Electronic Supplementary Information

Efficient co-delivery of microRNA 21 inhibitor and doxorubicin to cancer cells using core-shell tecto dendrimers formed *via* supramolecular host-guest assembly

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Part of experimental section:

Materials. Generation 3 (G3) and generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers were purchased from Dendritech (Midland, MI). Acetic anhydride, triethylamine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), and doxorubicin hydrochloride (DOX.HCl) were provided by Sigma-Aldrich (St. Louis, MO). 1-Adamantaneacetic acid (Ad-COOH), β-cyclodextrin (CD) and N,N-carbonyldiimidazole were purchased from J&K Scientific Ltd. (Shanghai, China). Enhanced green fluorescent protein plasmid DNA (EGFP pDNA) and 2'-O-methyl microRNA 21 inhibitor (miR 21i) (sequence: 5'-UCA ACA UCA GUC UGA UAA GCU A -3') were supplied by Shanghai Gene Pharma (Shanghai, China). Dimethyl sulfoxide (DMSO) was from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Regenerated cellulose dialysis membranes were from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Agarose was from Biowest (Nuaille, France). Luria-Bertani (LB) medium were from Sangon Biotech Co., Ltd. (Shanghai, China). The Primary Amino Nitrogen (PANOPA) Assay Kit was from Megazyme (Wicklow, Ireland). The Lighting-Link Rapid Cy3 Conjugation Kit was from Innova Biosciences (Babraham, UK). MDA-MB-231 cells were obtained from Institute of Biochemistry and Cell Biology (the Chinese Academy of Science, Shanghai, China). 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 purchased from 7 Sea Biotech Co., Ltd. (Shanghai, China). 4',6-Diamidino-2-phenylindole (DAPI) was acquired from BestBio Biotechnology Co., Ltd. (Shanghai, China). RNAeasy™ Plus Animal RNA Isolation Kit with Spin Column, cDNA synthesis kit, SYBR Green qPCR (polymerase chain reaction) Mix (2X, Low ROX), Western blot kit and HRP-labeled Goat Anti-Mouse IgG(H+L) were purchased 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 GAPDH, PTEN, PDCD4, p53, and Caspase-3 were obtained from Santa Cruz Biotechnology Co., Ltd. (Santa Cruz, CA). Water used in all experiments was purified using a MilliQPlus 185 water

purification system (Boston, MA) with a resistivity higher than 18 MΩ.cm. Upstream and downstream primers of genes (miR 21, GAPDH, PTEN, PDCD4, p53, and Caspase-3) and miR 21 stem ring sequence were designed and obtained from Generay Biotechnology Co., Ltd. (Shanghai China).

Cytotoxicity Assay. MDA-MB-231 cells were regularly cultured and passaged in DMEM supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL) in a 37 ℃ humidified incubator containing 5% CO_2. Cell Counting Kit-8 (CCK-8) was used to evaluate the cytotoxicity of G5-CD/Ad-G3 core-shell tecto dendrimers (CSTDs), CSTDs/pDNA and CSTDs/miR 21i polyplexes, and the therapeutic efficacy of free DOX.HCl, CSTDs/DOX and CSTDs/DOX/miR 21i polyplexes, respectively. Taking G5-CD/Ad-G3 CSTDs as an example, MDA-MB-231 cells were seeded into a 96-well plate at a density of 1.0×10^4 cells per well with 0.1 mL DMEM, and were cultured for 24 h at 37 ℃ and 5% CO_2. Then, medium of each well was replaced with 100 μL fresh medium containing 10 μL of CSTDs solution (in PBS) with different concentrations. Among them, 100 μL fresh medium containing 10 μL PBS were used as a control. After the cells were cultured for another 24 h, 10 μL of CCK-8 solution was added to each well, and cells were incubated for 2 h under regular culture conditions. Finally, the absorbance of each well was quantified using a Thermo Scientific Multiskan MK3 ELISA reader (Waltham, MA) at a wavelength of 450 nm. For cytotoxicity assays, G5-CD/Ad-G3 in each well have a final concentration range of 0 to 3000 nM for G5-CD/Ad-G3, CSTDs/pDNA, or CSTDs/miR 21i, respectively. The dose of pDNA or miR 21i used for polyplexe preparation was 1 μg. To test the therapeutic efficacy of DOX-loaded CSTDs or polyplexes, DOX in each well have a final concentration range of 0 to 50 μg/mL for free DOX.HCl, CSTDs/DOX, and CSTDs/DOX/miR 21i, respectively. The dose of miR 21i used for the polyplexes was always kept at an N/P ratio of 10.

Gene Transfection and Cellular Uptake of CSTDs/pDNA Polyplexes. To investigate the gene transfection efficiency of G5-CD/Ad-G3 at different N/P ratios in the absence of serum, pDNA encoding EGFP was used as a reporter gene, and Nikon Ti-S invert fluorescence microscope (Tokyo, Japan) was applied to evaluate the expression of EGFP. Briefly, MDA-MB-231 cells were seeded

into a 24-well plate at a density of 1.0×10^5 cells per well, and were cultured overnight at 37 ℃ and 5% CO_2. The cell medium of each well was replaced with 500 μL serum-free fresh medium containing CSTDs/pDNA polyplexes. After the cells were incubated for 4 h, the cell medium of each well was replaced with 500 μL fresh complete medium, and the cells were cultured for another 24 h under regular conditions. The morphology of cells was observed by fluorescence microscope.

To study the cellular uptake of CSTDs/pDNA polyplexes, BD FACSCalibur flow cytometer (BD Biosciences, Franklin, CA) was used. The Trachert intracellular nucleic acid localization kit was used to prepare Cy3-labeled EGFP pDNA. Then, the CSTDs/Cy3-pDNA polyplexes were prepared at different N/P ratios according to the above mentioned method. Afterwards, MDA-MB-231 cells were seeded into a 24-well plate at a density of 1.0×10^5 cells per well, and were cultured overnight at 37 ℃ and 5% CO_2. The cell medium of each well was replaced with 500 μL serum-free fresh medium containing CSTDs/Cy3-pDNA polyplexes. After the cells were incubated for 4 h, the cells were washed with PBS for three times, digested with trysin-EDTA, collected into the tube, resuspended in PBS after centrifugation, and finally measured in the FL2-H channel by flow cytometry.

Transfection of CSTDs/miR 21i Polyplexes. According to the above mentioned protocols, Cy3-miR 21i was prepared to form CSTDs/Cy3-miR 21i polyplexes, and the polyplexes were incubated with MDA-MB-231 cells for flow cytometry assay of the cellular uptake. To further observe the intracellular uptake of the polyplexes *via* ZEISS laser scanning confocal microscope (LSM-700, Jena, Germany), the MDA-MB-231 cells were incubated with the polyplexes according to the same procedures as described above. Then the cells were fixed with 2.5% glutaraldehyde at 4 ℃ for 15 min and stained by DAPI for 5 min. Finally, the cells were imaged using confocal microscope.

Wound healing assay was used to examine the migration of cancer cells after transfected with the CSTDs/miR 21i polyplexes following standard procedures reported in our previous work.[1] MDA-MB-231 cells were seeded into a 12-well plate at a density of 2.0×10^5 cells per well, and were cultured overnight at 37 ℃ and 5% CO_2. Then, a 100 μL pipette tip was used to make a scratch

in each well and the cells were washed with PBS (pH = 7.4) three times. The cell medium was replaced with 1 mL of serum-free fresh medium containing CSTDs/miR 21i polyplexes (N/P = 10, 2 μg miR 21i) in each well. The 10% FBS-containing medium was also added as a control group. The medium were exchanged with 10% FBS-containing medium after 4 h incubation, and the wound in each well was observed by Leica DM IL LED inverted phase contrast microscope at 0, 12, 24, and 48 h, respectively. Image J software were used to quantify of the migration area.

Western blot analysis was used to examine the regulation of the protein markers according to standard protocols reported in our previous work.[1] MDA-MB-231 cells were seeded into a 24-well plate at a density of 1.0×10^5 cells per well, and were cultured overnight at 37 ℃ and 5% CO_2. The cell medium of each well was replaced with 500 μL serum-free fresh medium containing CSTDs/miR 21i polyplexes (N/P=10, 1 μg miR 21i). The 10% FBS-containing medium was also added as a control group. The medium was exchanged with 10% FBS-containing medium after 4 h incubation. The cells were were further incubated for 48 h and washed with PBS for three times, and lysed to extract protein. The whole cell protein was electrophoresed on 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels and transferred onto PVDF membranes. The PVDF membrane was blocked in 5% non-fat milk, incubated with primary antibodies at 4 °C overnight, and incubated with secondary antibody (Beyotime, Shanghai, China). The density of target protein signals was observed using a chemiluminescence imaging system (Bio-Rad Laboratories, Hercules, CA). All antibodies were diluted in PBS, and the membrane was wahsed between steps.

Real-time PCR (RT-PCR) was used to quantify the gene levels of the cancer cells after transfection of the polyplexes. After MDA-MB-231 cells were incubated with the CSTDs/miR 21i polyplexes (N/P = 10, 1 μg miR 21i) for 48 h in a 24-well plate, the total RNA was extracted from cells using RNAeasy™ Plus Animal RNA Isolation Kit and was reversely transcribed into cDNA by cDNA synthesis kit. The obtained cDNA, SYBR Green qPCR mix, and the upstream and downstream primers of the gene were mixed into a 20-μL reaction system according to standard protocols. Amplification reaction was carried out using 7500 fast RT-PCR system (Applied Biosystems, Foster City, CA) and the PCR was performed at 95 °C for 2 min, and 40 reaction cycles

were applied for the process of 95 ^{o}C for 15s, 60 ^{o}C for 30s and 72 ^{o}C for 60s. Relative quantification was conducted using amplification efficiencies derived from 2nd-strand cDNA. Data are gained as fold changes ($2^{-\Delta\Delta Ct}$) and were analyzed using Opticon Monitor Analysis Software V 2.02 (Thermal, Waltham, MA). The upstream and downstream primer sequences of each gene are shown in Table S1.

Combinational Gene Therapy and Chemotherapy of Cancer Cells. To further improve the therapeutic effect of cancer cells, the DOX-loaded CSTDs/miR 21i polyplexes were used to treat cancer cells and the enhanced therapeutic effect was evaluated through CCK-8 cell viability assay (*vide supra*). DOX-loaded CSTDs without the miR 21i were also tested for comparison. Meanwhile, to check the cellular uptake and intracellular localization of the CSTDs/DOX complexes and CSTDs/DOX/miRNA 21i poplyplexes, flow cytometry and confocal microscopy experiments were performed under the protocols described above by virtue of the DOX fluorescence.

Statistical Analysis. The experimental results were analyzed through the one-way analysis of variance (ANOVA) statistical method. A value of 0.05 was set as the significance level, and the data were marked with (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$, respectively.

Table S1. The sequences of upstream, downstream, and stem loop primers.

Name	Sequences(5'~3')
GAPDH-F*	GGACCTGACCTGCCGTCTAG
GAPDH-R*	GTAGCCCAGGATGCCCTTGA
PDCD4-F	GGGAGTGACGCCCTTAGAAG
PDCD4-R	ACCTTTCTTTGGTAGTCCCCTT
PTEN-F	TTTGAAGACCATAACCCACCAC
PTEN-R	ATTACACCAGTTCGTCCCTTTC
p53-F	CAGCACATGACGGAGGTTGT
p53-R	TCATCCAAATACTCCACACGC
Caspase-3-F	CATGGAAGCGAATCAATGGACT
Caspase-3-R	CTGTACCAGACCGAGATGTCA
miR 21-F	CGGGCTAGCTTATCAGACTG
miR 21-R	CAGCCACAAAAGAGCACAAT
miR 21-S*	CCTGTTGTCTCCAGCCACAAAAGAGCAC AATATTTCAGGAGACAACAGGTCAACA T

* F, R, and S represent the upstream, downstream, and stem loop primer, respectively.

Table S2. Mean number of primary amines per G5-CD/Ad-G3 CSTD.

Vector	G3-Ad	G5-CD	G5-CD/Ad-G3
Mw[a]	7122.7	41786.5	65291.4
Mean number of primary amines per dendrimer	8.1	54.6	80

[a] Mws were estimated by ^{1}H NMR spectra.

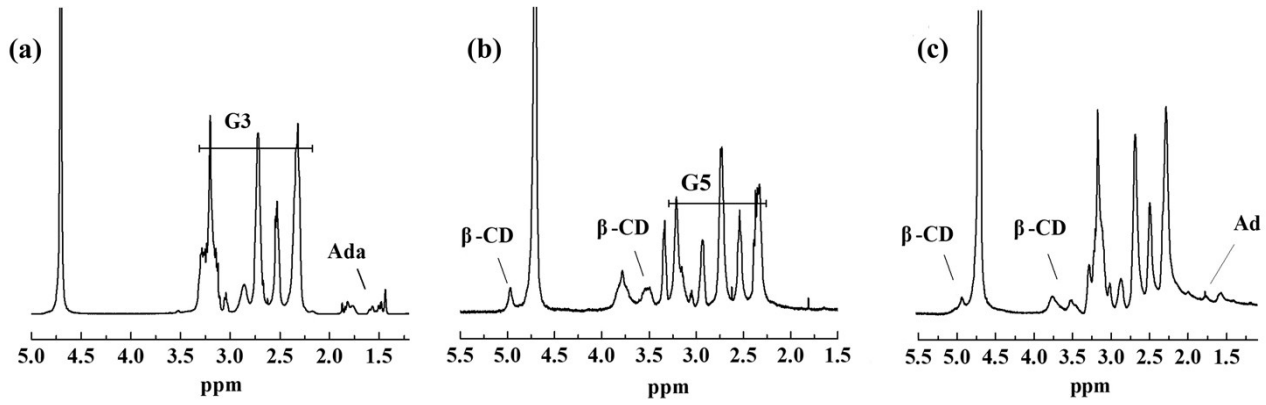

Figure S1. ^{1}H NMR spectra of G3-Ad dendrimers (a), G5-CD dendrimers (b), and G5-CD/Ad-G3 CSTDs (c), respectively.

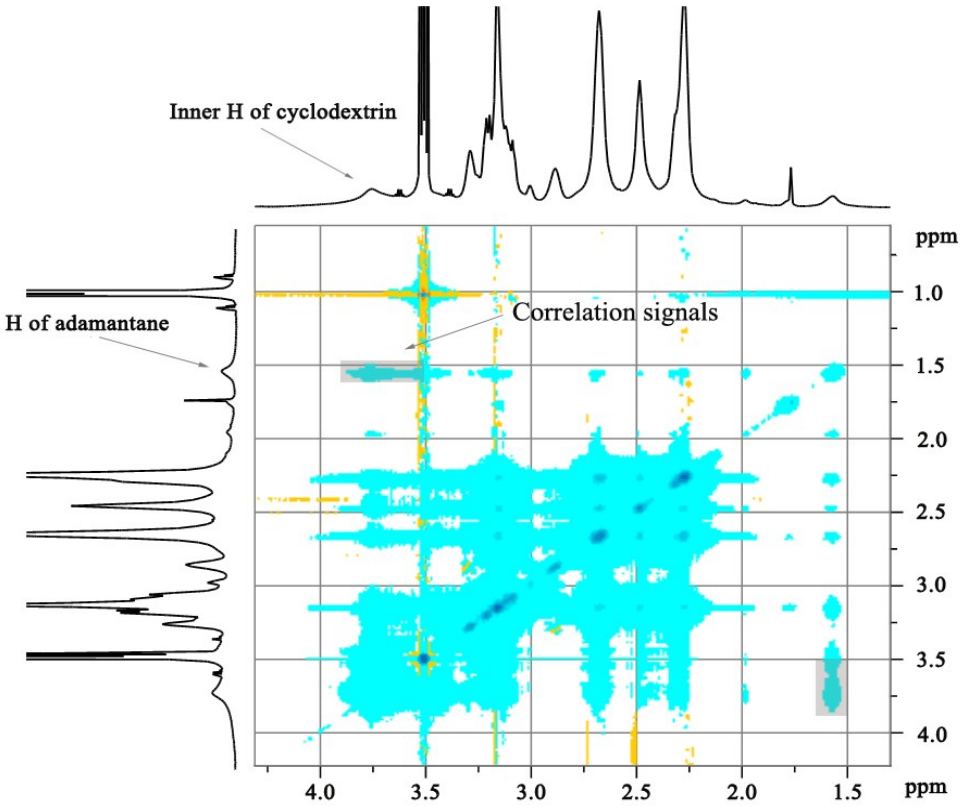

Figure S2. 2D NOESY spectrum of G5-CD/Ad-G3 CSTDs dissolved in D_2O.

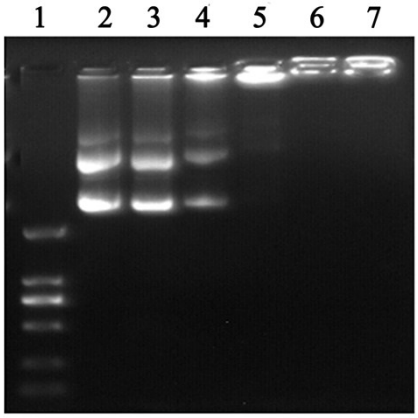

Figure S3. Agarose gel retardation assay of EGFP pDNA complexed with G5-CD/Ad-G3 CSTDs under different N/P ratios. Lane 1, DNA marker 2000; lane 2, N/P = 0.125 : 1; lane 3, N/P = 0.25 : 1; lane 4, N/P = 0.5 : 1; lane 5, N/P = 1 : 1; lane 6, N/P = 2 : 1; and lane 7, N/P = 5 : 1.

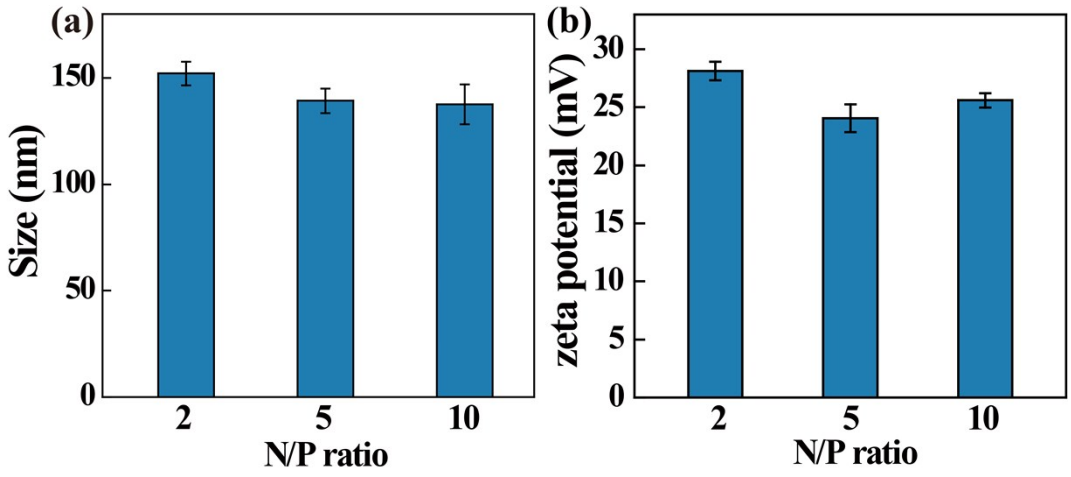

Figure S4. The hydrodynamic sizes (a) and zeta potentials (b) of the CSTDs/pDNA polyplexes under different N/P ratios (mean ± SD, n = 3).

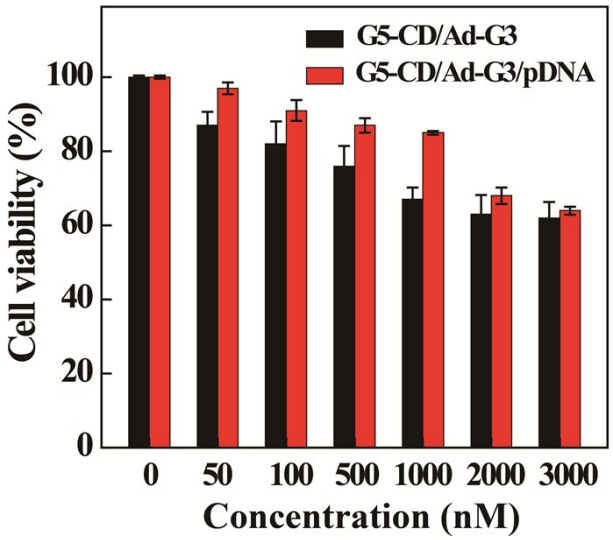

Figure S5. The cell viability of MDA-MB-231 cells after treated with the G5-CD/Ad-G3 CSTDs and CSTD/pDNA polyplexes for 24 h under different concentrations.

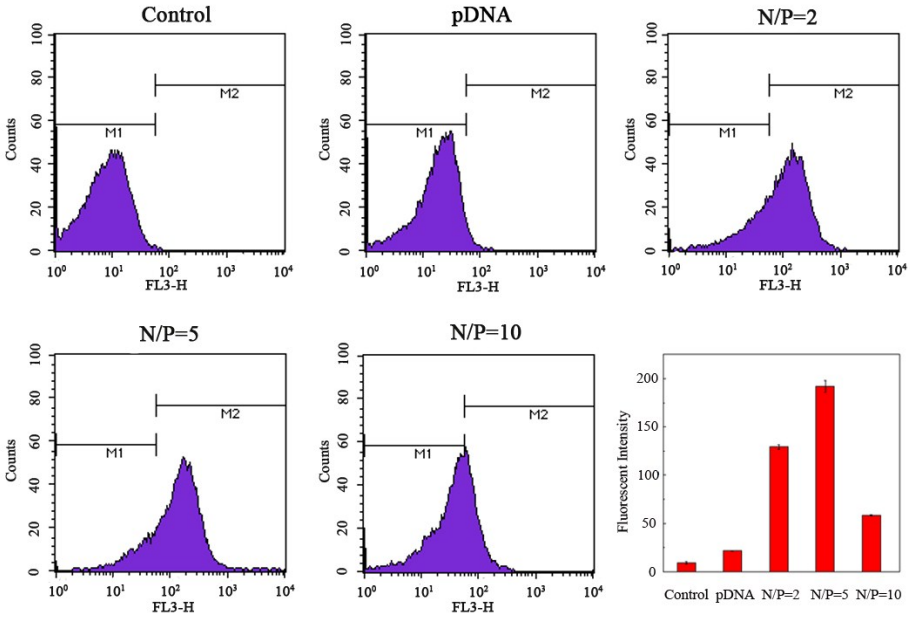

Figure S6. Flow cytometry assay of MDA-MB-231 cells treated with CSTDs/Cy3-pDNA polyplexes for 4 h under different N/P ratios.

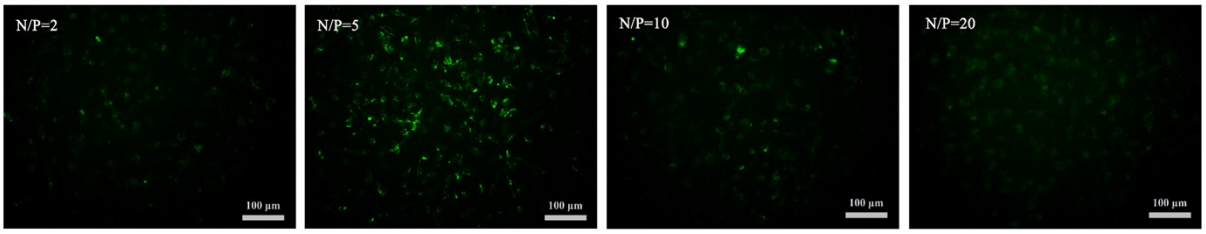

Figure S7. Fluorescence microscopic images of MDA-MB-231 cells transfected with CSTDs/pDNA polyplexes for 24 h under different N/P ratios.

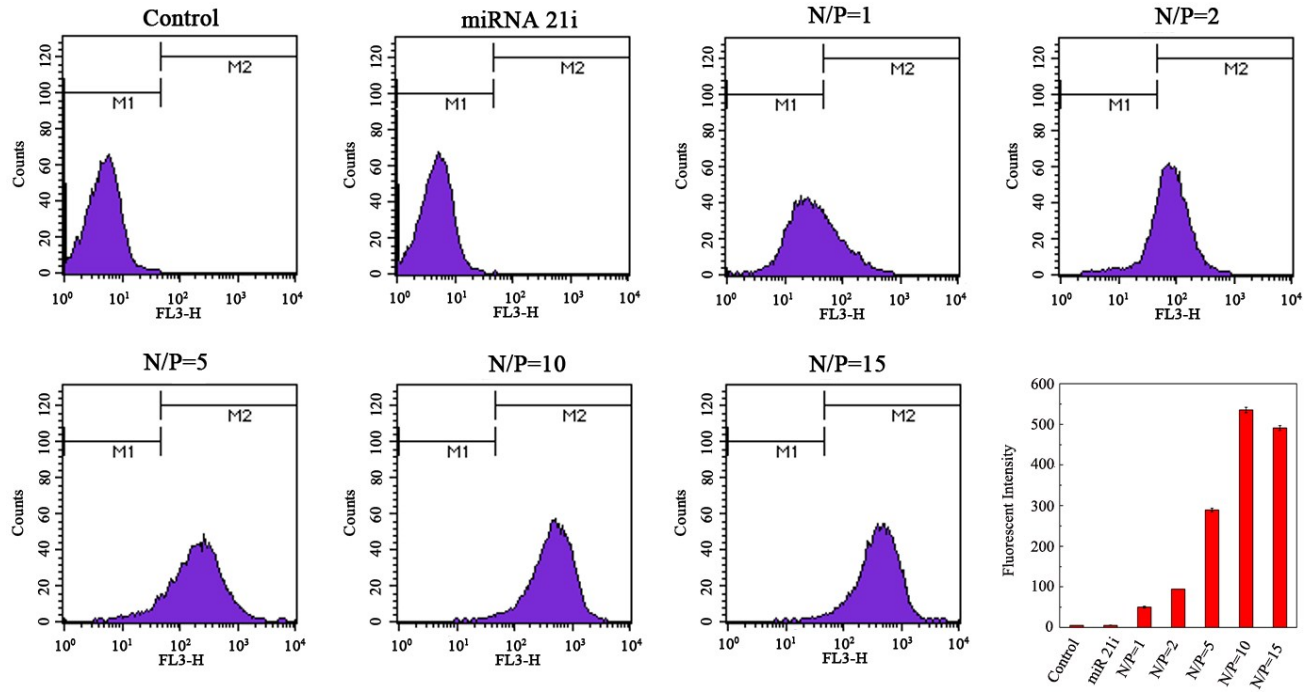

Figure S8. Flow cytometry assay of MDA-MB-231 cells treated with the CSTDs/Cy3-miR 21i polyplexes for 4 h under different N/P ratios.

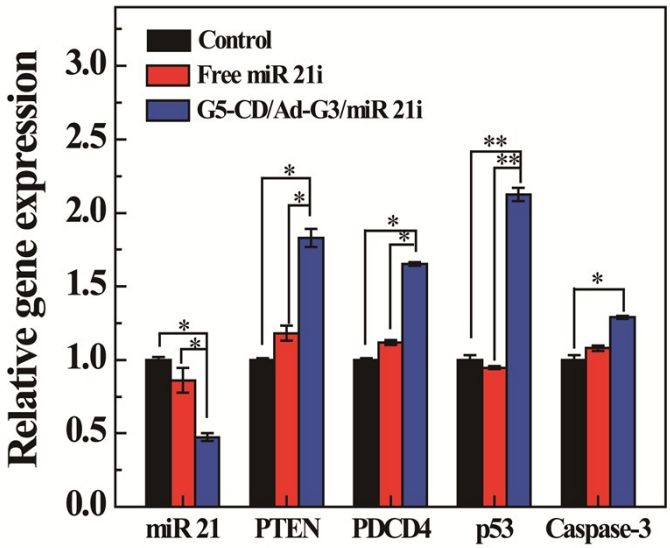

Figure S9. Quantitative analysis of gene expression based on RT-PCR assay.

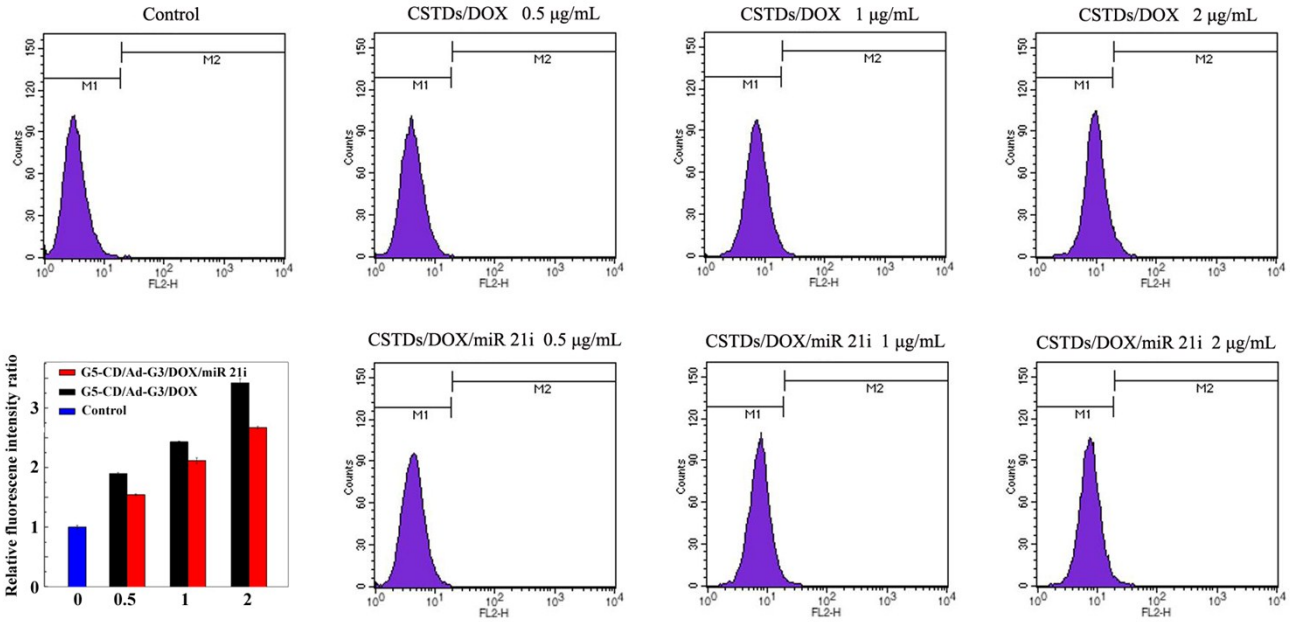

Figure S10. Flow cytometry assay of MDA-MB-231 cells treated with the CSTDs/DOX complexes or CSTDs/DOX/miR 21i polyplexes for 4 h under different DOX concentrations (0.5, 1, and 2 μg/mL, respectively).

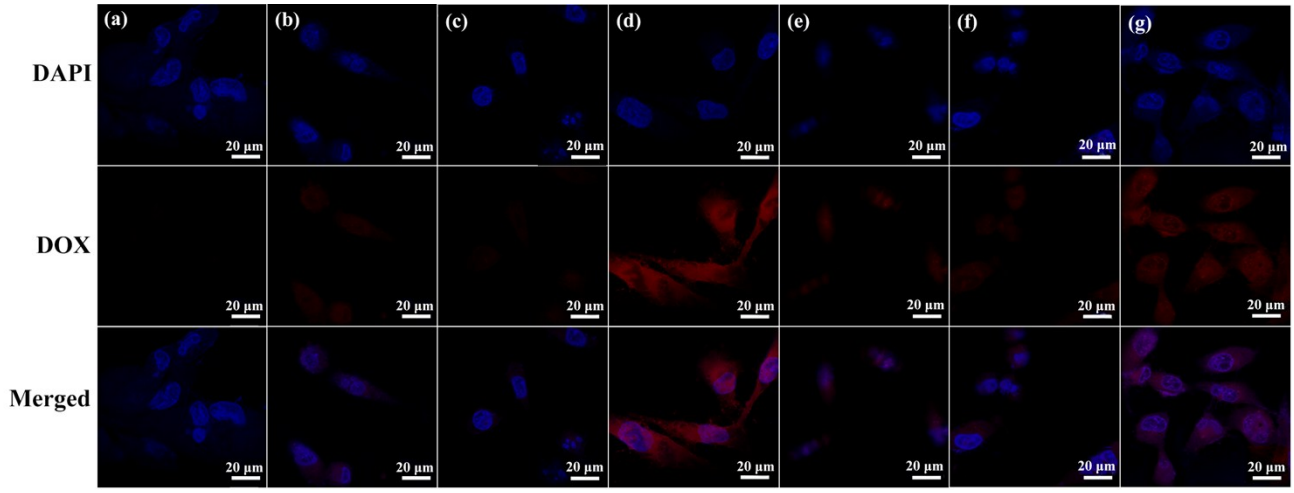

Figure S11. Confocal microscopic images of MDA-MB-231 cells treated with the CSTDs/DOX/miR 21i and CSTDs/DOX for 4 h: (a) cells without treatment; cells treated with the CSTDs/DOX/miR 21i polyplexes under the DOX concentration of (b) 0.5 μg/mL, (c) 1 μg/mL, and (d) 2 μg/mL; and cells treated with the CSTDs/DOX complexes under the DOX concentration of (e) 0.5 μg/mL, (f) 1 μg/mL, and (g) 2 μg/mL. Red represents the DOX fluorescence, while blue represents DAPI-stained cell nucleus.

References

1. Z. Xiong, C. S. Alves, J. Wang, A. Li, J. Liu, M. Shen, J. Rodrigues, H. Tomás and X. Shi, *Acta Biomater.*, 2019, **99**, 320-329.