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Supporting Information

The Effects of Spacer Length and Composition on Aptamer-Mediated Cell-Specific Targeting with Nanoscale PEGylated Liposomal Doxorubicin

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Materials

The oligonucleotides were purchased from Integrated DNA Technologies Inc. (Coralville, IA). HSPC, cholesterol, mPEG2000-DSPE, mPEG5000-DSPE, maleimide-PEG2000-DSPE, and extruder were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). SYBR Green II stain was obtained from Thermo Fisher Scientific Inc. (Grand Island, NY). Sephadex G-100 medium was obtained from GE Healthcare (Chalfont St. Giles, UK). MDA-MB-231 and MCF-7 cells were obtained from ATCC (Manassas, VA). Streptomycin was obtained from Invitrogen (Carlsbad, CA). Other reagents of analytical grade were obtained from Sigma-Aldrich Inc. (St. Louis, MO) and used without further purification.

Molecular Dynamics

Molecular dynamics simulation was performed using the Discover module in Material Studio 7.0. The polymer consistent force field (PCFF) was selected. The NPT ensemble was used, and simulations were run at a room temperature of 298 K, and an atmospheric pressure of 0.1 GPa, with Berendsen thermostat and barostat respectively. A large periodic cell was constructed including each long molecule under study, as well as randomly filled water molecules, to a final density of the system of 0.997 g/cm^3, the experimental density of liquid water at the temperature and pressure used. Configurations were calculated at 5 fm time steps, with data outputted every 100 steps, with a total duration of 100 pm was lasted to ensure equilibration of each system. Data were recorded after the first 10 000 steps.

Preparation of DNA-Lipid Conjugate

Lipids were activated by adding 300 nM DSPE-PEG2000-Maleimide in 1 mL of PBS buffer and heating to 50 ℃ for 15 min. DNA-lipid conjugate was prepared by mixing 300 nM activated lipids and 100 nM activated thiol-modified DNA into 1 mL of 25 mM HEPES buffer (pH 7.2) and shaking the reaction solution in room temperature for 48 h. The reaction mixture was purified using 4-20% gradient polyacrylamide gel electrophoresis. The purified DNA-lipid conjugate can be extracted from the gel by soaking in extraction buffer (0.5 M ammonium acetate; 10 mM magnesium acetate; 1 mM EDTA; pH 8.0; 0.1% SDS) at 50 ℃ for 30 minutes, twice. The resulting solution was desalted using PD-10 column, lyophilized overnight and dispersed in Millipore water for mass spectrometry and later usage.

Preparation of Liposomes

DNA-functionalized and cargo-loaded liposomes are prepared on the basis of our previously reported method with some modifications. HSPC (5 mg), cholesterol (1.67 mg), and mPEG2000-DSPE (1.67 mg) (or mPEG5000-DSPE (3.46 mg)) were dissolved in 300 μL of chloroform with a molar ratio of 1.5:1:0.14 in a scintillation vial. The mixture was blown dry with N_2 gas and further lyophilized overnight. 4 nmol of DNA was then added; the solution was frozen in liquid nitrogen and lyophilized for 1 h. 300 μL of chloroform was added and blown dry with N_2 gas, followed by overnight lyophilization. After addition of 300 μL hydration buffer (130 mM $(NH_4)_2SO_4$, 10 % w/v sucrose, pH 5.5), the mixture was incubated at 50 ℃ for 30 min, followed by being incubated at 37 ℃ for 2 h. Five freeze-thaw cycles were applied using liquid nitrogen and a 50 ℃ water bath. The mixture was incubated at 37 ℃ for 6 h. The obtained liposomal suspension was sized by extruding the lipid mixture through a filter of pore size 0.1 μm 11 times. The sized liposomes were dialyzed against external buffer (10 mM histidine, 300 mM sucrose, pH 6.5) to remove hydration buffer

outside liposomes using a column containing Sephadex G-100 medium to obtain ~ 1 mL of liposomes. 150 μL of doxorubicin solution (10 mg/mL doxorubicin in external buffer) was then added to the sized and dialyzed liposomes and incubated at 60 ℃ for 1 h. The doxorubicin-loaded liposome was separated from the free doxorubicin over a Sephadex G100 column eluted with external buffer and was cooled down to room temperature and stored at 4 ℃.

Characterization of Liposomes

After dialysis, liposome samples were characterized using cryo-EM, DLS, and zeta-potential measurements.

A Vitrobot was used to prepare the cryo-EM samples of liposomes. Briefly, the TEM grid covered with a drop of liposome solution was blotted and rapidly frozen into liquid ethane cooled by liquid nitrogen. A Gatan CT3500 Cryotransfer system, which was pre-cooled with liquid nitrogen, was used to transfer the as-prepared liposome sample, on a TEM grid, onto a cryo holder. The cryo-EM images were taken on a JEOL 2100 transmission electron microscope at an acceleration voltage of 80 kV.

For estimation of size and surface charge of the liposomes, DLS and zeta-potential were measured by using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Freshly prepared liposomes without drug loading were used for size and zeta-potential measurements. Error bars are obtained from three parallel experiments.

The concentrations of encapsulated doxorubicin were determined from UV-Vis spectra by comparison to a calibration curve of free doxorubicin. Liposome samples were standardized to the same corresponding encapsulated cargo concentration before any cell experiments.

The stability of the Dox-loaded liposomes can be characterized by monitoring the fluorescence of Dox at the emission wavelength of 556 nm. As the fluorescence of intact Dox-loaded liposomes can be significantly lower than released Dox due to self-quenching effects while encapsulated, the extent of fluorescence increase can indicate the stability of the liposomes. 60 μL liposomes were incubated with 90 μL of liposome elution buffer and 150 μL of human serum at 37 ℃. The fluorescence can be monitored by taking an aliquot of the solution in different time points.

Estimation of Lipids and DNA Concentration

Nile Red-stained liposomes without loaded cargo were prepared to measure lipid concentrations. The concentrations were analyzed by measuring the fluorescence of the prepared samples to determine whether the lipid concentrations were the same.

DNA-functionalized liposomes without cargo loading were prepared to measure the DNA concentration and density on the surface of liposomes. The SYBR Green II stain was utilized to measure the concentration and density of DNA. DNA-functionalized liposome samples were treated with Triton-X and heated at 80 ℃ for 30 min to rupture liposomes and release DNA strands prior to the staining process. A calibration curve was obtained from a series of standard DNA solutions following manufacturer's instructions.

Cell Culture

MDA-MB-231 cells were cultured in Dulbecco's Modification of Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS), 100 units/mL Penicillin G, 100 μg/mL streptomycin, and 1 nM estrogen.

MDA-MB-231 cells were seeded in a 4-well chamber slide at 37 ℃ with 5% CO_2 for 24 h. Cells were washed once with Opti-MEM, followed by being incubated at 37 ℃ for 2 h or 6 h with 1 mL Opti-MEM containing prepared liposomes (50 nM lipid equivalent). The cells were then washed with PBS buffer (3 ×1 mL), fixed with 4 % paraformaldehyde, and imaged on a confocal laser scanning microscope. Cells without added liposomes were imaged as control.

To examine cell uptake of doxorubicin-loaded liposomes, MDA-MB-231 cells were seeded in a 12-well plate for 24 h. 10 μL of doxorubicin-loaded liposomes were incubated with cells in 1 mL of Opti-MEM at 37 ℃ with 5% CO_2 for 24 h. The cells were washed with PBS buffer (3 ×1 mL) and then detached by trypsinization. Cells were fixed with 4% paraformaldehyde for flow cytometry analysis (10000 cells analyzed, λ_{ex} = 480 nm, λ_{em} = 593 nm). The fluorescence intensity of the fluorescence-positive cells and the percentage of the fluorescent cells relative to the total analyzed cells were analyzed. The marker was set such that 1.0% of non-treated cells were fluorescently positive. All experiments were performed in triplicate.

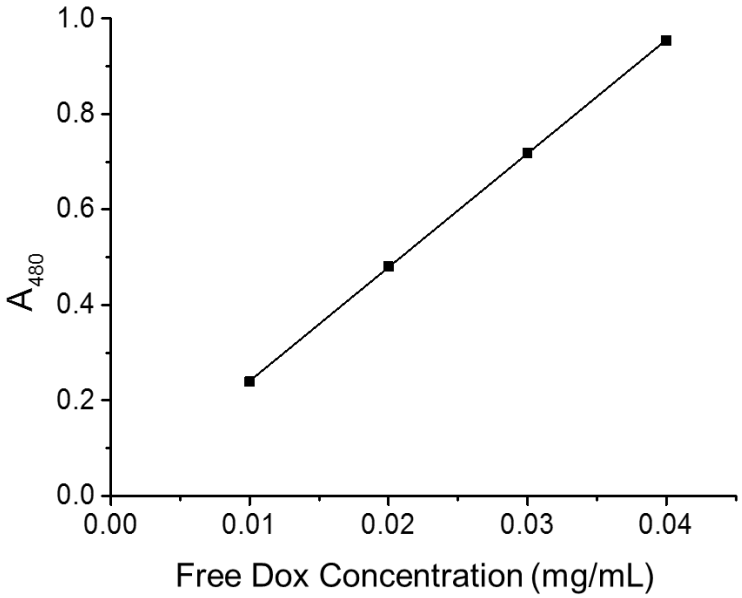

Figure S1. Standard curve for the quantification of doxorubicin. Absorbance at 480 nm vs. concentrations of free doxorubicin.

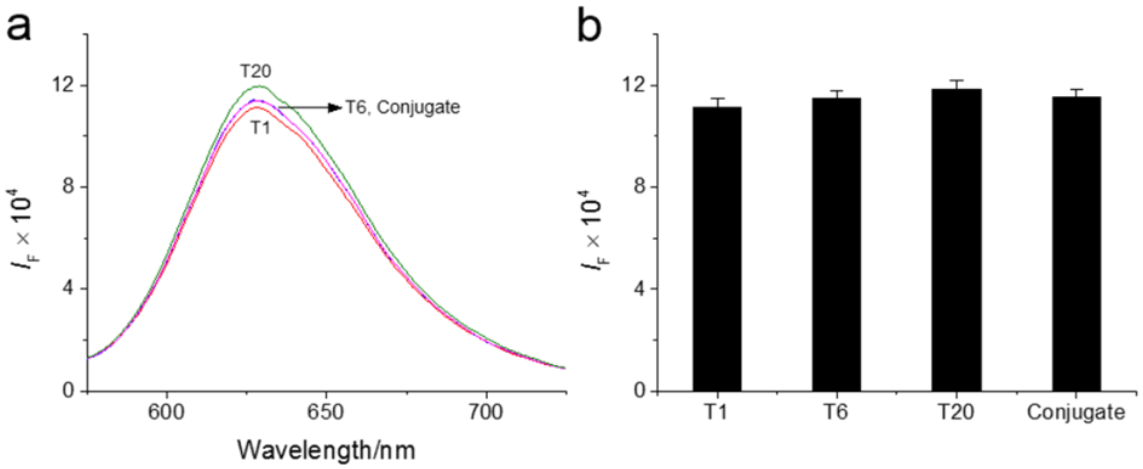

Figure S2. (a) Fluorescent spectra of Nile Red stained DNA-PEG-Lip samples in ethanol. λ_{ex} = 552 nm. λ_{em} = 627 nm. (b) Fluorescent intensities at 627 nm of different DNA-PEG-Lip samples. Bars show no significant difference at the level of $p > 0.05$ under Student *T*-test, indicating that there is no significant difference between concentrations of prepared liposome samples.

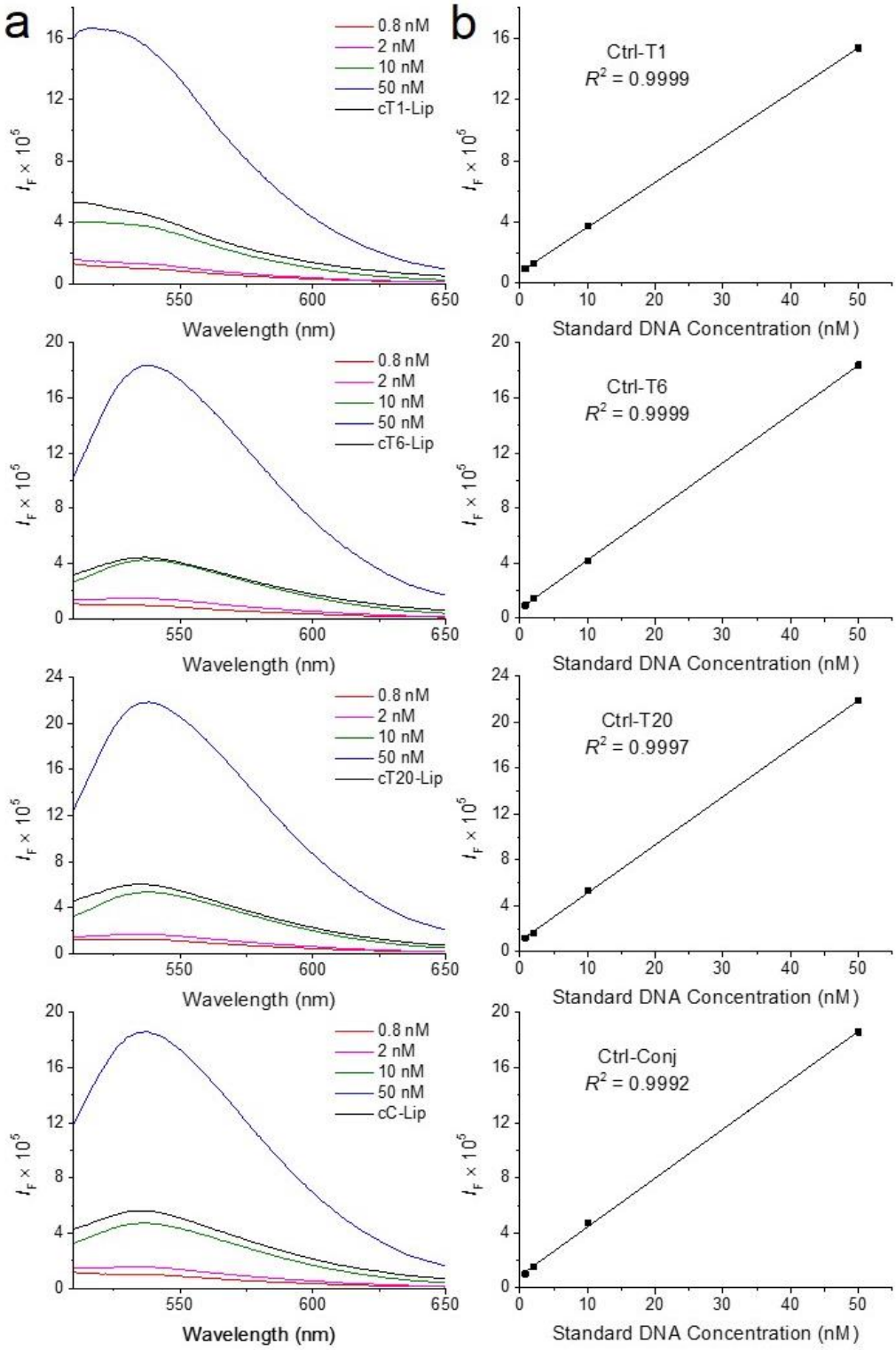

Figure S3. (a) Fluorescent spectra of SYBR Green II stained DNA-PEG-Lip samples. DNA-PEG-Lip solutions were treated to break lipid bilayers and release DNA strands before staining. λ_{ex} = 497 nm. λ_{em} = 538 nm. (b) Standard curves for quantification of DNA concentrations plotted from 0.8-50 nm standard DNA solutions.

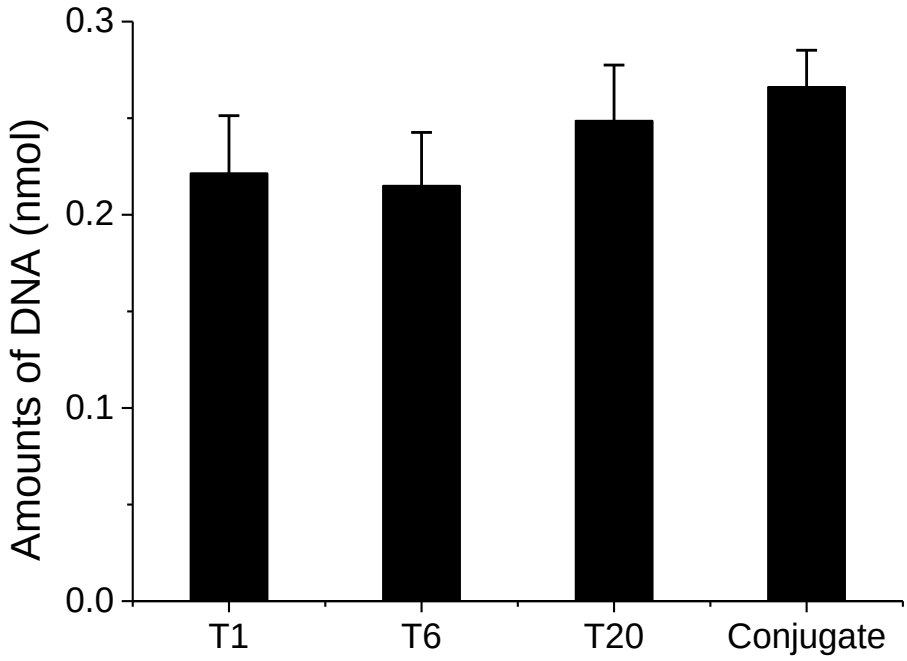

Figure S4. The amounts of DNAs in ~100 μL liposome samples measured by SYBR Green II. Bars show no significant difference at the level of $p > 0.05$ under Student *T*-test.

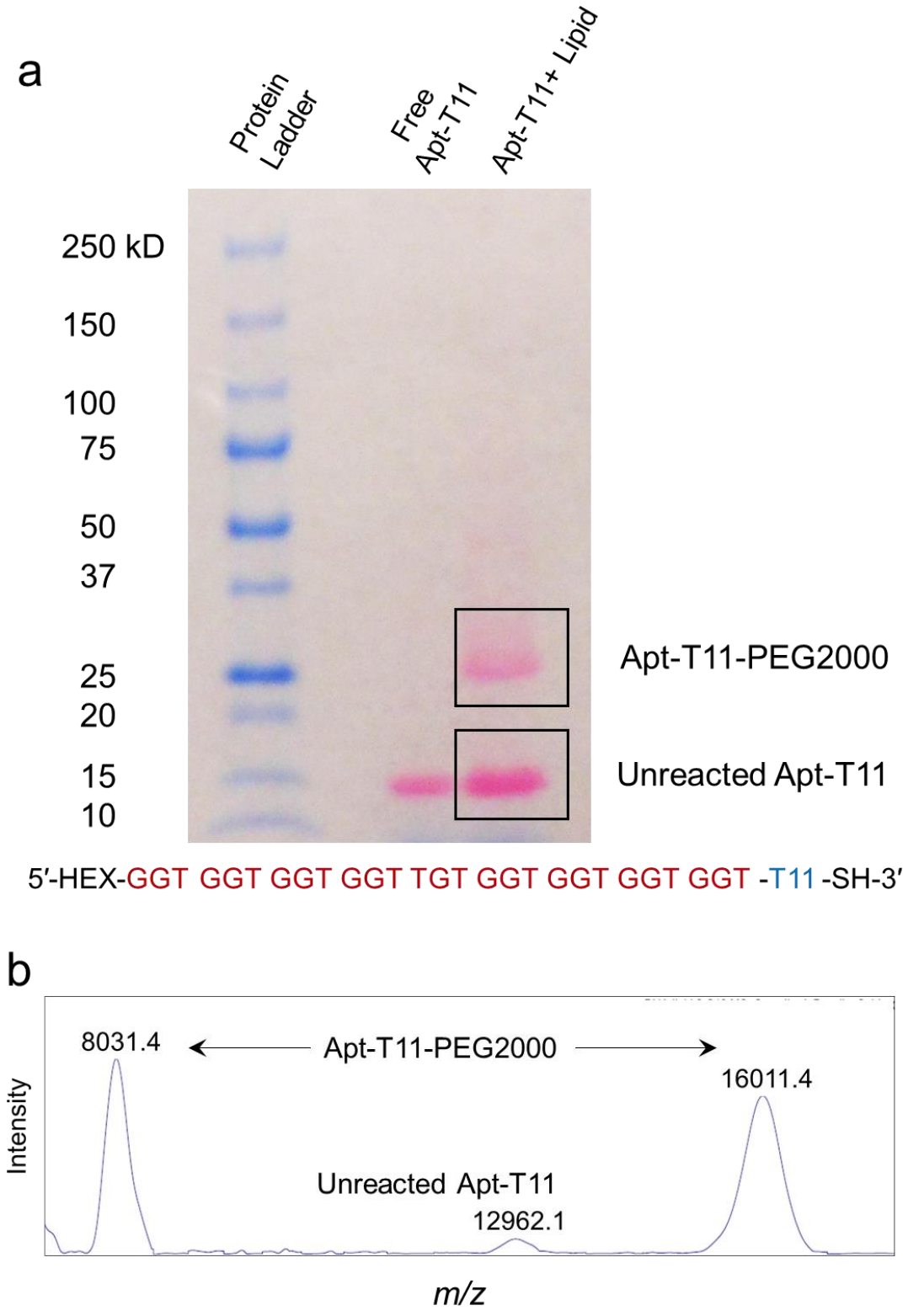

Figure S5. (a) SDS-PAGE characterization of Apt-T11-PEG2000 conjugate. Protein ladder (lane 1), Apt-T11 control (lane 2), and Apt-T11-PEG2000 conjugate (lane 3) samples were run on a 5-20% SDS-PAGE gradient gel. (b) MALDI-MS characterization of Apt-T11-PEG2000 conjugate.

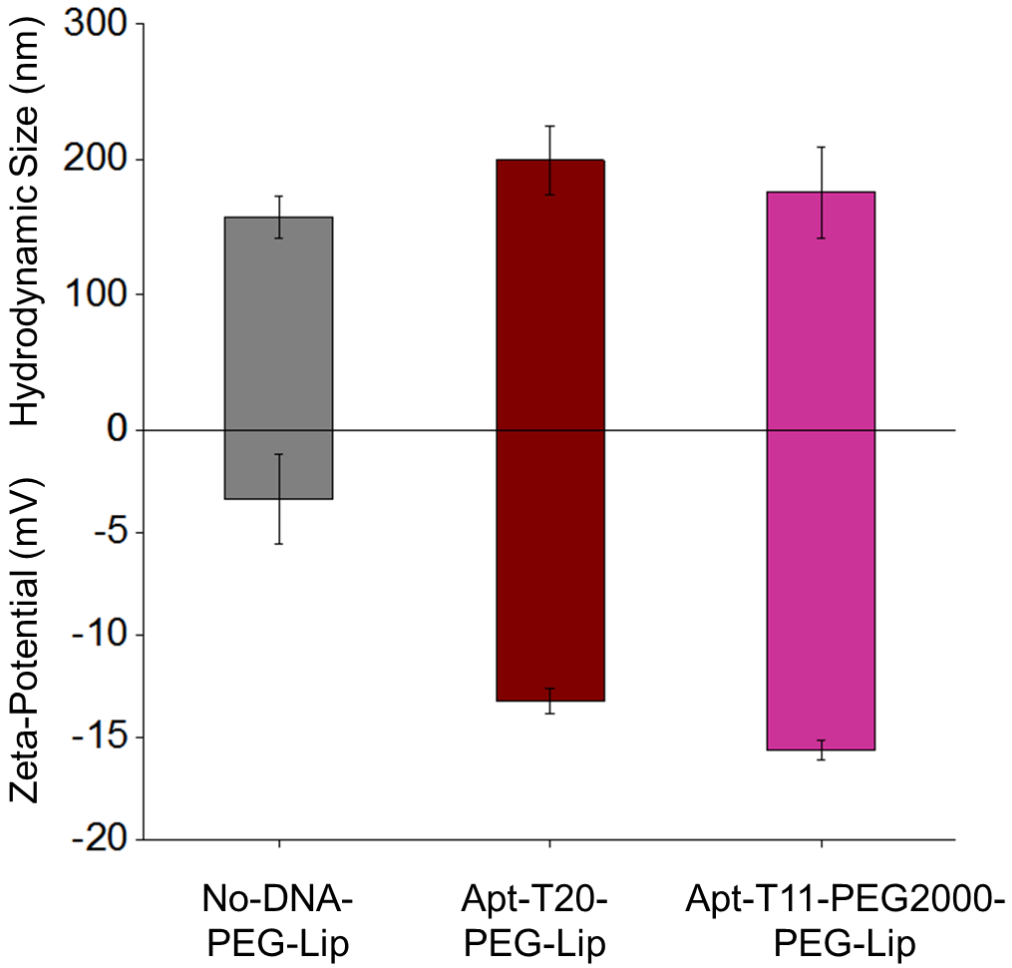

Figure S6. DLS and zeta-potential measurements of liposome samples (No-DNA-PEG-Lip, Apt-T20-PEG-Lip, and Apt-T11-PEG2000-PEG-Lip). The reported hydrodynamic size and zeta-potential of liposome samples were calculated from the average of three measurements. Bars represent standard deviation.