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Supplementary Materials

Selective Delivery of an Anticancer Drug with Aptamer-Functionalized Liposomes to Breast Cancer Cells *in Vitro* and *in Vivo*

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Experimental Details

Formulation of liposomes

DNA-functionalized, cargo-loaded liposomes are prepared according to a previously published method,[1] with some modifications. Stock solutions of 50 mg mL^{-1} of HSPC (3.0 mg), cholesterol (0.77 mg), and mPEG2000–DSPE (0.89 mg) in chloroform were mixed in a 2:1:0.16 molar ratio in a scintillation vial. This mixture was blown dry with N_2 and further dried under vacuum overnight. The liposome preparation buffer contained 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethane sulfonic acid (HEPES, pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. The buffer solution (100 μL) was added to the dry lipids followed by addition of 7 nmol of cholesterol-tagged DNA. After the mixture was incubated for 6 h at 37 °C, 400 μL of liposome preparation buffer containing 25 mg mL^{-1} doxorubicin was added. A doxorubicin stock solution was prepared by dissolving the drug in 25 mM HEPES preparation buffer at 50 °C followed by cooling to 37 °C for incubation with lipids. The overall lipid concentration was estimated to be ~4.7 mg mL^{-1} with a DNA concentration of 14 μM for the mixture. This solution was gently stirred and kept at 37 °C for 3 h. After that, the solution was stirred and then alternatively flash-frozen in liquid nitrogen and thawed at least 5 times each. The final solution was incubated at 37 °C overnight. The lipid mixture was then extruded to form liposomes of ~200 nm in diameter following the instructions from Avanti Polar Lipids, Inc. The liposomes were purified on a column containing Sephadex G-100 medium to remove free DNA and doxorubicin. Fluorescence spectra of fully decomposed liposome samples were used to determine the concentration of encapsulated fluorescent cargo by plotting a standard curve of fluorescence emission intensity versus concentration. DNA-functionalized liposome samples were standardized to the same corresponding cargo concentration before any imaging or therapy experiments. For the *in vivo* tumor efficacy study, the doxorubicin concentration of control and aptamer liposome samples was standardized to ~1 mg mL^{-1} for consistency. In a typical experiment, two vials of lipid mixture will generate approximately 500 μL of ~1 mg mL^{-1} doxorubicin-loaded liposome samples.

Typically, liposomes are characterized after extrusion. These characterization methods include: loading capacity estimation, stability and release profile test, size measurement by using cryo-EM and DLS, surface charge estimation by measuring the ζ-potential.

Microscope characterization

In cell staining and microscope images, in order to focus on the evaluation of pure internalization of the aptamer-modified liposome, the fluorescent dye uranin was loaded into liposomes instead of Dox to avoid cell death due to the cytotoxicity of Dox. Fluorescence microscope images of uranin-loaded

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liposomes (Urn-Lip) were acquired with a Zeiss Axiovert 200M inverted microscope equipped with an EC Epiplan 40× objective and CCD camera under mercury lamps for excitation. The digital camera was white-balanced using Zeiss Axiovision software before data acquisition. Measurement of fluorescence spectra of liposome samples was carried out in a Fluoromax-P fluorimeter (Horiba Jobin Yvon, Edison, NJ) at room temperature.

For MTT assay, the absorbance wavelength on a Victor$^{3}{}_{TM}$ V microplate reader (Perkin-Elmer, Waltham, MA) was set at 590 nm. Flow cytometric analysis of cells was conducted using a BD FACSCanto 6 color flow cytometry analyzer (BD, Franklin Lakes, NJ).

The confocal microscopy images for cell internalization studies were taken on a LSM700 Confocal Microscope (Carl Zeiss, Thornwood, NY) using a 63× / 1.4 oil lens with excitation wavelengths set at 405 nm, 488 nm and 555 nm.

For the *ex vivo* tumor penetration study, the flash frozen tumor tissue embedded with optimum cutting temperature (O.C.T.) compound (Sakura Finetek, USA) was sectioned (5 μm thick) with a Leica CM3050S cryostat and mounted on glass slides. Tissue sections were observed on an Axiovert 200M fluorescence microscope (Carl Zeiss, Thornwood, NY). Fluorescent images were taken on a Zeiss Axiovert 200M fluorescence microscope. A tiling image was taken with fixed exposure time to show the penetration of Urn-Lip in tumor sections.

For the *in vivo* tumor penetration study, flash frozen tumor sections measuring 10 μm in thickness were imaged on an Axiovert 200M fluorescence microscope and LSM700 confocal microscope. The nucleus was stained by DAPI. Fluorescence images were taken on a Zeiss Axiovert 200M fluorescence microscope to image the whole injection area. Cellular internalization in the selected area of tissue sections were further analysed with a LSM 700 confocal microscope. Tissue sections were imaged with a 63× and 0.3 NA lens. Doxorubicin fluorescence, representing Dox-Lipo, was visualized with 555 nm laser excitation.

Cryo-EM characterization

Cryo-EM samples of liposomes were prepared using a Vitrobot. Briefly, a drop of liposome solution was applied onto a TEM grid, the grid was then blotted and immediately plunge-frozen into ethane slush cooled by liquid nitrogen. The as-prepared sample, on TEM grid, was then transferred onto a cryo holder via a Gatan CT3500 Cryotransfer system, which was pre-cooled with liquid nitrogen. The images were obtained on a JEOL 2100 transmission electron microscope at an acceleration voltage of 80 kV.

DLS and ξ-potential measurement

To estimate the size and charge of the liposomes, dynamic light scattering and ξ-potential experiments were performed using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Freshly prepared unmodified or DNA-conjugated liposomes were dispersed in a buffer containing 10 mM NaCl, 5 mM HEPES at pH 7.4 to a lipid concentration of ~0.1 mg mL^{-1} for size and ξ-potential measurement. The reported hydrodiameter and ξ-potential of liposome samples was calculated from the algebraic average of twenty measurements. Errors represent standard deviation.

Cell Culture

MCF-7 and LNCaP cells (ATCC, Manassas, VA) were cultured in DMEM medium containing 10% Fetal Bovine Serum (FBS) and supplemented with 1000 units mL^{-1} aqueous Penicillin G, 100 μg mL^{-1} streptomycin (Invitrogen, Carlsbad, CA). For the culture of MCF-7 cells, 1 nM estrogen (Sigma-Aldrich Inc., St. Louis, MO) was added to the cell media.

Cellular internalization

MCF-7 cells (250,000) were seeded in a 4-well chamber slide for 24 h (37 °C, 5% CO_2). Cells were washed once with Opti-MEM and then incubated for 4 h (37 °C) with 1 mL Opti-MEM containing either Apt-Urn-Lip or Ctrl-Urn-Lip (50 nM lipid equivalent). The cells were then washed with PBS (3 x 1 mL), fixed with 4% paraformaldehyde, and subsequently imaged on a confocal laser scanning microscope. Nuclei were stained by DAPI. Cells without the addition of Urn-Lip were imaged as control.

To examine cell uptake of Urn-Lip, MCF-7 cells (200,000) were seeded in a 12-well plate for 24 h. Urn-Lip (50 nM or 500 nM lipids in equivalent) were incubated with the cells in opti-MEM (1 mL) for 2 h (37 °C, 5% CO_2). The cells were then washed with PBS (3 x 1 mL) and detached via trypsinization. Cells were fixed with 4% paraformaldehyde for flow cytometry analysis (10,000 cells analysed, green fluorescence, FITC channel). Both the percentage of the fluorescent cells relative to the total analysed cells and the fluorescence intensity of the fluorescence-positive cells were assessed. The marker was set such that 1.0% of non-treated cells were fluorescently positive. All experiments were performed in triplicate.

Cytotoxicity (MTT assay)

MCF-7 cells (3,000 per well) were seeded in 96-well plates and grown in DMEM medium containing 10% FBS and 1 nM estrogen at 37 °C for 24 h in a humidified 5% CO_2 atmosphere. The medium was then replaced with fresh medium containing Dox, Ctrl-Dox-Lip, or Apt-Dox-Lip in concentrations ranging from 10 nM to 500 nM of Dox or equivalent Dox. At each concentration six wells per plate were treated. The cells were treated for 6 h and then the drug-containing media was replaced with fresh

media. The cells were further incubated for 66 h. The cell viability was determined by the MTT assay. The standard MTT assay protocols were followed thereafter [2].

Additional Figures

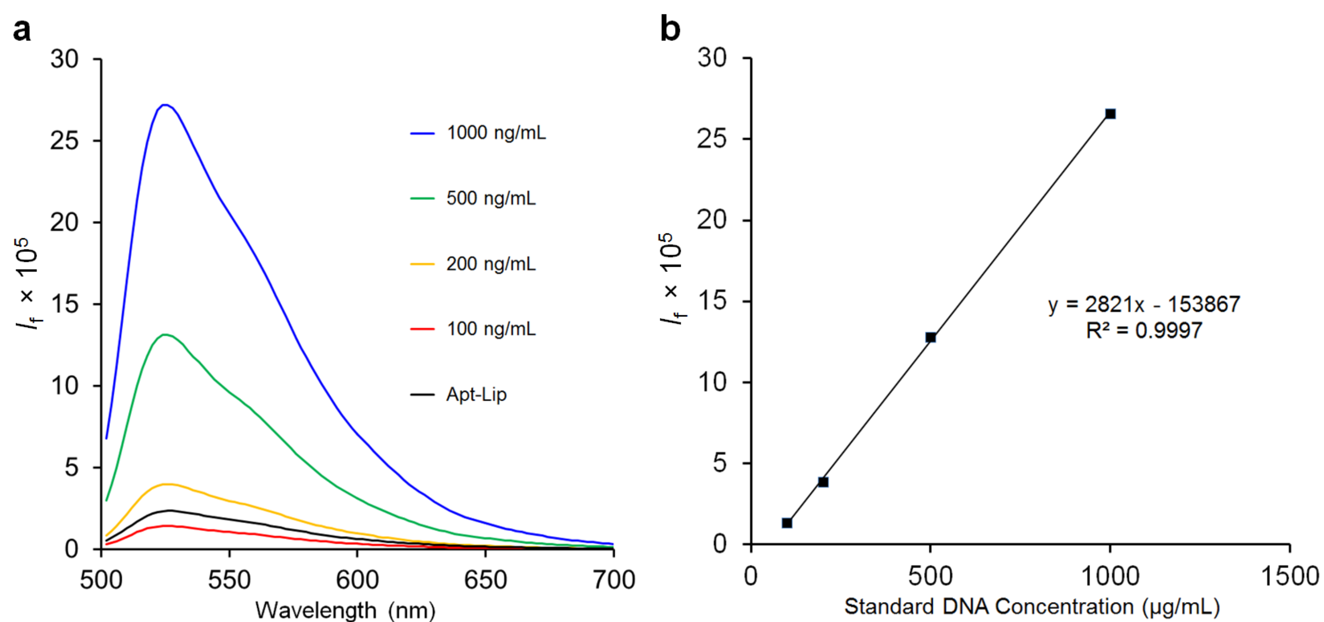

Figure S1. (a) Fluorescent spectra of OliGreen® ssDNA reagent stained 100-1000 ng mL^{-1} standard DNA solutions and Apt-Lip sample. Apt-Lip solution was treated to break the lipid bilayer and release DNA strands before staining. λ_{ex} = 480 nm. λ_{em} = 520 nm. (b) Standard curve for quantification of DNA aptamer concentration plotted from 100-1000 ng mL^{-1} standard DNA solutions. The NCL aptamer concentration in Apt-Lip sample was calculated to be ~9.7 μM. Therefore, in 1 mL Apt-Lip samples, there are approximately 9.7 nmol NCL aptamer strands with ~9.3 mg lipids. The conjugation efficiency is estimated to be [9.7 μM / (14 μM × 2)] × 100% = 34.6%.

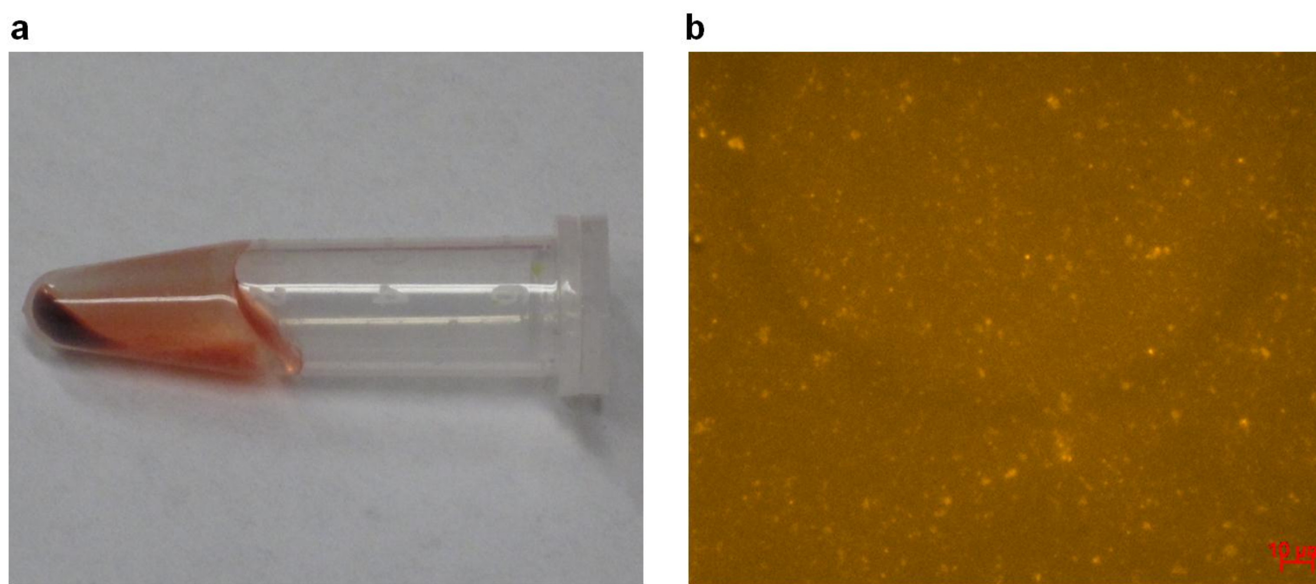

Figure S2. (a) Photograph of doxorubicin-loaded liposome sample. Liposome sample solution was centrifuged at 2000 g for 10 min to precipitate dark red Dox-Lip samples to the bottom of centrifuge tube. (b) Fluorescence microscope image of Rhodamine 6G-loaded liposome sample (Zeiss, Axiovert 200M). 40X objective lens and 555 nm excitation wavelength were used for imaging of liposomes.

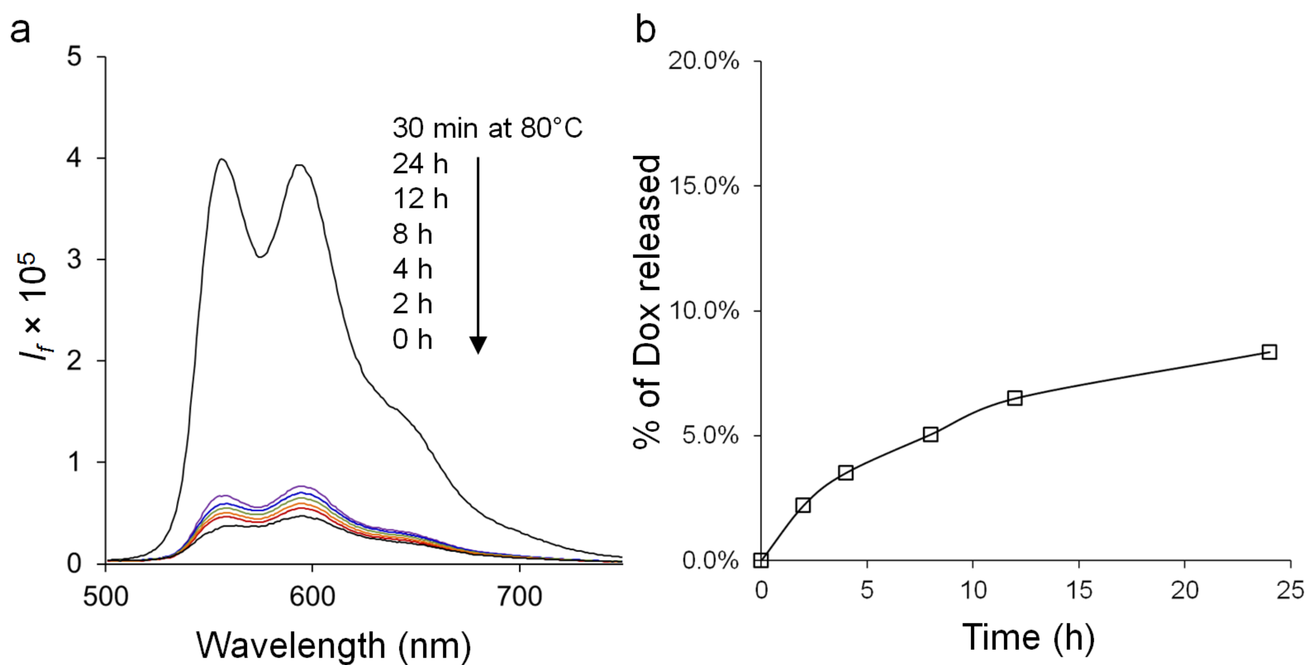

Figure S3. Release profile of doxorubicin-loaded liposome. (a) Fluorescent spectra of Apt-Dox-Lip sample solution at 1:500 dilution in 25 mM HEPES preparation buffer (pH 7.4) after incubation at 37 °C for different lengths of time. λex = 480 nm; λem= 592 nm. Heat treatment was used to facilitate the rupture of liposomes. (b) Timecourse study of doxorubicin release from liposomes at 37 °C in 25 mM HEPES preparation buffer.

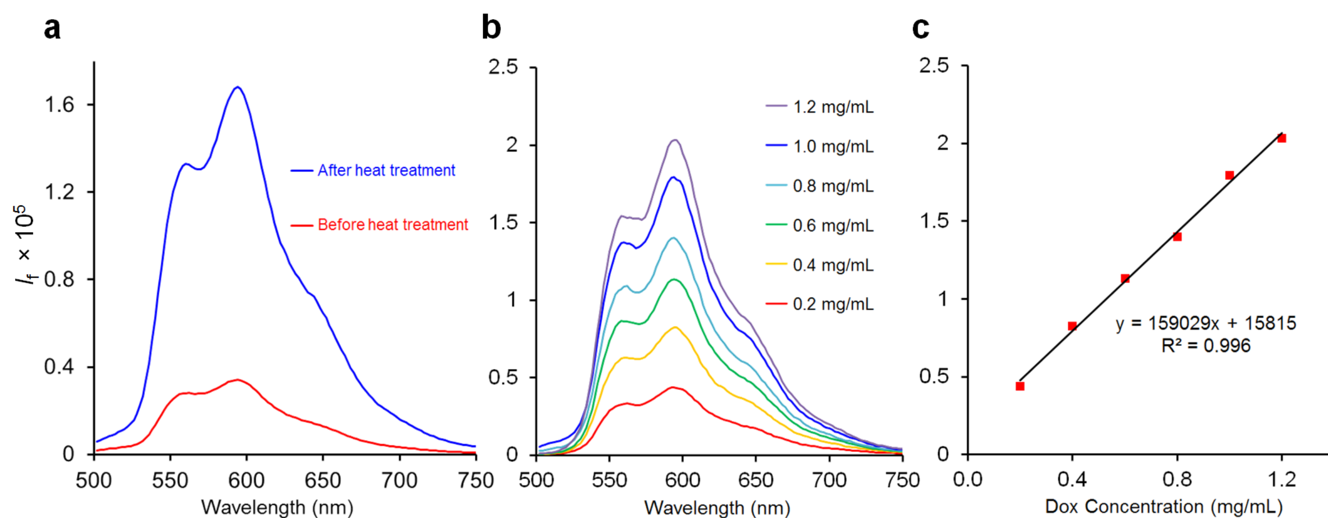

Figure S4. (a) Fluorescent spectra of Apt-Dox-Lip solutions before and after heat treatment. (b) Fluorescent spectra of free Dox solutions from 0.2-1.2 mg mL^{-1}. (c) Standard curve for quantification of doxorubicin concentration. The fluorescence emission intensity at 592 nm was plotted as a linear function of Dox concentration. The equivalent Dox concentration in Apt-Dox-Lip sample was determined to be 0.92 mg mL^{-1} with the drug-to-lipid ratio of 0.097 (wt/wt).

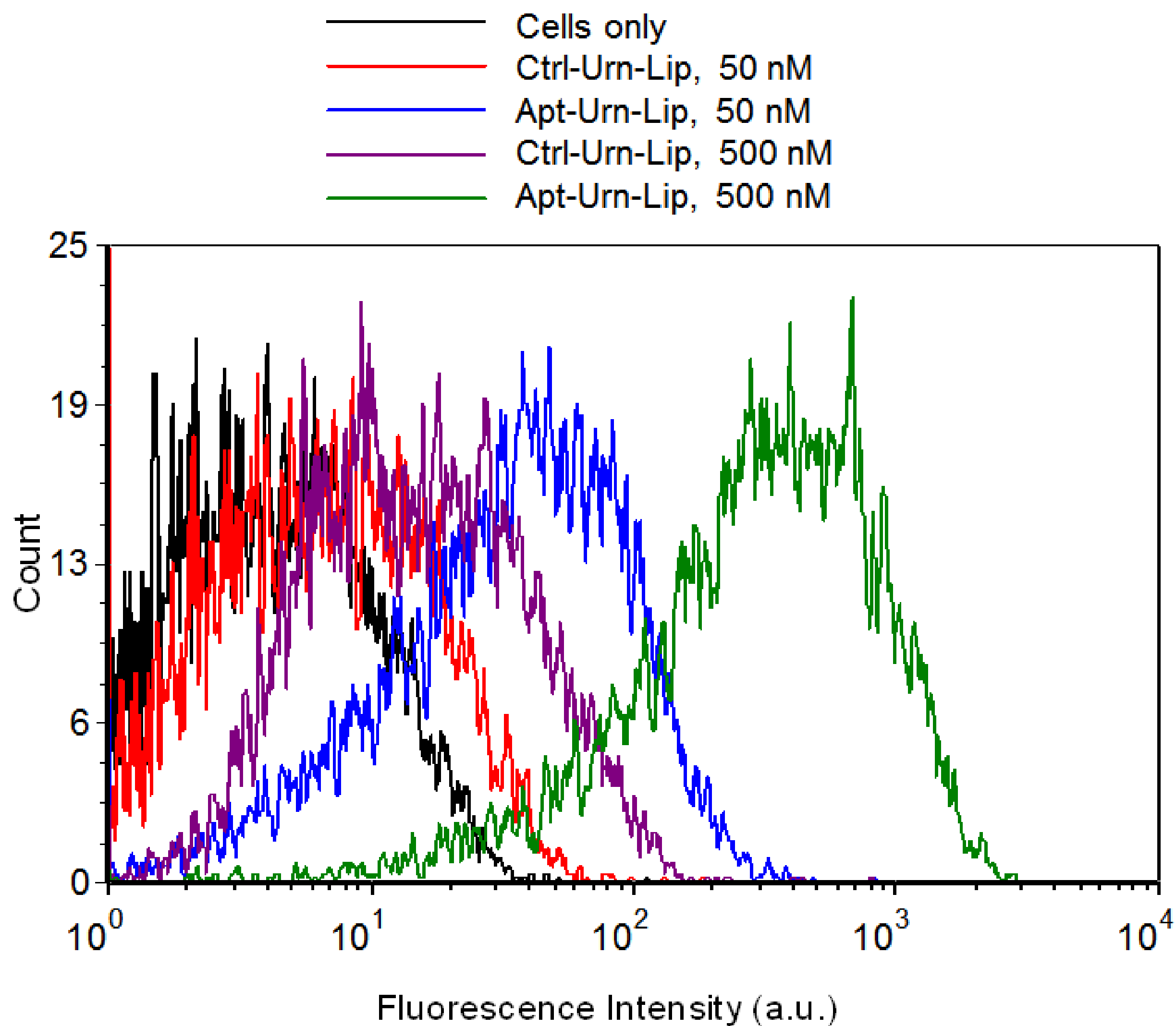

Figure S5. *In vitro* cellular targeting study of MCF-7 cells with bare Ctrl-Urn-Lip or Apt-Urn-Lip by flow cytometry. MCF-7 cells (200,000 per well) were plated in a 12-well plate. The cells were treated with liposome (50-500 nM lipids in equivalent) labeled with uranin at 37 ºC for 2 h. The cells were then washed, trypsinized and fixed in 4% paraformaldehyde solution for flow cytometry analysis.

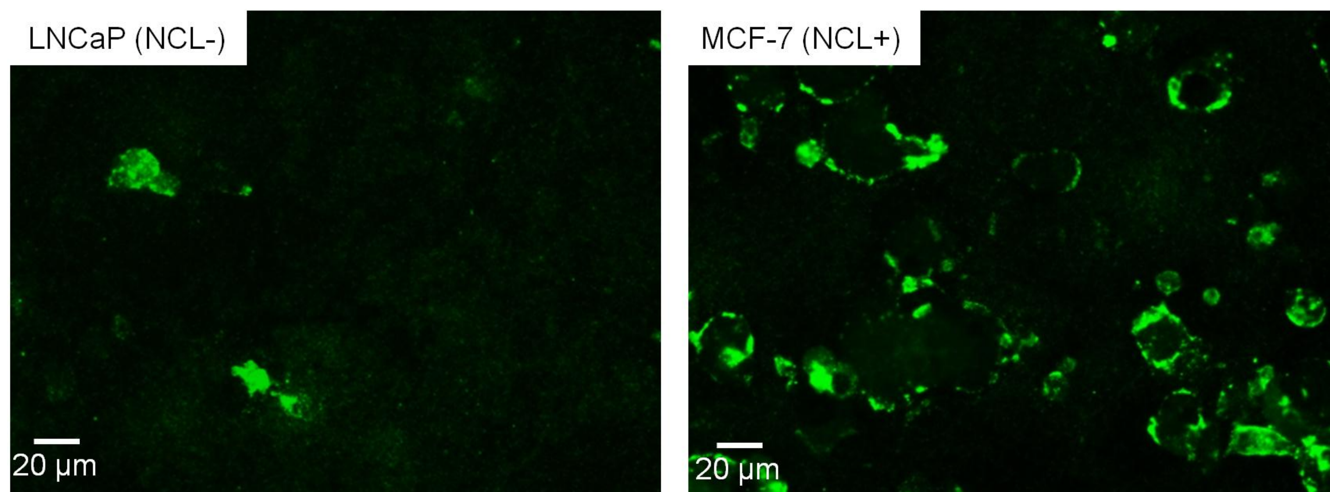

Figure S6. Fluorescence microscope images of LNCaP (left) and MCF-7 (right) cells treated with NCL aptamer-functionalized liposomes (Apt-Urn-Lip) containing uranin. LNCaP cells (200,000 per well) or MCF-7 cells (200,000 per well) were plated in a 12-well plate. The cells were treated with liposome (500 nM lipids in equivalent) labeled with uranin at 37 ºC for 2 h. The cells were then washed and fixed in 4% paraformaldehyde solution for fluorescence microscope imaging analysis.

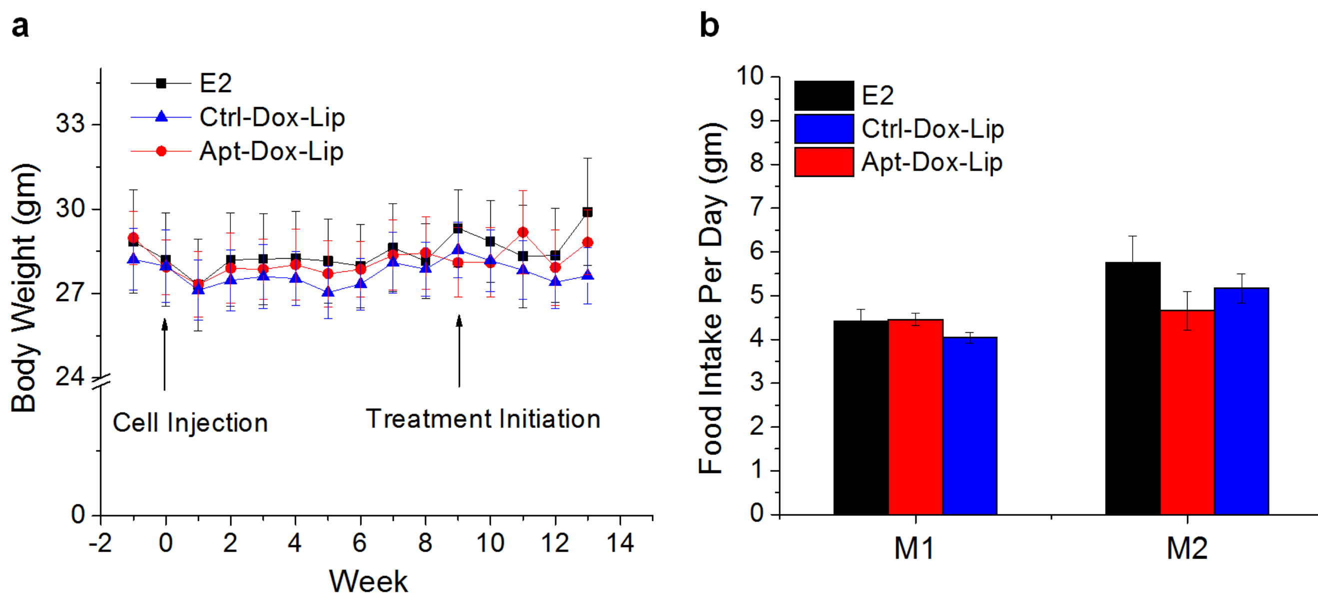

Figure S7. (a) Neither Apt-Dox-Lip nor Ctrl-Dox-Lip affected the body weight of mice bearing human breast MCF-7 tumors. The animals' body weight was monitored weekly throughout the study. Data were analyzed using the PROC MIXED of SAS, and the results showed no significant differences between the experimental groups. (b) Neither Apt-Dox-Lip nor Ctrl-Dox-Lip affected food intake (per day) of mice bearing human breast MCF-7 tumors. Food intake (per day) was measured twice, the first time at week 3 (M1) and the second time at week 12 (M2) post cell injection. Data were analyzed using one-way ANOVA with post hoc Fisher's LSD test, and the results showed no significant differences between the experimental groups.

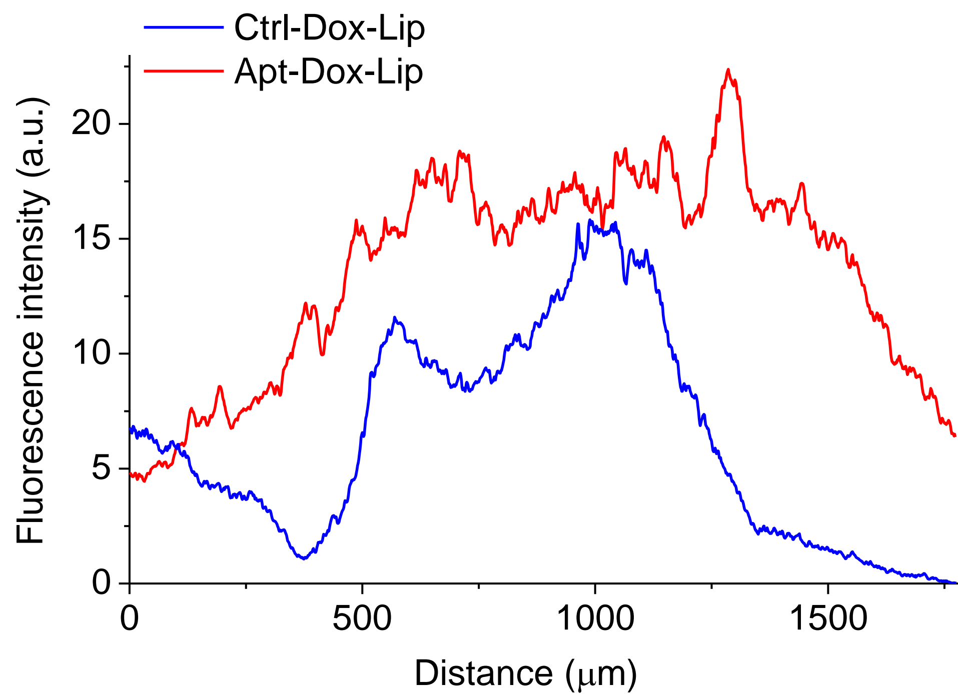

Figure S8. Fluorescence intensity profiles of tumor sections after intratumoral injection of Ctrl-Urn-Lip or Apt-Urn-Lip in MCF-7 tumors and the corresponding single-peak fits.

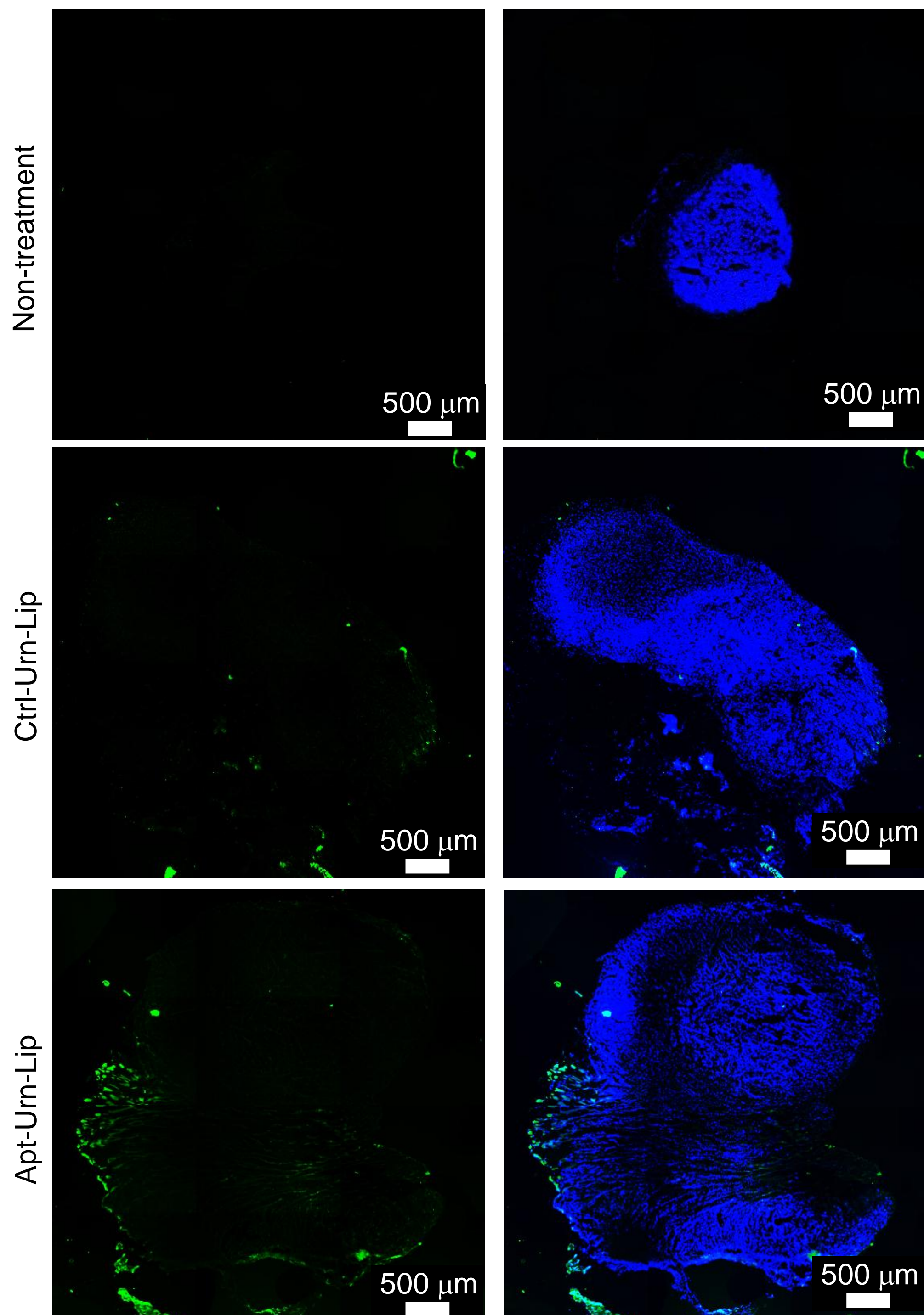

Figure S9. MCF-7 tumors (size: ~4.0 mm × 4.0 mm) were *ex vivo* cultured with PBS (as non-treatment group), Ctrl-Urn-Lip or Apt-Urn-Lip (10 μg uranin in equivalent per mL) in cell medium at 37 ºC for 24 h. Tumor sections (5 μm thickness) were collected by cryostat and mounted on glass slides. Nuclei were

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stained by DAPI. A tiling image was taken by fluorescence microscope (Zeiss, Apotome) with fixed exposure time to show the penetration of liposomes into tumor tissues.

Entry	Group	% FL Positive	Mean of FL Intensity
1	Non-treatment	1.1	22.6
2	Ctrl-Urn-Lip	57.0	44.5
3	Apt-Urn-Lip	93.6	291.6

Table S1. Flow cytometry analysis of MCF-7 cells treated with Ctrl-Urn-Lip or Apt-Urn-Lip. MCF-7 cells (200,000 per well) were plated in a 12-well plate. The cells were treated with liposome (50 nM lipids in equivalent) labeled with uranin at 37 ºC for 4 h. Percentage of fluorescence (FL) positive cells and mean fluorescence intensity are summarized in the table.

***P* value**		D4	D8	D12	D16	D20	D24
DL-Apt	E2		<0.01	<0.01	<0.01	<0.01	<0.01
DL-Apt	DL-random DNA			0.03	0.01	0.02	0.05
DL-random DNA	E2				<0.01	<0.01	<0.01

Table S2. Statistical *P* value between two treatment groups for each measurement. The first column lists the two treatment groups to be compared. The first row lists the time point for each measurement. Data were analyzed using one-way ANOVA. If the overall F ratio was significant, the means of different treatment groups were compared using Fisher's LSD test.

1. Z. H. Cao, R. Tong, A. Mishra, W. C. Xu, G. C. L. Wong, J. J. Cheng and Y. Lu, *Angew. Chem. Int. Ed.*, 2009, **48**, 6494-6498.
2. I. Martin-Kleiner, I. Svoboda-Beusan and J. Gabrilovac, *Immunopharm. Immunot.*, 2006, **28**, 411-420.