Templated synthesis of spherical RNA nanoparticles with gene silencing activity

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Supporting Information

SI-I. General

Unless otherwise stated, all starting materials were purchased from commercial suppliers and used without further purification. Magnesium chloride, triethylamine, tris(hydroxymethyl)-aminomethane (Tris), urea, EDTA, glycerol, formamide, diethylpyrocarbonate (DEPC), triphenylphosphine, iodine, guanosine, 1,12-dodecanediol, N-methyl-2-pyrrolidone (NMP), dichloromethane (DCM), sodium azide, L-PEI (linear polyethylenimine, 25 kDa), RNAzol RT, ammonium persulfate (APS), demethylformamide (DMF), ethanol, diethyl ether, and all other solvents were used as purchased from Sigma-Aldrich. FBS, antibiotic-antimyotic 100X, DMEM (Dulbeco's modified eagle medium), acetic acid and boric acid were purchased from Fisher Scientific. T7 RNA Polymerase (cat.# M0251), DNase I, 10x RNA polymerase buffer, and 10x DNase I buffer were purchased from New England Biolabs. Glo Lysis Buffer and Bright-Glo Luciferase Reagent were purchased from Promega (USA). GelRed™ nucleic acid stain was purchased from Biotium Inc. Concentrated ammonium hydroxide, acrylamide/bis-acrylamide (40% 19:1 solution) and TEMED were obtained from Bioshop Canada Inc. and used as supplied. 1 μmol Universal 1000Å LCAA- CPG supports and standard reagents used for automated DNA synthesis were purchased through Bioautomation. DBCO-dT-CE (cat.# 10-1539) and DBCO-TEG (cat.# 10-1941) phosphoramidites were purchased from Glen Research. iTaq™ Universal SYBR Green Supermix reagents for qPCR was purchased from Biorad. Mono-dimethoxytrityl-1,12-dodecanediol was synthesised from dodecanediol as per Skrzypczynski and Wayland[1], which was then converted to the phosphoramidite using 2-cyanoethyl N,N-diisopropylchloro-phosphoramidite as described by Beaucage[2]. 10x TAMg buffer consisted of 400mM tris(hydroxymethyl)aminomethane and 125mM magnesium acetate tetrahydrate at pH 8.0 (adjusted using acetic acid). 10x TBE buffer was composed of 900mM tris(hydroxymethyl)aminomethane, 900 mM boric acid, and 11 mM ethylenediaminetetraacetic acid with a pH of 8.0. TEAA buffer was made by adding 7 mL of trimethylamine to water before the adjusting the pH to 8.0 with glacial acetic acid and diluting to 1 L total. 5x siRNA buffer was composed of 300 mM KCl, 30 mM

HEPES – pH 7.5, and 1.0 mM $MgCl_2$. 5x siRNAMg buffer was the same as 5x siRNA, but with 10 mM $MgCl_2$ instead of 1.0 mM.

SI-II. Instrumentation

Standard automated oligonucleotide solid-phase synthesis was performed on a Mermade MM6 Synthesizer from Bioautomation. HPLC purification was carried out on an Agilent Infinity 1260. DNA and RNA quantification measurements were performed by UV absorbance with a NanoDrop Lite spectrophotometer from Thermo Scientific. Gel electrophoresis experiments were carried out on either 20 X 20 cm vertical Hoefer 600 or a Bio-Rad Mini-PROTEAN® Tetra Vertical electrophoresis units. Gel images were captured using a ChemiDoc™ MP system from Bio-Rad Laboratories. Firefly Luciferase luminescence was measured using a Biotek Synergy HT plate reader. Thermal annealing of all nucleic acids was conducted using an Eppendorf Mastercycler® 96 well thermocycler. Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS) was carried out using Dionex Ultimate 3000 coupled to a Bruker MaXis Impact™ QTOF. Dynamic light scattering (DLS) experiments were carried out using a DynaProTM Instrument from Wyatt echnology. Quantitative polymerase chain reactions (qPCR) experiments were performed on. Roche LightCycler® 96 instrument.

SI-III. Synthesis of RNA amphiphiles

III-a. Enzymatic synthesis of N_3-RNA

The DNA template strands used for *in vitro* transcription of RNA were synthesised using standard solid-phase synthetic methods as described in III-b. RNA transcription reaction mixes consisted of: 1xRNA polymerase buffer, 0.8xTAMg, 4mM DTT, 4uM of DNA template, 2.5 mM of rNTPs, 10 mM 5'-azido-5'-deoxyguanosine (from 150 mM stock in DMSO), 0.4 units/uL RNaseOUT^{TM}, 0.02 units/uL of thermostable inorganic pyrophosphatase, and 5 units/uL of T7 RNA polymerase. Reactions were incubated at 37°C for 20 hrs before being diluted 2x and supplemented with 10x DNase I buffer. 0.02 units/uL of DNase I was added and the mix was incubated at 37°C for a further 2 hrs. Excess rNTPs were removed by size-exclusion chromatography using Sephadex G-25.

III-b. Solid-phase synthesis of DNA and sequence-defined polymers

All solid-phase syntheses were performed on a 1 μmol scale using universal 1000 Å LCAA-CPG solid-supports. Coupling efficiencies were monitored following removal of the dimethoxytrityl (DMT) 5'-OH protecting group. DBCO-dT-CE and DMT-dodecane-diol phosphoramidites were dissolved in the appropriate solvents (anhydrous acetonitrile/dichloromethane 1:3 (v/v) and acetonitrile respectively) under a nitrogen atmosphere (<0.04 ppm oxygen and <0.5 ppm trace moisture) for a final concentration of 0.1M. The DMT-dodecane-diol was activated using 0.25M 5-(ethylthio)tetrazole in anhydrous acetonitrile and an extended coupling time of 5 minutes was employed. The DBCO-dT-CE and DBCO-TEG amidites were also activated with 0.25M 5-(ethylthio)tetrazole in anhydrous acetonitrile, but were manually coupled under an nitrogen atmosphere for an extended time of 15 minutes. 3% dichloroacetic acid in dichloromethane was used to remove the DMT protecting group on the DNA synthesiser. Strands containing the DBCO-dT-CE amidite at the 3' end were oxidised using 0.5M CSO (cat.# 40-4632, purchased from Glen Research). Sulfurization (including those containing DBCO) was executed prior to capping on the DNA synthesiser using Sulfurizing Reagent II (cat.# 40-4037, Glen Research). Following synthesis, strands were cleaved from the solid-support and deprotected using 28% aqueous ammonium hydroxide

solution for 20 hours at 60°C. Strands were dried under vacuum at 60°C, resuspended in Millipore H_2O, then filtered with 0.22 μm centrifugal filters. Strands were purified as stated in Supporting Table ST1.

III-c. Purification of N_3-RNA, DNA, and sequence-defined polymers

PAGE purification

Aqueous solution of oligomer was mixed with an equal volume of formamide prior to loading on gel in order to assist in denaturation. For transcribed RNA, 20% polyacrylamide/8M urea gels in 1xTBE were run for 30 minutes at 250V then 150 minutes at 500V. These conditions allowed isolation of the desired length RNA from oligos of length n±1. Oligomers synthesised by solid-phase methods or by conjugation were run on 15% polyacrylamide/8M urea gels in 1xTBE for 30 minutes at 250V, followed by 60 minutes at 500V.

Following gel electrophoresis, bands were imaged using a handheld UV illuminator (254nm) and excised, crushed, and suspended in ~5-10 mL of DEPC treated H_2O. This suspension was frozen by brief submersion in liquid nitrogen, before being incubated at 60°C for 16 hours. The supernatant was concentrated by evaporation, desalted using size-exclusion chromatography (Sephadex G-25), and quantified (OD_{260}) using a NanoDrop Lite spectrophotometer from Thermo Scientific.

HPLC purification

0.5 OD of sample in 20-50 μL of Millipore water were injected into a Hamilton PRP-1 5 μm 2.1x150mm column at 60°C. The mobile phases were TEAA and HPLC grade acetonitrile, with an elution gradient of 3-70% acetonitrile over 30 minutes for DBCO-HE_6, DBCO-HE_9, and DBCO-HE_{12}, or 3-50% for DNA1-HE_{12}-RNA1 and HE_6-RNA1. Strands were detected using a diode array detector monitoring absorbance at 260nm.

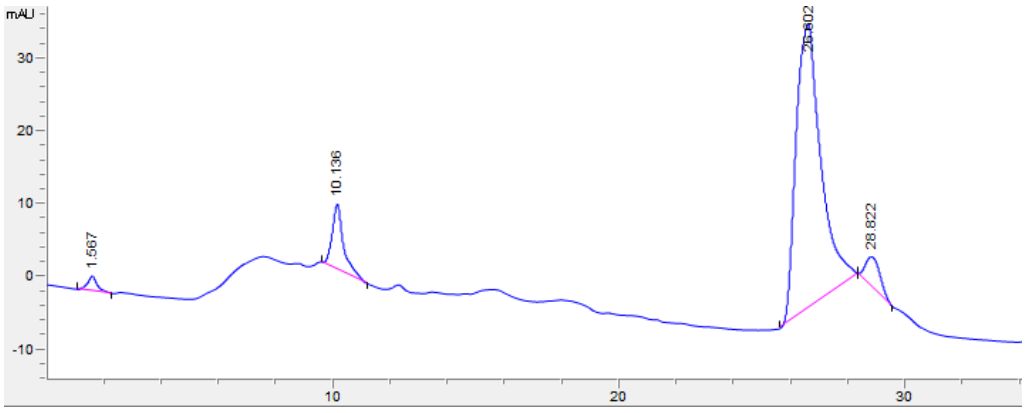
III-d. Conjugation of N_3-RNA to sequence-defined polymers

Synthesis and purification of DNA-HE_{12}-RNA conjugates

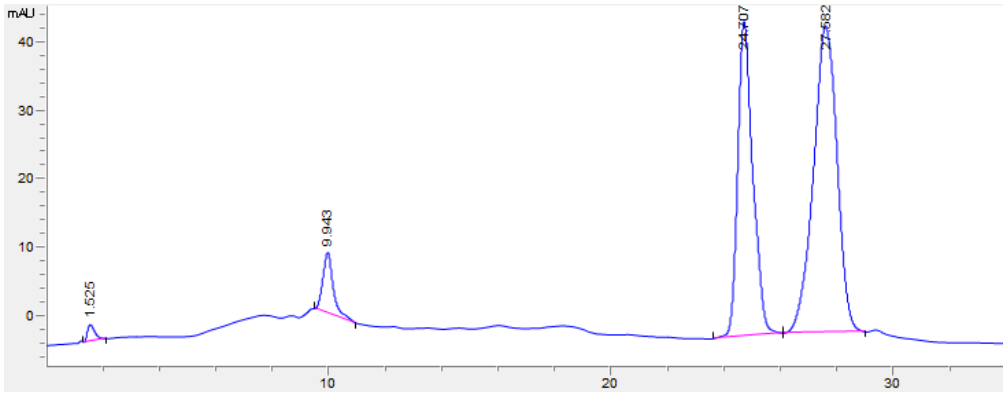
5 µM of 5'-N_3-RNA was combined with 10 µM of 5'-DNA-HE_{12}-DBCO in 1xTAMg (made with DEPC treated water). The reaction mix was left at room temperature for 16 hours. DNA1-HE_{12}-RNA1 was purified using reverse-phase HPLC, whereas LucASO-HE_{12}-rLucS was purified using denaturing PAGE (15% in 1xTBE).

Synthesis and purification of HE_6-, HE_9- and HE_{12}-RNA conjugates

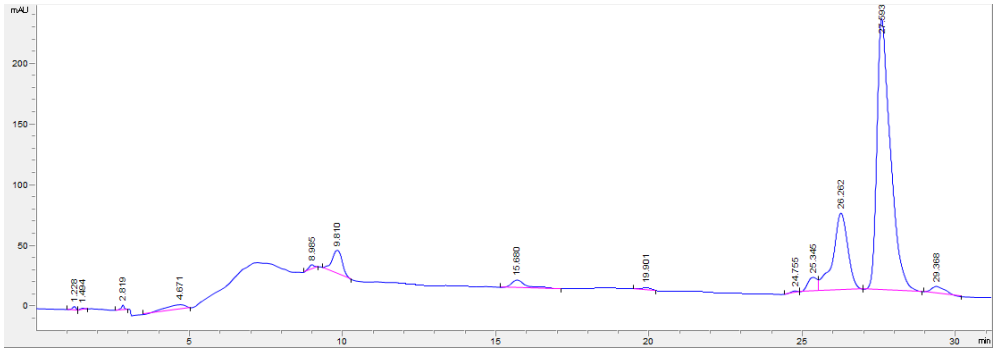
5 µM of 5'-N_3-RNA and 10 µM of DNA-micelle template (DNA1-HE_{12}, LucPc-HE_{12}, or ApoB'-HE_{12}) were combined with 30 µM of DBCO-HE_6, DBCO-HE_9, or DBCO-HE_{12} in DEPC treated H_2O prior to the addition of TAMg. The reaction mixes were then briefly vortexed and left at room temperature for 16 hrs. HE_6-RNA1 and HE_9-rApoBs were directly purified by reverse-phase HPLC, while HE_{12}-RNA1 and HE_{12}-rLucP were supplemented with 10x DNase I buffer and treated with 0.2 units/µL of DNase I for 2 hrs at 37°C. HE_{12}-RNA1 and HE_{12}-rLucP were then isolated using PAGE. For controlled reactions, the order of addition of reagents was kept consistent with the experimental.



Supporting Figure SF1| Representative reverse-phase HPLC trace for purification of DNA1-HE_{12}-RNA1. 50 uL of crude reaction mix was injected. Peak at ~10.1 minutes is unreacted N_3RNA1, peak at ~26.5 minutes is product DNA1-HE_{12}-RNA1, and peak at ~28.8 minutes is unreacted DNA1-HE_{12}-DBCO.



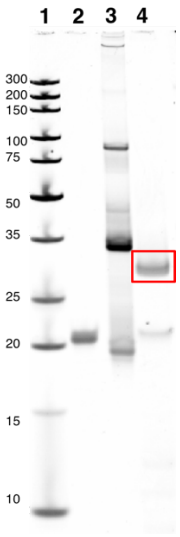
Supporting Figure SF2| Representative reverse-phase HPLC trace for purification of HE_6-RNA1. Absorbance at 260 nm. 50 uL of crude reaction mix was injected. Peak at ~10 minutes is unreacted RNA1 (without azide), peak at ~25 minutes is product HE_6-RNA1, and peak at ~28 minutes is micelle template DNA1-HE_{12}.



Supporting Figure SF3| Representative reverse-phase HPLC trace for purification of HE_9-ApoBs. Absorbance at 260 nm. 100 uL of crude reaction mix was injected. Peak just below 10 mins is RNA without azide (N_3ApoBs elutes at 10.1 mins). Peak at 26.3 mins is HE_9-ApoBs. Peak at 27.6 mins is DNA-micelle reaction template.

Application of DNase I on crude reaction mixes

Treatment of the crude reaction mix of N_3RNA1 with DNA1-HE_{12}-DBCO selectively degrades the DNA segment of DNA1-HE_{12}-RNA1. This can be seen in Supporting Figure SF3.



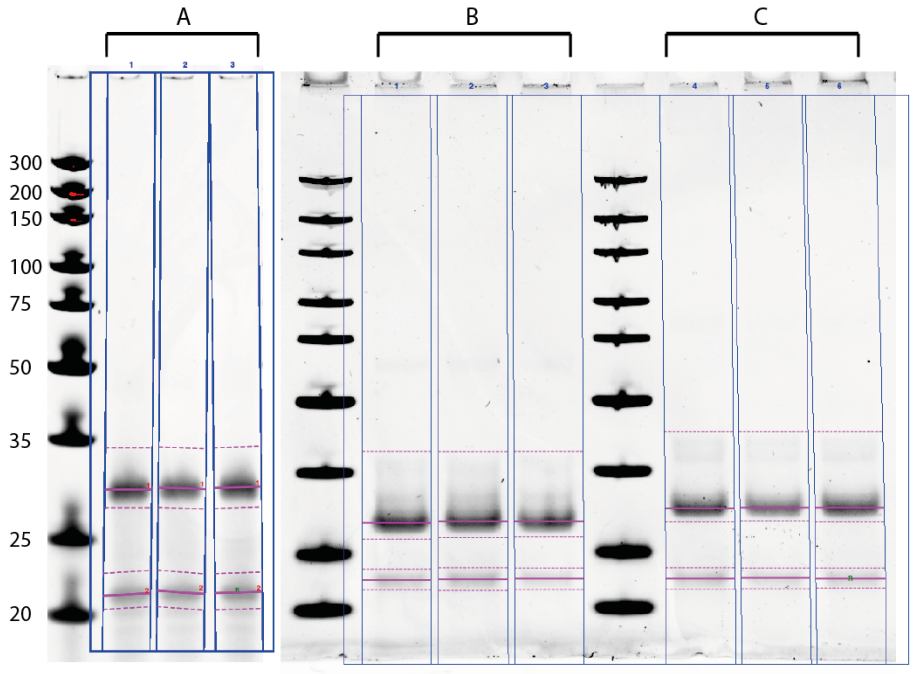
Supporting Figure SF4| PAGE (15% polyacrylamide/8M urea in 1xTBE). Representative gel showing the shift to higher mobility following treatment with DNase I as DNA1-HE_{12}-RNA1 is converted to HE_{12}-RNA1. Lane 1: ultra low range ladder; lane 2: N_3RNA1; lane 3: crude reaction mix (darkest band is DNA1-HE_{12}-RNA1); lane 4: crude reaction mix following DNase I treatment. Product following DNase I is highlighted by red box.

Supporting Table ST1| Sequences of polymers used. Atypical amidite/nucleotide codes: (**D** = dodecane-diol), (**N_3-G** = 5'-azido-5'-deoxyguanosine), (**dT-DBCO** = DBCO-dT-CE), (**DBCO** = DBCO-TEG), (lower case = phosphorothioated).

Name	Sequence (5'-xx-3')	Description	Purification
RNA1-T	TTTTTCAGTTGACCATATAGTCTCTCCCTATAGTGAGTCGTATTAATTTC	DNA templates for T7 transcription of RNA1, rLucG, rLucP, rLucS, rApoBs	PAGE
LucG-T	CCGC**TT**CAAGTCTTTAATTAAGGGTCCTATAGTGAGTCGTATTAATTTC		
LucP-T	GGACCCTTAATTAAAGACTTC**AA**GCTCCTATAGTGAGTCGTATTAATTTC		
LucS-T	ATATCCTTGTCGTATCCC-TATAGTGAGTCGTATTAATTTC		
ApoBs-T	ATTGGTATTCAGTGTGATGACTTCCCTATAGTGAGTCGTATTAATTTC		
P1	GAAATTAATACGACTCACTATAGGGAGA	DNA promoter for T7 transcription	PAGE
LucASO	atatccttgtcgtatccc	DNA Luciferase antisense	PAGE
LucS	GGGATACGACAAGGATAT	DNA Luciferase sense	PAGE
DNA1-HE_{12}	TGACCATATAGTCTCTCCC**DDDDDDDDDDDD**	DNA-amphiphile comp. to RNA1	PAGE
DNABT-HE_{12}	TTTTTCCATCTGGTATTAC**DDDDDDDDDDDD**	Micelle non-complementary to RNA1	PAGE
LucPc-HE_{12}	ATTAAAGACTTCAAGCTCC**DDDDDDDDDDDD**	DNA-amphiphile comp. to rLucP	PAGE
ApoBc-HE_{12}	TTCAGTGTGATGACTTCCC**DDDDDDDDDDDD**	DNA-amphiphile comp. to rApoBs	PAGE
LucASO-HE_{12}	atatccttgtcgtatccc**DDDDDDDDDDDD**	DNA-amphiphile comp. to rLucS; Luciferase ASO	PAGE
DNA1-HE_{12}-DBCO	TGACCATATAGTCTCTCCC**DDDDDDDDDDDD-dT-DBCO**	DNA-amphiphile comp. to RNA1 with DBCO	PAGE
LucASO-HE_{12}-DBCO	atatccttgtcgtatccc**DDDDDDDDDDDD-dT-DBCO**	DNA-amphiphile comp. to rLucS with DBCO; Luciferase ASO	PAGE
rLucG	GGACCCUUAAUUAAAGACUUGAAGCGG	RNA transcript; Luciferase siRNA antisense	PAGE
rApoBs	GGGAAGUCAUCACACUGAAUACCAAU	RNA transcript; ApoB siRNA sense	PAGE
rApoBas	AUUGGUAUUCAGUGUGAUGACUUCCC	Synthetic RNA; ApoB siRNA antisense	PAGE
N_3-RNA1	**N_3-G**GGAGAGACUAUAUGGUCAACUGAAAAA	5'-azido RNA transcript	PAGE
N_3-rLucP	**N_3-G**GAGCUUGAAGUCUUUAAUUAAGGGUCC	5'-azido RNA transcript; Luciferase siRNA sense	PAGE
N_3-rLucS	**N_3-G**GGAUACGACAAGGAUAU	5'-azido RNA transcript; sense for Luciferase ASO	PAGE
N_3-ApoBs	**N_3-G**GGAAGUCAUCACACUGAAUACCAAU	5'-azido RNA transcript; sense for ApoB siRNA	PAGE
DBCO-HE_6	**DBCO-dT-DDDDDD**	Sequence-defined hydrophobes with DBCO	HPLC
DBCO-HE_{12}	**DBCO-dT-DDDDDDDDDDDD**		
DBCO-HE_9	**DBCO-T-DDDDDDDDD**		
DNA1-HE_{12}-RNA1	TGACCATATAGTCTCTCCC**DDDDDDDDDDDD-dT-G**GGAGAGACUAUAUGGUCAACUGAAAAA	DNA/RNA hybrid with polymer linkage	HPLC
HE_6-RNA1	**DDDDDD-dT- G**GGAGAGACUAUAUGGUCAACUGAAAAA	RNA amphiphile	HPLC
HE_{12}-RNA1	**DDDDDDDDDDDD-dT-**GGGAGAGACUAUAUGGUCAACUGAAAAA	RNA amphiphile	PAGE
LucASO-HE_{12}-rLucS	atatccttgtcgtatccc**DDDDDDDDDDDD-dT-G**GGAUACGACAAGGAUAU	DNA/RNA hybrid with polymer linkage; DNA ASO with RNA comp.	PAGE
HE_{12}-rLucP	**DDDDDDDDDDDD-dT-G**GAGCUUGAAGUCUUUAAUUAAGGGUCC	RNA amphiphile; Luciferase siRNA antisense	PAGE
HE_9-rApoBs	**DDDDDDDDD-T-G**GGAAGUCAUCACACUGAAUACCAAU	RNA amphiphile; ApoB siRNA antisense	HPLC

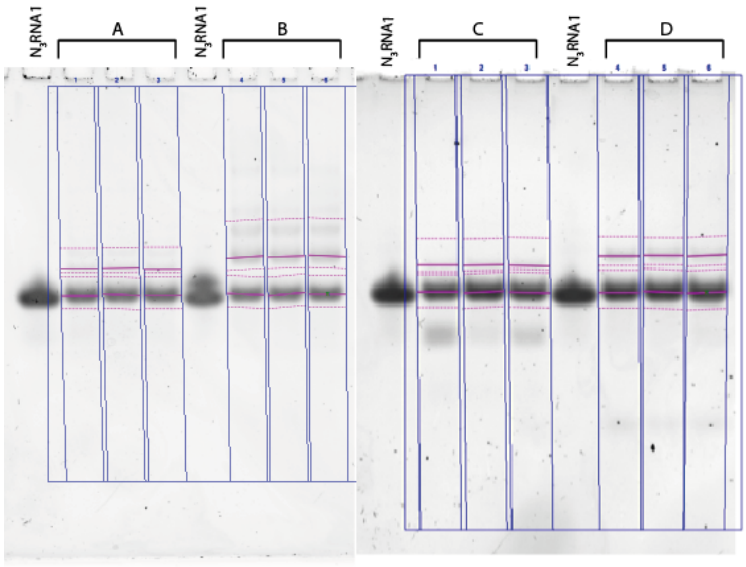
III-e. Yields of conjugations

To quantify the yields of conjugations, reactions were supplemented with 10x DNase I buffer and treated with 0.2 units/µL of DNase I for 2 hours at 37°C, before being analysed by denaturing PAGE. This ensured selective degradation of DNA and preservation of RNA, which allowed direct comparison of the ratio of starting material to product using the intensity of GelRed™ nucleic acid stain fluorescence. Image Lab™ software was employed for this purpose. Yield was determined by comparing the total fluorescence within indicated band boundaries (**SF1**, **SF2**, **SF3)** of product (lower mobility) to the remaining N_3RNA1 (higher mobility). In **SF3**, the highest mobility bands lightly visible in several lanes are DNA micelle template that were incompletely degraded and hence were excluded from yield calculations. Using Image Lab software, standard deviations of yields were calculated to be <5% in all cases, however due to the variability inherent to GelRed staining and electrophoresis in general, standard deviations were assumed to be 5%.

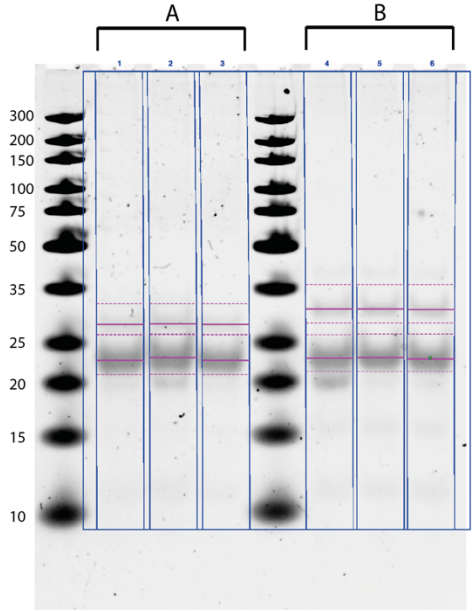


Supporting Figure SF5| PAGE (15% polyacrylamide/8M urea in 1xTBE). Results of triplicate conjugation reactions of N_3RNA1 with DNA1-HE_{12}-DBCO (A),

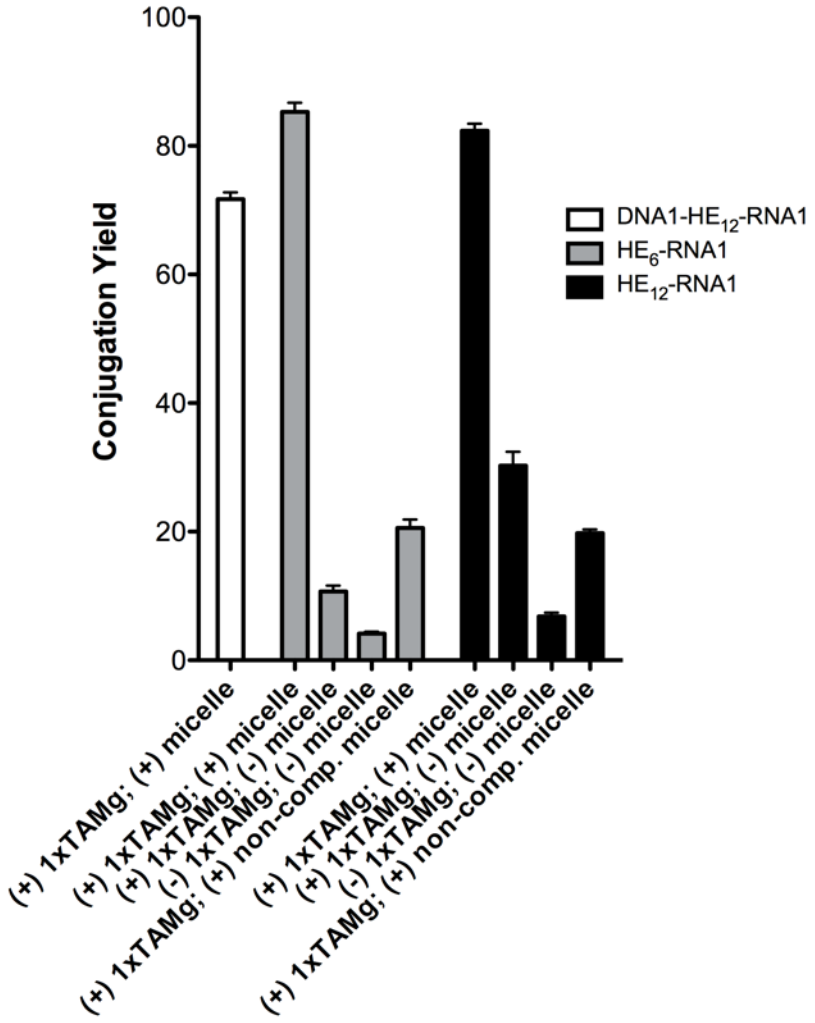
DBCO-HE_6 (B), DBCO-HE_{12} (C). Band of lower mobility indicates the conjugate, while band of higher mobility indicates the starting material (N_3RNA1).



Supporting Figure SF6| PAGE (15% polyacrylamide/8M urea in 1xTBE). Results of triplicate control reactions (with no DNA micelle template) of N_3RNA1 with DBCO-HE_6 (A) and DBCO-HE_{12} (B) in 1xTAMg, and DBCO-HE_6 (C) and DBCO-HE_{12} (D) in DEPC treated H_2O.



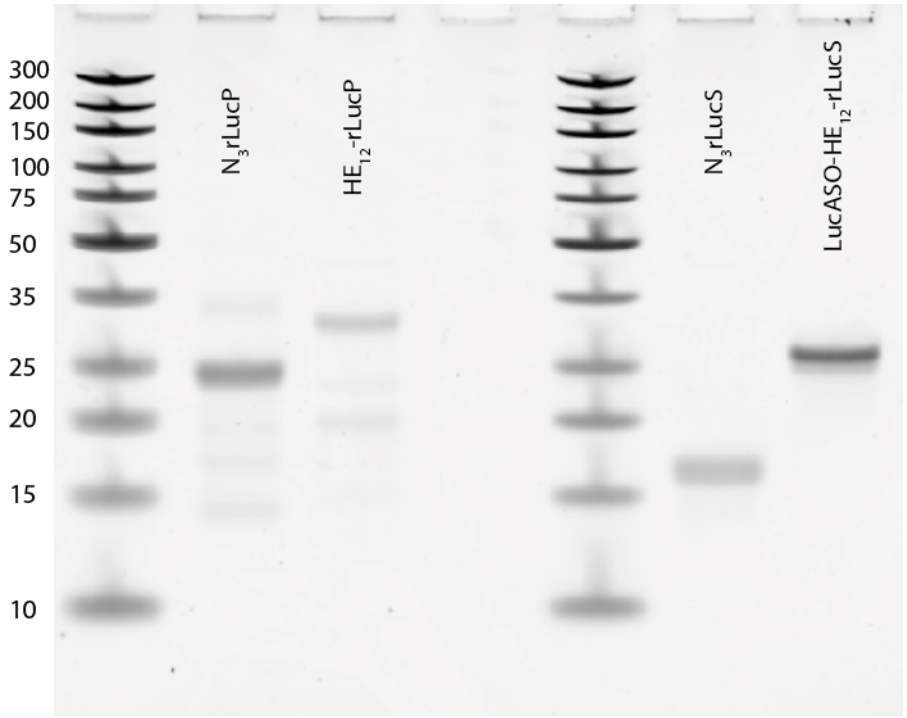
Supporting Figure SF7| PAGE (15% polyacrylamide/8M urea in 1xTBE). Results of triplicate control reactions (with non-complementary DNA micelle template: DNABT-HE_{12}) of N_3RNA1 with DBCO-HE_6 (A) and DBCO-HE_{12} (B).



Supporting Figure SF8| Yields of HE_6-RNA1 and HE_{12}-RNA1 amphiphiles under different conditions. All experiments were performed in triplicate.

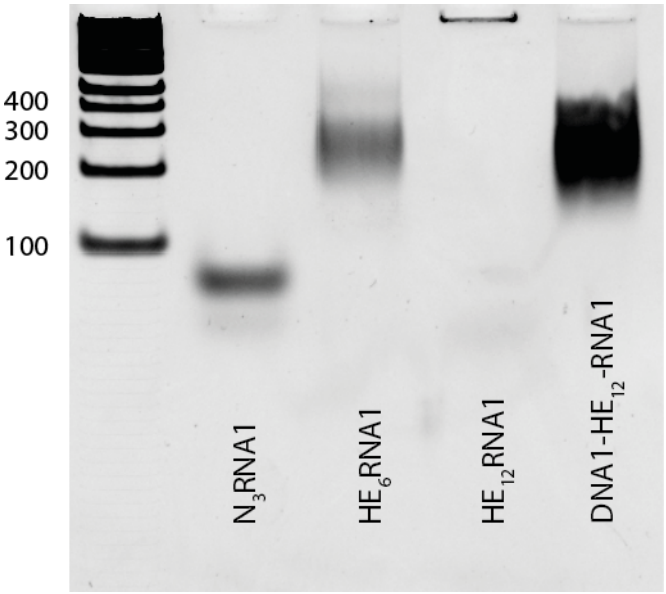
SI-IV. Characterisation and self-assembly of RNA amphiphiles

IV-a. Gel Electrophoresis

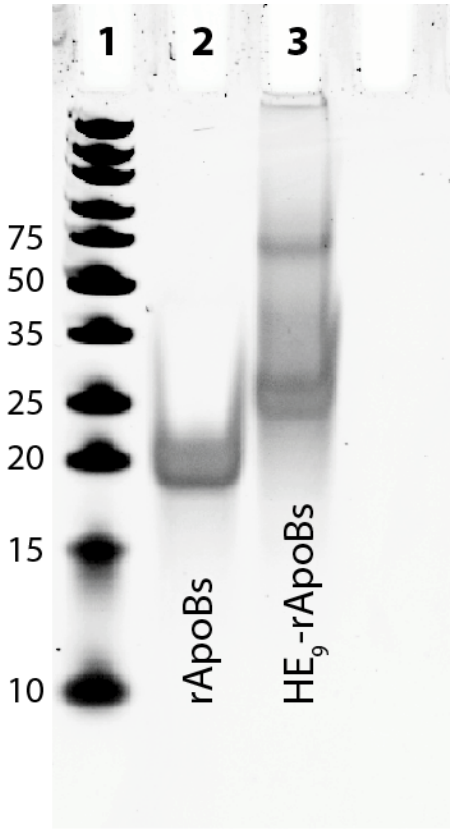


Supporting Figure SF9| PAGE (15% polyacrylamide/8M urea in 1xTBE).

Characterisation of purified therapeutic conjugates compared to starting material.



Supporting Figure SF10l PAGE (6% polyacrylamide in 1xTAMg)



Supporting Figure SF11l PAGE (17.5% polyacrylamide in 1xTBE). Lowr mobility bands in Lane 3 due to incomplete denaturation of secondary RNA-RNA interactions and the hydrophobic effect.

IV-b. LC-MS

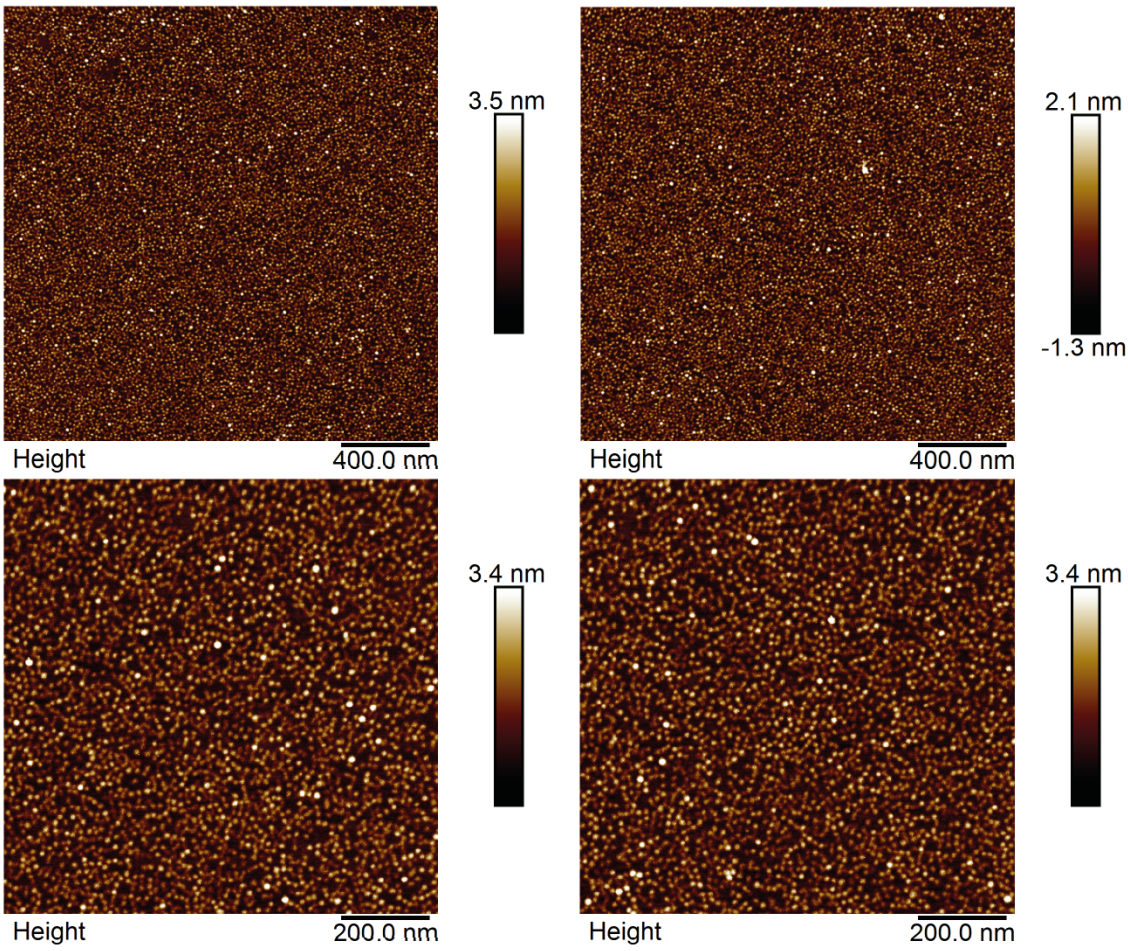
Supporting Table ST2l ESI-MS Table. Calculated and experimental m/z values for amphiphilic RNA conjugates.

Molecule	Calculated mass	Experimental mass (negative ion mode)
DNA1-HE_{12}-RNA1	18765.75	18764.47
HE_6-RNA1	11419.23	11418.19
HE_{12}-RNA1	13005.04	13004.33
HE_{12}-rLucP	12888.84	12888.28
LucASO-HE_{12}-rLucS	15502.67	15502.72
HE_9-ApoBs	11542.85	11545.75

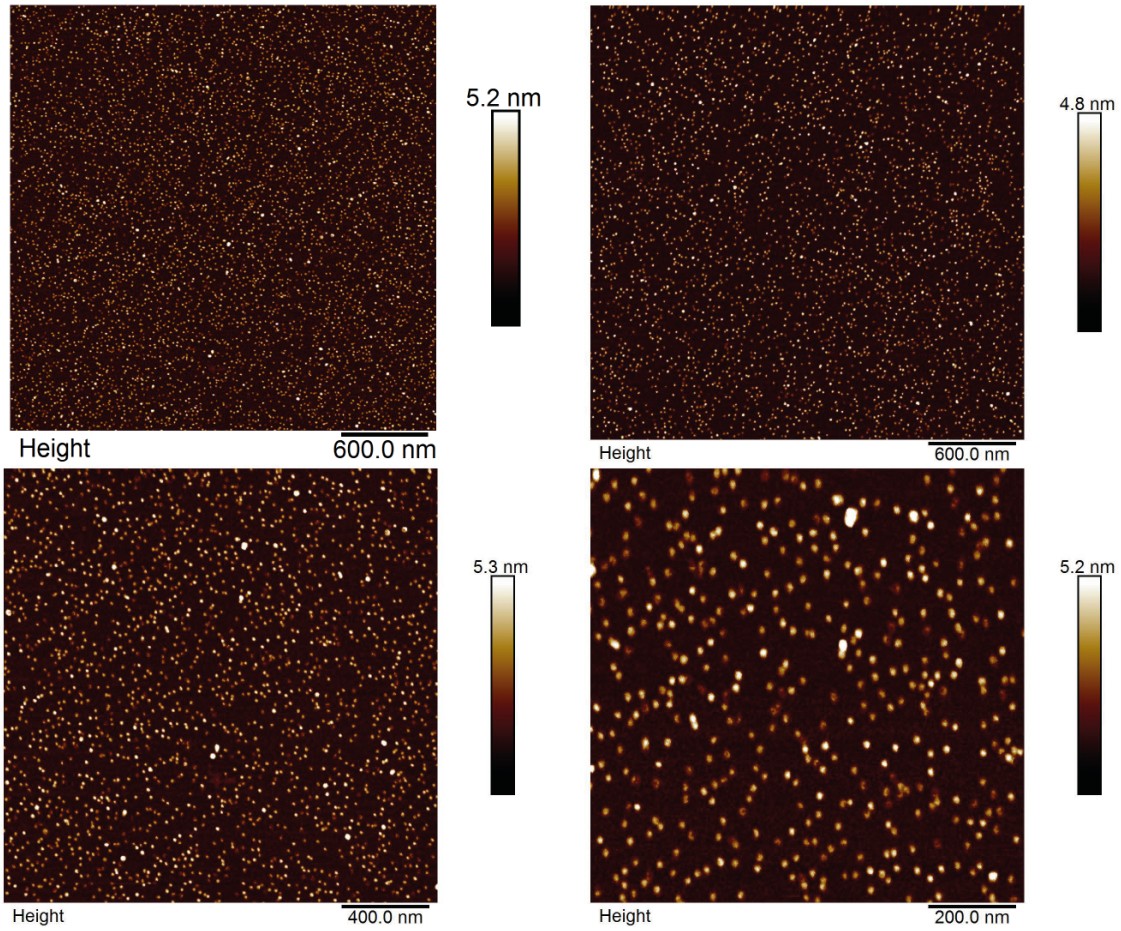
IV-c. Atomic Force Microscopy

10 μM of each strand (20 μM total RNA for HE_{12}-LucP + rLucS) in either 1xTAMg or siRNA buffer were annealed from 95 to 4°C over one hour. Samples were diluted to 2.5 μM with the relevant buffer before 4 μL were deposited on freshly cleaved mica (ca. 7 x 7mm). After 10 seconds, the surface was washed by adding 50 μL of 0.22 μm filtered Millipore water that was immediately removed with filter paper. This washing procedure was repeated an additional four times before a light to moderate flow of argon was used to expel excess liquid from the surface. Samples were dried under vacuum for a minimum of 30 minutes prior to imaging. Imaging was performed under dry conditions using ScanAsyst mode with ScanAsyst Air triangular silicon nitride probe (tip radius = 2nm, k = 0.4 N/m, f_0 = 70 kHz; Bruker).

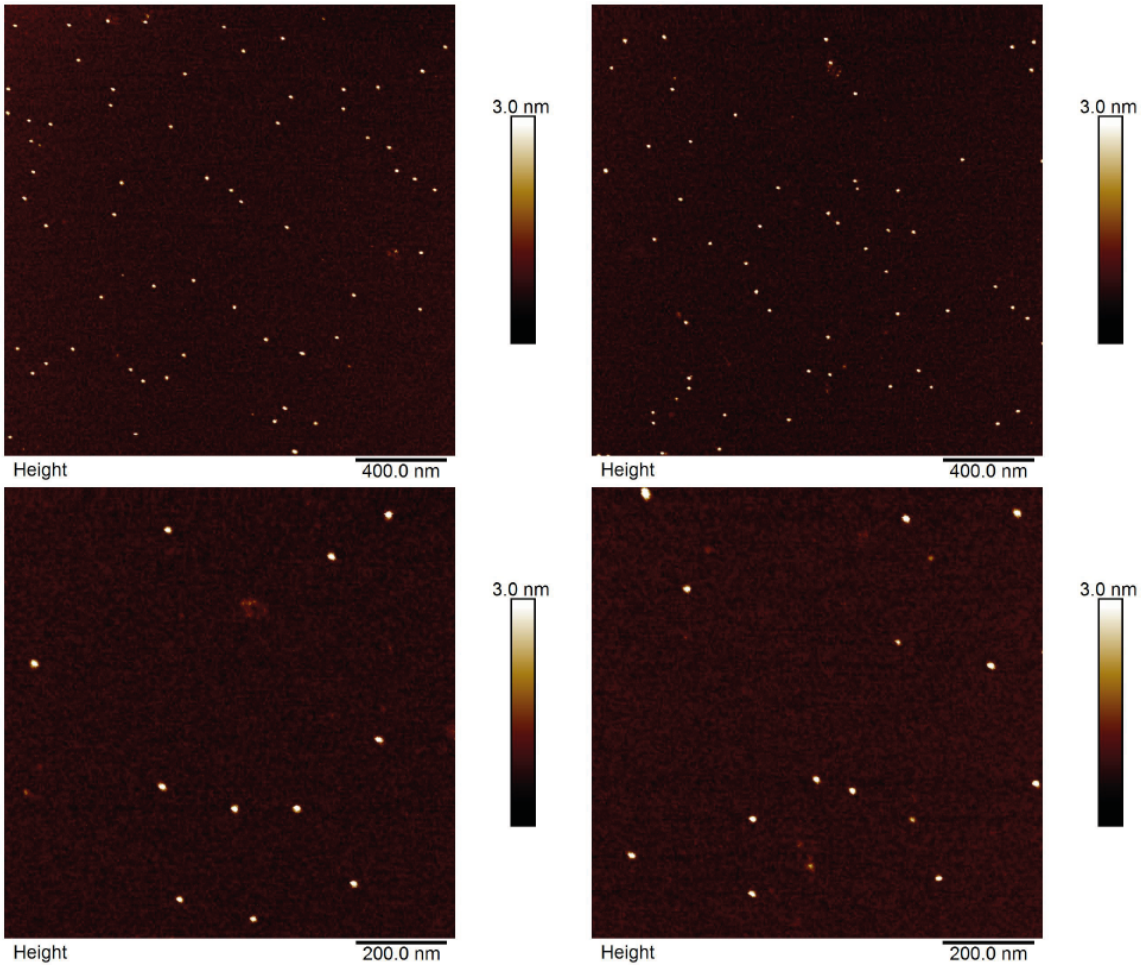
Images were processed using Nanoscope Analysis 1.40 software. The Flatten tool was used to correct tilt, bow, and scanner drift, while average heights were obtained using the Particle Analysis tool. To find average diameters, images were exported to ImageJ software, where between 50 and 120 data points were measured using the AFM scan size as scale.



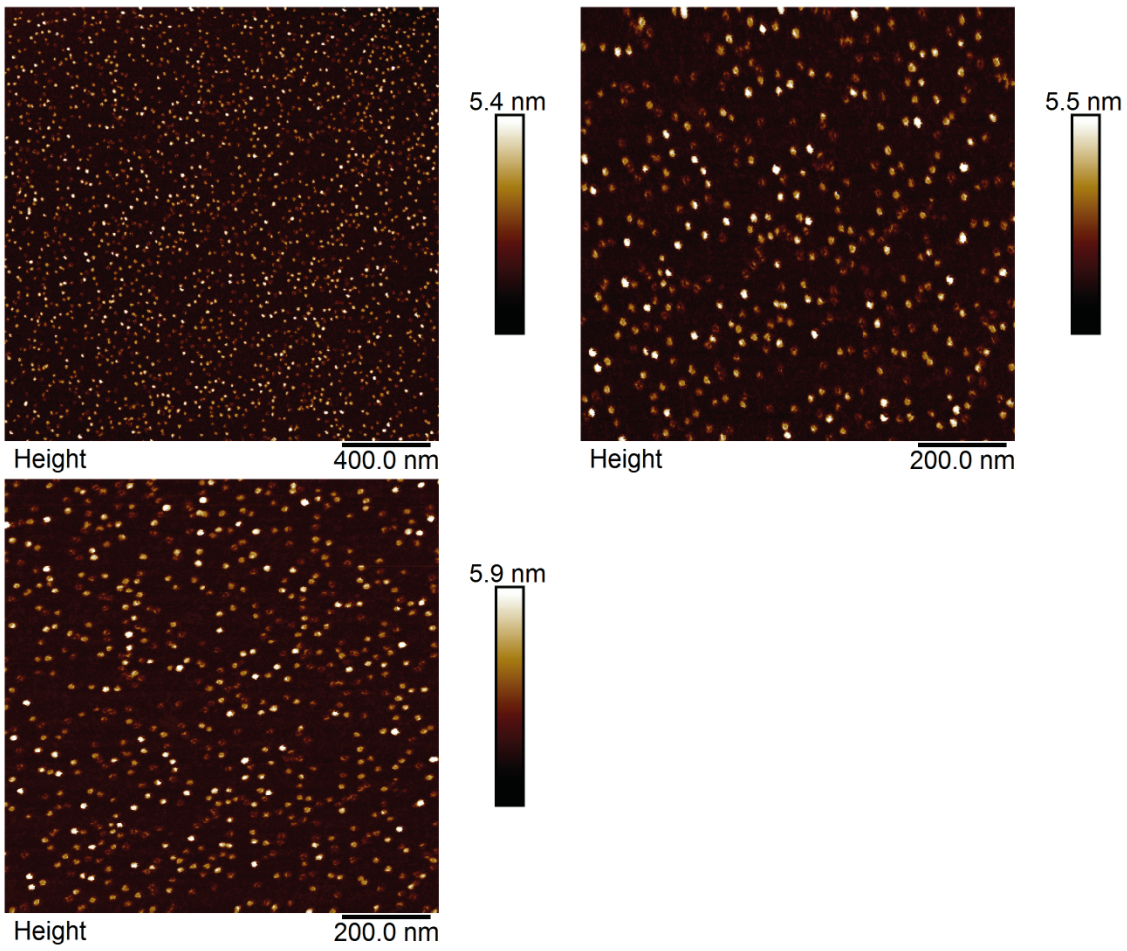
Supporting Figure SF12| AFM images of DNA1-HE$_{12}$-RNA1. Structures were assembled in 1xTAMg. Diameter = 13.8 ± 1.6 nm (N = 108).



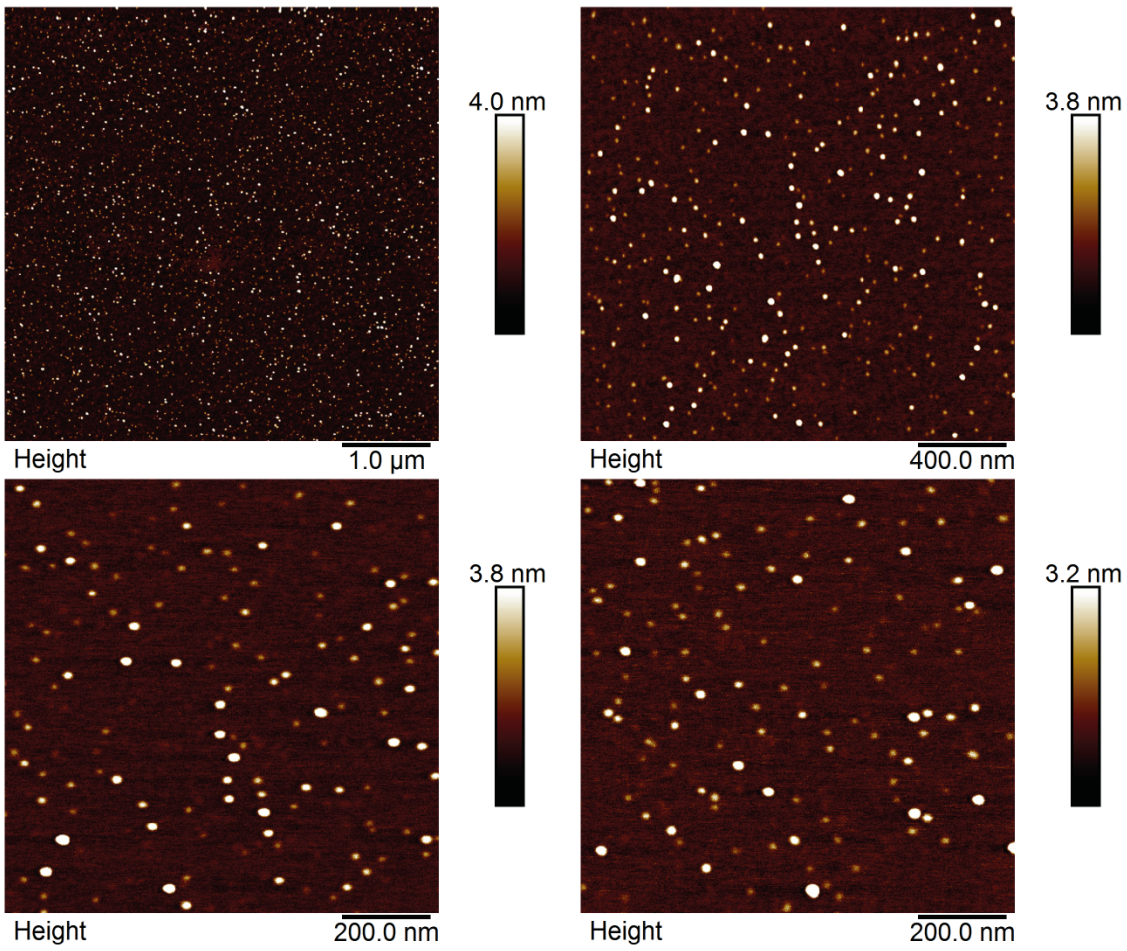
Supporting Figure SF13| AFM images of HE_6-RNA1. Structures were assembled in 1xTAMg. Diameter = 16.3 ± 1.9 nm (N = 73), height = 3.1 ± 0.8 nm (N = 352).



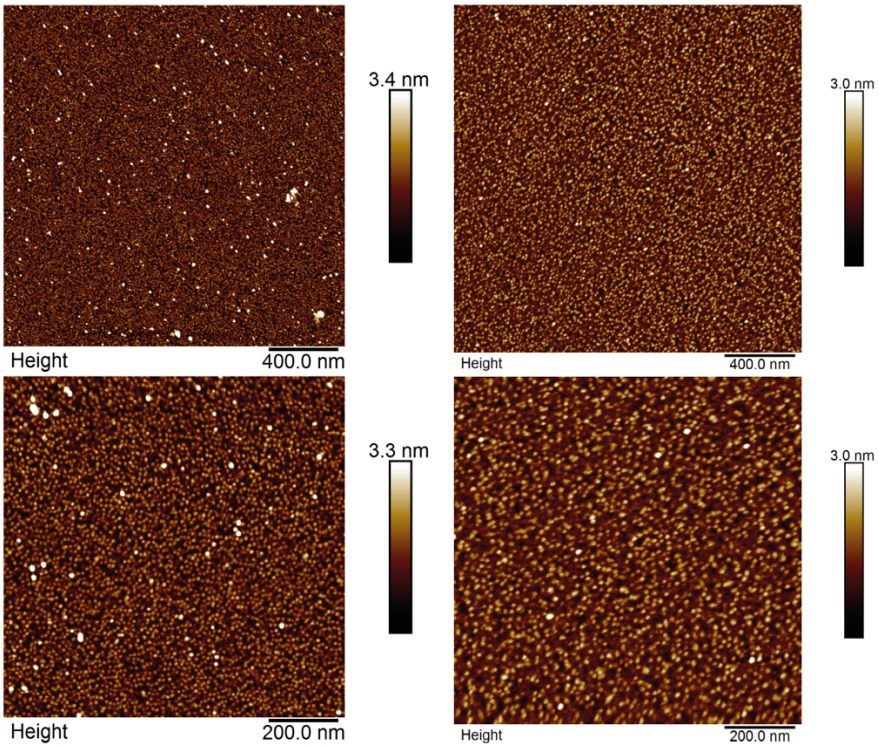
Supporting Figure SF14| AFM images of HE_{12}-RNA1. Structures were assembled in 1xTAMg. Diameter = 17.1 ± 2.9 nm (N = 102), height = 3.3 ± 0.5 nm (N = 26).



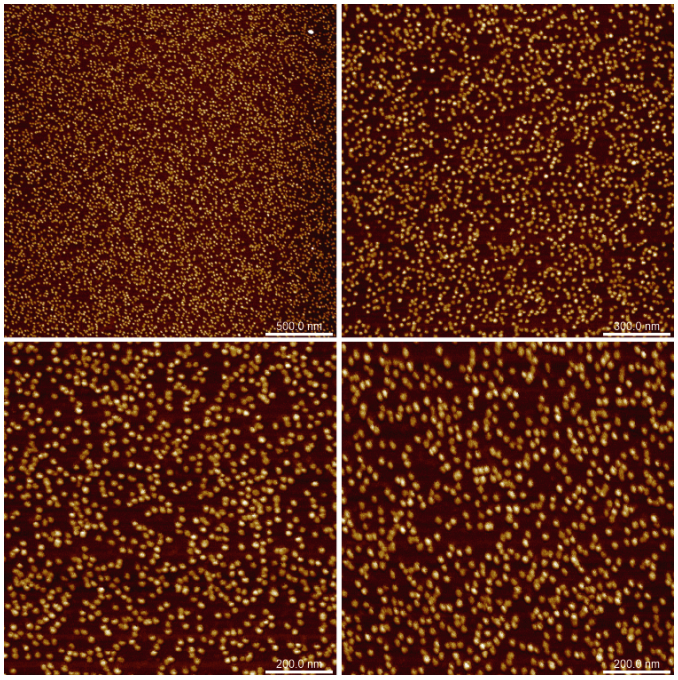
Supporting Figure SF15| AFM images of HE_{12}-RNA1. Assembled in 1xsiRNA+Mg buffer.



Supporting Figure SF16l AFM images of HE12-rLucP + rLucS. Assembled in 1xsiRNA+Mg buffer. Diameter = 19.8 ± 3.1 nm (N = 65), height = 2.7 ± 1.5 nm (N = 119).



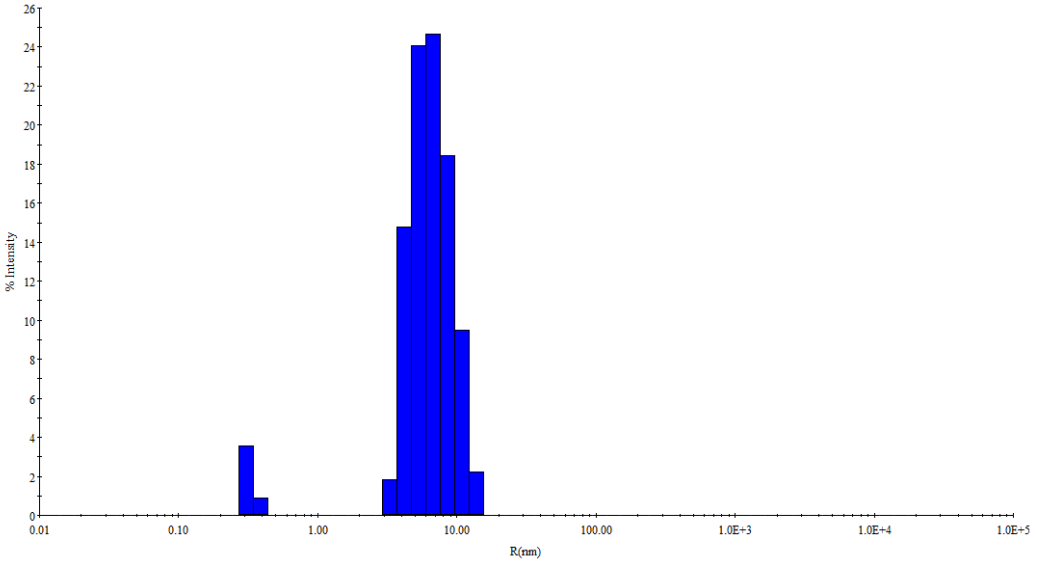
Supporting Figure SF17| AFM images of LucASO-HE$_{12}$-rLucS. Assembled in 1xTAMg. Diameter = 13.1 ± 1.7 nm (N = 102).



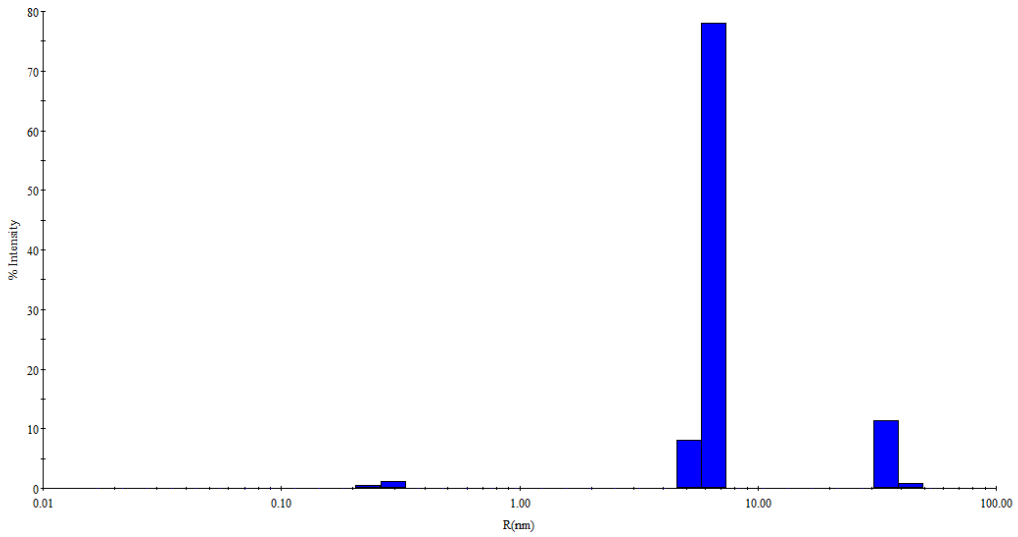
Supporting Figure SF18| AFM images of HE$_9$-rApoBs + rApoBas. Assembled in 1xTAMg. Diameter = 17.9 ±1.8 nm (N = 68), height = 1.9 ± 0.3 nm (N = 390).

IV-d. Dynamic light scattering

A cumulants fit model was used to confirm the presence and determine the size of the biologically active RNA-amphiphiles. DEPC treated water and 1xTAMg buffer were filtered using a 0.45 µm nylon syringe filter before use in DLS sample preparation. 15 µL of sample (concentration: 5 to 10 µM) was used in each measurement. Measurements were performed at 25°C. Each measurement consisted of 30 acquisitions, with each acquisition lasting 10 seconds. Data was filtered to exclude acquisitions with a baseline above 1.010 and a SOS error above 100.



Supporting Figure SF19| Dynamic light scattering of HE_9-rApoBs + rApoBas. Assembled in 1xTAMg. Diameter = 13.8 ± 2.3 nm.



Supporting Figure SF20| Dynamic light scattering of ASO-HE$_{12}$-rLucs.
Assembled in 1xTAMg. Diameter = 12.8 ± 0.4 nm.

SI-V. Gene silencing assays

V-a. Cell Culture

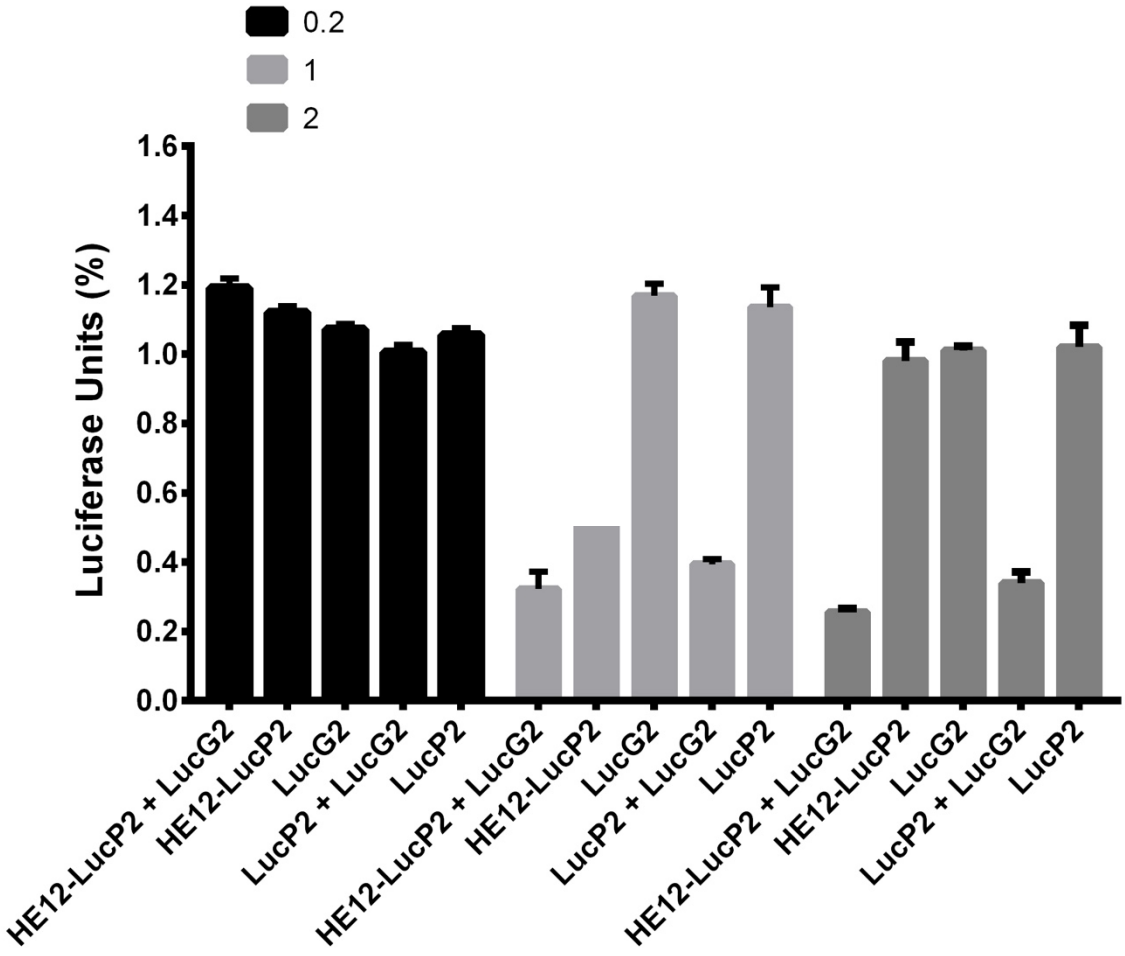
X1/5 is a HeLa cell line stably transfected with a tet repressor fused with the activating domain of virion protein 16 of herpes simplex virus thus generating a tetracycline-controlled transactivator (tTA). This stimulates transcription from a minimal promoter sequence derived from the human cytomegalovirus promoter IE combined with tet operator sequences. Integration of a luciferase gene controlled by a tTA-dependent promoter into this tTA producing HeLa cell line leads to high levels of tetracycline-sensitive luciferase expression. The luciferase activity can be regulated over up to five orders of magnitude depending on the concentration of tetracycline in the culture medium (0-1ug/ml). Specific and stringent control of the activity of an individual gene (luciferase) in the mammalian cell can be monitored. It is also suitable for creation of on/off situations and analysis of mRNA decay rates under physiological conditions. It is a generous gift from Dr. Pelletier (McGill).

HeLa cells were maintained in DMEM supplemented with 10% FBS and antibiotic-antimycotic (AB-AM) and cultured in 5% CO_2 at 37°C. Cells were typically split in 1:4 ratio every three days.

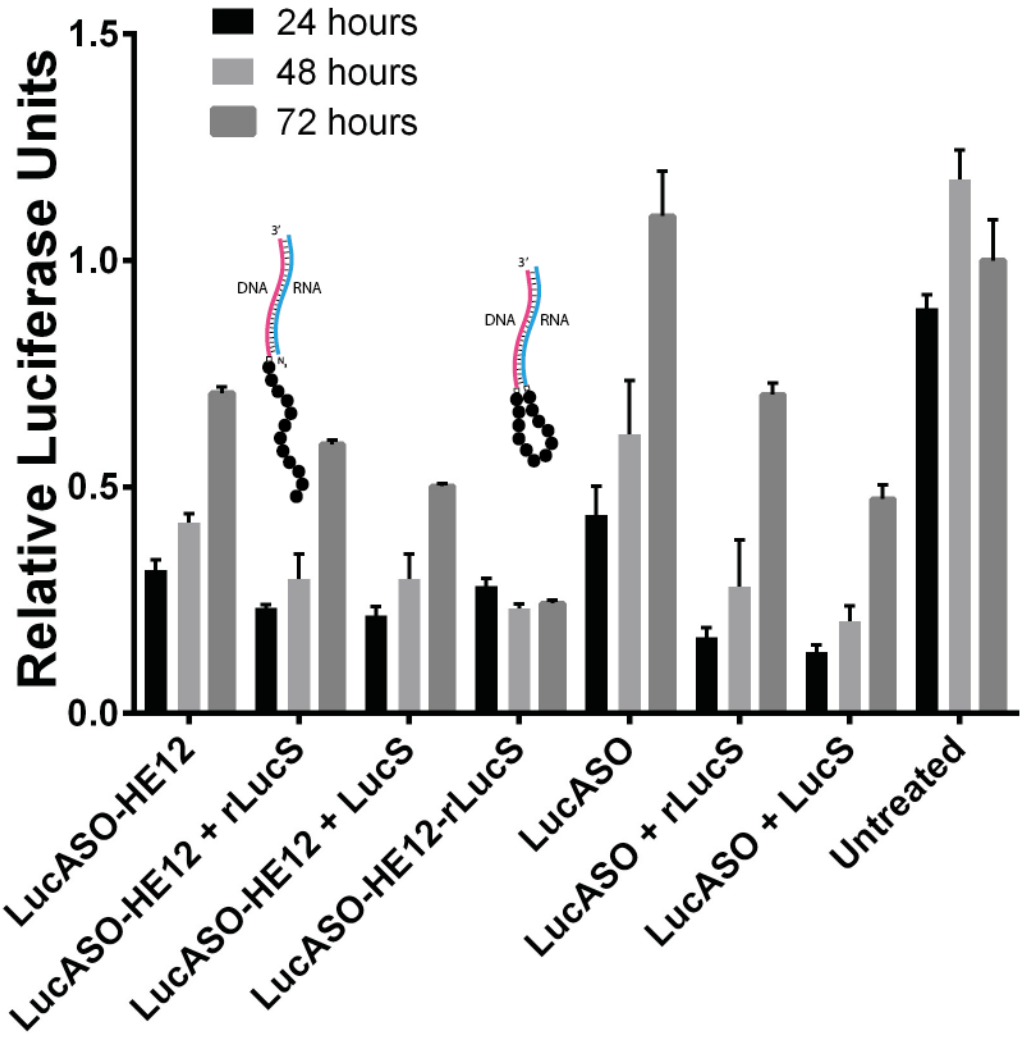
V-b. Firefly luciferase knockdown assays

HeLa cells were counted and seeded at a density of 5000 cells per well (for 24 hr experiment) or 2000 cells per well (for 72 hr experiment) in a 96-well plate before being left to adhere overnight. Prior to the application of sample, media was replaced with 80 uL of DMEM. DNA/RNA-polymer conjugates and control PEI preparations were then diluted up to 20 uL with DMEM and transfection reagent (N:P = 10 or 20) and added to the appropriate well (for total volume of 100 uL and nucleic acid concentration of either 50 or 100 nM). The cells were incubated for a total of either 24 or 72 hours. Following incubation the cells were washed with 1x PBS and lysed with Glo Lysis Buffer, before 100 uL of Bright-Glo Luciferase reagent was added to each well. Luminescence was

measured using a Biotek Synergy HT plate reader. Data was acquired with the Gen5 software suite.



Supporting Figure SF21| Firefly Luciferase Knockdown Activity of siRNA-Polymer/PEI Complexes at N:P = 2 (legend: 0.2), 10, and 20. Firefly Luciferase activity was measured after treatment with a range of single and double stranded siRNA containing polymers. Data are expressed as mean value ± s.e.m. Total concentration of each strand in well was 100 nM. Samples were in 1xsiRNA buffer.



Supporting Figure SF22| Firefly Luciferase Knockdown Activity of siRNA-Polymer/PEI Complexes at N:P = 10. Firefly Luciferase activity was measured at 24, 48, and 72 after treatment with a range of single and double stranded siRNA containing polymers. Data are expressed as mean value ± s.e.m. Total concentration of each strand in well was 100 nM. Samples were in 1xTAMg buffer.

V-c. ApoB knockdown assays

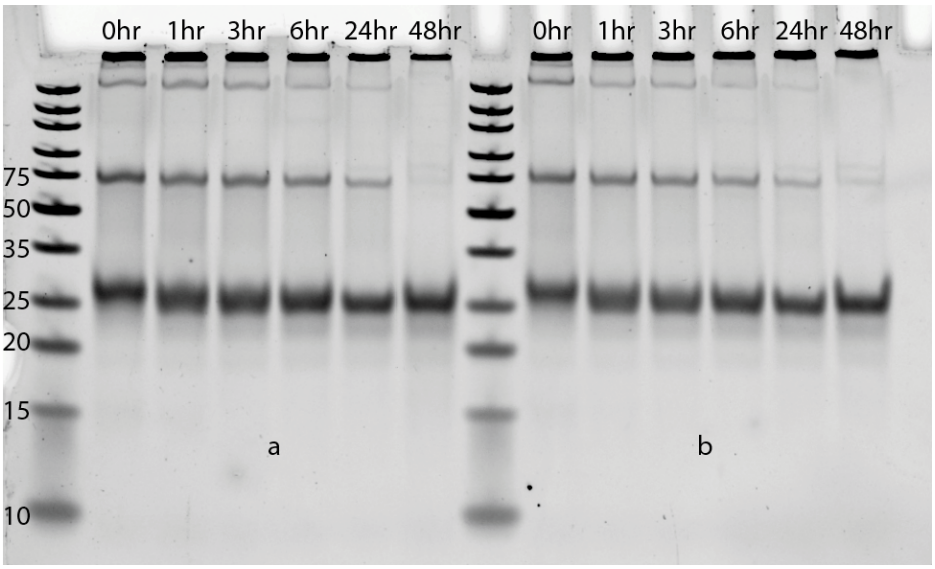
HepG2 cells were plated in 6-well plates at a density of 10^6 cells/well 24 hours prior to the beginning of the experiment. Media was removed and DMEM supplemented with 10% FBS was added to the cells before the samples were transfected with Lipofectamine 3000 Reagent™ as per the provided protocol for a final RNA concentration of 80 nM.

Following incubation, the media was removed, and the cells were lysed directly in the plate with RNAzol RT™. RNA was obtained as per the recommended procedure for isolating large RNA provided by RNAzol RT™.

V-d. Serum stability

LucASO-HE_{12}-rLucS was assembled in 12 uL 1xTAMg at 8uM and then mixed with 12 uL of DMEM with 10% FBS and incubated for 48 hours at 37°C. At 0, 1, 3, 6, 24, and 48 hours, 4 uL was taken and 1 uL of Proteinase K (from Sigma Aldrich) was added to this aliquot. Following incubation for 30 minutes at 37°C, 5 uL of formamide was added aliqouts were stored at -20°C prior to analysis by denaturing PAGE. Experiment was performed in duplicate.

Supporting Figure SF23 shows that over time, there is a slight shift towards higher mobility of the band. Also, the presence of lower mobility bands decreases. These lower mobility bands are due to the formation of dimers and trimers between molecules, as the RNA and DNA segments are complementary. As the strand is degraded, the general mobility of the band increases and the potential to form these dimers and trimers is nullified. Because the DNA segment is phosphothioated, it is likely the RNA degrades first. Hence, the initial protection of the DNA ASO by the complementary RNA, then its the subsequent increased exposure of over time as the RNA degrades could be a reason as to why the silencing is maintained for a longer time frame. This is observed in Supporting Figure SF22.



Supporting Figure SF23| Denaturing PAGE (17.5% polyacrylamide in 1xTBE). Aliquots of LucASO-HE_{12}-rLucS mixed with serum were taken at 0, 1, 2, 6, 24, and 48 hours and analysed by denaturing PAGE. (a) and (b) denote duplicate experiments.

V-e. Quantitative polymerase chain reaction (qPCR)

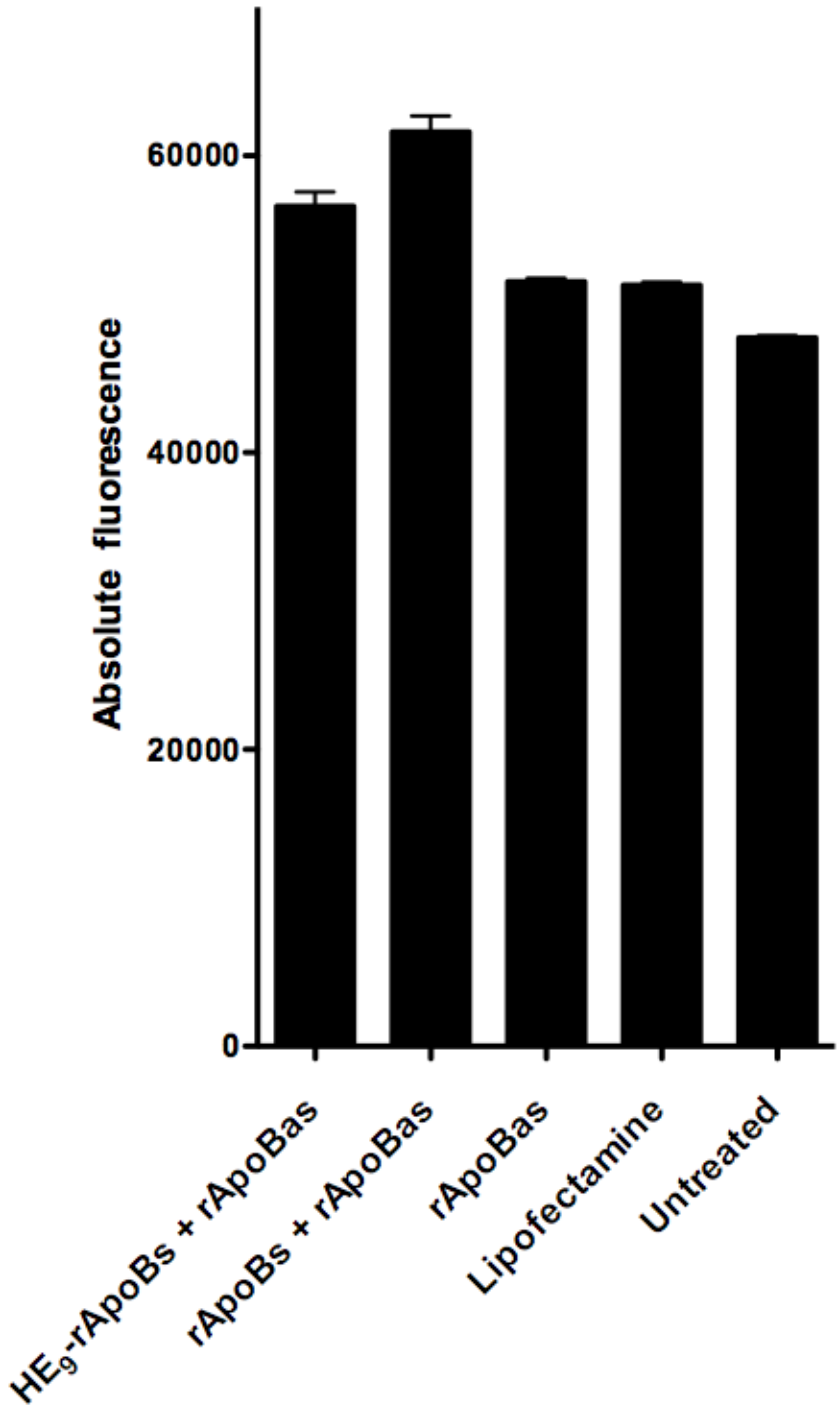
Reverse transcription and subsequent amplification were run in one pot using iTaq™ Universal SYBR Green Supermix. Each reaction used 200ng of RNA and had a total volume of 10µL. The relative amount of ApoB mRNA was quantified against the amount of the endogenous control GAPDH. The primers used for ApoB and GAPDH and shown in Supporting Table ST3. The thermal cycling conditions were as follows: 30 sec at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C. A negative control (distilled water), and RT-negative controls (no reverse transcriptase) were included in each run. Three independent biological experiments (biological triplicates) were performed, and for each of these triplicates, measurements of gene expression were obtained by performing three qPCR reactions (PCR triplicates). The means and errors from the PCR triplicates for each of the biological triplicates were combined to obtain the final data. The ΔΔCt method was used for relative quantification.

Supporting Table ST3| Primers used in qPCR experiments

Gene	Forward	Reverse
ApoB	5'-TTTGCCCTCAACCTACCAAC-3'	5'-TGCGATCTTGTTGGCTACTG-3'
GAPDH	5'-GGAGCGAGATCCCTCCAAAAT-3'	5'-GGCTGTTGTCATACTTCTCATGG-3'

V-f. Cell viability

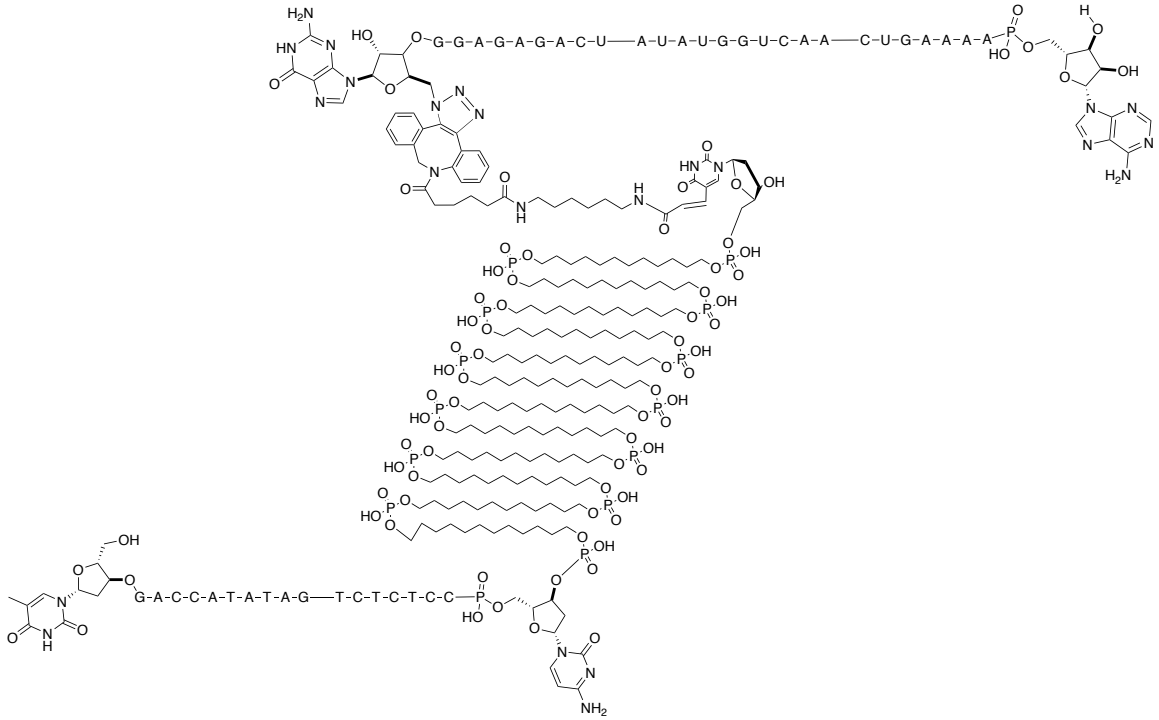
Cell viability of HepG2 cells after treatment with RNA-amphiphiles, was measured using the Cell-Titer Blue assay (Promega, USA). Briefly, cells were seeded in 96-well plates at a density of $1x10^5$. After 24 hours, RNA-amphiphiles were added (final concentration 80 nM) with Lipofectamine (as described in SI-5-b). Following incubation for 24 hours, plates were analyzed with a Bio Plater Reader using 560 nm Ex/590 nm emission.



Supporting Figure SF24| MTS assay for cell viability on HepG2 cells transfected with the siRNA-micelle HE$_9$rApoBs + rApoBas. Each sample was transfected in four separate wells, and the data was combined.

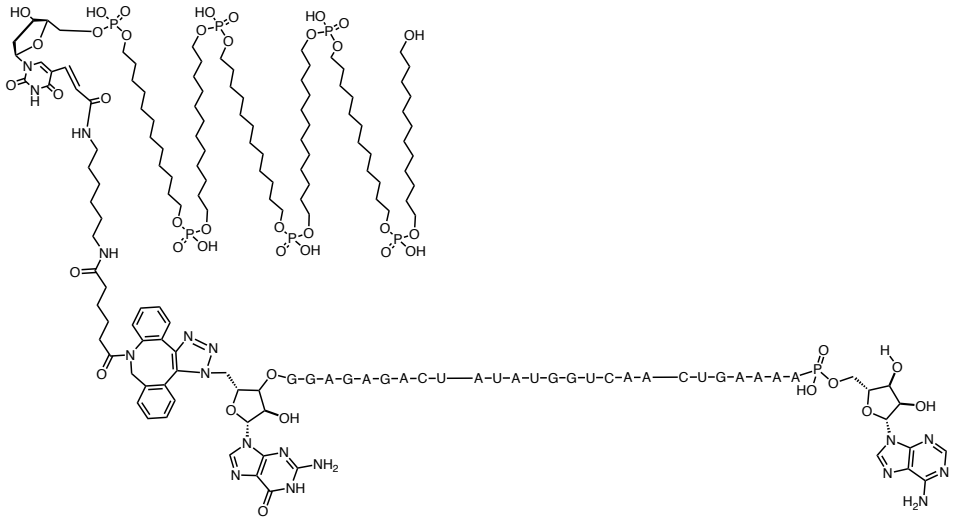
SI-VI. Chemical structures of conjugates

VI-a. DNA1-HE$_{12}$-RNA1



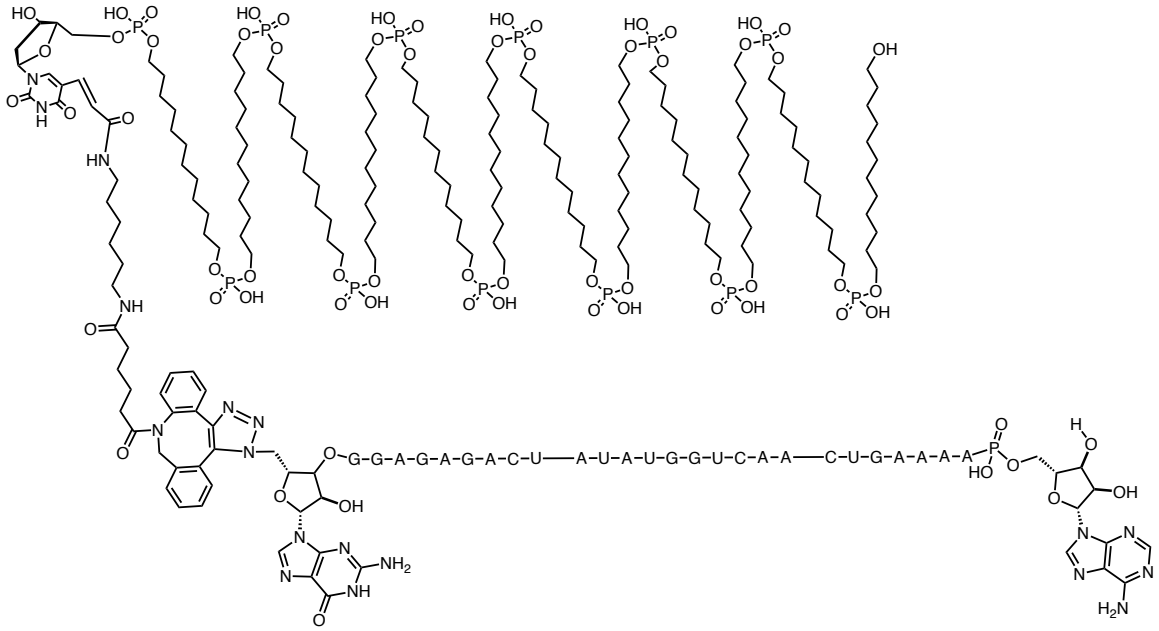
Supporting Figure SF25| Chemical structure of DNA1-HE$_{12}$-RNA1. A, U, T, G, and C represent the bases adenine, uracil, thymine, guanine, and cytosine respectively as parts of an RNA (top of image) or DNA (bottom of image) oligonucleotide.

VI-b. HE$_6$-RNA1



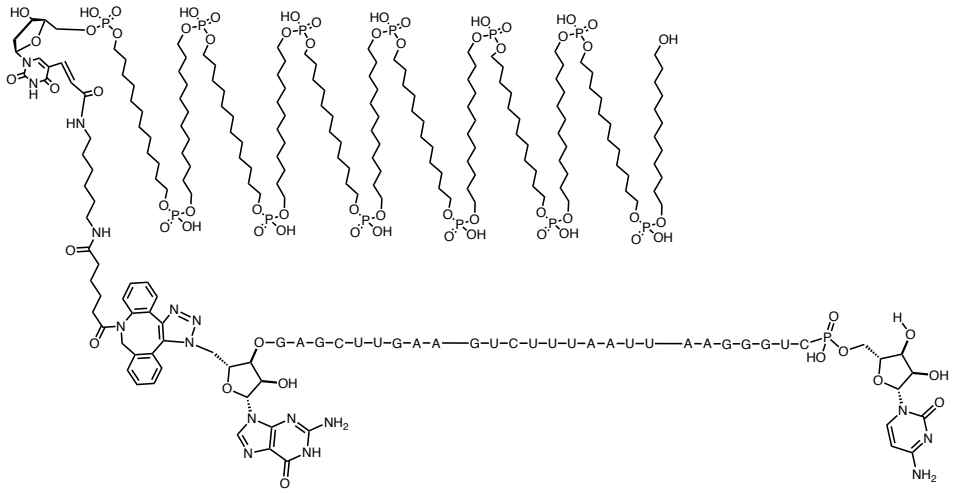
Supporting Figure SF26| Chemical structure of HE_6-RNA1. A, U, G, and C represent the bases adenine, uracil, guanine, and cytosine respectively as parts of an RNA oligonucleotide.

VI-c. HE_{12}-RNA1



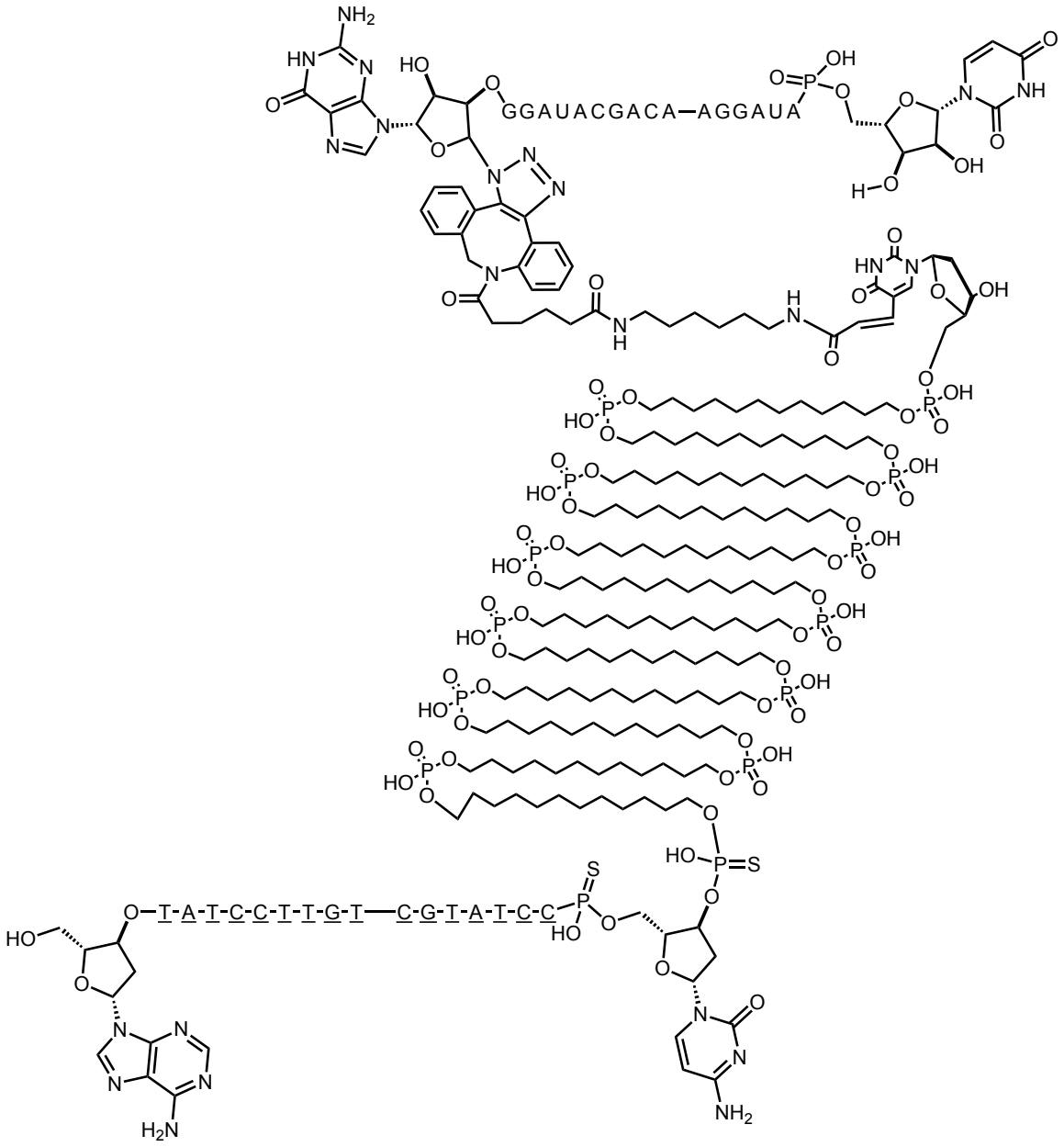
Supporting Figure SF27| Chemical structure of HE_{12}-RNA1. A, U, G, and C represent the bases adenine, uracil, guanine, and cytosine respectively as parts of an RNA oligonucleotide.

VI-d. HE_{12}-rLucP (9)



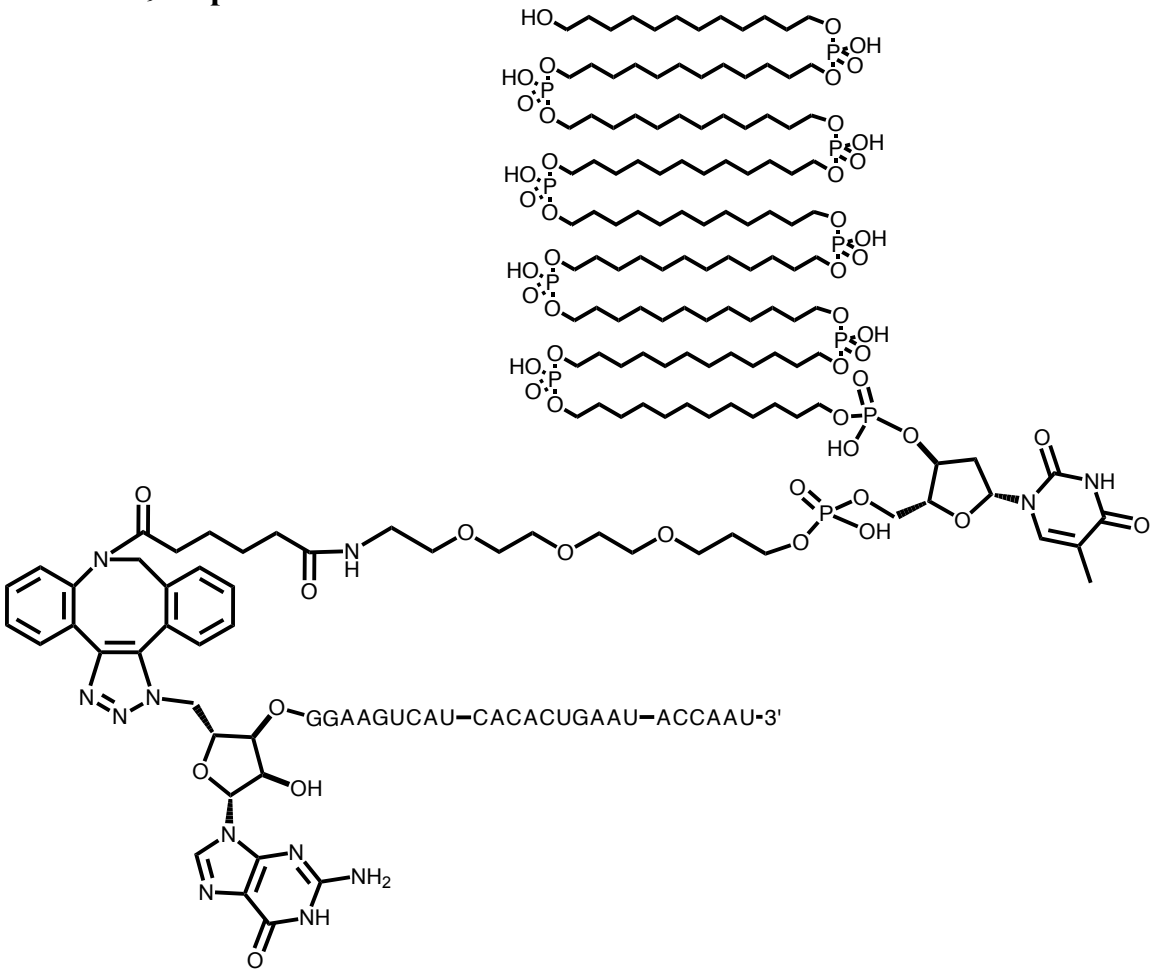
Supporting Figure SF28| Chemical structure of HE_{12}-rLucP. A, U, G, and C represent the bases adenine, uracil, guanine, and cytosine respectively as parts of an RNA oligonucleotide.

VI-e. LucASO-HE_{12}-rLucS



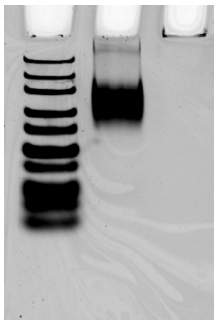
Supporting Figure SF29| Chemical structure of LucASO-HE_{12}-rLucS. A, U, T, G, and C represent the bases adenine, uracil, thymine, guanine, and cytosine respectively as parts of an RNA (top of image) or DNA (bottom of image) oligonucleotide. <u>Underline</u> denotes phosphorothioate linkage.

VI-f. HE_9-rApoBs

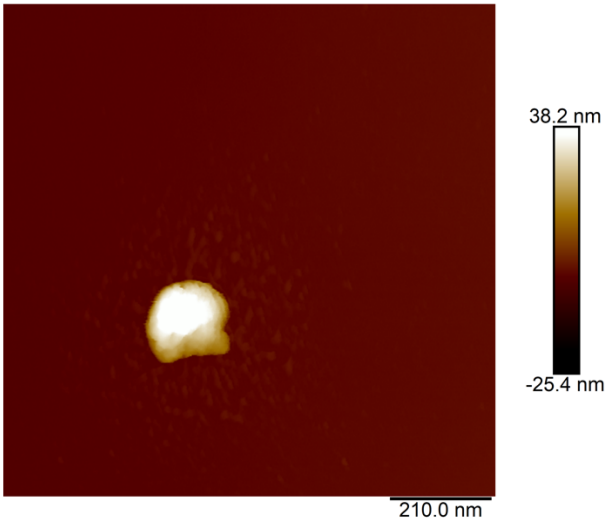


Supporting Figure SF30l Chemical structure of HE_9-ApoBs. A, U, G, and C represent the bases adenine, uracil, guanine, and cytosine respectively as parts of an RNA oligonucleotide.

SI-VII. Complexation with transfection agent



Supporting Figure SF31| 15% native PAGE in 1xTAMg. Lanes 1 and 6: ultra low range ladder. Lanes 2 and 3: LucASO-HE_{12}-rLucS without and with Lipofectamine.



Supporting Figure SF32| AFM in air on MICA. LucASO-HE_{12}-rLucS + Lipofectamine.

References

1. Z. Skrzypczynski and S. Wayland, *Bioconjugate Chem*., 2003, **14**, 642-652.
2. S. L. Beaucage, *Methods Mol Biol*, 1993, **20**, 33-61.