Electronic Supplementary Information

Drug delivery by a self-assembled DNA tetrahedron for overcoming drug resistance in breast cancer cells

Kyoung-Ran Kim,[a] Da-Rae Kim,[a] Eungjin Ahn,[c] Ji Young Yhee,[a] Byeong-Su Kim,[d] Ick Chan Kwon[a] and Dae-Ro Ahn *[a,b]

[a] Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, Hwarangno 14-gil 5, Seongbuk-gu, Seoul 136-791, Republic of Korea. Fax: +82 2 958 5909; Tel: +82 2 958 6645; E-mail: drahn@kist.re.kr

[b] KIST campus, University of Science (UST-KIST), Hwarangno 14-gil 5, Seongbuk-gu, Seoul 136-791,Republic of Korea.

[c] School of NanoBioscience and Chemical Engineering, Ulsan National Institute of Science and Engineering (UNIST), Ulsan 689-798, Republic of Korea.

[d] Interdisciplinary School of Green Energy and KIER-UNIST Advanced Center for Energy, Ulsan National Institute of Science and Engineering (UNIST), Ulsan 689-798, Republic of Korea.

Table S1. DNA oligonucleotides used to construct Td.

DNA	Sequence
S1	5'CCAGGCAGTTGAGACGAACATTCCTAAGTCTGAAATTTATCACCCG CCATAGTAGACGTATCA
S2	5'CTTGCTACACGATTCAGACTTAGGAATGTTCGACATGCGAGGGTCC AATACCGACGATTACAG
S3	5'GGTGATAAAACGTGTAGCAAGCTGTAATCGACGGGAAGAGCATGC CCATCCACTACTATGGCG
Cy5-S4	5'Cy5-CCTCGCATGACTCAACTGCCTGGTGATACGAGGATGGGCATG CTCTTCCCGACGGTATTGGAC
FAM-S4	5'FAM-CCTCGCATGACTCAACTGCCTGGTGATACGAGGATG GGCATGCTCTTCCCGACGGTATTGGAC

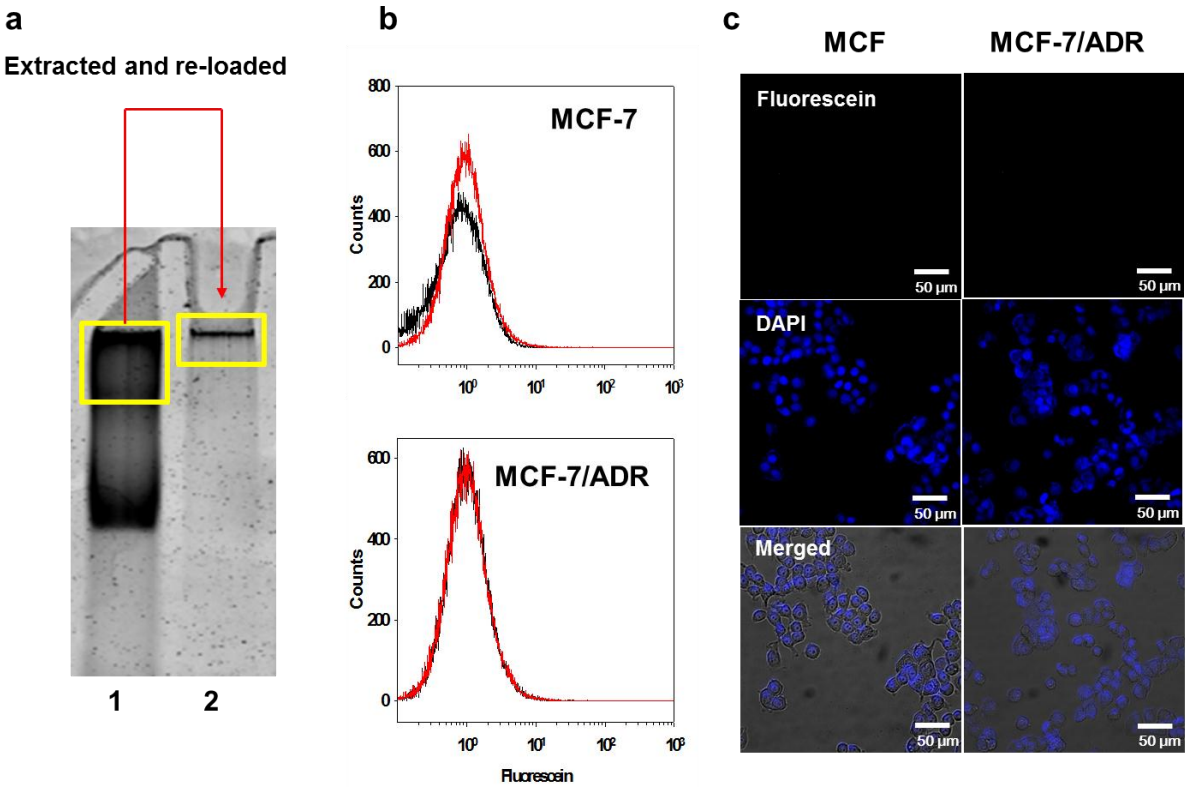

Figure S1. The polymeric mis-assembled product could not be delivered into cells (a) The mis-assembled portion (lane 1) was extracted from the gel and reloaded to confirm the retained the polymeric state (lane 2) (b) Flow cytometry analysis for the cellular uptake of the mis-assembled portion into MCF-7 (top) or MCF-7/ADR cells (bottom). The black and red traces represent the untreated cells and the cells treated with the mis-assembled portion, respectively. (c) Fluorescence microscopic images of MCF-7 (left) and MCF-7/ADR (right) cells treated with the mis-assembled portion. The cellular uptake experiments were performed by using the same manner adopted in the transfection of Td into the cells.

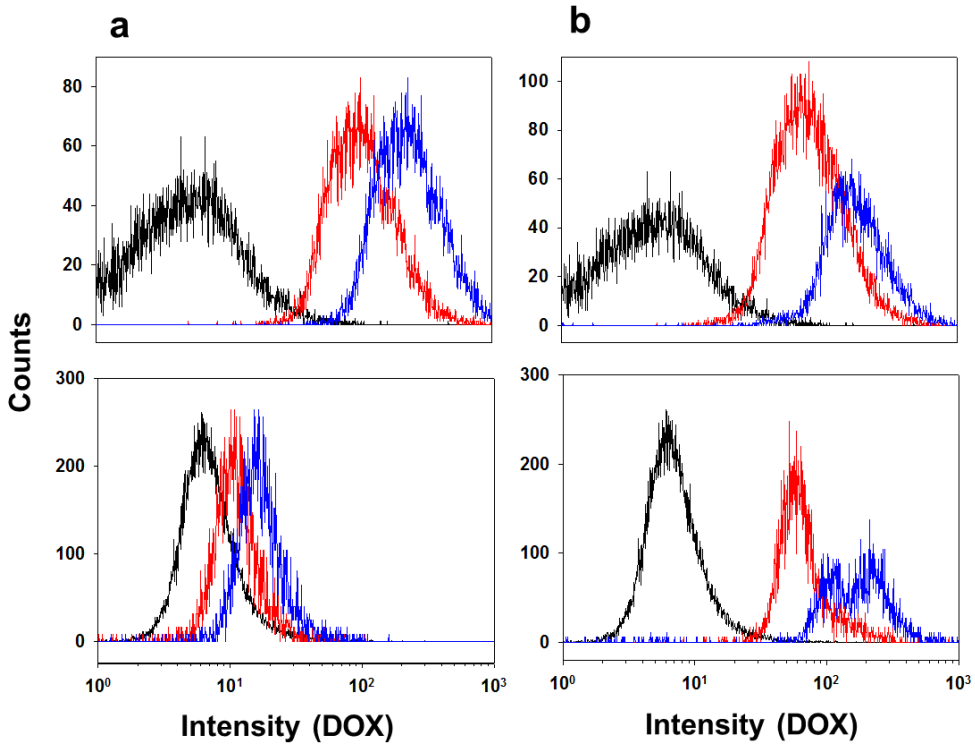

Figure S2. Flow cytometry data showing delivery of (a) free DOX and (b) DOX@Td into MCF-7 cells (top) or MCF-7/ADR cells (bottom) at different concentrations of the drug: 0 nM (black), 10 nM (red), 50 nM (blue).

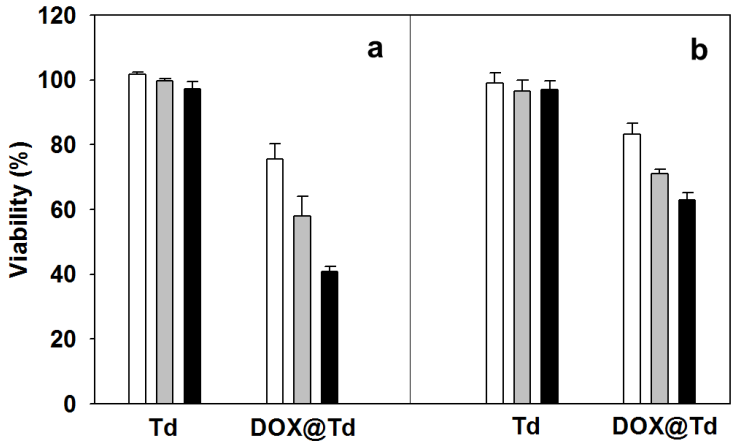

Figure S3. Cytotoxicity of Td and DOX@Td at 5 (white bars), 50 (gray bars), and 500 nM DNA (black bars) in (a) MCF-7 and (b) MCF-7/ADR cells.

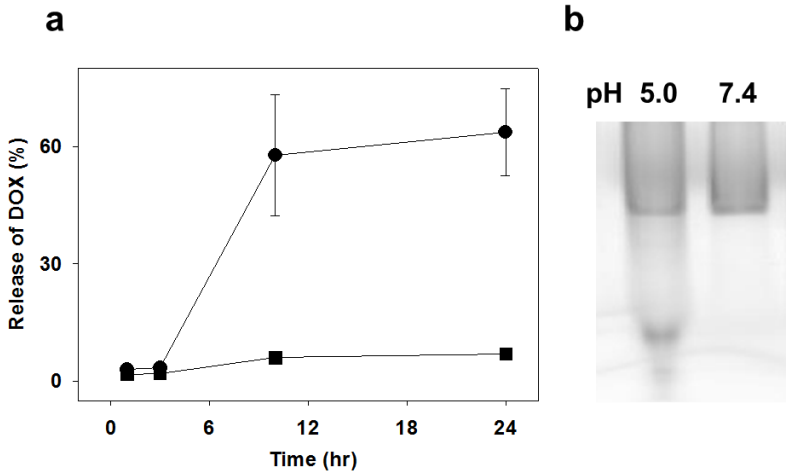

Figure S4. (a) *In vitro* release of DOX from DOX@Td at pH 7.4 (squares) and at pH 5.0 (circles). (b) PAGE analysis of Td assembled at pH 7.4 and at pH 5.0.

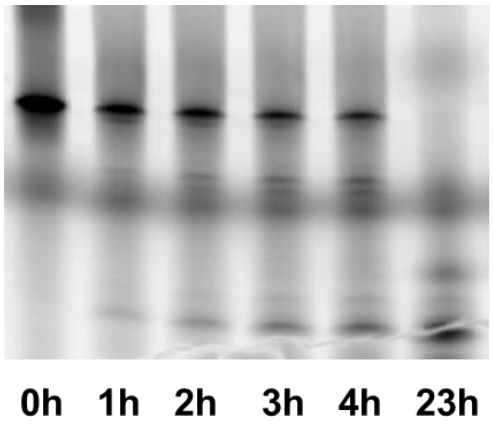

Figure S5. Gel electrophoretic analysis of the stability of Td in 10% human serum.

EXPERIMENTAL SECTION

Preparation of Td: All DNA oligonucleotides were purchased from Bioneer (Daejeon, Korea). Td was assembled by mixing S1, S2, S3 and Cy5-S4 (or FAM-S4). Four DNA sequences (250 nM for the final concentration of each sequence) were mixed in TM buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH=8). The mixture was sequentially incubated at 95 °C for

5 min and at 54 °C for 30 min, and then cooled to 4 °C over approximately 30 sec. Oligonucleotide sequences and schematic diagrams of the tetrahedron are shown in Table S1 and Figure 1, respectively.

Gel electrophoresis: Non-denaturing polyacrylamide gel (6%) was run in TBE buffer with 100V at 4 °C for 40 min. After electrophoresis, the image was visualized using a fluorescence scanner (Typhoon9400, GE healthcare, USA).

Dynamic light scattering: The hydrodynamic size of the DNA tetrahedron was measured in Zetasizer (Malvern, UK) by following a literature procedure.[1]

AFM imaging: Freshly cleaned Si wafer (10 mm×10 mm) was spin-coated with the solution of Td (250 nM, 100 μL) in TM buffer at 3000 rpm for 1 min. The DNA sample was imaged in tapping mode on a Dimension AFM instrument (Dimension D3100, Veeco, USA) in air, using 0.01 – 0.025 Ohm·cm Antimony (n) doped Si probes having a resonance frequency in the range of 332–376 kHz (Veeco-TESP). AFM imaging was performed at 25 °C. AFM data were processed with NanoScope 7.20 software.

Transfection of Td into breast cancer cells: MCF-7 cells used in this study were purchased from the Korean Cell Line Bank (Seoul, Korea). MCF-7/ADR cells were kindly donated from Dr. Dae-Duk Kim (College of Pharmacy, Seoul National University, Seoul, Korea).[2] Cells were plated in glass-bottomed 35 mm petri dishes with RPMI1640 media (Gibco, USA) containing 10% heat inactivated fetal bovine serum, 1% penicillin and streptomycin. After cells (2.5×10^4) were seeded in each dish, the dishes were incubated overnight at 37 °C in humidified atmosphere containing 5% CO_2. Growth medium was removed from each cell

sample, and the cells were washed twice with PBS (Gibco, USA). Td (10 nM) in fresh media (250 μL) lacking serum and the antibiotics was then added to a sample of cells and incubated for 6 h at 37 °C in humidified atmosphere containing 5% CO_2. Cells then were washed twice with PBS and used for microscopy experiments.

Microscopic imaging of Td in breast cancer cells: For microscopic examination, the nuclei were stained using 3 μg/mL Hoechst 34580 (Invitrogen, USA), and the cells were washed with PBS (200 μL) twice. PBS (200 μL) was then added. Live cells were imaged using a fluorescence microscopy (DeltaVision RT, Applied Precision, USA). Excitation/emission filters used for Cy5, doxorubicin (or fluorescein), and Hoechst 34580 (or DAPI) were 630-650/665-705 nm, 480-500/509-547 nm, and 340-380/432-482 nm, respectively.

Flow cytometry analysis: For flow cytometry analyses shown in Figure 3, Figure 4 and Figure S1, FAM-S4 strand instead of Cy5-S4 was used for the preparation of fluorescently labeled Td. Cells were seeded on 24-well culture plates at a density of 10^5 cells/mL and cultured for 24 h at 37 °C with 5% CO_2 and then washed twice with PBS. They were incubated with the fluorescein-labeled Td for 6 h at 37 °C with 5% CO_2, harvested, and washed three times with PBS. Then, 0.2 mL of trypsin replacement (TrypLE™, Gibco, USA) was added to each sample, and the samples were incubated for 5 min at 37 °C. Then, 1 mL of media was added to each sample, and the resulting cell suspensions were transferred to conical tubes (Falcon™ tubes, BD Biosciences, USA) and centrifuged for 3 min at 2500 rpm. Supernatant was removed, and the cell pellets were resuspended in 1 mL of PBS. Fluorescence intensity of the cells was determined by a flow cytometry (FC500, Beckman coulter, USA) with emission at 488 nm and excitation at 525 nm, respectively. Samples of at least 1000 cells were analyzed in triplicate.

Preparation of DOX@Td: For DOX intercalation into the Cy5-labeled Td, DOX (1 mM) was mixed with the fluorescent DNA tetrahedron (10 μM) for 1 h and then run through NAP10 (G25-DNA grade column, GE Healthcare, USA) to remove the free DOX remaining in solution. The number of intercalated DOX molecules per DNA tetrahedron was calculated using the extinction coefficient of Cy5 at 633 nm (ε=230400 $M^{-1}cm^{-1}$) and that of DOX at 480 nm (ε=10410 $M^{-1}cm^{-1}$), respectively.

Endocytosis mechanism: MCF-7 cells (2×10^5 cells/well) were seeded with RPMI1640 media (2 mL) on a 6-well plate and incubated overnight at 37 °C in humidified atmosphere containing 5% CO_2. For the treatment of inhibitors, the media were replaced with the fresh media (2 mL) containing amiloride (2 mM, an inhibitor for macropinocytosis), chloroproamzine (10 μg/mL, CPZ, an inhibitor for clathrin-mediated endocytosis), or genistein (200 μM, an inhibitor for caveolae-mediated endocytosis). After 30 min, Td (10 nM) was added into the cell media and incubated at 37°C for 2 h. After washing with PBS, the cells were trypsinized, centrifuged and lysed in RIPA buffer, and fluorescence intensity of Cy5 labeled on Td was measured using an fluorescence microplate reader (AppliskanTM, Thermo Fisher Scientific, USA) for quantitation of intracellularly delivered Td *via* endocytosis. The amount of Td in cell lysates was normalized to the total cellular protein content of cells, which was determined by protein assay kit (BIO-RAD, Hercules, CA, USA).

Cellular uptake of free DOX and DOX@Td: MCF-7 and MCF-7/ADR cells (2.5×10^4) were plated in glass-bottomed 35 mm petri dishes with RPMI1640 media containing 10% heat inactivated fetal bovine serum, 1% penicillin and streptomycin, and incubated at 37 °C overnight in humidified atmosphere containing 5% CO_2. The culture media were replaced

with the fresh media containing free DOX (10 nM) or 10 nM DOX (10 nM)-loaded Td (DOX@Td), and then incubated at 37 °C for 6 h in the CO_2 chamber. The cells were examined under the fluorescence microscopy using the same procedure described above.

For flow cytometry analysis, MCF-7 and MCF-7/ADR cells were seeded with complete RPMI1640 media (1 mL) onto 24-well plates at a density of 10^5 cells/well and incubated at 37 °C in the CO_2 chamber for 24 hour. The media were replaced with the fresh media containing free DOX (10 and 50 nM) or DOX (10 and 50 nM)-loaded Td (DOX@Td), and then incubated at 37 °C for 6 h in the CO_2 chamber. The cells were washed with PBS twice, harvested and suspended in PBS (1 mL) for the flow cytometry analysis.

Cell viability assays: Cytotoxicity of Td was estimated using an MTT assay. Briefly, 8×10^3 mammalian cells were seeded with media (100 μL) in 96-well plates and cultured overnight to reach ~80% confluency. The cells were then incubated in the fresh media containing Td at 37 °C for 6 h in the CO_2 chamber. For cytotoxicity by the DOX treatment, alternatively, the cells were incubated in fresh media containing free DOX or DOX@Td were incubated at 37 °C for 24 h in the CO_2 chamber. Then, thiazolyl blue tetrazolium bromide (MTT, TACS, Germany) solution (10 μL) was added to each well, followed by 4 h incubation at 37 °C. Next, cells were lysed with 200 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA). After overnight incubation at room temperature, the absorbance was measured at 580 nm using a microplate reader (SpectraMax Plus^{TM}, Molecular Devices, USA).

***In vitro* release of DOX:** To estimate *in vitro* drug release, DOX@Td containing 10 μM DOX was added into a conical filter (Amicon Ultra-0.5, MWCO=10,000, Millipore, USA). The filter was placed in the tube containing PBS reservoir (1 mL, pH=5.0 or 7.4). The tube was incubated at 37 °C under light sealed condition. After 3, 10, and 24 h, fluorescence

intensity of the PBS reservoir was measured to estimate the drug release through the filter by using a fluorescence microplate reader (Appliskan™, Thermo Fisher Scientific, USA). Excitation at 480 nm and emission at 595 nm were employed for the measurement, respectively.

Nuclease resistance. For the stability test, 10% human serum (10 μL, Gibco, USA) were added to DNA solutions (90 μL, 1 μM), and the mixtures were incubated at 37°C. At each time point, the solutions were quenched by adding the stop solution (20 μL) composed of 98% deionizedformamide, 10 mM EDTA, 0.5 mg/mL bromophenol blue and xylencyanol, and then analyzed on a denaturing 12% PAGE (7 M urea). The amount of undamaged DNA structures was estimated by visualization of Cy5-S4 on a fluorescence scanner (Typhoon9400, GE healthcare, USA).

[1] K. A. Afonin, W. W. Grabow, F. Walker, E. Bindewald, M. Dobrovolskaia, B. A. Shapiro, L. Jaeger, *Nat. Protocols* **2011**, *6*, 2022.

[2] H.-J. Cho, H. Y. Yoon, H. Koo, S.-H. Ko, J.-S. Shim, J.-H. Lee, K. Kim, I. C. Kwon, D.-D. Kim, *Biomaterials* **2011**, *32*, 7181.