Electronic Supporting Information

pH-Responsive, Two-in-One Doxorubicin and Bcl-2 siRNA-Loaded Micelleplexes for Triple-Negative Breast Cancer Therapy

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Materials

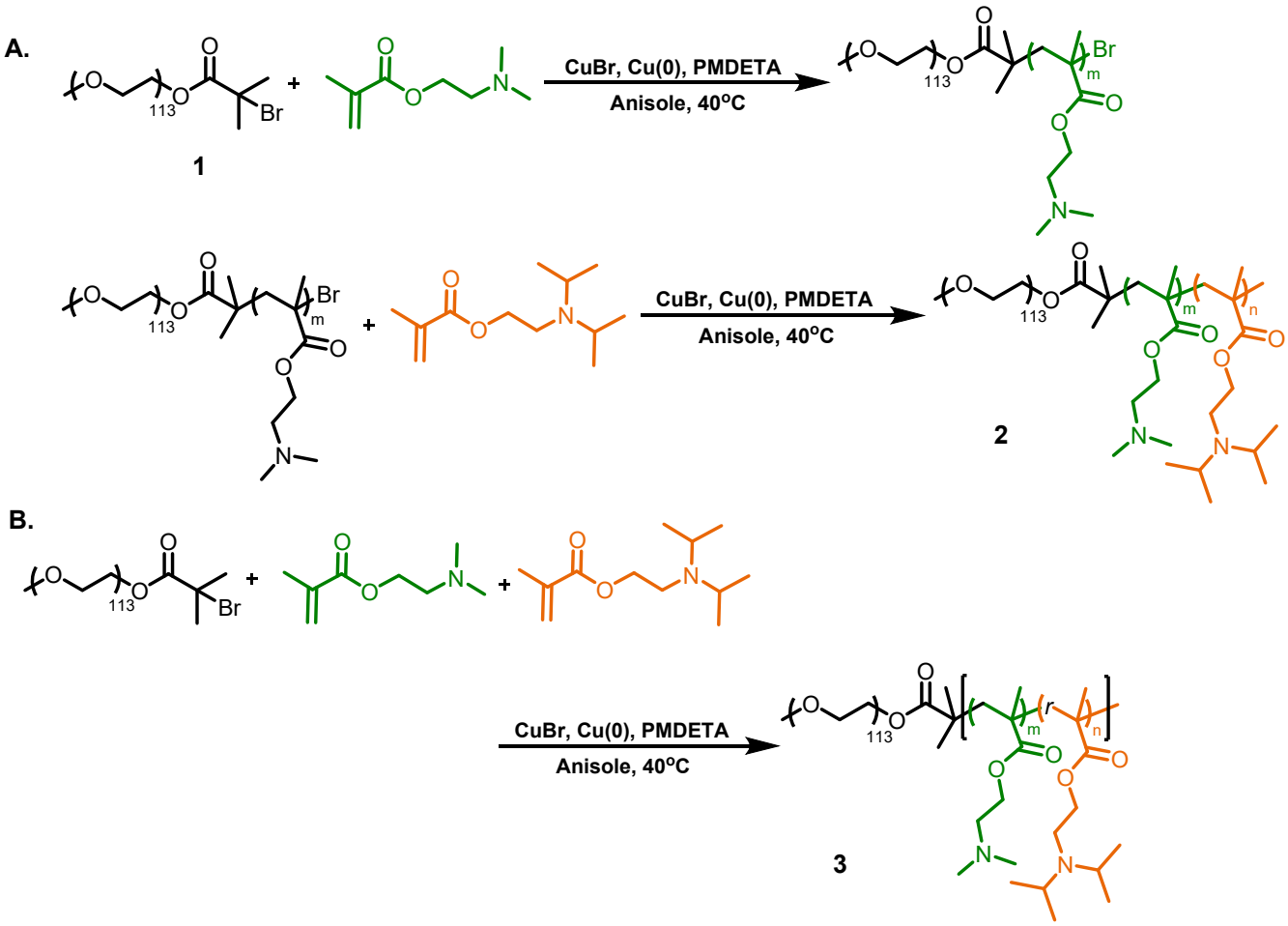
2-(Dimethylamino) ethyl methacrylate (DMAEMA, Sigma-Aldrich, contains 700-1000 ppm monomethyl ether hydroquinone as inhibitor, 98%) and 2-(diisopropylamino)ethyl methacrylate (DPA, Sigma-Aldrich, contains ~100 ppm monomethyl ether hydroquinone as inhibitor, 97%) was passed through basic Al_2O_3 to remove inhibitors. Poly(ethylene glycol) methyl ether (M_n = 5000 g/mol) (Aldrich), α-bromoisobutyryl bromide (BiBB, Alfa Aesar, 97%), triethylamine (TEA, Alfa Aesar, 99%), *N,N,N′,N″,N‴*-pentamethyldiethylenetriamine (PMDETA, Aldrich, 99%). Doxorubicin hydrochloride (DOX·HCl, Sigma-Aldrich, Pharmaceutical Secondary Standard; Certified Reference Material). Copper wire (1 cm) was soaked in HCl to clean the oxide off of copper. RNase/DNase-free water was prepared by adding diethyl pyrocarbonate (DEPC, purchased from MDBio Inc., Taiwan) into DIW (0.1% v/v). The solution was stirred for 1 day and then was autoclaved to remove DEPC. Negative control siRNA oligo duplex and FAM-labeled negative control siRNA oligo duplex were purchased from MDBio Inc., Taiwan.

Instrument

NMR spectroscopy was used for characterization of chemical structure and calculation of monomer conversion. The spectra were recorded on a Bruker-400 spectrometer at 298 K. The chemical shifts in ^{1}H NMR were shown in ppm refer to residual solvent in $CDCl_3$ as δ 7.24 ppm. Molecular weight distribution of PEG-*b*-PDMAEMA-*b*-PDPA were determined via gel permeation chromatography (GPC) (UltiMate® 3000 HPLC system, Thermo Scientific Dionex) with Waters columns (Styragel® HR 2 DMF, Styragel® HR 4 DMF, and Styragel® HR 5 DMF) using DMF with LiBr (0.1M) as the eluent at 80 °C with flow rate of 1 mL /min and Refractive Index Detector (RI-101, Thermo Scientific Dionex). The calibration was based on linear poly(ethylene glycol) (PEG) with molecular weight (2000, 5000, 17800, 31400, 72750 g/mol) with low polydispersity ($Đ$ = 1.01~1.05). UV-vis spectra were measured by ultraviolet-visible spectrophotometry, (SHIMADZU UV-1800). Fluorescence spectra were measured by fluorescence spectrophotometer (JASCO FP-6500). Hydrodynamic diameter was measured by dynamic light scattering (DLS) with a single-angle (173°) Malvern Zetasizer Nano S (He-Ne laser, λ = 633 nm) (Malvern Instruments, Worcestershire, U.K.). Condensation efficiency of siRNA by polyplexes

and micelleplexes using ethidium bromide is quantified by gel electrophoresis and imaged using the ChemiDoc MP imaging system (Biorad, Hercules, Ca, USA). The cumulative of drug release were measured by MultiMode Microplate Reader (SpectraMAX® M3, λ_{ex} = 480 nm, λ_{em} = 592 nm). Transmission electron microscope (TEM) images were captured by Philips, TECNAI 20 at 160 kV. Human epithelial breast cancer cells (MDA-MB-231) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum FBS and 1% penicillin-streptomycin (HyClone, Logan, UT) and 1 mM sodium pyruvate.

Methods



Scheme S1 (A) Synthesis of the block copolymer PEG-*b*-PDMAEMA-*b*-PDPA by ATRP. (B) Synthesis of the random copolymer PEG-*b*-(PDMAEMA-*r*-PDPA) by ATRP.

Synthesis of PEG_{113}-Br macroinitiator (1).

Typically, PEG_{113}-OH (1 g, 0.2 mmol) was dissolved in 10 mL of anhydrous DCM (10 mL) followed by the addition of TEA (84 μL, 0.6 mmol). BiBB (74.2 μL, 0.6 mmol) was then added into the solution under ice bath. This solution was stirred for 24 h at 25 °C and then extracted by DCM with 0.1M HCl and with NaCl (5% w/w) twice. The crude product was purified by precipitation in ether to obtain the white powder (yield = 88%). ^{1}H NMR ($CDCl_3$), δ (ppm): 4.33 (d, 2 H, -OCH_2COO-), 3.73 (m, 452H, -CH_2-), 3.36 (s, 3H, -OCH_3), 1.92 (s, 6H, $(CH_3)_2$CBrCOO-).

Preparation of poly(ethylene glycol)-*b*-poly(2-dimethylaminoethyl methacrylate)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (PEG-*b*-PDMAEMA-*b*-PDPA) triblock copolymer by ATRP (2).

DMAEMA (406 μL , 2.4 mmol), PMDETA (3.8 μL, 0.018 mmol), and anisole (4 mL) were added into a schlenk flask. After three cycles of freeze-pump-thaw process, the mixture was injected into another schlenk flask with PEG_{113}-Br (300 mg, 0.6 mmol), $CuBr_2$ (1.4 mg, 0.006 mmol) and 1 cm length of copper wire under nitrogen. The polymerization was carried out at 40 °C. When the conversion is over 90% determined

by ^{1}H-NMR, the reaction was recovere to 25 °C. Then, DPA (674 μL , 3.6 mmol) and anisole (2 mL) treated with three cycles of freeze-pump-thaw process in another schlenk flask were injected into reaction. The polymerization was carried out at 40 °C for 12 h. The polymer product was obtained from the precipitation using cold hexane after the copper complex was removed by neutral alumina chromatography.

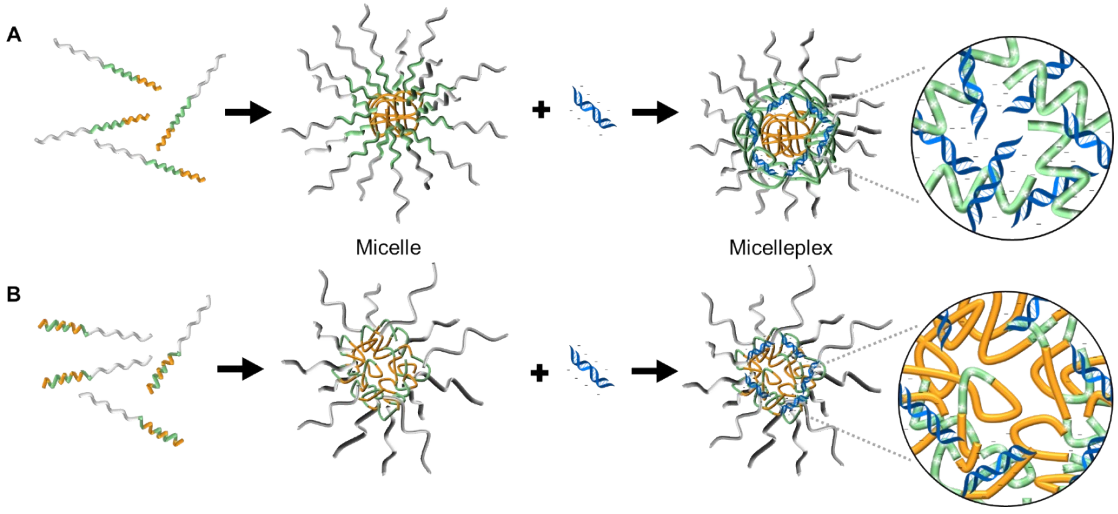
Preparation of poly(ethylene glycol)-*b*-(poly(2-dimethylaminoethyl methacrylate)-*r*-poly(2-(diisopropylamino)ethyl methacrylate)) (PEG-*b*-(PDMAEMA-*r*-PDPA)) by ATRP (3).

DMAEMA (406 μL , 2.4 mmol), DPA (674 μL , 3.6 mmol), PMDETA (3.8 μL, 0.018 mmol), and anisole (6 mL) were added into a schlenk flask. After three cycles of freeze-pump-thaw process, the mixture was injected into another schlenk flask with PEG_{113}-Br (300 mg, 0.6 mmol), $CuBr_2$ (1.4 mg, 0.006 mmol) and copper wire (1 cm) under nitrogen. The polymerization was carried out at 40 °C for 24 h. The polymer product was obtained from the precipitation using cold hexane after the copper complex was removed by neutral alumina chromatography.

Fabrication and colloidal property of PEG-*b*-PDMAEMA-*b*-PDPA and PEG-*b*-(PDMAEMA-*r*-PDPA) copolymer micelles.

The triblock copolymer micelles were prepared by nanoprecipitation. PEG-*b*-PDMAEMA-*b*-PDPA in THF (40 μg/mL, 100 μL) was added to phosphate buffer solution (3 mL, pH=7.4, 20 mM) under sonication, then the mixture was loaded into dialysis bag (molecular weight cut off, MWCO: 12000-14000 g/mol) against phosphate buffer solution (3 mL, pH=7.4, 10 mM) to remove THF for 24 h. Hydrodynamic diameter of the block and random copolymer micelles (1 mL, 0.5 mg/mL) were measured by dynamic light scattering (DLS) at 25 °C.

siRNA-condensed PEG-*b*-PDMAEMA-*b*-PDPA and PEG-*b*-(PDMAEMA-*r*-PDPA) copolymer micelleplexes.



Scheme S2 Proposed illustration of the condensation of siRNA by (A) PEG-*b*-PDMAEMA-*b*-PDPA and (B) PEG-*b*-(PDMAEMA-*r*-PDPA) copolymer self-assembled micelles through electrostatic interaction between positively-charged PDMAEMA and negatively-charged siRNA.

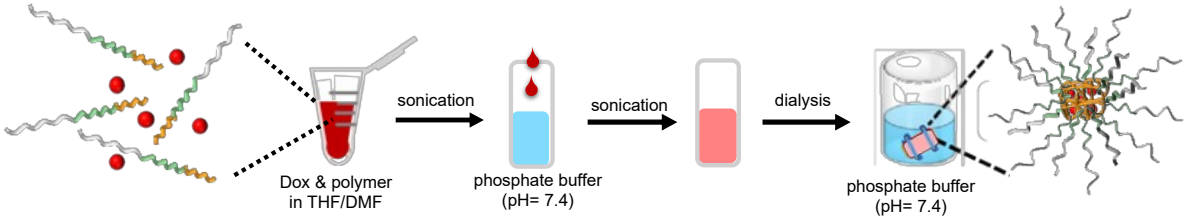
Micelleplexes were prepared by addition of as-prepared micelle solution (10 μL) to siRNA solution (2 μM, 10 μL) in RNase-free water to form complexes at various N/P ratios and incubated in water bath at 25 °C for 30 minutes.

siRNA loading efficiency of polyplexes and micelleplexes at various N/P ratios ranging from 1 to 20 by agarose gel electrophoresis.

The condensation efficiency of siRNA by micelleplexes were determined by agarose gel electrophoresis. The complexes were formulated by adding 5 μL of micelle solution (PEG-*b*-PDMAEMA-*b*-PDPA) to siRNA solution (2 μM, 5 μL) in RNase-free water to form complexes at various N/P ratios and incubate for 30 minutes. Solution of micelleplexes or polyplexes was mixed with 6× DNA loading dye (1 μL). Then 5 μL of the mixture was loaded onto 2% agarose gel containing 5 μg/mL ethidium bromide .

Electrophoresis was carried out at 120 V voltage for 25 min in 1× TBE running buffer. Finally, the results were analyzed by ChemiDoc MP imaging system (Biorad, Hercules, Ca, USA). The siRNA condensation efficiency were evaluated by using the following equation: siRNA condensation efficiency (%) = {1-[(band intensity of certain N/P)/(band intensity of N/P = 0)]}*100%

Preparation of Dox-loaded micelles.



Scheme S3 Illustrated procedure of preparing the doxorubicin-loaded micelles.

Hydrophonic doxorubicin was obtained by stiring DOX·HCl (10 mg) in DMF (5 mL) with 1:1.4 weight ratio of triethylene amine (TEA) at 25 °C for 12 h. The encapsulation of doxorubicin in micelles was prepared by nanoprecipitation technology. Briefly, PEG-*b*-PDMAEMA-*b*-PDPA (4 mg) in THF (200 μL) was mixed with Dox solution (300 μL) and added dropwisely into PB buffer (pH 7.4, 20 mM, 4

mL) under sonication. This solution was dialyzed (molecular weight cut off, MWCO: 12000-14000) against PB buffer to remove organic solvent and final volume was adjusted to 5 mL.

Dox encapsulation efficiency of micelles.

To measure the encapsulation efficiency of Dox in micelles, freeze-dring Dox-loaded micelles were dissolved in DMF to break the self-assemblies, then the absorption of liberated doxorubicin was determinated by UV-Vis spectrophotometry (SHIMADZU UV-1800) at 480 nm and quantified against calibration curve. The calibration curve was established by using free Dox at different concentration (0 − 20 μM) in DMF. Drug loading content (DLC) and drug loading efficiency (DLE) were thus calculated according to the following equations:

DLC (%) = [weight of loaded Dox / (weight of loaded Dox + weight of polymer in feed)] × 100%

DLE (%) = [weight of loaded Dox / (weight of Dox in feed)] × 100%

pH-Responsive Dox release profile.

Evaluation of pH-responsive Dox release behavior was determinated by dialysis

method. Briefly, 1 mL Dox-loaded nanoparticles solution (1 mg/mL) was sealed in dialysis bag (molecular weight cut off, MWCO: 12000-14000) and incubated in 30 mL PB bufer (pH = 5.0 or pH = 7.4) at 37 °C water bath under shaking at the speed of 100 rpm. At pre-determined time (1, 2, 4, 8, 12, 24, 36, 48, 60, 72 h), 1 mL of solution outside dialysis bag was withdrawn for testing and replenished with equal volume of fresh PBS. The fluoresence intensity of released Dox was detected by MultiMode Microplate Reader (SpectraMAX® M3) , and the concentration was calculated according to the calibration curve.

The cumulative Dox release was calculated by the following equation:

$$E\ (\%) = \frac{V_a \sum_1^{n-1} C_b + V_i C_n}{W_{Dox}} \times 100$$

Where E (%) represnts accumulative released percentage, W_{Dox} is the total amount of Dox in the micelles, V_i is the total volume of the release media (V_i = 30 mL), V_a is the volume of the replenished media (V_a = 1 mL), C_n represents the concentration of Dox of the n^{th} sample and C_b represents the concentrations of Dox from first to $(n-1)^{th}$ samples. The experiment is repeated in triplet for each time point.

Evaluation of siRNA release from micelleplexes at phosphate buffer (pH 7.4 and 5.0, *I* = 0.14 M).

The micelleplexes were formulated by adding 10 μL of PEG_{113}-*b*-$PDMAEMA_{54}$-*b*-$PDPA_{63}$ micelles (0.42 mg/mL) to 10 μL of FAM-labled siRNA solution (2 μM) at N/P ratio of 20 in RNase-free water and incubate for 30 minutes. The micelleplexes were diluted to 200 μL by phosphare buffer solution (pH 7.4 or 5.0, 0.14 M) and transfer to centrifugal filters (Amicon® Ultra 0.5 mL, MWCO: 50 kDa) in water bath at 37 °C under shaking at 100 rpm. At pre-determined time (1, 2, 4, 8, 12, 24 h), the solution was centrifugated to separate released FAM-labled siRNA (4 °C, 10,000 rcf) for 10 min, and free FAM-labled siRNA in collection was quantified by fluorescence spectrophotometer (JASCO FP-6500) (λ_{ex} = 494 nm · λ_{em} = 520 nm) against calibration curve and refer the siRNA cumulative release efficiency. The pellet was resplenished to 200 μL and continue to monitor the siRNA release from micelleplexes. In a control group (Fig. S5) according to abovementioned protocol, free FAM-labled siRNA was demonstrated to test the viability of experimental method. Within first hour, near 90% of release FAM-labled siRNA was collected in the bottom layer after

centrifugation, which showed viable way to evaluated the siRNA release from micelleplexes.

Quantitative reverse-transcriptase polymerase chain reaction

The expression of Bcl-2 mRNA was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). MDA-MB-231 (2×10^6 cells) were seeded in two 12-well plates containing complete RPMI-1640 medium with 10% FBS, 1% penicillin-streptomycin and 1 mM sodium pyruvate, and incubated at 37°C with 5% CO_2 for 24 h. The cells were replaced with serum-free RPMI-1640 medium before treatment with different concentration of Bcl-2 (50 and 100 nM) and then incubated at 37 °C with 5% CO_2 for 4 h. Then, medium was washed by PBS for three times and replaced with complete RPMI1640 medium, and incubated for 24 h. Total RNA was extracted from MDA-MB-231 cells using the RNeasy Mini Kit (Qiagen, CA). cDNAs were synthesized using the High-Capicity cDNA Reverse Transcription Kit (Applied Biosystems, CA). Primers specific for Bcl-2, GAPDH and β-actin were used, and relative gene expression was determined using Real-Time SYBR Green PCR Master Mix (Applied Biosystems, CA) by the qPCR System. The comparative threshold cycle

method was used to calculate fold change in gene expression, which was normalized to *β*-actin as a reference gene. Total RNA was prepared with TRIzol reagent (Life Technologies), and complementary DNA (cDNA) was reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR primer sequences were as follows:

Name	**Sequences**
β-actin	For : CATGTACGTTGCTATCCAGGC Rev: CTCCTTAATGTCACGCACGAT
GAPDH	For: AATCCCATCACCATCTTCCA Rev: TGGACTCCACGACGTACTCA
Bcl-2(7)	For: ACAACATCGCCCTGTGGATGA Rev: CATGCTGGGGCCGTACAGT
Bcl-2(10)	For: TTTGTGGAACTGTACGGCCC Rev: CACTTGTGGCCCAGATAGGCA

Cell viability.

MDA-MB-231 cells were seeded at 3000 cells per well in a 96-well plate for 24 h before transfection. Cells were treated with LB micelles at concentrations from 1.5 μg/mL to 15 μg/mL in serum-free RPMI-1640 for 6 h. RPMI-1640 medium was removed and replaced with fresh RPMI-1640 to further incubate 24 h. Then, 15 μL of 5 mg/mL MTT (in phosphate buffered saline, PBS) was added to each well and incubated for 3 h at 37 °C. The medium was then replaced with 50 μL DMSO in each well. The absorbance was measured by Multiskan (Thermo, USA) at 570 nm. All data were normalized based on background intensity. All experiments were carried out with six replicates, while control groups (without micelles) were twelve replicates. The cell viability (%) was calculated using the following equation: Cell viability (%) = (samples / mean of controls) * 100%

Apoptosis assay *in vitro.*

MD-MB231 cells were seeded at a density of 10^6 in 12-well plates. Six hours after treatment with different formulations, the cells were harvested. Further analysis was performed using a FITC Annexin V assay (556419, BD Biosciences) and flow

cytometry. Flow cytometry data were obtained from a BD FACSAria III flow cytometer (Becton Dickinson) and analyzed with FACSDivaTM software.

Cellular uptake.

MDA-MB-231 cells were cultured in complete RPMI-1640 medium with 10% FBS, 1% penicillin-streptomycin and 1 mM sodium pyruvate, and were seeded 10^5 cells/well in a volume of 1 mL/well to a 12-well plate with a coverslip each well at 37°C for 24 h. Before treatment, medium was replaced with serum-free RPMI-1640 medium. MDA-MB-231 cancer cells were respectively treated with free FAM-labled siRNA (75 nM), micelleplexes containing FAM-labled siRNA (75 nM), and Dox-loaded micelleplexes containing FAM-labled siRNA (75 nM) for 4 h and then the medium was fixed with 4% paraformaldehyde, followed by washing PBS for three times. The coverslips containing the cells were mounted with 3 μL of DAPI to dye cell nucleus and imaged by the confocal laser scanning microscopy (LSM 780, Zeiss, Germany). The images were analyzed by Image J. For cellular uptake *via* flow cytometry, 10^6 of MD-MB-231 cells were seeded in 12-well plates, and treated with different NP formulations for 4 h. The cells were then collected, followed by three

washes with PBS. The cellular uptake of NPs was quantified with mean fluorescence intensity (MFI) using a FACS-based analysis.

Supporting Figures

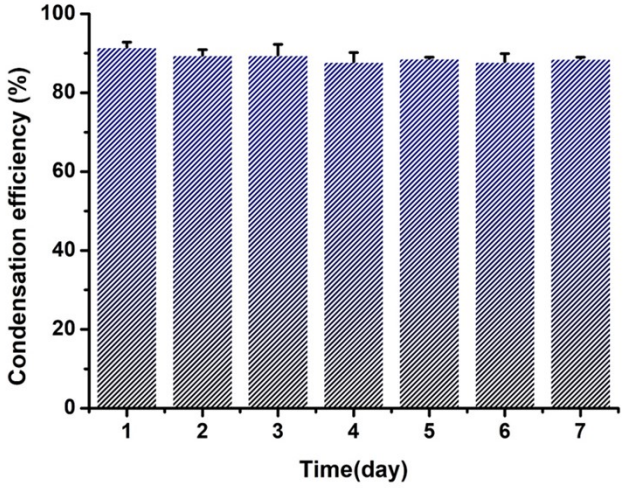


Fig. S1 siRNA condensation efficiency of LB micelles at N/P ratio of 20 in DEPC-treated DIW at 4 °C in a week.

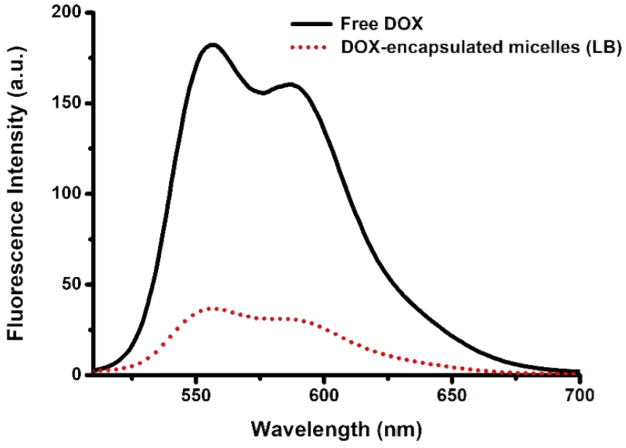


Fig. S2 Fluorescence spectra (λ_{ex} = 480 nm) of free doxorubicin (20 μg/mL) and doxorubicin encapsulated in micelles (LB as a representative) in phosphate buffer solution (pH = 7.4, 20 mM).

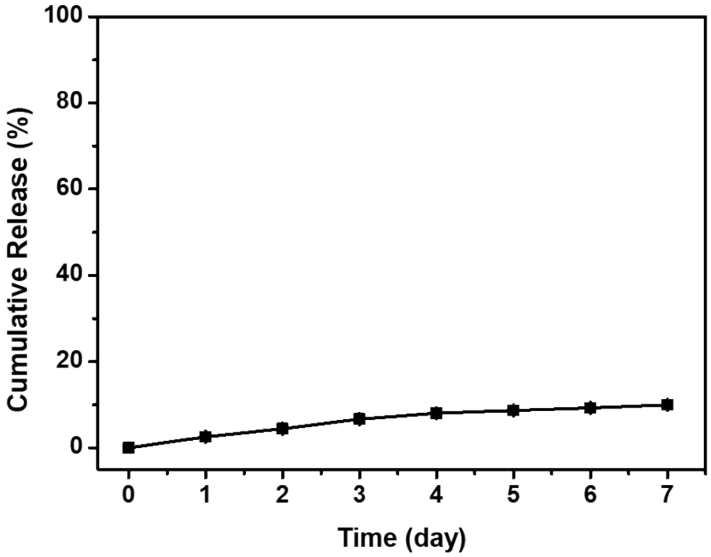


Fig. S3 Cumulative release profile of doxorubicin-loaded LB copolymer micelles in phosphate buffer solution (pH = 7.4, 20 mM) at 4 °C in a week.

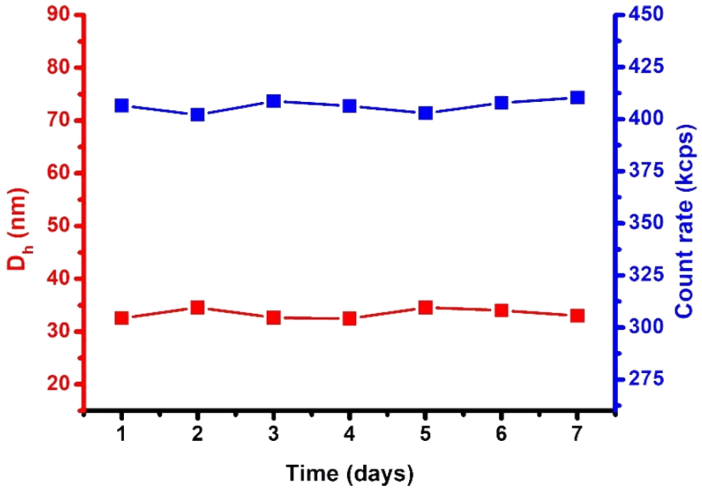


Fig. S4 Time-dependent hydrodynamic diameter (D_h) and count rate of doxorubicin-loaded LB micelles measured by dynamic light scattering (DLS) in phosphate buffer solution (pH = 7.4, 20 mM).

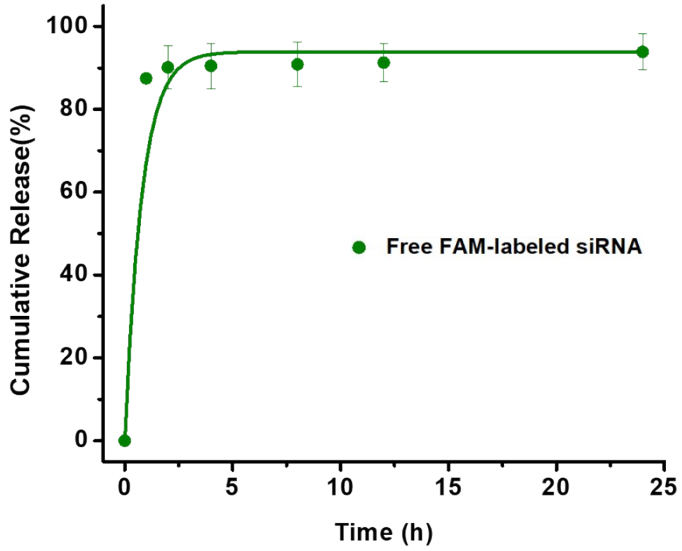


Fig. S5 Cumulative release of free FAM-labeled siRNA as a control group micelleplexes at 37 °C in phosphate buffer solution (pH 7.4, 20 mM, ionic strength = 0.14 M) by centrifugal filters (Amicon® Ultra 0.5 mL, MWCO: 50 kDa).

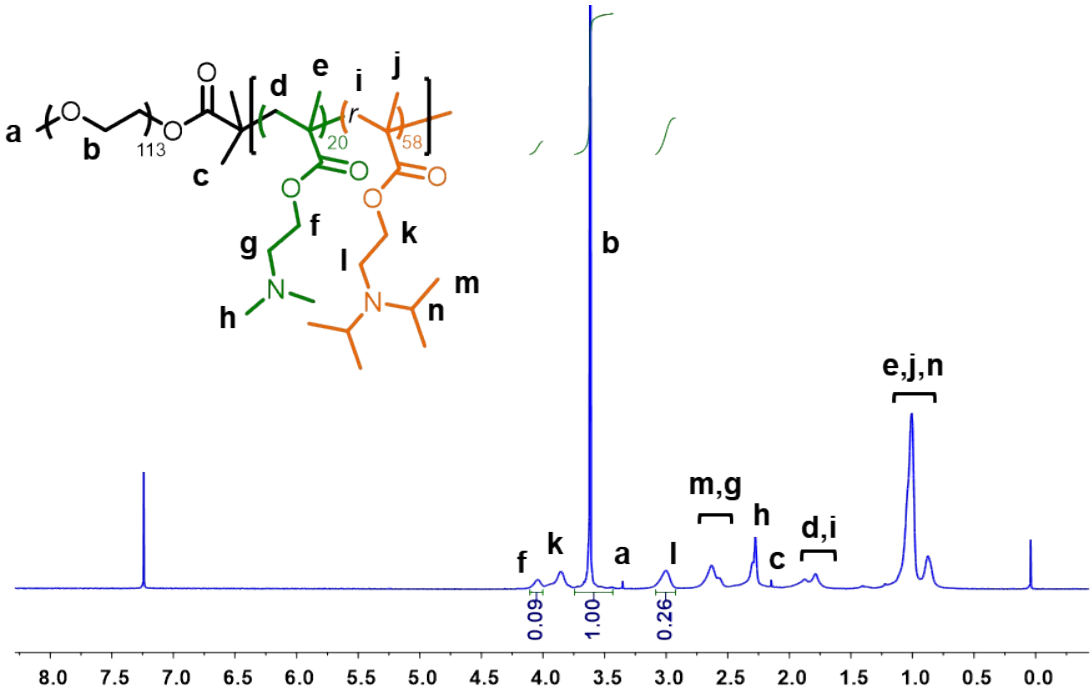


Fig. S6 ^{1}H-NMR spectrum of PEG_{113}-*b*-($PDMAEMA_{20}$-*r*-$PDPA_{58}$) in $CDCl_3$.

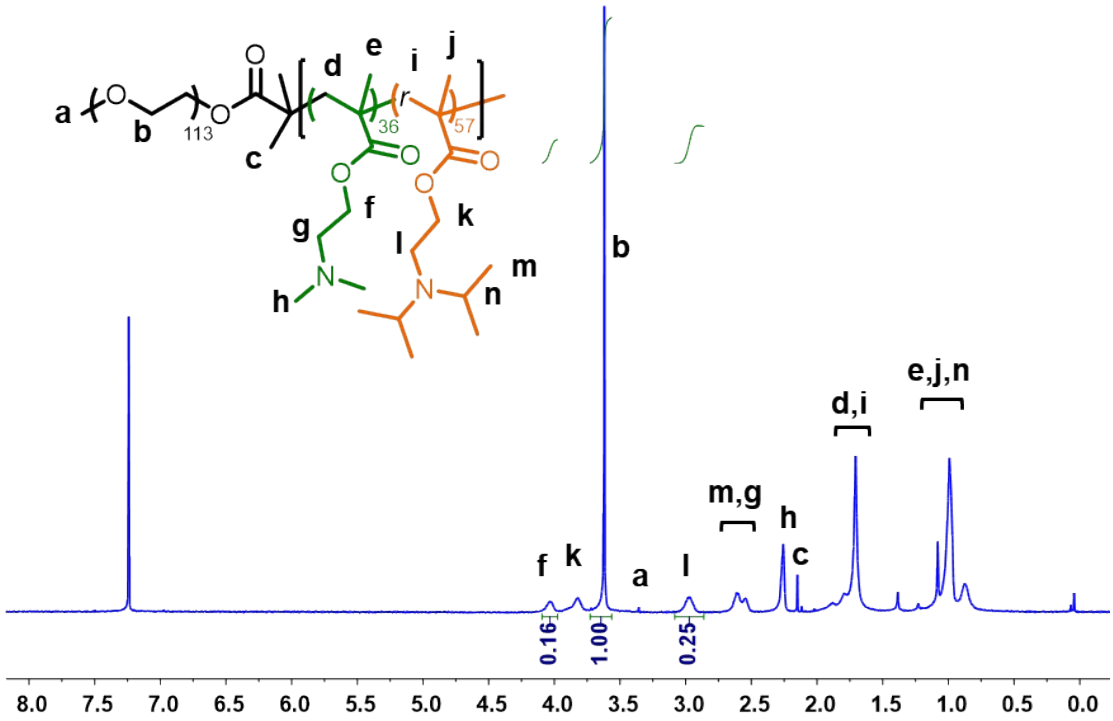


Fig. S7 ^{1}H-NMR spectrum of PEG_{113}-*b*-($PDMAEMA_{36}$-*r*-$PDPA_{57}$) in $CDCl_3$.

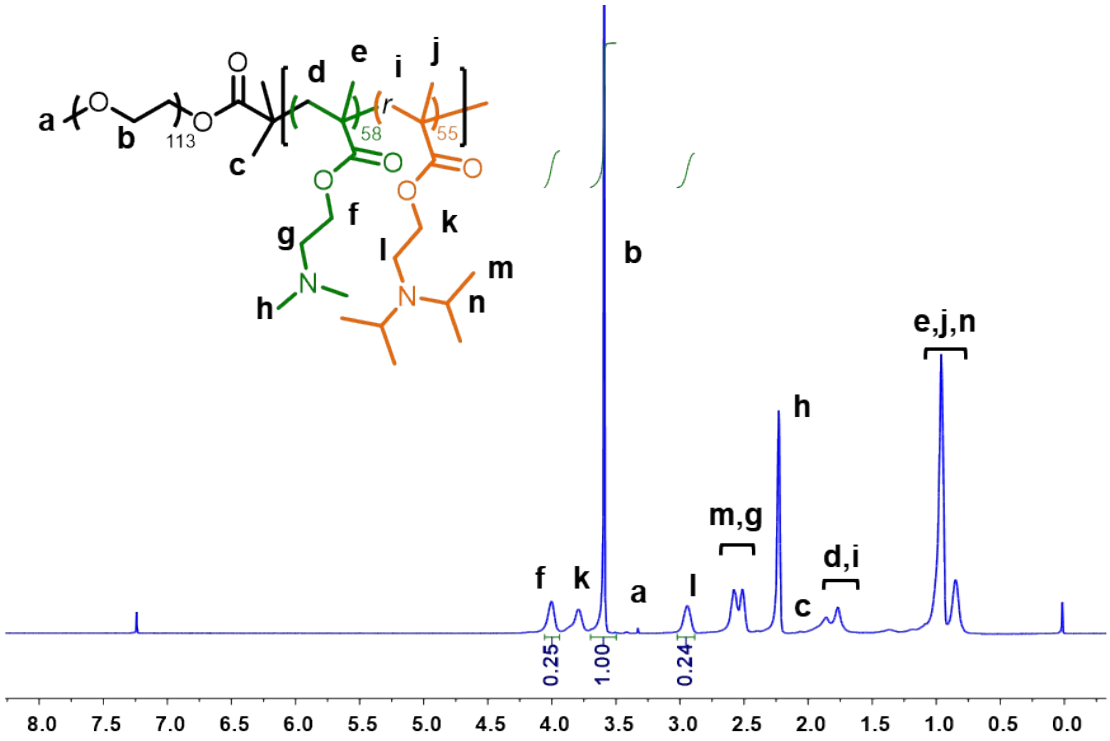


Fig. S8 ^{1}H-NMR spectrum of PEG_{113}-*b*-($PDMAEMA_{58}$-*r*-$PDPA_{55}$) in $CDCl_3$.

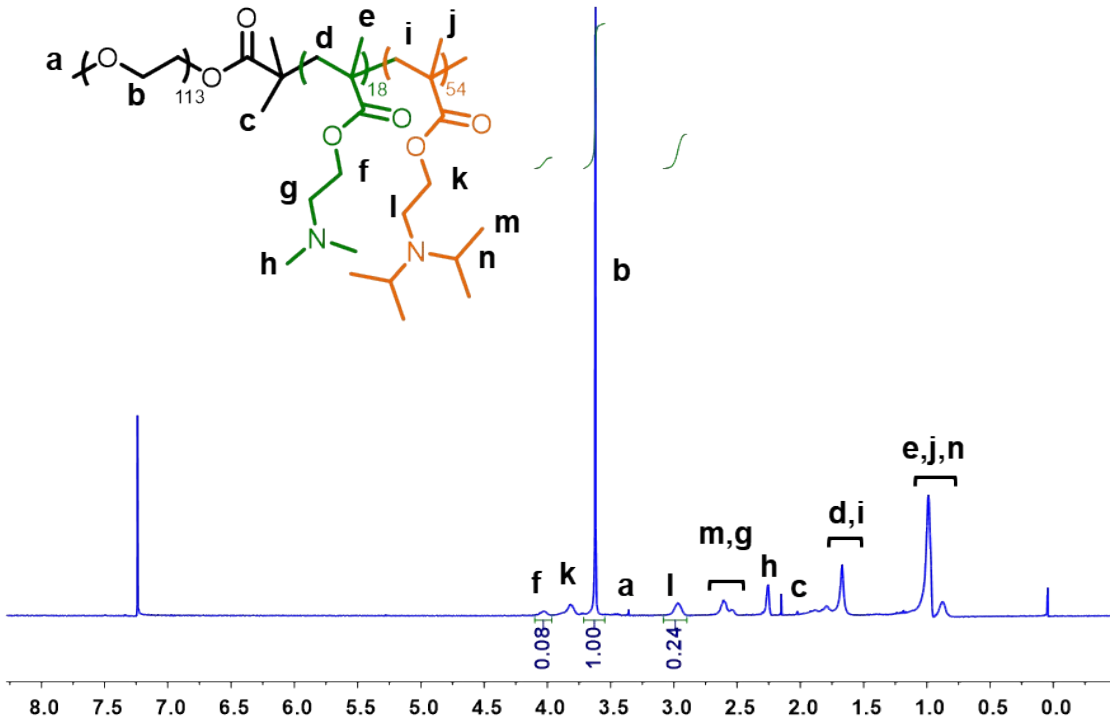


Fig. S9 ^{1}H-NMR spectrum of PEG_{113}-*b*-$PDMAEMA_{18}$-*b*-$PDPA_{54}$ in $CDCl_3$.

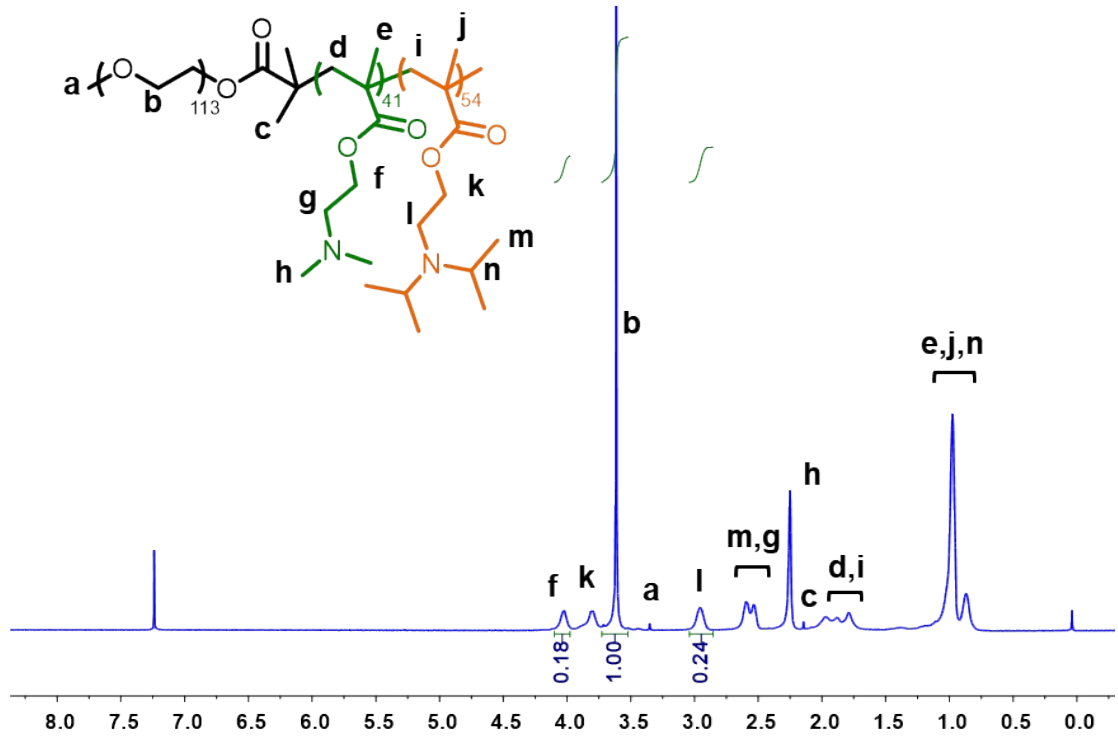


Fig. S10 ^{1}H-NMR spectrum of in $CDCl_3$.

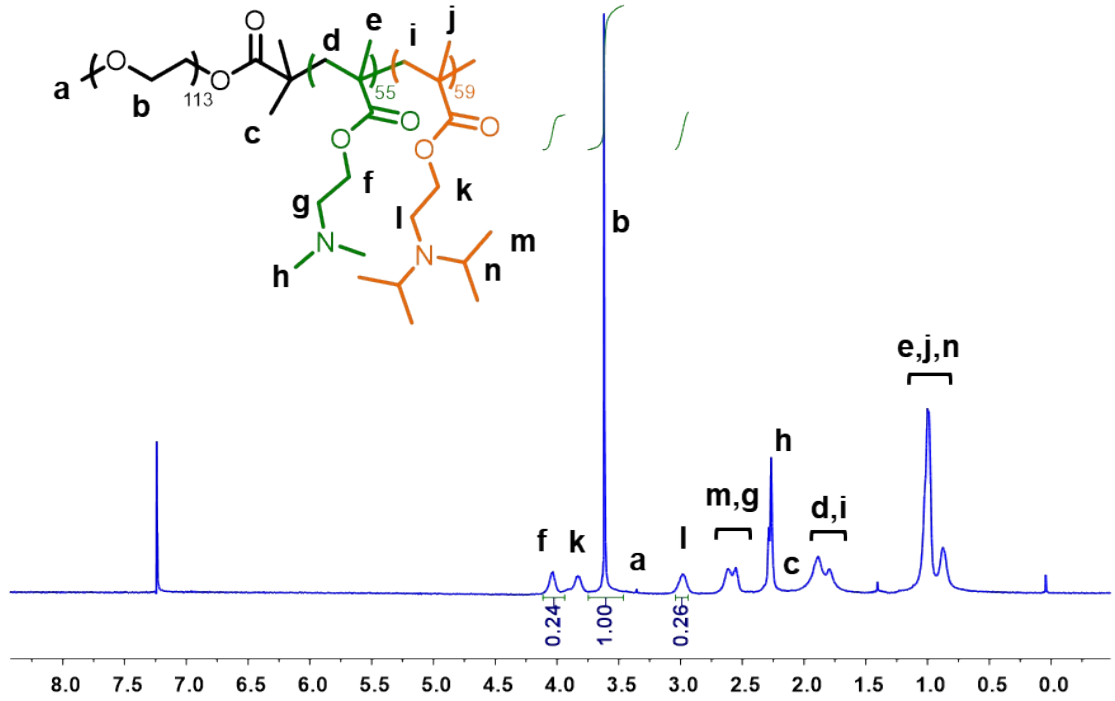


Fig. S11 ^{1}H-NMR spectrum of PEG_{113}-*b*-$PDMAEMA_{55}$-*b*-$PDPA_{59}$ in $CDCl_3$.

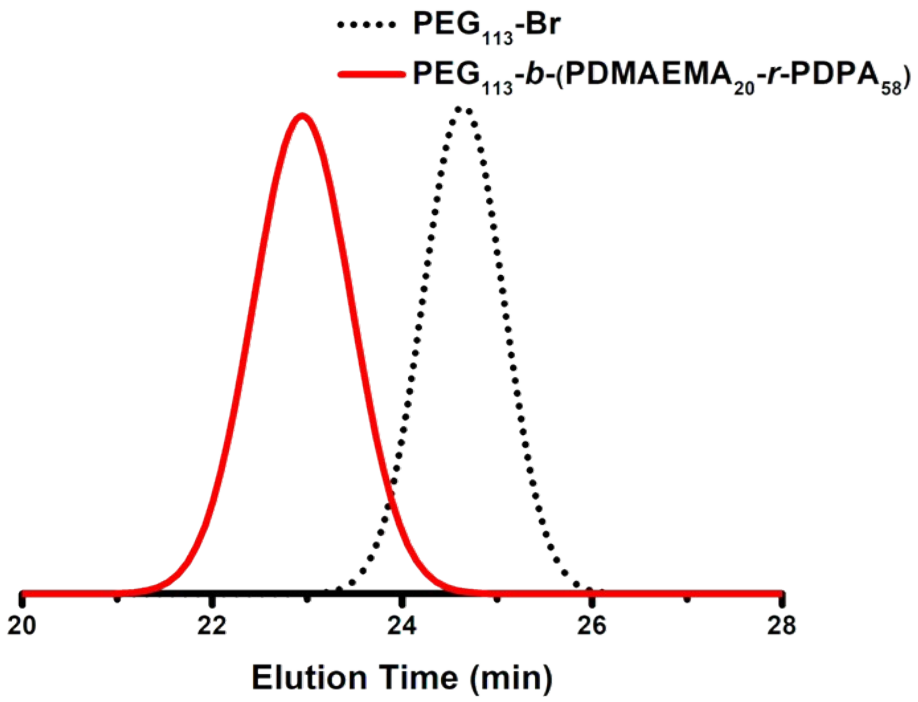


Fig. S12 GPC trace of PEG_{113}-*b*-($PDMAEMA_{20}$-*r*-$PDPA_{58}$).

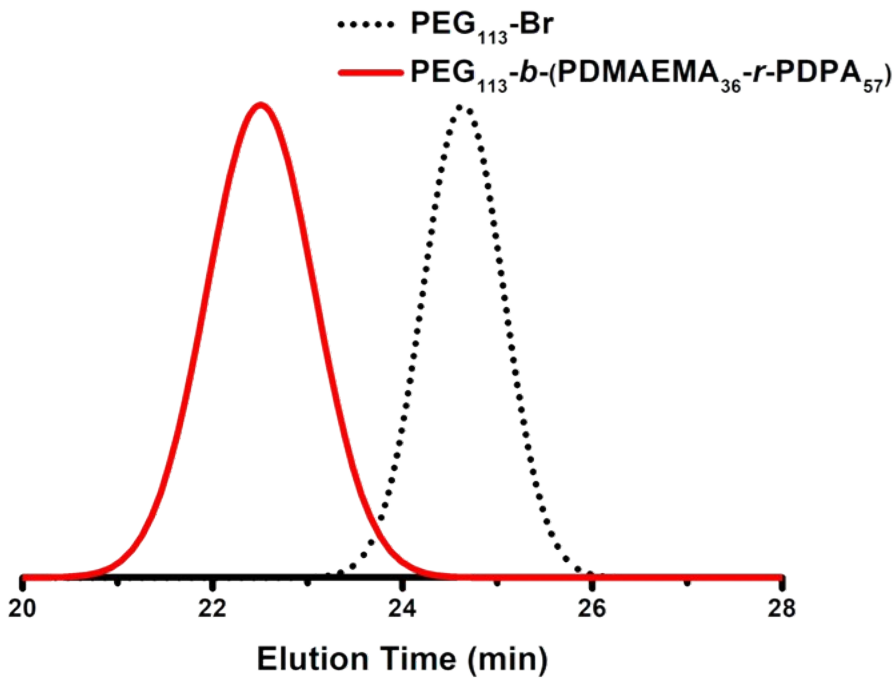


Fig. S13 GPC trace of PEG_{113}-*b*-($PDMAEMA_{36}$-*r*-$PDPA_{57}$).

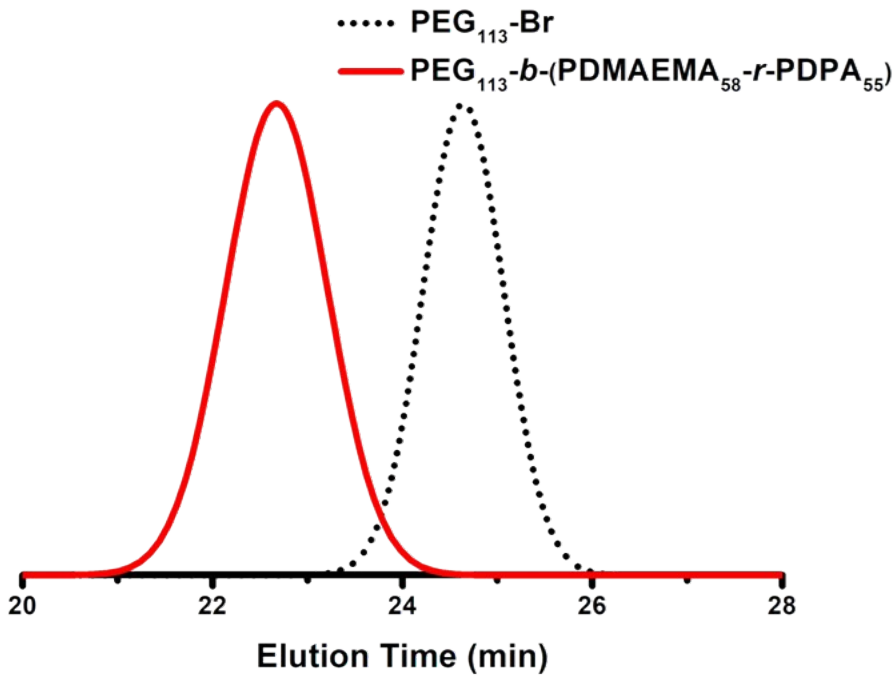


Fig. S14 GPC trace of PEG_{113}-*b*-($PDMAEMA_{58}$-*r*-$PDPA_{55}$).

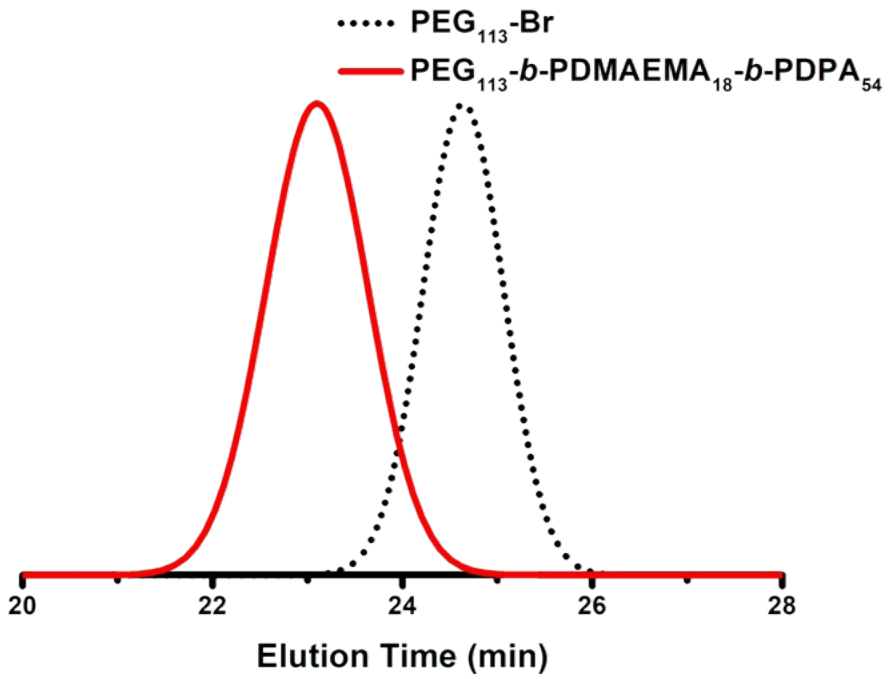


Fig. S15 GPC trace of PEG_{113}-*b*-$PDMAEMA_{18}$-*b*-$PDPA_{54}$.

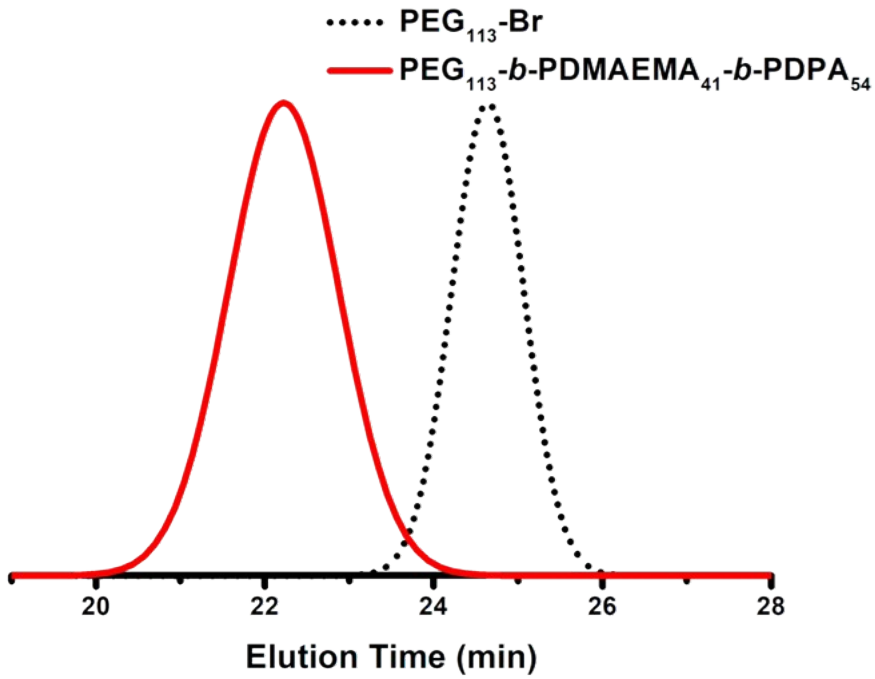


Fig. S16 GPC trace of PEG_{113}-*b*-$PDMAEMA_{41}$-*b*-$PDPA_{54}$.

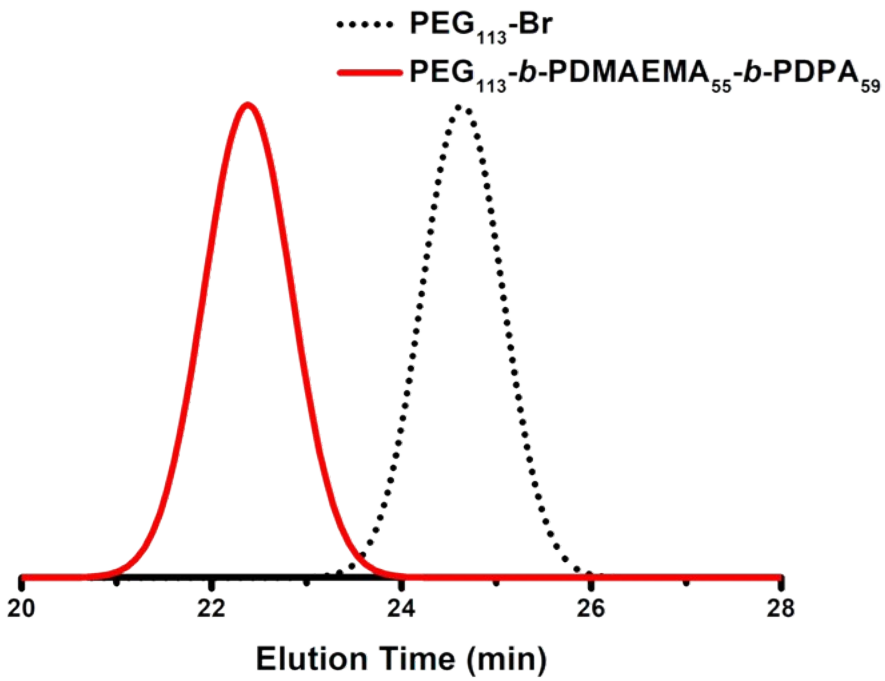


Fig. S17 GPC trace of PEG_{113}-*b*-$PDMAEMA_{55}$-*b*-$PDPA_{59}$.

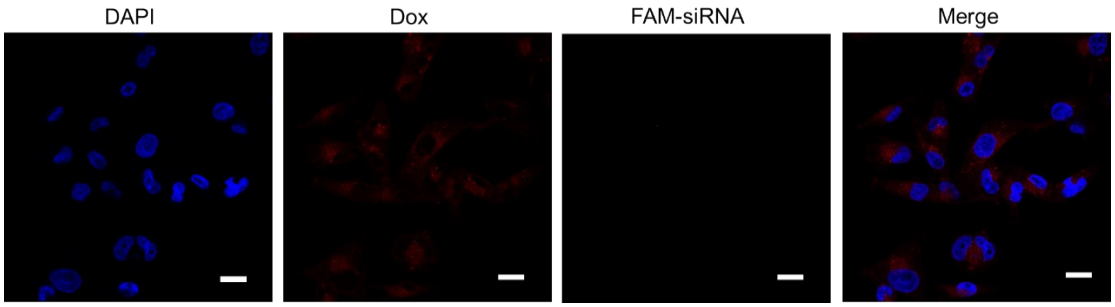


Fig. S18 CLSM images of free Dox (3 μg/mL) in MDA-MB-231 cancer cells. FAM-labeled siRNA was represented in green, Dox was shown in red, and the nucleus stained by DAPI was represented in blue. Scale bar = 20 μm.

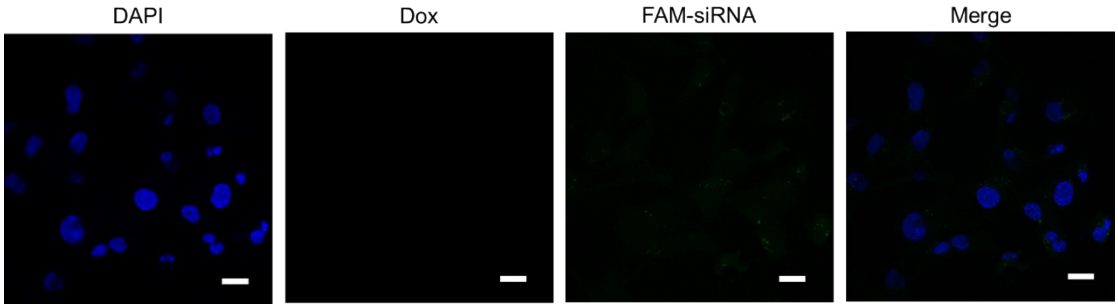


Fig. S19 CLSM images of free FAM-labeled siRNA (75 nM) in MDA-MB-231 cancer cells. FAM-labeled siRNA was represented in green, Dox was shown in red, and the nucleus stained by DAPI was represented in blue. Scale bar = 20 μm.

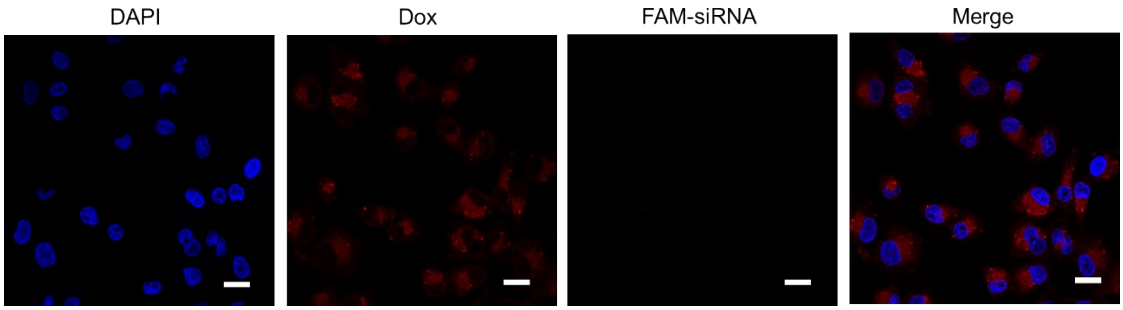


Fig. S20 CLSM images of Dox/LB micelles in MDA-MB-231 cancer cells. FAM-labeled siRNA was represented in green, Dox was shown in red, and the nucleus stained by DAPI was represented in blue. Scale bar = 20 μm.

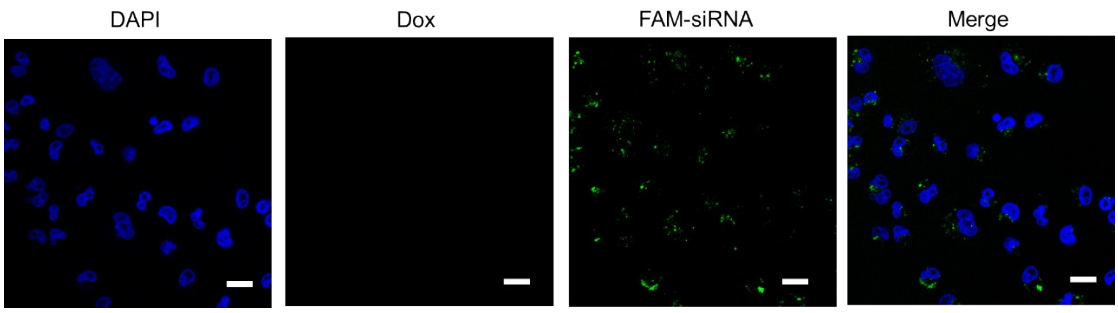


Fig. S21 CLSM images of FAM-labeled siRNA/LB micelleplexes in MDA-MB-231 cancer cells. FAM-labeled siRNA was represented in green, Dox was shown in red, and the nucleus stained by DAPI was represented in blue. Scale bar = 20 μm.

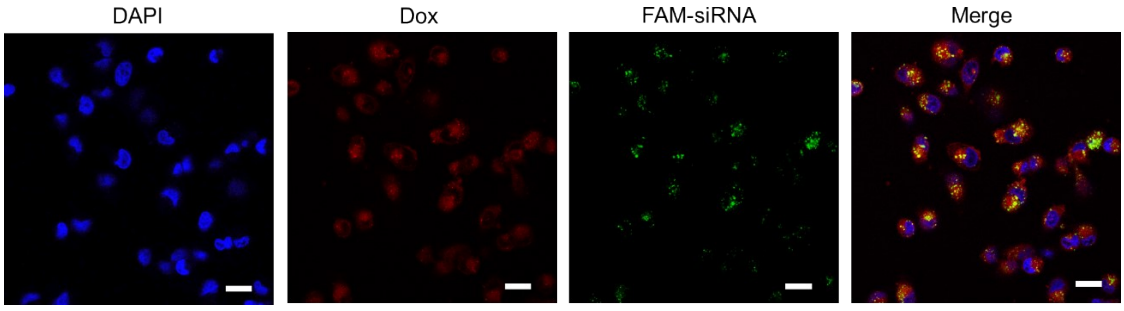


Fig. S22 CLSM images of FAM-labeled siRNA/Dox/LB micelleplexes in MDA-MB-231 cancer cells. FAM-labeled siRNA was represented in green, Dox was shown in red, and the nucleus stained by DAPI was represented in blue. Scale bar = 20 μm.

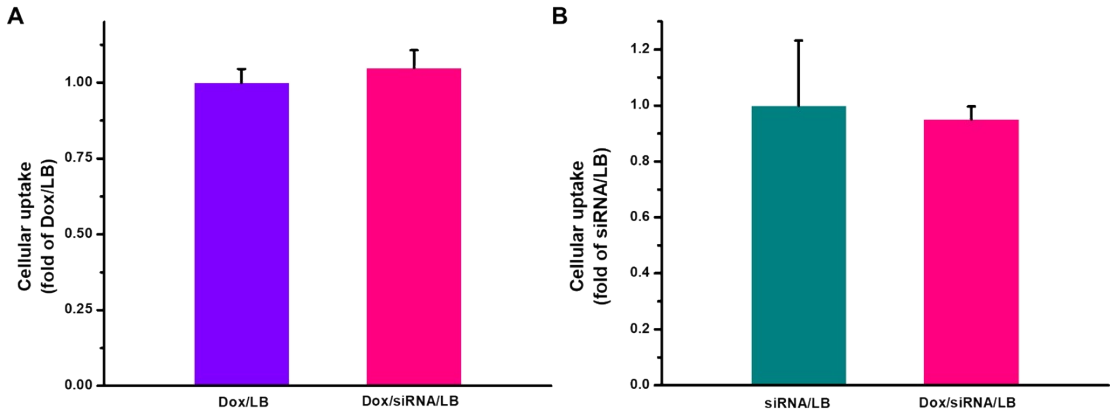


Fig. S23 Cellular uptake of (A) Dox and (B) FAM-labeled siRNA in MDA-MB-231 cancer cells *via* flow cytometry.

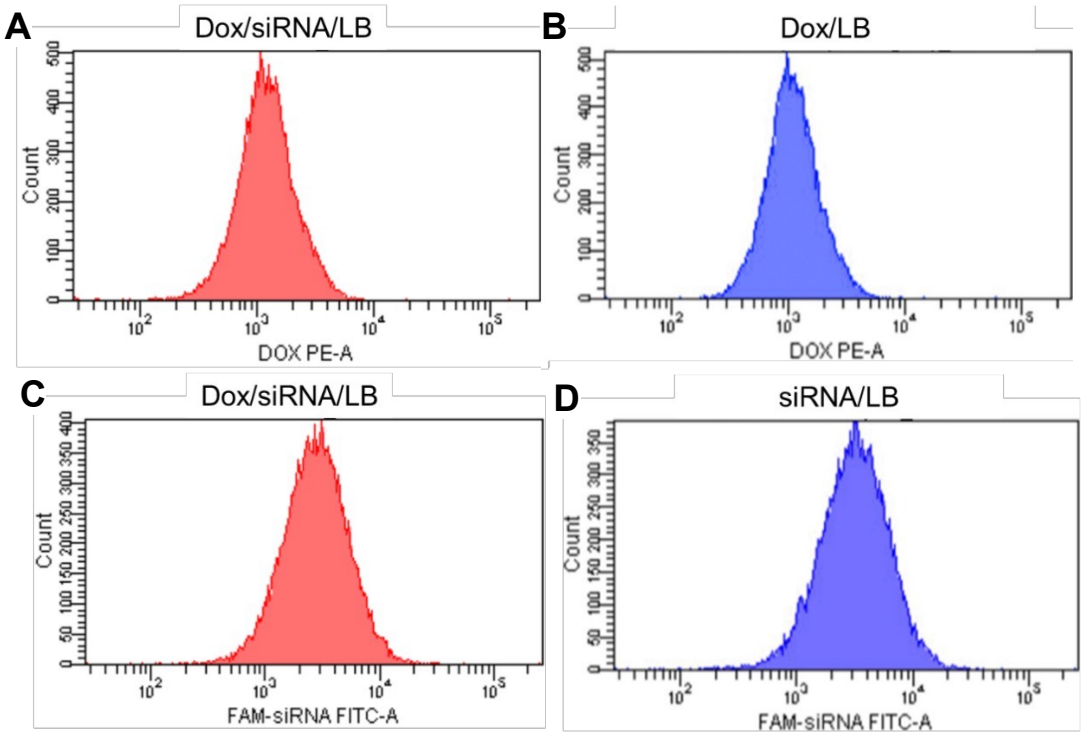


Fig. S24 Representative flow cytometric histogram of (A) Dox/siRNA/LB and (B) Dox/LB at Dox channel, and (C) Dox/siRNA/LB and (D) siRNA/LB at FAM channel.

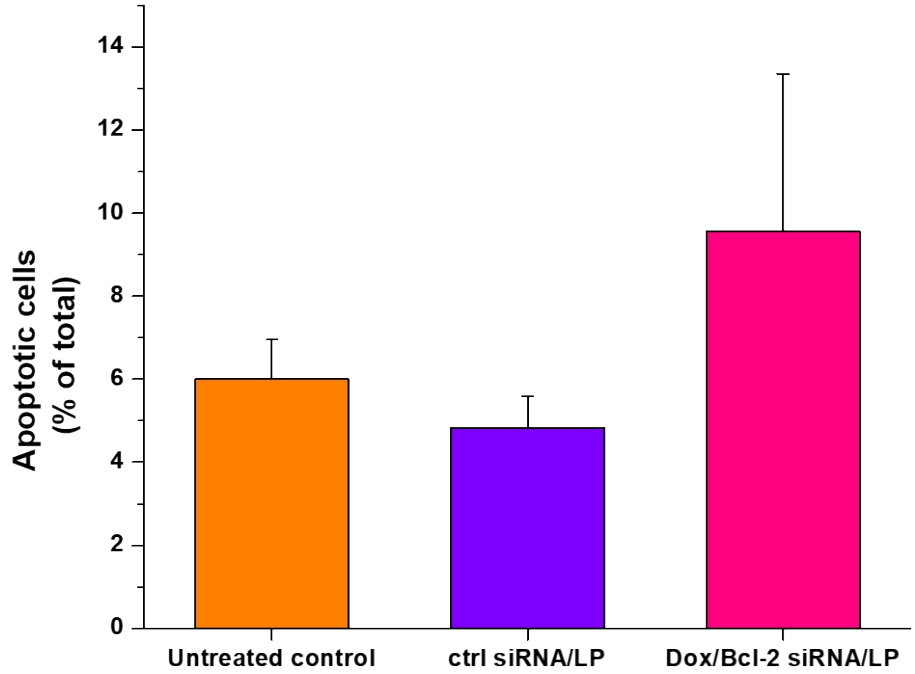


Fig. S25 Cytotoxicity determined by FITC Annexin V assay *via* flow cytometry.

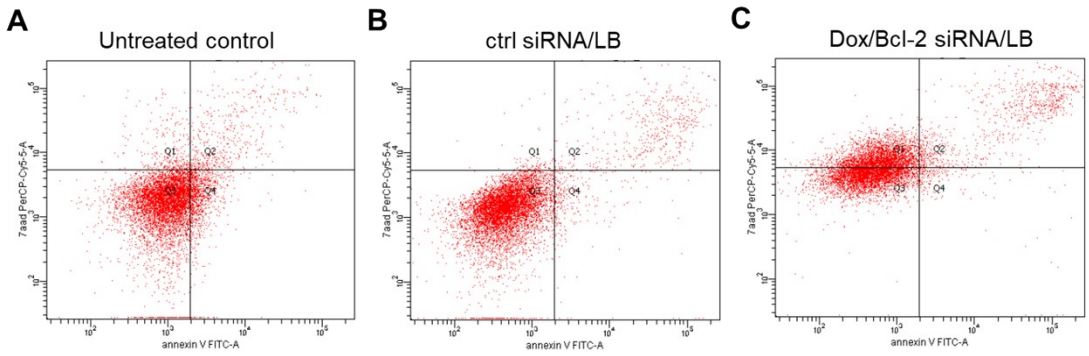


Fig. S26 Flow cytometry dot plots of Annexin V/7-AAD staining cells (A) without treatment and with treatment of (B) ctrl siRNA/LB or (C) Dox/Bcl-2 siRNA/LB.