Supporting Information

Liposomal Spherical Nucleic Acids

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Materials. 1,2-dioleoyl-sn-glycero-3-phosphocholine lipid monomer (DOPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) either in dry powder form or in a chloroform solution and used without further purification. Phosphoramidites and other DNA synthesis reagents were purchased from Glen Research, Inc., (Sterling, VA, USA) and used as received. Ultrapure deionized water (18.2 MΩ cm resistivity) was obtained from a Millipore system (EMD Millipore, Billerica, MA, USA).

Instrumentation. Lyophilization was carried out using a Freezone Lyophilizer (Labconco, Kansas City, MO, USA). Sonication was conducted using a titanium-alloy solid probe sonicator (500 watt Vibra-Cell™ VC 505, Sonics & Materials, Inc., Newtown, CT, USA) set at 40% intensity of 20 kHz without pulsing. Ultracentrifugation was carried out using a Beckman-Coulter Avanti J-30I (Beckmann-Coulter, Inc., Indianapolis, IN, USA) centrifugation apparatus.

Transmission electron microscopy (TEM) was performed using a Hitachi-2300 STEM electron microscope. Dynamic light scattering (DLS) measurements were collected using a Malvern Zetasizer Nano-ZS (Malvern Instruments, UK) equipped with a He-Ne laser (633 nm). Matrix-assisted laser desorption/ionization (MALDI-TOF) analysis was performed using a Bruker Autoflex III SmartBeam spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). Fluorescence measurements were carried out on a Fluorolog-3 system (HORIBA Jobin Yvon Inc., NJ, USA). UV-Vis spectra were collected using a Cary 5000 UV-Vis spectrophotometer (Varian, Inc., Palo Alto, CA, USA). Phosphorus concentrations of the synthesized materials were determined using inductively coupled plasma mass spectrometry (ICP-MS) on a Thermo Fisher X Series II (Thermo Fisher Scientific Inc., Waltham, MA, USA) instrument.
Oligonucleotide Synthesis. The oligonucleotides used in this study (Table S1) were synthesized using automated solid-support phosphoramidite synthesis on an Expedite 8909 Nucleotide Synthesis System (MM48 Synthesizer, Bioautomation, Plano, TX, USA) with DCI as an activator. The tocopherol phosphoramidite was coupled to the nucleic acid using the standard automated protocol with an extended coupling time of 15 minutes. After the completion of solid phase synthesis, the oligonucleotide strands were cleaved from the solid support by overnight treatment with aqueous ammonium hydroxide (28-30 wt% aqueous solution, Aldrich Chemicals, Milwaukee, WI, USA), after which the excess ammonia was removed using a gentle flow of nitrogen gas (house nitrogen was used). The oligonucleotides were purified using a Microsorb C18 column on a reverse-phase high pressure liquid chromatography system (Varian ProStar Model No. 210, Varian, Inc., Palo Alto, CA, USA) using a gradient of TEAA (triethylammonium acetate) buffer and acetonitrile (10% v/v to pure acetonitrile over 30 min). The product-containing fractions were collected and concentrated on a lyophilizer. The oligonucleotides were re-suspended in ultrapure deionized water, and purity was analyzed using MALDI-TOF and denaturing acrylamide gel electrophoresis techniques.

Table S1. The oligonucleotides and oligonucleotide-modified materials used in this study.

| Name of the strand | Application | Sequence |
|--------------------|-------------|----------|
| Cy5-T25 strand     | Size analysis, DNA density determination, and stability studies | 5’-Cy5-T25-tocopherol-3’ |
| Particle A         | Melt analysis | 5’-AGG TTC TCT-A10-tocopherol-3’ |
| Particle B         | Melt analysis | 5’-tocopherol-A10-CAG ATG CGT-3’ |
| Linker strand      | Melt analysis | 5’-AGA GAA CCT ACG CAT CTG-3’ |
| HER2 antisense     | Gene regulation | 5’-CTC CAT GGT GCT CAC-T10-tocopherol-3’ |
| Cy5-labeled HER2 antisense | Imaging and Cellular uptake | 5’-Cy5-CTC CAT GGT GCT CAC-T10-tocopherol-3’ |
| Scrambled antisense | Gene regulation | 5’-GAG CTG CAC GCT GCC GTC A-T10-tocopherol-3’ |
**Synthesis of small unilamellar vesicles (SUVs).** SUVs were synthesized using a modification of a published protocol. An aliquot from the lipid monomer stock solution (1-2 mL, 25 mg/mL concentration) was added into a 25 mL glass vial, and the solvent was carefully evaporated using a stream of nitrogen. The obtained lipid monomer was further dried overnight under vacuum to remove the residual chloroform. The resulting lipid film was then hydrated with 20 mM HEPES-buffered saline (HBS, 5.0 mL) followed by vortexing to form a liposomal suspension. This suspension was further probe-sonicated for 30 min keeping the temperature of the lipid mixture below 10 °C with an ice-water bath. After sonication, the suspension was subjected to ultracentrifugation at 100,000 g for 90 min at 12 °C. After centrifugation, the clear supernatant containing the desired small unilamellar vesicles (SUVs) was collected, and the pellet was discarded.

To obtain particles with a narrower size distribution, the obtained SUVs were further extruded through a polycarbonate membrane (30 nm pore size, Avanti Polar Lipids, Inc., Alabaster, AL, USA). The extruded SUVs were then analyzed using dynamic light scattering (DLS). The hydrodynamic diameters (D\(_H\)) of the nanoparticles were calculated with Malvern Zetasizer software using the Stokes-Einstein equation (D\(_H\) = kB/T/3\(\pi\eta\)D, where \(k_B\) is the Boltzmann constant, T is the absolute temperature, and \(\eta\) is the solvent viscosity). The polydispersity index (PDI) was calculated as the width of the size distribution using cumulants analysis via the following formula: PDI = (standard deviation/mean diameter). The size of the SUVs was further confirmed by TEM (Figure S1). The final phospholipid concentration in a given sample was determined by analyzing its phosphorus content by inductively coupled plasma mass spectrometry (ICP-MS).
**Scheme S1.** A schematic representation of the synthesis of small unilamellar vesicles. The larger size liposomes are sonicated into SUVs using a probe sonicator, separated from heavy impurities by ultracentrifugation.\(^1\).

**Figure S1.** TEM image of SUVs after isolation and purification.

**Preparation of DNA-functionalized liposomal SNAs.** To prepare liposomal SNAs, the desired 3’-tocopherol modified oligonucleotide (8 nmol) was added to an aliquot of the SUV solution (500 µL of a solution containing 1.3 mM phospholipid) and allowed to shake overnight. The solution was then purified by size-exclusion chromatography on a cross-linked sepharose column (Sepharose CL 4B, Aldrich Chemicals, Milwaukee, WI, USA); and the particle size distribution was analyzed using DLS. To observe the liposomal SNAs using TEM, the samples were placed onto plasma-cleaned carbon TEM grids and further stained with an aqueous solution of uranyl acetate (2 wt%) for 2 min, washed with water, and allowed to dry. The dried grid was imaged using a Hitachi-2300 STEM electron microscope (Figure S2). The TEM-determined average particle diameter (25 ± 7 nm) is slightly smaller than that determined by DLS due to drying effects.
Figure S2. TEM image of liposomal-SNAs negatively stained with uranyl acetate.

**Gel electrophoresis of liposomal SNAs.** All gel electrophoresis experiments were conducted in a 1% agarose gel in 1x TBE (Tris/borate/EDTA) buffer. The samples were loaded in the wells with aqueous glycerol (5 µL of a 30 vol% solution) as a loading agent. The gel chamber was filled with 1x TBE and was precooled with ice. The gels were run at 70 V for 1 h at 10 °C and imaged with Fluorchem Q (BioRad, Hercules, CA) with a Cy5 filter. The results of a typical gel electrophoresis experiment are described in details in Figure S7.

**Quantification of the number of DNA strands on the surface of liposomal SNAs.** To determine the average loading of DNA on the surface of liposomal SNAs, 5’-Cy5-labeled, 3’-tocopherol-modified DNA (7.5 nmol) was incubated overnight with an aliquot of SUVs (500 µL of a solution containing 1.3 mM of phospholipid). The liposomal SNAs were purified by size-exclusion chromatography on a cross-linked sepharose column (Sepharose CL 4B, Aldrich Chemicals, Milwaukee, WI, USA) as described above; and then analyzed using gel electrophoresis. To quantitatively determine the average number of nucleic strands loaded onto the surface of a liposome, the liposomal SNAs solution (2.5 µL) was mixed with Triton X-100 (10 µL of a 5 wt% aqueous solution) and an aliquot of HBS buffer (100 µL). After vortexing the mixture for 1 min, the final nucleic acid concentration was determined by measuring the extinction at 260 nm relative to a calibrated oligonucleotide standard. The number of liposomes in the corresponding solution was calculated using the third equation shown in Scheme S2, with the assumption that the phospholipid concentration of the liposomes remains constant after functionalization and purification. From this number and the nucleic acid concentrations, the
average number of DNA per particle can be estimated to be ~70. We note that this surface coverage is lower than that in a typical gold-based SNA structure (a surface of 30 nm Au nanoparticle can be functionalized with up to 600 DNA strands).²

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\text{DNA loading} = \frac{N(\text{DNA})}{N(\text{liposomes})}
\]

\[
N(\text{total}) = \frac{4\pi(d/2)^2 + 4\pi(d/2 - h)^2}{\alpha}C(\text{lipid}) \cdot N_A \cdot V
\]

\[
N(\text{liposomes}) = \frac{N(\text{total})}{N(\text{total})} - \text{number of lipids per liposome}
\]

\[
N_A - \text{Avogadro's number}
\]

\[
d - \text{particle diameter by DLS}
\]

\[
h - \text{thickness of the lipid bilayer (5 nm)}
\]

\[
\alpha - \text{footprint of the lipid head group (DOPC 0.71 nm}^2)
\]

**Scheme S2.** The calculation of the total number of liposomes in a given solution. Concentration of the lipid can be determined using ICP-MS. For most of the studies performed in this manuscript, the working lipid concentration 1.3 mM gives \(1.361 \times 10^{17}\) liposomes/L.

**Melting assays.** A two-nanoparticle system was formed using liposomal SNAs that were functionalized with strands complementary to the linker strand as described in Table S1. The aggregates were formed by hybridizing two DNA-functionalized liposomal SNAs to a linker strand in a 1:1:1 ratio (total DNA concentration = 1.5 µM, total volume = 1 mL). The extinction spectra for the liposomal SNAs with the linker was collected using a Cary 5000 UV-Vis spectrometer and compared to the extinction spectra of liposomal SNAs without the linker (see Figure 3 in the manuscript). The solution containing the aggregates was then gradually heated from 20 to 65 °C at a rate of 0.25 °C/min while monitoring the extinction at 260 nm. The
melting transition of the tocopherol-DNA hybridized with a linker was measured as a control (Figure S3).

![Figure S3](image)

**Figure S3.** (A) Melting transition profile for a two-nanoparticle system comprising two different liposomal SNAs and a linker strand. (B) Melting transition profile for the corresponding free-DNA system comprising two different free tocopherol-DNA strands and a linker DNA strand. Both profiles were monitored as functions of changes in extinction at 260 nm.

**Preparation of rhodamine-encapsulated SUVs and liposomal SNAs.** Dry DOPC monomer (25 mg) was re-suspended in a 20 mM solution of Sulforhodamine B in HBS (5 mL). The resulting suspension was gradually extruded through a series of polycarbonate membranes (100, 80, 50, and 30 nm in pore size; Avanti Polar Lipids, Inc., Alabaster, AL, USA). The rhodamine-encapsulated liposomes were separated from the free rhodamine via size-exclusion chromatography on a cross-linked sepharose column (Sepharose CL-4B, Aldrich Chemicals, Milwaukee, WI, USA). The purified SUVs were either used immediately or treated with DNA-tocopherol conjugates to form the corresponding rhodamine-encapsulated liposomal SNAs following the procedure described above.

**Serum-stability assays.** To analyze the serum stability of SUVs and liposomal SNAs, the sulforhodamine-encapsulated versions of these constructs were suspended in a solution comprised of 10 vol% fetal bovine serum (FBS) in HBS at 37 °C. The release of the dye at 37 °C, as represented by the intensity of the solution fluorescence at 480 nm, was monitored continuously for a period of 200 minutes with sample excitation at 420 nm. To prove that the
degradation of SUVs is primarily due to their interactions with serum proteins, a control experiment was also performed where sulforhodamine-encapsulated SUVs were incubated in phosphate-buffered saline (PBS) at 37 °C. The three fluorescence profiles are shown in Figure S3.

![Figure S3](image.png)

**Figure S3.** The degradation of sulforhodamine-encapsulated SUVs (red traces) and liposomal SNAs (blue traces) in the presence of 10 vol% fetal bovine serum at 37 °C, as measured by increases in the fluorescence intensity of released sulforhodamine. The fluorescent profile of sulforhodamine-encapsulated SUVs in phosphate-buffered saline (PBS) at 37 °C (green traces) was also included as a control.

**Cell culture studies.** SKOV-3 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in McCoy’s 5A medium supplemented with fetal bovine serum (10 vol%), penicillin (0.2 units/mL), and streptomycin (0.1 µg/mL). Cells were maintained at standard cell culture conditions (at 37 °C in a humidified atmosphere with 5 % CO₂). For cellular studies, the cells were plated 24 hours prior to the treatment at 50% confluency.

**Confocal microscopy.** For visualization of the cellular internalization of liposomal SNAs, the SKOV-3 cells were plated on 35 mm FluoroDish™ chambers at 30% confluency. The cells
were incubated with Cy5-labeled liposomal SNAs (0.1 µM DNA) in OptiMEM serum-free medium (Invitrogen, Grand Island, NY, USA) for 20 h and then washed three times with 1x PBS containing 0.01 vol% of Tween-20. The cells were then further incubated with normal serum-containing McCoy’s 5A medium (Life Technologies, Grand Island, NY, USA) for 1 h before confocal imaging. The nuclei were stained with Hoechst 3342 (Invitrogen, Grand Island, NY, USA) following the manufacturer’s protocol. The live cells were then imaged with a Zeiss LSM 510 inverted laser-scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA) equipped with a Mai Tai 3308 laser (Spectra-Physics, Santa Clara, CA, USA) at 40 x magnification. The Hoechst dye was excited at 780 nm and emission data were collected at 390-495 nm; the Cy5 dye was excited at 640 nm and emission data were collected at 650-710 nm.

**Flow cytometry experiments.** To compare the cellular uptake of liposomal SNAs vs free-DNA strands, SKOV-3 cells were plated on a 96 well plate in McCoy’s 5A medium (supplemented with fetal bovine serum (10 vol%), penicillin (0.2 units/mL), and streptomycin (0.1 µg/mL); 100 µL of media/well) and incubated with either free-DNA or liposomal SNAs (final DNA concentration = 0.1 µM) for 24 h. Untreated cells were used as a negative control for these experiments. After incubation, the cells were washed 3 times with 1x PBS containing 0.01 vol% of Tween-20 and then trypsinized to form a suspension. Flow cytometry was performed on the resulting cellular suspension using the Cy5 intensity channel on a Guava easyCyte 8HT (Millipore, Billerica, MA, USA). The signal from the untreated cells was subtracted as background. The error-values were calculated using the standard error of the mean of median signal from different wells representing a single sample.

**Cytotoxicity studies.** To evaluate the cytotoxicity of the liposomal SNAs, the SKOV-3 cells were plated on a 96 well plate, as described in the flow cytometry experiments above, 24 h before the experiment. The cells were then incubated with either liposomal SNAs or DNA-encapsulated DharmaFECT® 1 (Dharmacon), a commercially available transfection agent, over a range of final DNA concentrations (0.1, 0.25, 0.5, 1, and 2 µM) for 24 h. After the 24 h incubation period, the cells were washed three times with 1x PBS and incubated in alamarBlue® solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37 °C for 4 h under a humidified atmosphere with 5 vol% CO₂. The fluorescence emission at 590 nm was recorded.
using a BioTek Synergy H4 Hybrid Reader (BioTek, Winooski, VT, USA). After normalized to the signals for untreated cells, the relative cytotoxicity of liposomal SNAs and DNA-encapsulated DharmaFECT®1 can be compared.

**Western Blotting to quantify HER2 protein knockdown.** The SKOV-3 cells were plated on a 6 well plate at a density of 100,000 cells per well and incubated overnight at 37 °C under a humidified atmosphere with 5% CO2. The cells were then incubated with either anti-HER2 antisense liposomal SNAs or scrambled liposomal SNAs (1 µM final DNA concentration) in OptiMEM serum-free medium (Invitrogen, Grand Island, NY, USA). After 24 h, the medium was replaced with normal serum-containing McCoy’s 5A medium (Life Technologies, Grand Island, NY, USA), and the cells were allowed to grow for an additional 48 h.

To analyze the HER2 protein knockdown, the cells were collected and re-suspended in an aliquot (100 µL) of mammalian cell lysis buffer (Cell Signaling, Danvers, MA, USA) containing protease and phosphatase inhibitors (Thermo Scientific, Inc., Waltham, MA, USA). The protein concentration of the cell lysates was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Inc., Waltham, MA, USA). Equal amounts (40 µg) of protein were fractionated by 4-15% Precast gradient gel (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane (Thermo Scientific, Inc., Waltham, MA, USA). The membrane was blocked using 5% dry non-fat milk solution (w/v) in Tris-buffered saline (TBS). The proteins were labeled with primary rabbit antibodies against HER2 (1:500, Cell Signaling 2242S; Cell Signaling Technologies, Danvers, MA, USA) and GAPDH (1:500, Santa Cruz sc-25778; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), followed by treatment with fluorophore-labeled anti-rabbit secondary antibodies (1:10,000; LI-COR Biosciences, Lincoln, NE, USA). The fluorescence signal was recorded using the Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

**Nuclease degradation assay.** To understand the fate of the DNA on the surface of liposomal SNAs, a 2 µM solution of liposomal SNAs in 1x DNAse reaction buffer (New England BioLabs, Ipswich, MA, USA) was first prepared and allowed to equilibrate at room temperature. To an aliquot (500 µL) of this solution was added DNAse I (final concentration = 25 units/mL, Invitrogen Grand Island, NY, USA) and the resulting mixture was stirred for 8 h at 37 °C. The
DNAse-I-treated liposomal SNAs were then analyzed by DLS and compared to a control of non-treated liposomal SNAs. A 9 nm decrease in diameter for the DNAse I-treated liposomal SNAs was observed (Figure S5), which is consistent with degradation of the nucleic acid shell.

In two separate experiments, Cy5-labeled liposomal SNAs and Cy-5-labeled tocopherol-DNA (as a control), respectively, were incubated with DNAse I (final concentration = 50 unit/mL) for 8 h at 37 °C (Figure S6). After the treatment, the liposomal SNA-treated particles were isolated and digested using a 1 vol% SDS solution. The content was lyophilized and re-suspended in chloroform. DNA strands were isolated from the mixture by extraction with ultrapure deionized water. This free DNA-containing aqueous solution was analyzed using agarose gel electrophoresis (3% w/v agarose in 1x TBE buffer, 200 V, 40 min). Non-DNAse I-treated liposomal SNAs were also studied as a control (Figure S6). The absence of a full-length DNA in the DNAse I-treated liposomal SNAs suggests that there is no significant amount of DNA entrapped in the core of the original liposomal SNA.

**Figure S5.** Change in the average diameter of liposomal SNAs before and after the DNAse I treatment, as measured by DLS.
**Figure S6.** 3% agarose gel electrophoresis of liposomal SNAs. The first and second lanes are the intact Cy5-labeled DNA (from the free-strand control experiment and the digestion of liposomal SNAs, respectively) prior to DNAse I treatment. The third and fourth lanes show the result of free-strand and liposomal-SNA treatment with DNAase I (8 h at 37 °C) and the presence of degraded DNA strands. The absence of full-length DNA in lane 4 is consistent with the conclusion that there is no DNA entrapped in the core of the SUV.
Figure S7. 1% agarose gel electrophoresis of liposomal SNAs. (Left) A schematic representation of the FITC-encapsulated liposomal SNAs functionalized with 5’-Cy5-labeled DNA strands. (Middle) The FITC channel showing movement of the liposomal core in lane 2 on the gel. Due to the presence of the negatively charged DNA corona in the FITC-encapsulated SNA, the band in lane 3 moves further. We note that there is no band in lane 1 because there is no FITC. The DNA shell on the liposomal surface allows the particles to move through the gel under the influence of applied voltage. (Right) The Cy5 channel confirms the difference in size and charge between the free strand and the liposomal SNA construct, which is comprised of many strands.

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