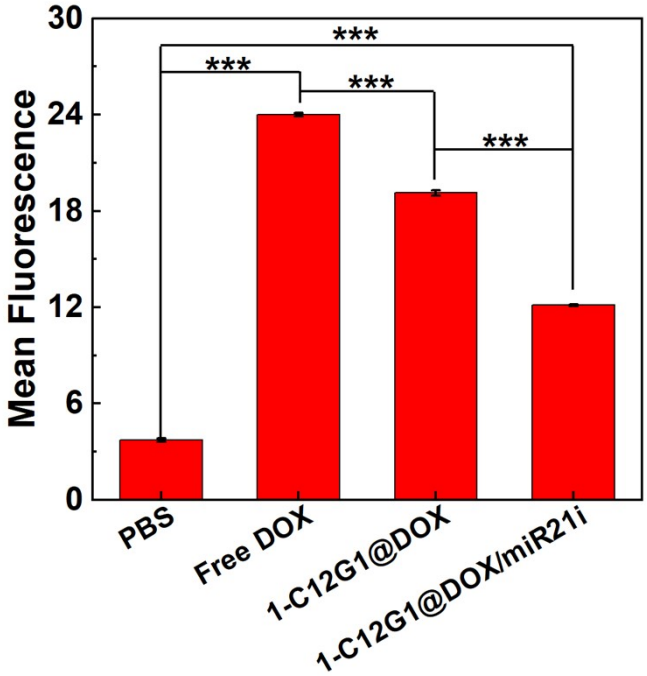

Figure S13. The mean fluorescence intensity of NIH-3T3 cells incubated with free DOX, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i (the equivalent concentration of DOX was 1 μM) assayed by flow cytometry.

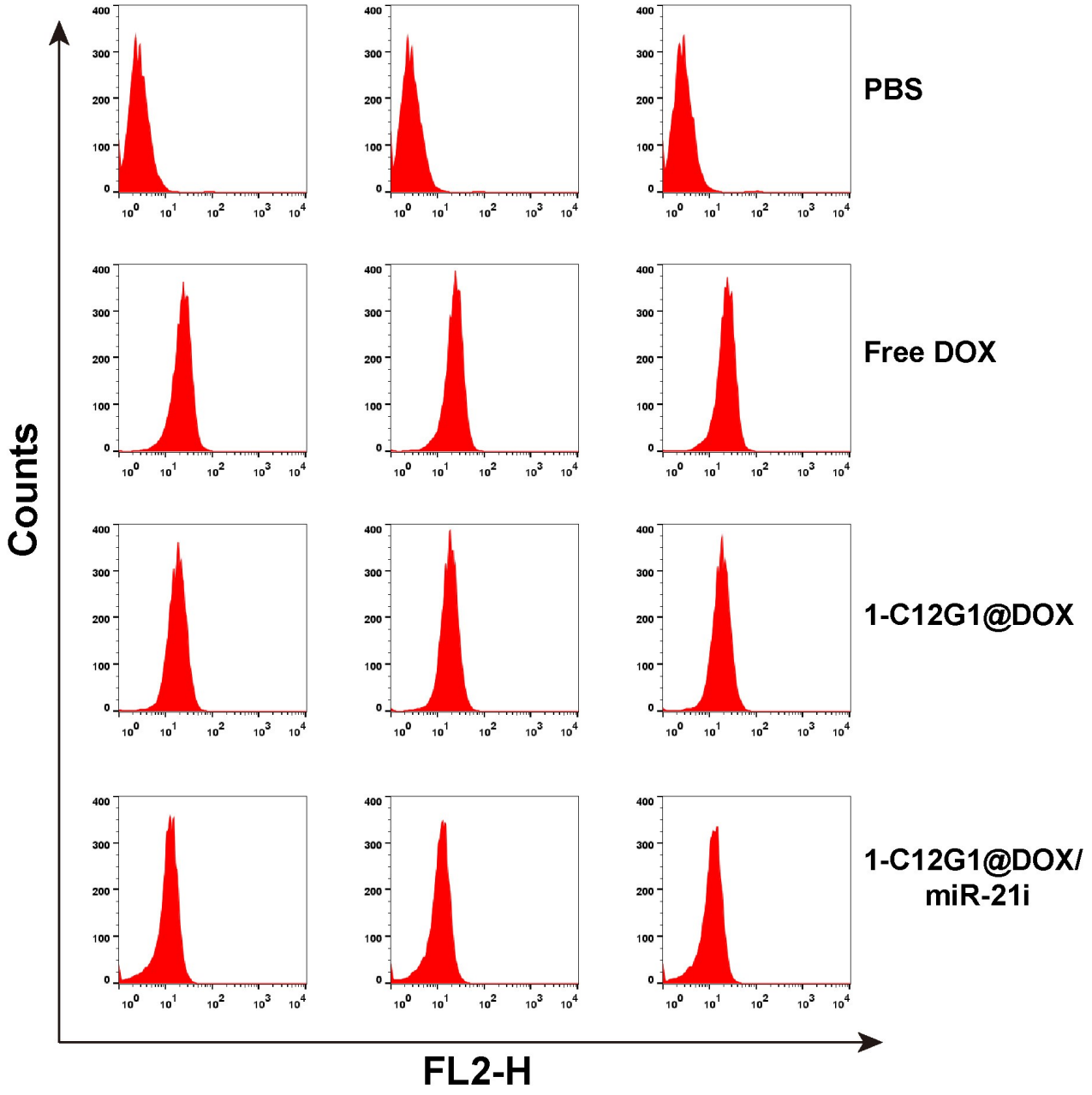

Figure S14. Flow cytometry analysis of NIH-3T3 cells after treatment with free DOX, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i for 4 h (the equivalent concentration of DOX was 1 μM).

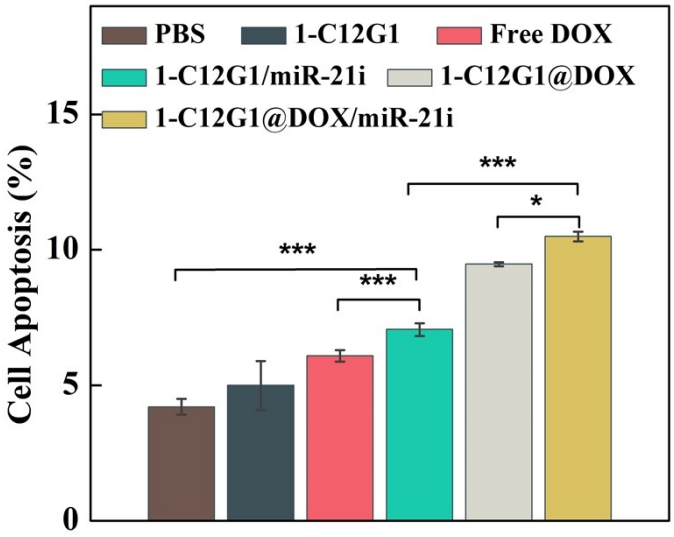

Figure S15. Percentages of apoptotic MDA-MB-231 cells treated with 1-C12G1, free DOX, 1-C12G1/miR-21i, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i (the equivalent concentration of DOX was 1 μM) analyzed by flow cytometry using annexin V-FITC/PI kit.

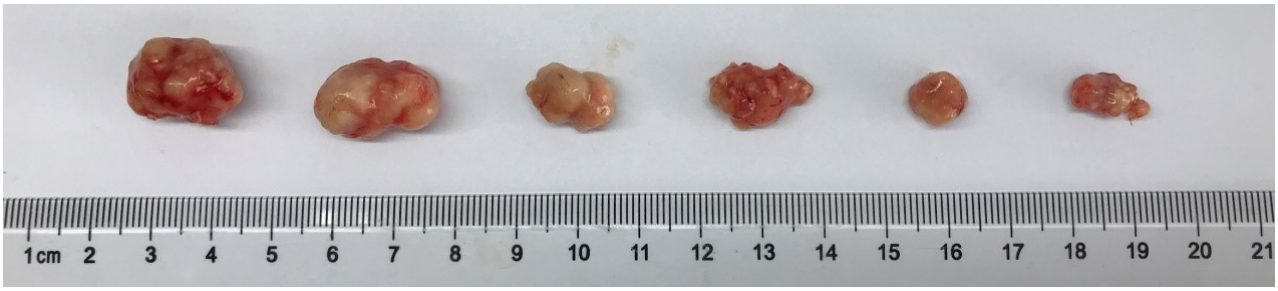

Figure S16. Representative photographs of tumor tissues in NS, 1-C12G1, free DOX, 1-C12G1/miR-21i, 1-C12G1@DOX and 1-C12G1@DOX/miR-21i groups on the 16th day.

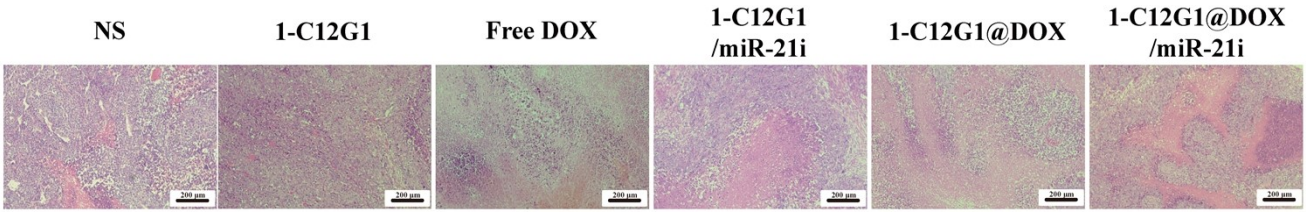

Figure S17. H&E-stained xenografted tumor sections from mice injected with NS, 1-C12G1, free DOX, 1-C12G1/miR-21i, 1-C12G1@DOX or 1-C12G1@DOX/miR-21i at 16 days post-injection. Scale bar in each panel represents 200 μm.

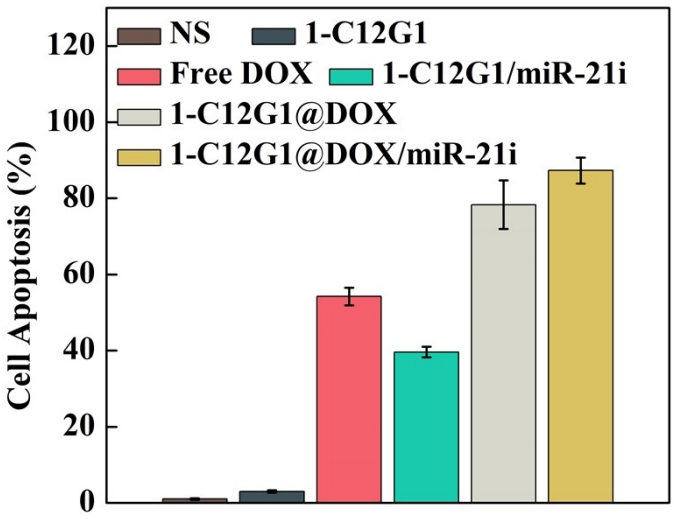

Figure S18. Apoptosis rates of tumor cells after different treatments by quantification of the TUNEL-positive tumor cells in tumor sections.

References

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2. L. Chen, L. Cao, M. Zhan, J. Li, D. Y. Wang, R. Laurent, S. Mignani, A.-M. Caminade, J.-P. Majoral and X. Y. Shi, *Biomacromolecules*, 2022, **23**, 2827-2837.

3. C. Song, Y. Xiao, Z. Ouyang, M. Shen and X. Shi, *J. Mater. Chem. B*, 2020, **8**, 2768-2774.