[Supporting Information (SI) to accompany *J. Am. Chem. Soc.* manuscript ja-2016-13359p]

Cross-linked micellar spherical nucleic acids from thermoresponsive templates

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S1. Materials and Instrumentation

Materials. Unless otherwise noted, all reagents were purchased from commercial sources and used as received. For oligonucleotide synthesis, all phosphoramidites and reagents were purchased from Glen Research Co. (Sterling, VA, USA). The $BS(PEG)_5$ (PEGylated bis(sulfosuccinimidyl)suberate) linker and buffer solutions were purchased from Thermo Fisher Scientific, Inc. (Richardson, TX, USA). Gold nanoparticles were purchased from Ted Pella Inc. (Redding, CA, USA). Amicon® Ultra centrifugal filter units were purchased from EMD Millipore (Billerica, MA, USA). All other reagents were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). The water used in all experiments is ultrapure deionized (DI) grade (18.2 MΩ·cm resistivity), obtained from a Milli-Q Biocel system (Millipore, Billerica, MA, USA).

Instrumentation. UV-vis absorbance spectra and thermal denaturation curves were collected on a Varian Cary 5000 UV-vis spectrometer (Varian, Inc., Palo Alto, CA, USA) using quartz cuvettes with a 1 cm path length.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometric data was obtained on a Bruker AutoFlex III MALDI-ToF mass spectrometer (Bruker Daltonics Inc., MA, USA). For MALDI-ToF analysis, the matrix was prepared by mixing an aqueous solution of ammonium hydrogen citrate (0.6 μL of a 35 wt % solution (15 mg in 30 μL of H_2O)) and 2-hydroxypicolinic acid (Fluka # 56297, 2 mg in H_2O:MeCN (30 μL of a 1:1 v/v mixture). An aliquot of the DNA (~0.5 μL of a 150 μM solution) was then mixed with the matrix (1:1) and the resulting solution was added to a steel MALDI-ToF plate and dried at 25 C for 1 h before analysis. Samples are detected as negative ions using the linear mode. The laser was typically operated at 10-20% power with a sampling speed of 10 Hz. Each measurement averaged for five hundred scans with the following parameters: ion source voltage 1 = 20 kV, ion source voltage 2 = 18.5 kV, lens voltage = 8.5 kV, linear detector voltage = 0.6 kV, deflection mass = 3000 Da.

Centrifugation was carried out in a temperature-controlled Eppendorf centrifuge 5430R (Eppendorf, Hauppauge, NY, USA).

Transmission electron microscopy (TEM) experiments were carried out on a Hitachi H2300 transmission electron microscope (Hitachi High-Technologies America, Schaumburg, IL, USA) operating at an accelerating voltage of 200 kV.

Dynamic light scattering (DLS) and zeta potential measurements were collected on a Zetasizer Nano ZS instrument (Malvern Instruments, UK) equipped with a He-Ne laser (633 nm).

S2. Synthesis of oligonucleotides, lipid-conjugated oligonucleotides, and micellar SNAs

Oligonucleotide synthesis. Oligonucleotides were synthesized on CPG support using an automated Expedite Nucleotide system (MM48 Synthesizer, Bioautomation, Plano, TX, USA). Whenever a modified (i.e., non-nucleoside-bearing) phosphoramidites is used, the coupling time is extended to 20 min compared to the usual 90 seconds for a typical phosphoramidite coupling. After synthesis, the completed DNA was cleaved off the CPG support through an overnight exposure to aqueous 8 M ammonium hydroxide (28-30 wt %). Excess ammonium hydroxide was removed from the cleaved DNA solution by passing a stream of dry nitrogen gas over the content of the vial until the characteristic ammonia smell disappears. The remaining solution was then passed through a 0.2 μm cellulose acetate membrane filter to remove

the solid support and then purified on a Varian ProStar 210 (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with reverse-phase semi-preparative Varian column ((Agilent Technologies, 250 mm × 10 mm, Microsorb 300 Å/10 µm/C4), gradient = 100:0 v/v 0.1 M TEAA (aq):MeCN (TEAA (aq) = triethylammonium acetate, aqueous solution), and increasing to pure acetonitrile in 30 min, flow rate = 3 mL/min for each 1 µmol DNA). The product fractions collected were concentrated using lyophilization. The lyophilized oligonucleotides were then re-suspended in ultrapure deionized water and their concentrations were measured using UV-vis spectroscopy. The purity of synthesized oligonucleotides was assessed using MALDI-ToF mass spectrometry.

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

Particle Type	Application	Sequence
T_{30}	Characterization of SNAs	5'-T_{30}-$(NH_2)_5$-(Spacer18[a])$_2$-DBCO[b]-3'
Cy5-T_{30}	Characterization and cellular uptake	5'-Cy5-T_{30}-$(NH_2)_5$-$(Spacer18)_2$-DBCO-3'
BHQ-T_{20}	Serum-stability study	5'-T_{20}-$(NH_2)_5$-$(Spacer18)_2$-BHQ[c]-DBCO-3'
Melt A	Melt analysis	5'-DBCO-$(Spacer18)_2$- $(NH_2)_5$-T_4-AATCCTTATCAATATTT-3'
Melt B	Melt analysis	5'-DBCO-$(Spacer18)_2$- $(NH_2)_5$-T_4- AAATATTGATAAGGATT-3'
IS-1826	Immunostimulation	5'- TCCATGACGTTCCTGACGTT-T_5-$(Spacer18)_2$-DBCO-3'
Scrambled	Immunostimulation	5'-T_{20}-$(NH_2)_5$-$(Spacer18)_2$-DBCO-3'
IS-7909	Immunostimulation	5'- TCGTCGTTTTGTCGTTTTGTCGTT-T_5-$(NH_2)_5$-$(Spacer18)_2$-DBCO-3'
Scrambled	Immunostimulation	5'-T_{20}-$(NH_2)_5$-$(Spacer18)_2$-DBCO-3'

[a]Spacer18 = 18-*O*-Dimethoxytritylhexaethyleneglycol,1-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite. [b]DBCO = 5'-Dimethoxytrityl-5-[(6-oxo-6-(dibenzo[b,f]azacyclooct-4-yn-1-yl)-capramido-*N*-hex-6-yl)-3-acrylimido]-2'-deoxyuridine,3'-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite. [c]BHQ = '-Dimethoxytrityloxy-5-[(*N*-4''-carboxyethyl-4''-(*N*-ethyl)-4'-(4-nitro-phenyldiazo)-2'-methoxy-4'-methoxy-azobenzene)-aminohexyl-3-acrylimido]-2'-deoxyuridine-3'-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite

Synthesis of lipid-conjugated oligonucleotides. For the synthesis of lipid-conjugated oligonucleotides, the purified DBCO-terminated oligonucleotides (1 µmol, see Table S1) were dissolved in an aliquot of water (250 µL). In a separate Eppendorf tube, 10 µmol of DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(6-azidohexanoyl) (ammonium salt), Avanti Polar Lipids, Inc., Alabaster, AL, USA) was dissolved in ethanol (250 µL). The lipid solution was then added to the oligonucleotide solution and the resulting mixture was allowed to shake at rt overnight on a benchtop Thermomixer R 5355 (Eppendorf AG North America, Hauppauge, NY, USA) instrument at 850 rpm. The following day, the content was dried on a Labconco centrivap (Labconco, Kansas City, MS, USA). The obtained dried pellet was resuspended in ultrapure deionized water (300 µL) and the resulting mixture was extracted with chloroform (3 × 300 µL) to remove excess lipids. The remaining suspension of lipid-conjugated DNA was lyophilized before being purified from the unconjugated DNA via HPLC or PAGE purification[S1] (Sigma-Aldrich).

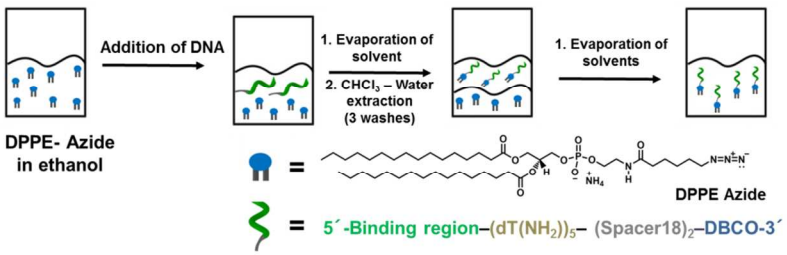

Figure S1. A schematic representation of the synthesis of lipid-conjugated oligonucleotides.

Synthesis of micellar SNAs. For the preparation of micellar SNA, free lipid-conjugated DNA strands (10 nmol for T_{30} sequence) was added to an aliquot of aqueous Pluronic F127 (1 mL of a 2 wt % solution in 1× HBS (20 mM of HEPES buffer, 150 mM aqueous NaCl). The resulting solution was allowed to shake at room temperature overnight on a benchtop Thermomixer R 5355 (Eppendorf AG North America) instrument. To cross-link the DNA strands, the $BS(PEG)_5$ linker (2.5 equiv of DNA concentration, or 50% of the amino group concentration for achieving a theoretical 100% cross-link density) was next added and the resulting solution allowed to shake for an additional 6 h or overnight. To remove the unincorporated Pluronic F127, the reaction mixture was cooled to 4 °C and then centrifuged in an Amicon® Ultracentrifugal filter units (100,000 MWCO, 7500 *g*) at 4 °C until ~100 µL of solution was retained. This solution of the desired micellar SNA products were subjected to a minimum of three washes with 1× HBS and passed through a 0.1 µm syringe filter (GE Healthcare Bio-Sciences, Co., Marlborough, MA, USA) before use.

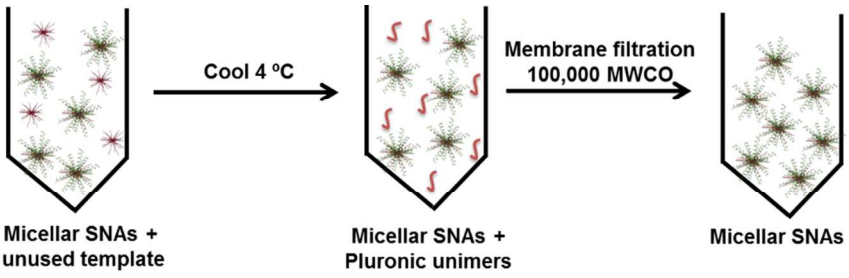

Figure S2. A schematic representation of the purification of micellar SNAs.

S3. Quantification of Pluronic F127 in micellar SNAs

To quantify the amount of Pluronic F127 in the micellar SNAs, we analyze both the filtrate and retentate fractions from the membrane centrifugation operation using a previously reported colorimetric assay method with minor modifications.[S2] Briefly, cobalt nitrate hexahydrate (0.3 g) and ammonium thiocyanate (1.2 g) were dissolved in water (3 mL) to make a cobalt thiocyanate reagent. Into an Eppendorf tube were combined an aliquot (100 µL) of the cobalt thiocyanate solution, an aliquot (40 µL) of the solution to be analyzed, ethyl acetate (200 µL), and ethanol (80 µL). The resulting semi-cloudy mixture was vortexed gently and centrifuged at 14000 *g* for 1 min. The blue supernatant was removed and the left-over blue pellet was washed several (~5) times with diethyl ether until the supernatant became colorless. The resulting pellet was then dissolved in acetone (1 mL) and subjected to UV-vis measurement. The absorbance value at 623 nm was compared to a calibration curve prepared from the colorimetric assay of standard Pluronic F127 solutions over a 0-2.5 wt % concentration range. The DNA:Pluronic F127 molar ratio in the purified solution is 0.55 (assuming the molecular weight of Pluronic F127 to be 10,000 and the DNA sequence to be Cy5-T_{30}, see Table S1).

The concentration of DNA strands in the cross-linked micellar SNAs (Figure S3B, red trace) is estimated against the UV-vis extinction coefficient for DNA at 260 nm (ε = 243600 L/mol cm for a T_{30} sequence based on https://www.idtdna.com/calc/analyzer) after disassembly (by sonicating with 0.1 M HCl and 0.01 wt % SDS solution for 30 sec and left in a 37 ºC water bath for 10 min). As the initial Pluronic F127 template at a high 2 wt % concentration does not show a significant absorption at 260 nm (Figure S3B, black trace), the small amount that is retained in the dissembled cross-linked micellar SNAs should not interfere with calculations of the DNA concentrations.

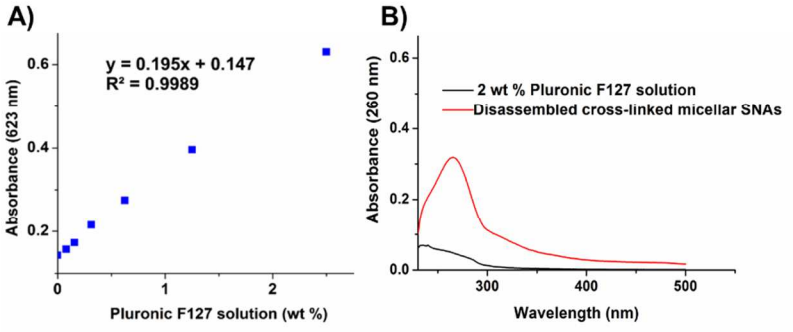

Figure S3. (A) Calibration curve used to determine the amount of Pluronic F127 in the micellar SNA. Pluronic F127 and cobalt thiocyanate formed a dark blue complex (absorbance at 623 nm). (B) A plot with the absorbance spectra of the template (2 wt % Pluronic F127 in 1× HBS, black trace) indicating a minimal absorbance of the Pluronic template at 260 nm. For comparison, the absorbance spectrum of disassembled (with 0.1 M HCl and 0.01 wt % SDS solution for 10 min) 0.15 wt% cross-linked micellar SNAs (red trace) is also included.

S4. Characterization of micellar SNAs

a. **Dynamic light scattering (DLS)**. The particle size distribution and surface charge (ζ potential) of the micellar SNAs were carried out via dynamic light scattering. To measure the size of nanoparticles, non-invasive backscatter method (detection at 173º scattering angle) was used. The collected data were fitted, using the method of cumulants, to the logarithm of the correlation function, yielding the diffusion coefficient D. The calculated diffusion coefficient was applied to the Stokes-Einstein equation ($D_H = k_BT/3\pi\eta D$, where k_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity (η = 0.8872 cP for water at 25 ºC)), to obtain the hydrodynamic diameters (D_H) of the nanoparticles (NPs). The reported DLS size for each sample was based on six measurements, each of which was subjected to non-negative least squares analysis.

b. **Scanning and transmission electron microscopy (STEM) imaging**. In a typical experiment, the micellar SNAs sample was first "stirred up" gently using a micropipetting technique to ensure complete homogeneity (even though there was no visual evidence of precipitation). An aliquot (1.5 μL) of this solution was then placed on a TEM carbon sample grid (Ted Pella, Inc. # 01820). After 1 min, the excess solution was gently wicked away from the grid with a piece of filter paper. The excess of salt was removed from the grid by touching it briefly with a drop of ultrapure DI water and then gently wicking away the excess solution. The grid was allowed to air-dry for 1 h before being stained with a drop (1.5 μL) of uranyl acetate solution (2 wt % in water). After 20 s, the excess staining agent was gently wicked away from the grid with a piece of filter paper and the grid was allowed to air-dry prior to analysis.

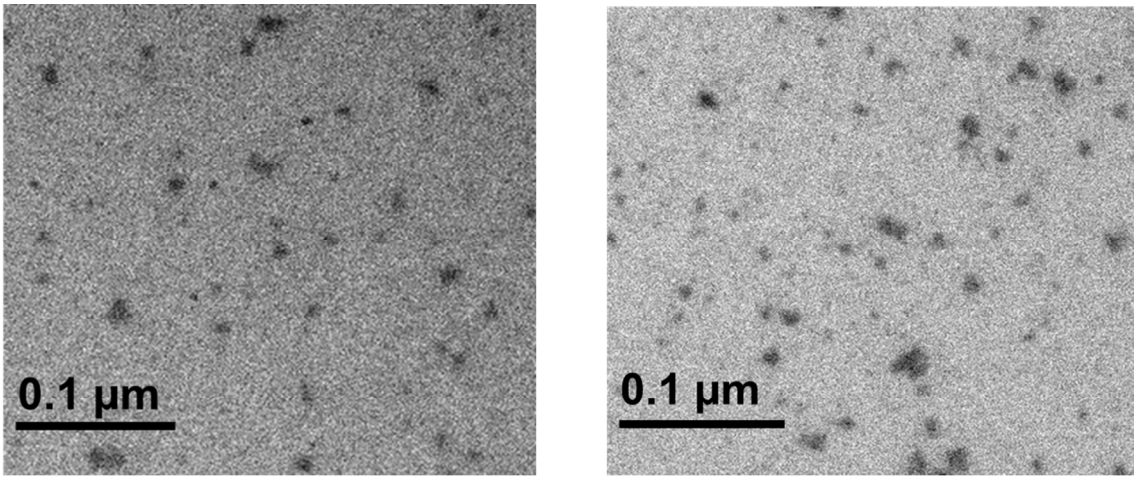

Figure S4. TEM images of micellar SNAs with different greyscales.

c. **Atomic force microscopy (AFM) imaging**. AFM imaging was carried out by drop-casting a small drop (10 μL of 2 μM solution) of the micellar SNAs in HBS buffer (150 mM NaCl, 10 mM HEPES, 2 mM $MgCl_2$, pH 7.4) on a piece of freshly cleaved mica (Ted Pella, Inc.). After 5 minutes, a small aliquot (200 μL) of HBS buffer was added to the sample. The samples were imaged with a Dimension Icon AFM (Bruker Nano, Inc., Santa Barbara, CA, USA) in peak force tapping mode using Scanasyst Fluid+ Cantilevers (Bruker Nano, Inc.) with the forces held to <500 pN.

S5. Melt analysis for micellar SNAs. A DNA-hybridized nanoparticle aggregate (Figure 2A in the main text) was formed using two different micellar SNAs possessing sequences Melt A and Melt B (Table S1) and a 1:1 DNA stoichiometry. Briefly, aliquots of the solutions of the two particles were added together and combined with enough water and conc aq NaCl to make a mixture suitable for melt analysis (final DNA concentration of one component = 1.5 μM, total volume = 1 mL, final NaCl concentration = 500 mM). The resulting mixture was allowed to hybridize overnight and then subjected to heating (from 25 to 90 °C) inside the UV-vis spectrometer at a rate of 0.5 °C/min, while the absorbance at 260 nm was continuously monitored. Data is shown in Figure 2B in the main text

S6. Gel electrophoresis. The gel electrophoresis experiments were carried out on a 1% agarose gel made in 1× TBE (Tris/borate/EDTA) buffer. An aqueous solution of glycerol (30% v/v) was used as a loading agent. The gel was allowed to develop at 80 V in 1× TBE buffer for 60 min. After the run was complete, the gel was imaged on a Flourchem Q (Bio-Rad Laboratories, Inc., Hercules, CA, USA) instrument equipped with a Cy5 filter.

S7. Thermal stability studies: An aliquot (1 mL of a 10 μM stock solution) of the Cy5-labeled micellar SNAs was transferred into an 1.5 mL safe-lock Eppendorf and diluted in 1× PBS (final concentration 1 μM). The resulting solution was incubated at 37 °C and 100 μM aliquots were collected after one week for analysis by gel electrophoresis.

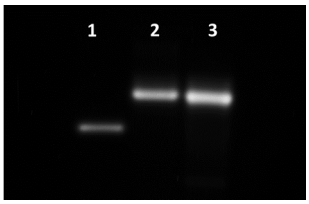

Figure S5. An image of a 1% agarose gel electrophoresis of micellar SNAs. The negatively charged DNA corona of the micellar SNA surface allows the particles to move through the gel under the influence of the applied voltage. Differences in the size and charge between the free strand (lane 1) and micellar SNAs (freshly prepared, lane 2; after 7 days of incubation at 37 °C, lane 3) are reflected though the distances they traveled on the gel, as visualized using the Cy5 channel. That lanes 2 and 3 each only shows a single band at a similar distance from the top of the gel confirms no thermal degradation and no dissociation of the DNA from the construct after 7 days of incubation at physiological temperature.

S8. Serum-stability studies

The serum stability of cross-linked micellar SNAs was assessed using purified $DilC_{18}$-encapsulated micelles that were functionalized with the BHQ-T_{20}-lipid material (Table S1) to form a $DilC_{18}$-encapsulated, BHQ-2-labeled micellar SNA sample (10 μM final DNA concentration, volume = 3 mL). An aliquot (1.5 mL) of this materials was removed and added to a separate Eppendorf tube. The $BS(PEG)_5$ cross-linker was added to the remaining mixture to form cross-linked micellar SNAs. The two samples were purified using size-exclusion chromatography with Sepharose CL-4B (Sigma-Aldrich). The quenching of the $DilC_{18}$ dye due to the proximity of the BHQ-2-labeled DNA was confirmed by the decrease of its fluorescence. To analyze the serum stability of non-cross-linked and cross-linked micellar SNAs, the $DilC_{18}$-encapsulated versions of these constructs were suspended in a solution comprising 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 560 nm, was monitored continuously for a period of 200 minutes with sample excitation at 540 nm on a BioTek Synergy H4 Hybrid Reader (BioTek, Inc., Winooski, VT, USA).

In another experiment, into an Eppendorf tube was combined an aliquot from a stock solution of the Cy5-labeled micellar SNAs (10 μM DNA concentration) and 10 vol % fetal bovine serum in 1× PBS to achieve a solution with 2.5 μM final DNA concentration. The resulting mixture was incubated at 37 °C and 100 μL aliquots were collected after 2, 4, and 8 h for analysis by gel electrophoresis.

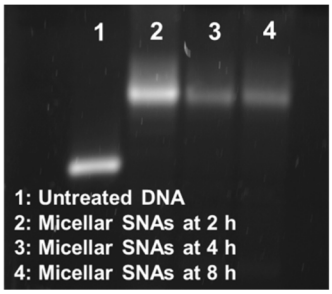

Figure S6. Image of a 1% agarose gel electrophoresis of cross-linked micellar SNAs after being incubated in a 10% FBS solution in HBS for 2, 4, and 8 h (lanes 2-4). For reference, free DNA (not treated with the media) was also included in lane 1. Dissociation of the DNA from the construct was not observed after 8 h of incubation.

S9. Cell culture studies. HEK-Blue™-mTLR9 cells (InvivoGen, San Diego, CA, USA) and Ramos-Blue cells (InvivoGen), derivatives of HEK-293 cells and Ramos cells, respectively, both stably expressing a secreted alkaline phosphatase (SEAP) inducible by NF-κB, were cultured as recommended by the supplier.

Confocal microscopy. HEK-Blue cells (~ 100,000 cells/well) were plated on 35 mm FluoroDish™ chambers at 30% confluency, incubated with Cy5-labeled micellar SNAs (0.1 μM DNA) in OptiMEM serum-free medium (Invitrogen brand

of Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 4 h, and then washed three times with 1× PBS. After the incubation, the cell media was switched to 4 wt % solution of paraformaldehyde in 1× PBS (Sigma-Aldrich) for 1 h before imaging. The resulting cell suspension was centrifuged and the supernatant was removed. The nuclei of incubated and untreated cells were stained with Hoechst 3342 (Invitrogen) following the manufacturer's protocol. The pellet was resuspened in mounting media (ProLong® Gold Antifade Mountant, ThermoFisher Scientific; 50 µL final volume). A 10 µL solution was added on a glass slide and allowed to dry in a dark chamber for 48 h. Confocal microscopy imaging of these cells were carried out on an a Zeiss LSM 810 microscope inverted laser-scanning confocal microscope (Carl Zeiss, Inc., USA) equipped with a Mai Tai 3308 laser (Spectra-Physics, Santa Clara, CA, USA) at 40× 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.

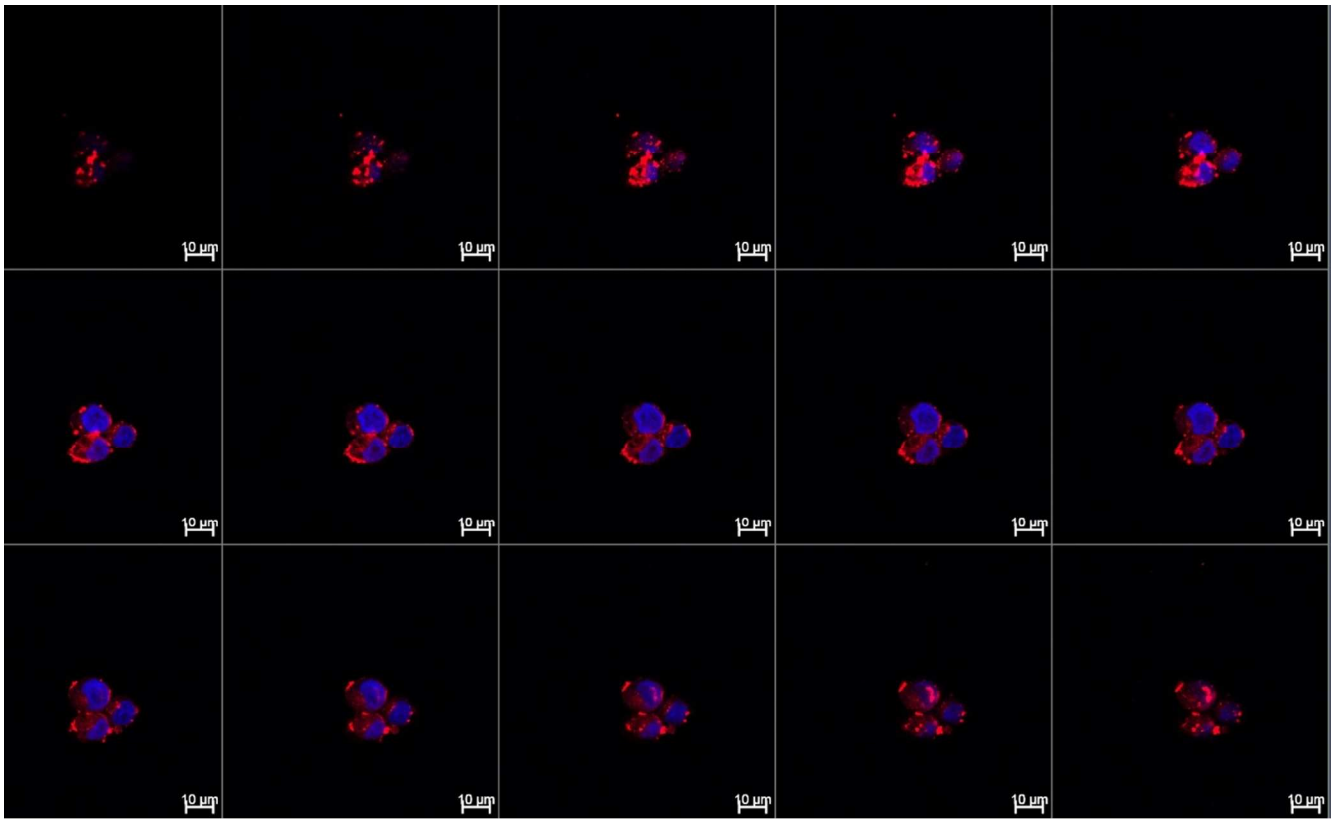

Figure S7. A Z-stack confocal microscopy image series (15 slices of 0.05 µm thickness from top to bottom) of a HEK-Blue cell culture that have been incubated with Cy5-labeled micellar SNAs for 4 h. The Cy5-labeled micellar SNAs (red) are rapidly internalized by the HEK-Blue cells (nuclei-blue stain).

Flow cytometry experiments. A comparative cell-uptake study between the micellar SNAs and free DNA was carried out using HEK-Blue cells. Cells (~ 100,000 cell/well) were plated on a 96 well plate in DMEM medium (supplemented with fetal bovine serum (10 vol%), penicillin (0.2 units/mL), and streptomycin (0.1 µg/mL), Normocin™ (100 µg/mL), L-glutamine (2 mM concentration); 100 µL of media/well) and incubated with either free-DNA or micellar SNAs (final DNA concentration 0.1 µM) for 16 h. The fluorescence was normalized using untreated cells as a negative control for these time-points. At the end of incubation period, the cells were washed 3 times with 1× PBS. The resulting cell suspension was subjected to flow cytometry using the Cy5 intensity channel on a Guava easyCyte 8HT instrument (Millipore). Error values were calculated using the standard error of the mean of median signal from different wells representing one type of sample.

Cytotoxicity studies. HEK-Blue cells (~ 100,000 cell/well) were plated onto a 96 well plate, as described in the flow-cytometry experiments above, for 24 h before the experiment. The cells were then incubated with micellar SNAs at different concentrations for 24 h, washed three times with 1× PBS, and incubated in alamarBlue® solution (Thermo Fisher Scientific, Inc.) for 4 h at 37 °C under a humidified atmosphere with 5 vol% CO_2. The fluorescence emission at 590 nm was recorded using a BioTek Synergy H4 Hybrid Reader and normalized to the signals for untreated cells.

In-vitro cell-stimulation studies. HEK-Blue (~60,000 cells/well) or Ramos-Blue (400,000 cells/well) cells were plated in 96 well plates in DMEM medium (supplemented with fetal bovine serum (10 vol%), penicillin (0.2 units/mL), and streptomycin (0.1 μg/mL), Normocin™ (100 μg/m), L-glutamine (2 mM concentration); 200 μL of media/well). Immediately after the plating, the cells were treated with the micellar SNAs test reagents and the appropriate controls. The plates were then incubated at 37 °C in 5 vol % CO_2 for 16 h before analysis.

For analysis, a 180 μL aliquot of QUANTI-Blue™ solution (InvivoGen, prepared per the manufacturer's protocol) was added to each well in a separate 96 well plate. To each well in this plate was then added a 20 μL aliquot of the supernatant of treated HEK-Blue cells (20 μL supernatant of untreated HEK-cells was used as a negative control). After 4 h incubation, the change in color due to SEAP activity was quantified by reading the OD at 620-655 nm using a BioTek Synergy H4 Hybrid Reader.

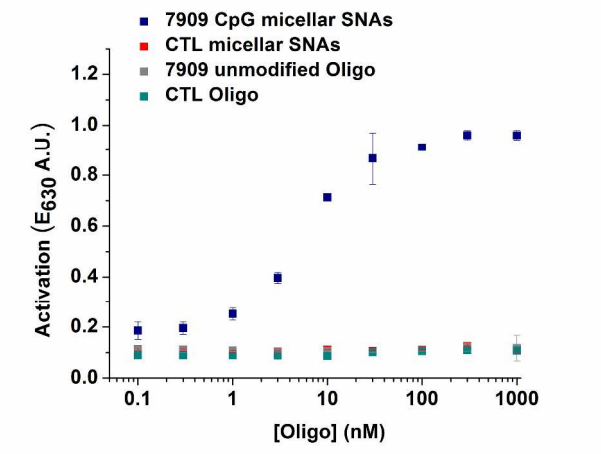

Figure S8. Plot of the amounts of secreted alkaline phosphatase (SEAP) by Ramos-Blue cells, as visualized by a colorimetric assay, showing enhanced immunostimulatory activity by micellar SNAs in comparison to control micellar SNAs bearing a T_{20} sequence and unmodified linear nucleic acids.

S10. References

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(S2) Zhang, Y.; Jeon, M.; Rich, L. J.; Hong, H.; Geng, J.; Zhang, Y.; Shi, S.; Barnhart, T. E.; Alexandridis, P.; Huizinga, J. D.; Seshadri, M.; Cai, W.; Kim, C.; Lovell, J. F. *Nat. Nanotechnol.* **2014**, *9*, 631-638.