Supporting Information

Functional Biodegradable Nitric Oxide Donor-Containing Polycarbonate-Based Micelles for Reduction-Triggered Drug Release and Overcoming Multidrug Resistance

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Experimental Section

Materials.

3-Methyl-3-oxetanemethanol (97%, Energy Chemical), hydrobromic acid (HBr, 48 wt% in H_2O, Energy Chemical), silver nitrate ($AgNO_3$, Nanjing chemical reagent co. Ltd), triethylamine (Et_3N, 99.5%, Energy Chemical), zinc bis[bis(trimethylsilyl)amide] (97%, Aldrich), reduced glutathione (GSH, 98%, Beyotime Biotechnology), and doxorubicin hydrochloride (DOX·HCl, 98%, Energy Chemical) were used as received. Methoxy poly(ethylene glycol) (mPEG, M_n = 5.0 kg/mol, Fluka) were dried by azeotropic distillation from anhydrous toluene. ε-Caprolactone (ε-CL, 99%, Alfa Aesar) was dried over CaH_2 and distilled under reduced pressure prior to use. Ethyl chloroformate was freshly distilled before use. Dichloromethane (DCM) was dried by refluxing over CaH_2 under an argon atmosphere prior to distillation. Toluene was dried by refluxing over sodium wire under an argon atmosphere prior to distillation. For cell culture experiments, MCF7/DOX^R cells were cultured in Dulbecco's modified eagle's medium (DMEM) with high glucose, supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, and sodium pyruvate. The medium and supplements were purchased from Life Technologies (Nanjing, China). Cells were cultured at 37 °C in a constant humidity condition with 5% CO_2.

Characterization

^{1}H NMR spectra were measured on a Bruker ECX 400 (Germany), and the chemical shifts were calibrated against residual solvent peaks as the internal standard. Gel permeation chromatograph (GPC) fitted with a column (GPC-300, 7.8 × 300mm, 5 μm, 300 Å, Sepax) was used to determine the molecular weight and polydispersity of the copolymers. The measurements were performed using N,N-dimethylformamide as a mobile phase at the flow rate of 1.0 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the column. Dynamic light scattering (DLS, Anton Paar Litesizer 500) and transmission electron microscopy (TEM, FEI Philips Tecnai 20) were utilized to determine the size of the micelles. Fluorescence images were recorded by a fluorescence microscope (IX73, Olympus, Japan). Flow cytometer analysis was performed using a FACSCalibur (BD Accuri C6). In vivo imaging was performed using a near-infrared fluorescence imaging system (Vilber Lourmat).

Synthesis of cyclic nitrate trimethylene carbonate (NTC)

NTC was synthesized in three steps. Briefly, 3-methyl-3-oxetanemethanol (10.20 g, 99.87 mmol) were dissolved in tetrahydrofuran (THF, 100 mL), followed by dropwise addition of HBr (48 wt%, 40 mL) at 0 °C. After stirring at room temperature for 12 h, the reaction mixture was diluted with distilled H_2O (150 mL), and extracted with DCM (3 × 150 mL). The combined organic phase was dried by anhydrous Na_2SO_4, and the filtrate was concentrated under reduced pressure to give the desired product (2-(bromomethyl)-2-methylpropane-1,3-diol) as a white solid. Yield: 17.37 g (95%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.28 (s, 2H, –(*OH*)$_2$), 3.63 (s, 4H, –C(*CH*$_2$OH)$_2$), 3.50 (s, 2H, –*CH*$_2$Br), 0.92 (s, 3H, –*CH*$_3$).

2-(Bromomethyl)-2-methylpropane-1,3-diol (10.00 g, 54.63 mmol) dissolved in acetonitrile (100 mL) was added with $AgNO_3$ (27.84 g, 163.89 mmol), and the mixture was stirring at 75 °C for 12 h. After filtration, the filtrate was diluted with distilled H_2O (200 mL) and extracted with DCM (3 × 200 mL). The combined organic phase was dried by anhydrous Na_2SO_4, and the filtrate was concentrated under reduced pressure to yield 3-hydroxy-2-(hydroxymethyl)-2-methylpropyl nitrate as a pale-yellow oil. Yield: 7.76 g (86%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.43 (s, 2H, –*CH*$_2$NO$_2$), 3.27 (s, 4H, –C(*CH*$_2$OH)$_2$), 0.83 (s, 3H, –*CH*$_3$).

Finally, a stirred solution of 3-hydroxy-2-(hydroxymethyl)-2-methylpropyl nitrate (7.00

g, 42.39 mmol) and ethyl chloroformate (9.20 g, 84.77 mmol) in dried THF was dropwise added with Et_3N (9.87 g, 97.49 mmol) at 0 °C. The reaction mixture was stirring under room temperature. After 12 h, the mixture was diluted with H_2O and extracted with DCM. The combined organic phase was dried by anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was further purified by recrystallization in THF/diethyl ether to give the desired product NTC as an offwhite solid. Yield: 2.43 g (30%). m.p.: 113-114 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.60 (s, 2H, –CH_2NO_2), 4.30 (q, 4H, –C($CH_2O)_2CO$), 1.04 (s, 3H, –CH_3). ^{13}C NMR (300 MHz, DMSO-d_6): δ 147.79, 74.36, 73.12, 31.97, 16.46. MS(ESI):m/z $C_6H_9NO_6$ $[M+H]^+$=192.0531 (Calcd for $C_6H_9NO_6$ 192.0508). Elemental analysis, calculated: C, 37.70; H, 4.75; N, 7.33. Found: C, 37.75; H, 4.54; N, 7.24.

Ring-opening polymerization

The ring-opening polymerization of NTC was carried out in dried DCM at 40 °C using mPEG (M_n = 5.0 kg/mol) as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst. Typically, in a glovebox under a nitrogen atmosphere, to a stirred solution of mPEG and NTC in dried DCM at a desired M_n was quickly added with zinc bis[bis(trimethylsilyl)amide] catalyst. Then, the reaction flask was sealed and placed in an oil bath at 40 °C with magnetic stirring for 12 h. The reaction mixture cooled down to room temperature, followed by the addition of acetic acid as the terminator. The resulting copolymer was isolated by precipitation in cold diethyl ether and dried in vacuo at room temperature.

Preparation and characterization of NO micelle (NO-M)

NO-M was prepared by the solution exchange method. Typically, 1.0 mL of phosphate buffer (PB, 10 mM, pH 7.4) was dropwise added to 0.2 mL of polymer solution in DMF (10 mg/mL) under sonication at room temperature. The resulting solution was dialyzed against PB with a MWCO (molecular weight cut off) of 3500 to remove organic solvent. The size and size distribution of NO-M were characterized by DLS at 25 °C and TEM.

GSH-triggered micelle disassembly and NO release

The size change of NO-M in the presence of 10 mM GSH at 25 ºC was monitored by DLS. NO-M samples at a concentration of 2.0 mg/mL were prepared as above-mentioned and divided into two parts with equal volume (1.0 mL). 10 μL of GSH solution (1.0 M in deionized water) was added into one part with a final GSH concentration of 10 mM. The

sample was stirred gently at 25 ºC. The NO-M sample without GSH was used as a control and performed under otherwise the same conditions.

The amount of NO released from NO-M in PB was determined by Griess reagent (Beyotime Biotechnology). Briefly, NO-M (approximately 100 μM NO) in PB was placed into a shaker at 120 rpm at 37 ºC in the presence or the absence of 10 mM GSH. At desired time intervals, 50 μL of release media was collected to mix with Griess reagent and incubated at room temperature for 10 min. After that, the mixture was measured by UV at 540 nm. NO release experiments were conducted in triplicate.

Loading and GSH-triggered release of DOX

DOX solution in DMSO (20 mg/mL) was first added into 0.1 mL of NO-M solution in DMF (20 mg/mL) at a DOX feeding ratio of 5,10, 15 and 20%, followed by dropwise addition of 1.0 mL of PB under sonication. The resulting DOX-loaded NO-M (NO-M@DOX) was dialyzed against PB with a MWCO of 3500 at room temperature for 6 h to remove organic solvent and unloaded DOX. The drug loading content (DLC) and drug loading efficiency (DLE) were determined by UV at 480 nm and calculated according to a DOX standard curve. DLC and DLE were calculated according to the following formulas:

DLC (wt %) = (weight of loaded drug/total weight of polymer and loaded drug) × 100%

DLE (%) = (weight of loaded drug/weight of drug in feed) × 100%

The DOX release profiles of NO-M@DOX were studied at 37 °C in two different media: PB (10 mM, pH 7.4) and PB (10 mM, pH 7.4) containing 10 mM GSH. Briefly, 1.0 mL of NO-M@DOX solution was sealed in a dialysis bag (MWCO: 12000 Da) and placed into 20 mL of the corresponding release medium. At the predetermined time intervals, 5.0 mL of release medium was collected and replenished with an equal volume of fresh medium. The amount of DOX in release medium was measured by a microplate reader at the excitation of 480 nm and calculated according to a DOX standard curve in the corresponding medium. Release experiments were performed with three times, and the results were presented as the average ± standard deviation.

Intracellular NO release

The intracellular NO release from NO-M was determined by Griess reagent in MCF7/DOX^R. Briefly, MCF7/DOX^R cells (5000 cells/well) were seeded in a 96-well plate. After 12 h, the culture medium was replaced by 100 μL of phenol red free Dulbecco's Modified Eagle

medium (DMEM, Life Technologies) containing NO-M (approximately 8 μM NO). At the predetermined time points, 50 μL of medium was collected to mix with Griess reagent for 10 mins. The amount of NO was determined by a microplate reader at the absorbance of 540 nm. To more clearly detect the intracellular NO production, MCF7/DOX^R cells were seeded in 96-well plate (5000 cells/well) for 12 h, and the medium was replaced by 100 μL of DMEM with 4-amino-5-methylamino-2',7'-difluoresceindiacetate (DAF-FM DA, Beyotime Biotechnology, 5 μM) for further 30 min incubation at 37 ºC. After that, the cells were washed twice with PBS, and 10 μL of NO-M (10 mg/mL) were added for another 4 h incubation. The cells were rinsed thrice with PBS and fixed with 4% paraformaldehyde. The cells were washed three times with PBS and stained with DAPI. The cells were further washed with PBS. Fluorescence images of cells were acquired by a fluorescence microscope (IX73, Olympus, Japan) with the excitation/emission at 470/585 nm.

Western blot analysis

MCF7/DOX^R cells were incubation with different concentrations of NO-M for 24 h, and then the cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM sodium vanadate, 1% Nonidet P-40, and protease inhibitors (Selleck). The cell lysates were harvested by centrifuging at 10000 rpm for 20 mins under 4 °C. The supernatants were mixed with loading buffer and separated by SDS-PAGE (10 % gel). The purified protein was transferred onto a polyvinylidene difluoride membrane (Roche), and then incubated in skimmed milk solution, followed by incubation in 1/1000 of anti-ABCB1 rabbit antibody (Cell Signaling Technology) overnight to detect the desirable proteins, followed by HRP-conjugated secondary antibody and ECL detection kit. Finally, the membranes were photographed using Alpha Innotech Fluor Chem FC2 imaging system (Gel Doc ^{TM} EE imager, CA).

Intracellular uptake

MCF7/DOX^R cells (5000 cells/well) were seeded in a 96-well plate. After 12 h incubation, 10 μL of NO-M (10 mg/mL) were added to treat the cells for 24 h. After that, the culture medium was replaced by 100 μL of fresh medium containing free DOX (5.0 μg/mL), and the cells were incubated for another 3 and 6 h at 37 °C. Afterward, the medium was removed and the cells were washed twice with PBS, followed by fixing with 4% paraformaldehyde. The cells were washed three times with PBS, and then the cell nuclei were stained with DAPI. Fluorescence images of cells were acquired by fluorescence microscope. The amount of DOX

accumulated in the cells was further quantified by flow cytometry. The cellular uptake of DOX into the cells without NO-M treatment of was also investigated under the same otherwise conditions.

Intracellular DOX release

MCF7/DOX^R cells (5000 cells/well) were seeded in a 96-well plate, and after 12 h incubation, NO-M@DOX and free DOX (DOX concentration: 5.0 μg/mL) were added. After 6 or 24 h incubation, the cells were washed twice with PBS and fixed with 4% paraformaldehyde, and the cell nuclei were stained with DAPI. Fluorescence images of cells were acquired by fluorescence microscope (IX73, Olympus).

Cytotoxicity by MTT assay

The cytotoxicity of NO-M was investigated by MTT assay using MCF7/DOX^R cells. MCF7/DOX^R cells (1.0×10^4 cell/well) were seeded in a 96-well plate, and after 24 h incubation, 10 μL of NO-M samples with various concentrations (1.0, 2.0, 5.0, 10, and 20 mg/mL) were added. The cells were incubated for 24 h, and then 10 μL of MTT (5.0 mg/mL) was added. After incubation for another 4 h, the medium was removed and 150 μL of DMSO were added to dissolve the resulting purple crystals. The absorbance of the solution at 570 nm was measured by a microplate reader. The cells cultured without any treatment were used as a control. All the experiments were carried out in triplicate.

To estimate the cytotoxicity of NO-M@DOX, various concentrations of free DOX or DOX-loaded in micelles were co-cultured with MCF7/DOX^R cells for 24 h. The following experimental procedure was performed in the same way as the cytotoxicity test of NO-M.

Blood circulation

The blood circulation experiments were carried out in healthy nude mice (Model Animal Research Center of Nanjing University, Nanjing, China). All the animal experiments were handled in compliance with the Animal Management Rules (Ministry of Health, People's Republic of China) and the guidance for Care and Use of Laboratory Animals (China Pharmaceutical University). NO-M@DOX and free DOX (7.5 mg DOX/kg) were injected through the tail vein of nude mice. At designed time points post-injection, 10 μL of blood were acquired from retro-orbital sinus of nude mice. The blood samples were dissolved in 50 μL of 1% Triton X-100 under sonication, followed by addition of 0.3 mL of DMSO to extract

DOX. After centrifugation at 11000 rpm for 20 mins, the supernatant was collected and measured the fluorescence intensity by a microplate reader with the excitation of 480 nm. The DOX level was obtained according to a standard curve of DOX.

In vivo biodistribution and imaging

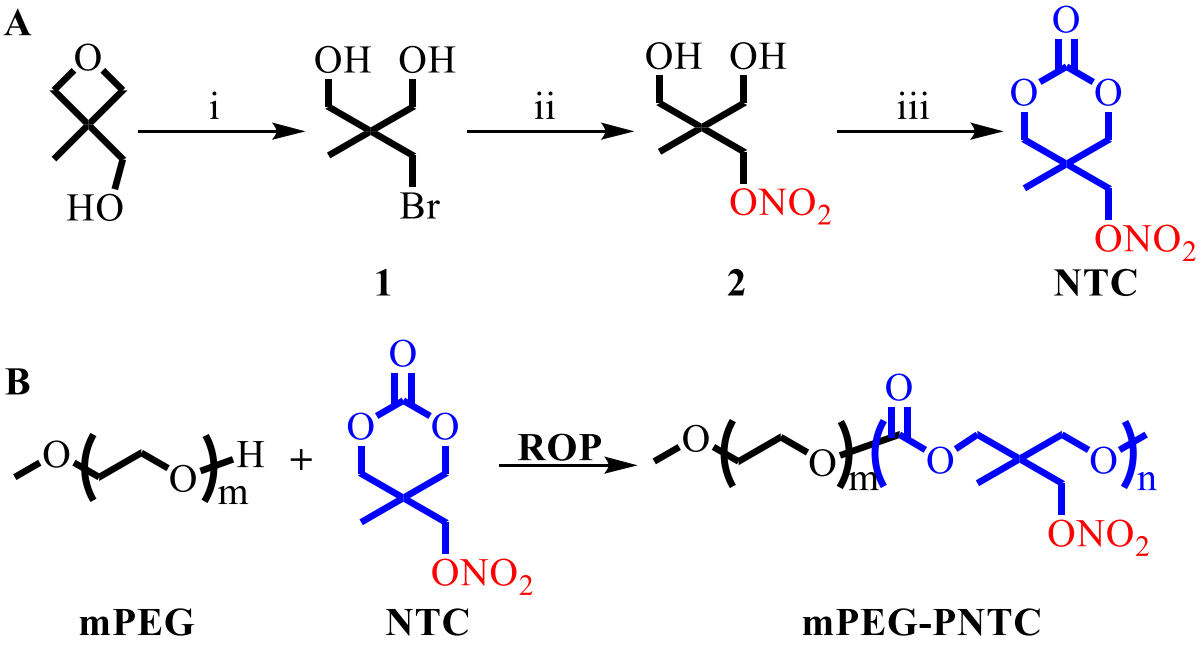
The MCF7/DOX^R breast tumor model was established by subcutaneous inoculation of MCF7/DOX^R cells (1.0×10^7 per mouse) in 50 μL of PBS into the right hind flank of the nude mouse. After 30 days, the tumor size reached approximately 150-200 mm^3, and the tumor-bearing mice were randomly grouped and injected with a single dose of NO-M@DOX and free DOX (7.5 mg/kg) in 200 μL of PBS via tail vein. At 10 h post-injection, the tumor-bearing mice were sacrificed. The tumor blocks and major organs including heart, liver, spleen, lung, and kidney were excised, collected, washed, weighted, and homogenized in 0.5 mL of 1% Triton X-100. The extraction solution of HCl/isopropanol (1.0 mL) was added to the tissue lysates. After extraction overnight, the samples were centrifuged for 30 min. The fluorescence intensity of supernatant was determined by microplate reader to calculate the amount of DOX.

In order to monitor the fluorescence imaging of NO-M in vivo, DIR was loaded into NO-M (NO-M@DIR) with a final DIR concentration of 20 μg/mL. MCF7/DOX^R tumor-bearing nude mice were intravenously injected with NO-M@DIR in 200 μL of PBS. At designed time intervals (4, 12, 24 h) post-injection, the fluorescent imaging was monitored using a FUSION FX7 SPECTRA multiapplication imager at excitation of 747 nm and emission of 774 nm (Vilber Lourmat).

In vivo therapeutic efficiency

MCF7/DOX^R breast tumor-bearing nude mice with tumor volume about 50 mm^3 were randomly assigned into four groups (5 mice in each group, n = 5) and treated with PBS, free DOX (7.5 mg DOX/kg), NO-M (20 mg NO-M/kg), and NO-M@DOX (7.5 mg DOX/kg and 20 mg NO-M/kg). All the samples were intravenously administrated on days 0, 3, 6, 9, 12 and 15. The body weight and the tumor volume were noted every 2 days during the treatment. The tumor volume was calculated as the formula $V= W^2 \times L/2$ (W and L were the width and length of tumor, respectively). The relative tumor volume was obtained as V/V_0 (V_0 is the tumor volume on day 0). The relative body weight of the mice was calculated as W/W_0 (W_0 is the body weight of the mouse on day 0). On day 16, the mice were sacrificed and the tumors and organs including, heart, liver, spleen, lung, and kidney were collected, followed by

hematoxylin and eosin (H&E) staining and immunohistochemical examinations (P-gP, Ki67 and Caspases 3). The sliced organ and tumor tissues mounted on the glass slides were observed by a digital microscope (Olympus CX23).



Scheme S1. (A) Synthetic route of NTC monomer: (i) 40% hydrobromic acid, room temperature, overnight, THF; (ii) silver nitrate, 75 ºC, 12 h, CH_3CN; (iii) ethyl chloroformate, Et_3N, 0 ºC, 4 h, THF; (B) Synthesis of biodegradable mPEG-PNTC copolymer by ring-opening polymerization in dried DCM at 40 ºC (initiator: mPEG with M_n of 5.0 kg/mol; catalyst: zinc bis[bis(trimethylsilyl)amide]).

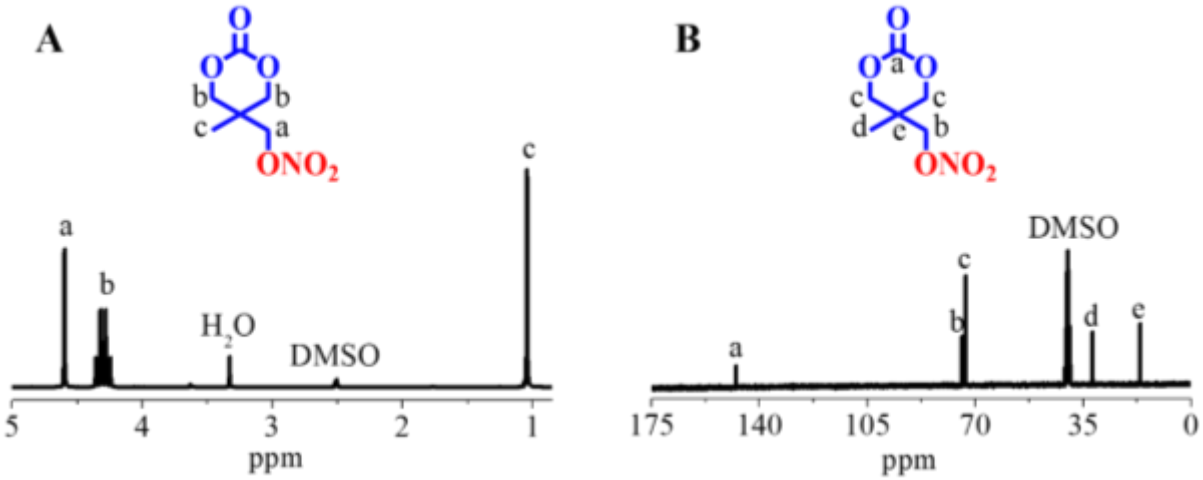
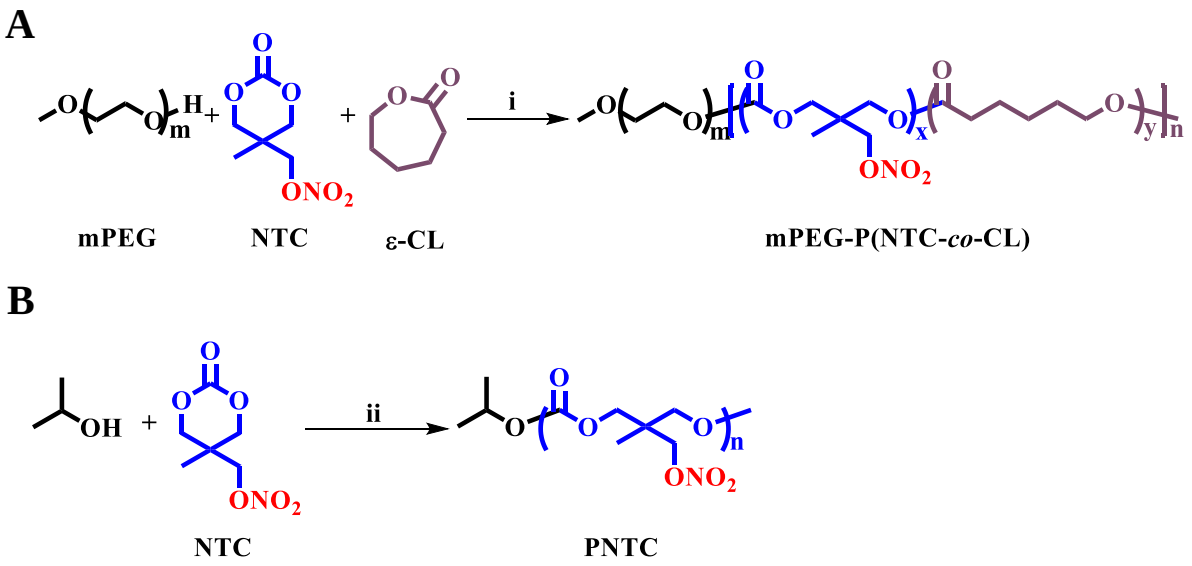


Figure S1. ^{1}H NMR spectrum (A) and ^{13}C NMR (B) spectrum of NTC monomer (300 MHz, DMSO-d_6).



Scheme S2. Synthesis of biodegradable mPEG-P(NTC-*co*-CL) (A) and PNTC (B) polymers by ring-opening polymerization using mPEG (M_n = 5.0 kg/mol) and isopropanol as an initiator, respectively. Conditions (i and ii): zinc bis[bis(trimethylsilyl)amide] as a catalyst, dried DCM, 40 ºC.

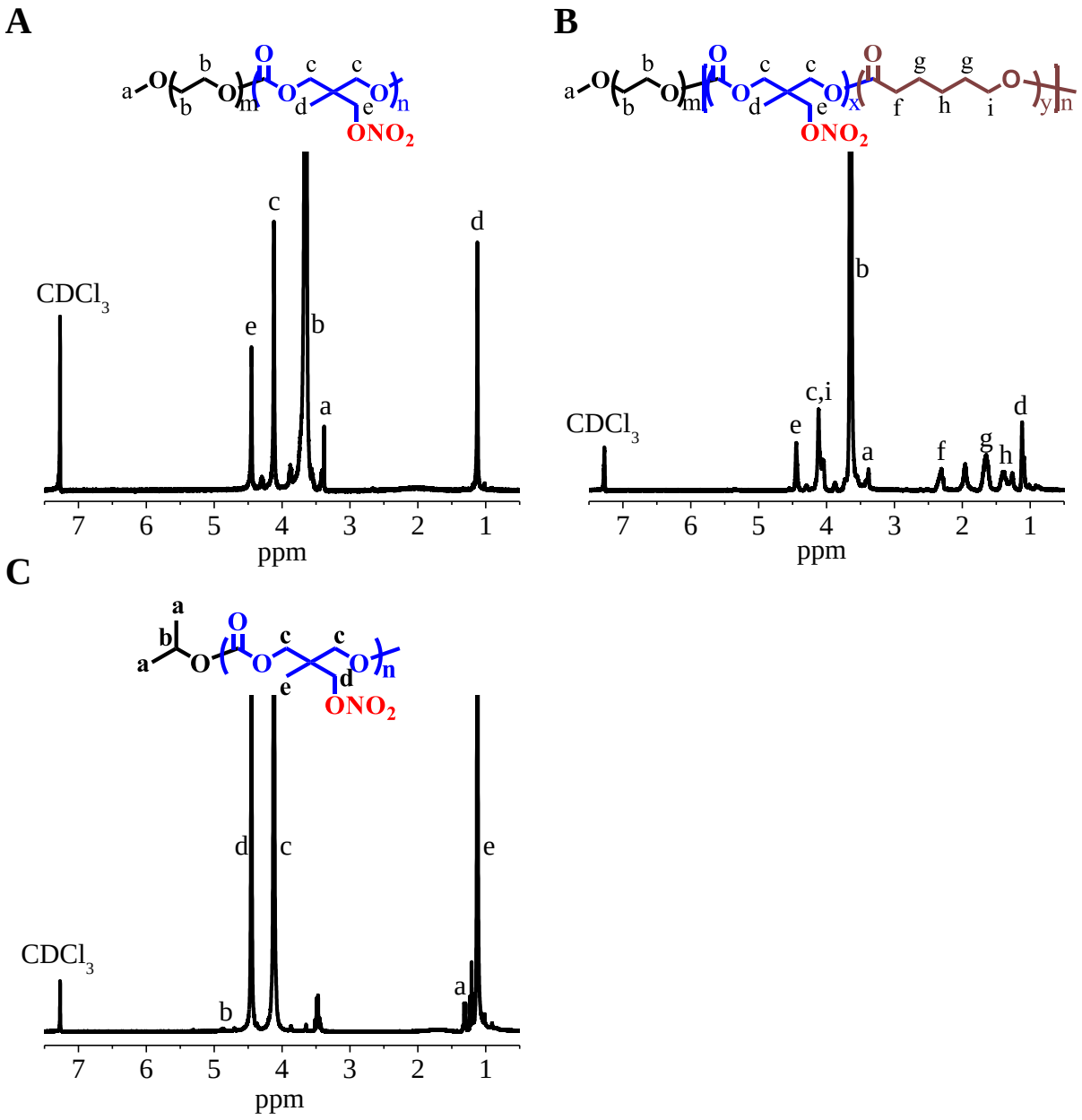


Figure S2. ^{1}H NMR spectra of biodegradable mPEG-PNTC (A), mPEG-P(NTC-*co*-CL) (B) and PNTC (C) (300 MHz, $CDCl_3$).

Table S1. Synthesis of biodegradable mPEG-PNTC and mPEG-P(NTC-*co*-CL) copolymers by ring-opening copolymerization.

Entry	polymer	f [(a)] (%)	F [(b)] (%)	M_n (kg/mol)			M_w/M_n GPC[(c)]
				Theory	^{1}H NMR	GPC[(c)]	
1	mPEG-PNTC$_{3.1k}$	37.5	38.7	5.0-3.0	5.0-3.1	8.7	1.12
2	mPEG-PNTC$_{4.8k}$	50.0	48.0	5.0-5.0	5.0-4.8	9.9	1.33
3	mPEG-PNTC$_{9.4k}$	66.7	62.4	5.0-10.0	5.0-9.4	15.1	1.20
4	mPEG-PNTC$_{14.3k}$	75.0	74.2	5.0-15.0	5.0-14.3	19.8	1.28
5	mPEG-P(NTC$_{2.1k}$-*co*-CL$_{2.3k}$)	25.0	23.9	5.0-(2.5-*co*-2.5)	5.0-(2.1-*co*-2.3)	9.6	1.22
6	PNTC$_{8.0k}$	100	100	7.6	8.0	8.1	1.02

[a] Mass fraction of NTC monomer in feed; [b] Mass fraction of NTC units in the resulting copolymer determined by ^{1}H NMR (300 MHz, $CDCl_3$); [c] Determined by GPC (eluent, DMF; flow rate, 1.0 mL/min; standards, polystyrene).

Table S2. Characteristics of NO-M@DOX [a].

DLC (wt %)		DLE (%)	Size [c]	PDI [c]
Theory	Determined [b]			
5	2.8	56.0	125.5±0.5	0.20
10	6.9	68.6	128.8±1.4	0.23
15	10.1	67.1	130.1±3.0	0.17
20	12.8	64.0	130.4±4.1	0.21

[a] The concentration of micelles was set at 2.0 mg/mL; [b] Drug loading content (DLC) and drug loading efficiency (DLE) were determined by fluorescence measurements; [c] Determined by DLS.

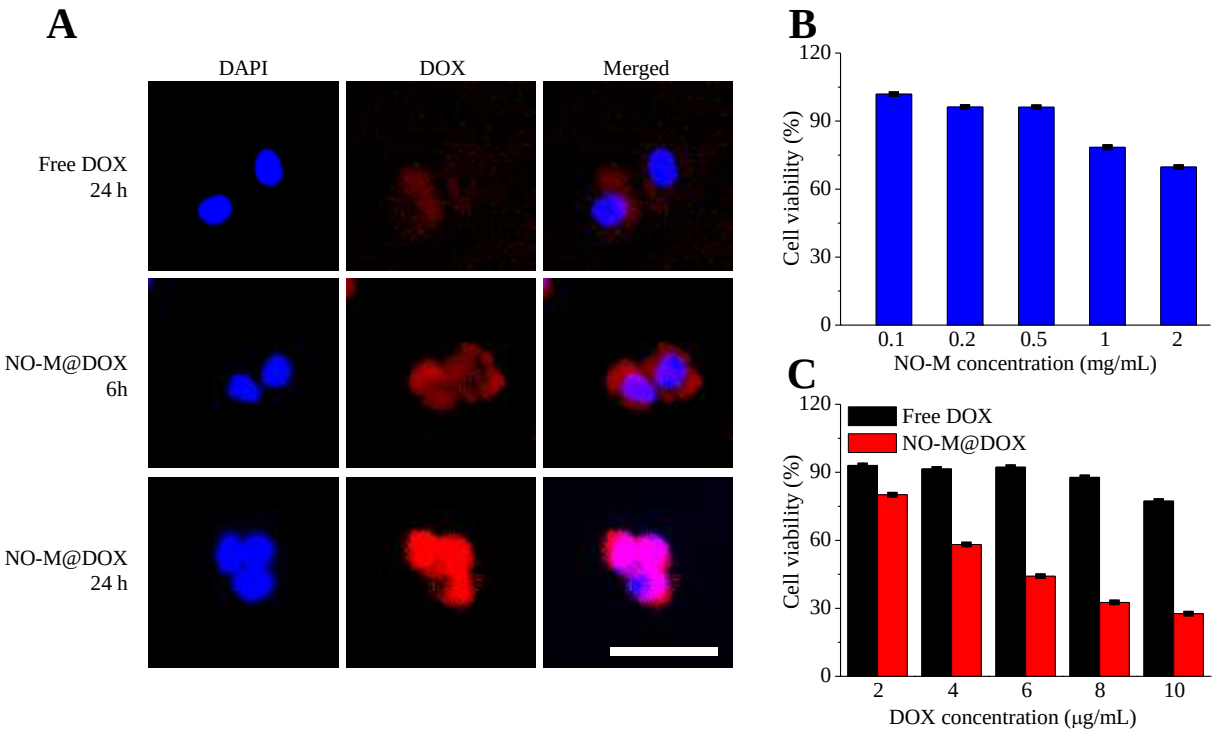


Figure S3. (A) Intracellular uptake of free DOX and NO-M@DOX in MCF7/DOX^R cells observed by fluorescence microscopy (DOX concentration: 5.0 μg/mL, the scale bar represents 50 μm for each image); In vitro cytotoxicity of NO-M with different concentrations (B) and NO-M@DOX with different DOX concentrations (NO-M concentration at 1.0 mg/mL, C) against MCF7/DOX^R cells for 24 h incubation determined by MTT assay.

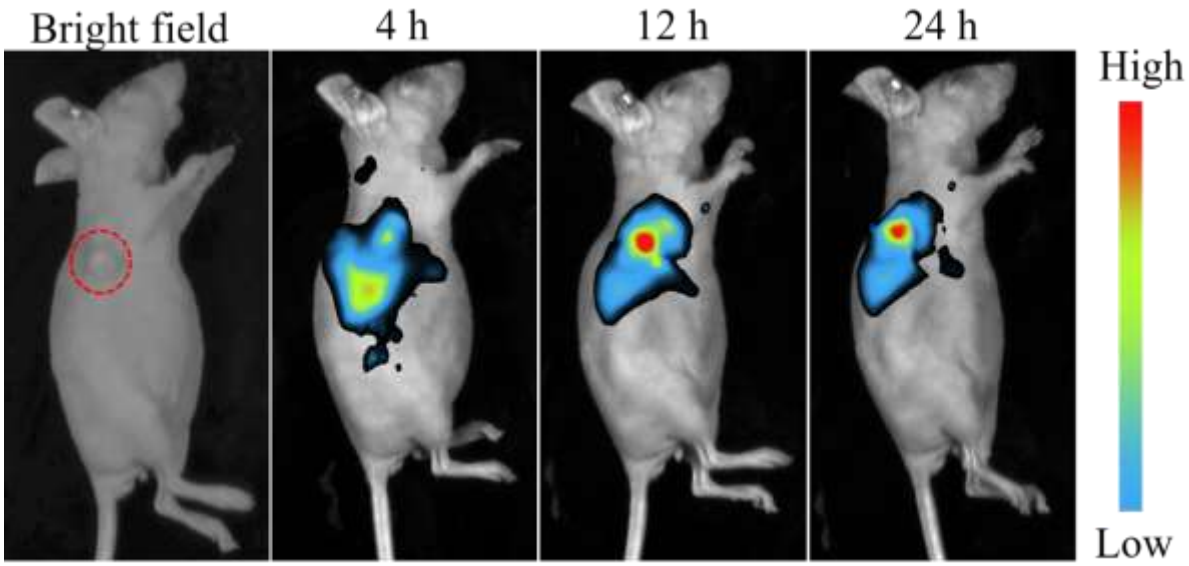


Figure S4. In vivo fluorescence images of MCF7/DOX^{R} breast tumor-bearing nude mice at different time points following intravenous injection with NO-M@DIR (DIR concentration: 20 μg/mL). The images were acquired and analyzed using a FUSION FX7 SPECTRA multiapplication imager.

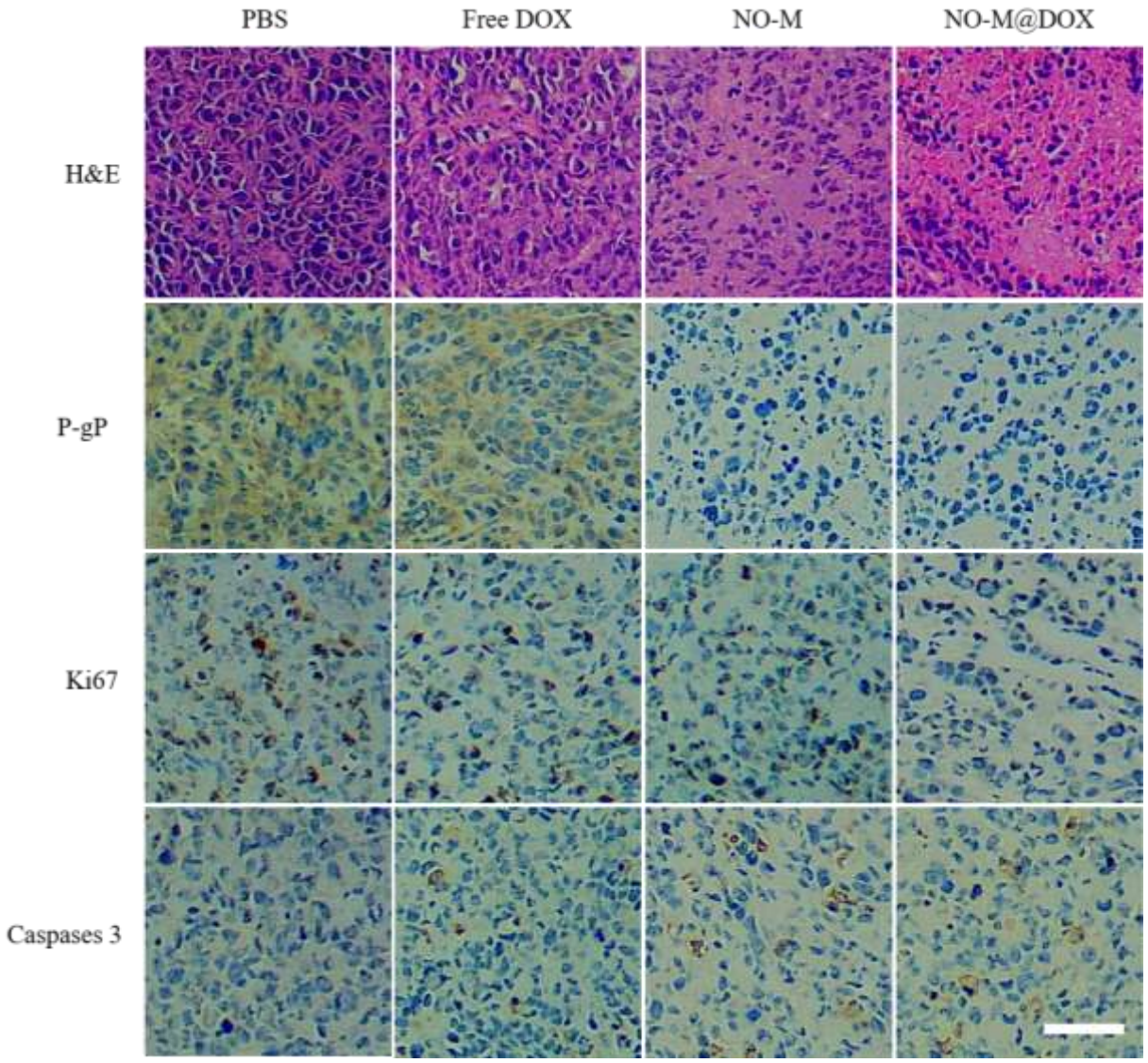


Figure S5. Histopathological (H&E staining) and immunohistochemical (P-gP, Ki67 and Caspase 3) analysis on tumor tissues from MCF7/DOX^{R} breast tumor-bearing nude mice after 16 days treatment with PBS, free DOX, NO-M, and NO-M@DOX, respectively. The scale bar represents 100 μm for each image (the images were observed by an Olympus CX23 microscope at a magnification of 100).

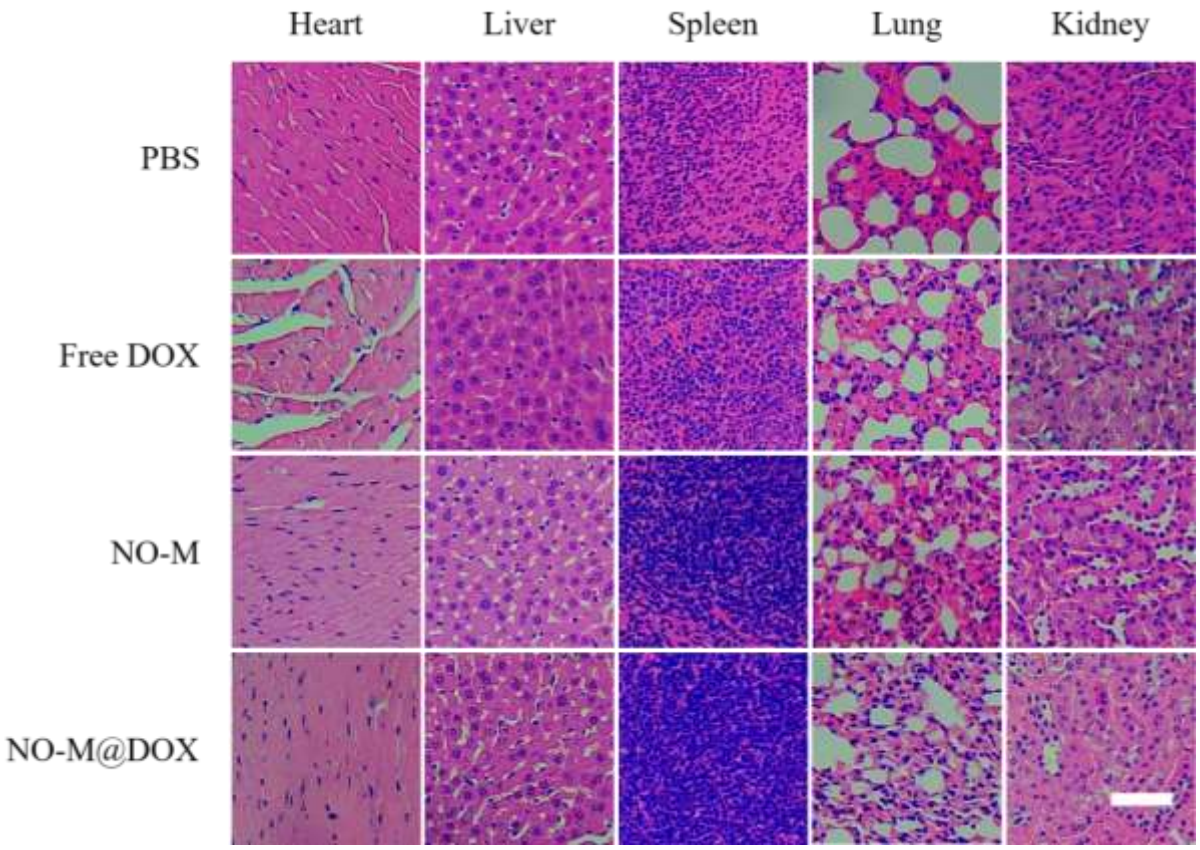


Figure S6. Hematoxylin & eosin (H&E) staining images of normal organs including heart, liver, spleen, lung and kidney observed by microscopy. The scale bar represents 100 μm for each image (the images were observed by an Olympus CX23 microscope at a magnification of 100).