Electronic Supplementary Material (ESI) for Nanoscale.


# Antisense-polymer micelles require less poly(ethylenimine) for efficient gene knockdown

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Supporting Information

## S-I General

Magnesium acetate and triethylamine were purchased from Sigma and used without further purification. Acetic acid and boric acid were used as purchased from Fisher Scientific. Acrylamide/Bis-acrylamide (40% 19:1 solution), ammonium persulfate, TEMED, tris(hydroxymethyl)-aminomethane (Tris), urea, EDTA and agarose were obtained from Bioshop Canada Inc. and used as supplied. Universal 1000Å LCAA-CPG supports with loading densities of 25-40 μmol/g and standard reagents used for automated DNA synthesis were purchased through Bioautomation. Sephadex G-25 (super fine, DNA grade) was purchased from Glen Research. GelRed™ nucleic acid stain was purchased from Biotium Inc. 1xTBE buffer is composed of 90mM Tris and Boric acid and 2 mM EDTA with pH 8.3. 1xTAMg buffer is composed of 45 mM Tris and 12.5 mM $Mg(OAc)_2 \cdot 6H_2O$ with pH adjusted to 8.0 using glacial acetic acid.

## S-II Instrumentation

DNA synthesis was performed on a Mermade MM6 synthesizer from Bioautomation. HPLC purification was carried out on an Agilent Infinity 1260. DNA quantification measurements were performed by UV absorbance with a NanoDrop Lite spectrophotometer from Thermo Scientific. Polyacrylamide gel electrophoresis experiments were carried out on a 20 X 20 cm vertical Hoefer 600 electrophoresis unit and for agarose gel electrophoresis experiments Thermo Scientific™ Owl™ EasyCast™ B1 Mini Gel Electrophoresis Systems were used. Gel images were captured using a ChemiDoc™ MP System from Bio-Rad Laboratories. Thermal annealing of all DNA structures was conducted using an Eppendorf Mastercycler® 96 well thermocycler. Oligonucleotide mass determination by Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS) was carried out using a Dionex Ultimate 3000 coupled to a Bruker MaXis Impact™ QTOF. DLS experiments were carried out using a DynaPro™ Instrument from Wyatt Technology. AFM was carried out using a MultiMode3™ SPM connected to a Nanoscope™ IIIa. controller from Veeco. TEM Micrographs were obtained using a Phillips Tecnai 12 120 kV microscope.

## S-III Synthesis, purification and characterization of polymer-ASO conjugates

### IIIa. Solid-phase synthesis

The DNA synthesis was based on previous work by Edwardson et al., using universal 1000 Å CPG solid-supports (BioAutomation, cat.# MM1-3500-1). DMT-dodecane-diol (cat.# CLP-1114) and DMT-hexaethyloxy glycol (cat.# CLP-9765) phosphoramidite were purchased from ChemGenes. All non-standard amidites were dissolved to 0.07 M in anhydrous acetonitrile, with extended coupling times of 5 minutes. Coupling efficiency was monitored after removal of the dimethoxytrityl (DMT) 5′-OH protecting groups, using 3% DCA in dichloromethane. Completed syntheses were deprotected in 1 mL of 1:1 v/v mixture of 40% aqueous Methylamine and 28% aqueous ammonium hydroxide solution for 3 hours at 65$^{o}$C. The crude deprotected solution was separated from the solid support and concentrated under reduced pressure at 60$^{o}$C. This crude solid was re-suspended in 0.2 mL sterile water in preparation for purification.

**Table ST1 – DNA Sequences.**

Non-standard amidite codes: (***3***=Cy3), (***D***=DMT-dodecane-diol), (***H***=DMT-hexaethyloxy glycol).

| Name | Sequence (5'-xx-3') |
|---|---|
| **Luc-ASO** | atatccttgtcgtatccc |
| **HE$_{12}$-Luc-ASO** | ***DDDDDDDDDDDD***atatccttgtcgtatccc |
| **(HE-HEG)$_6$-Luc-ASO** | ***DHDHDHDHDHDH***atatccttgtcgtatccc |
| **Cy3-1** | ***DDDDDDDDDDDD3***TTTTTCAGTTGACCATATA |

## IIIb. PAGE purification and analysis

The crude syntheses were purified on a 1.5mm 20 % polyacrylamide/8 M urea PAGE (half of a crude 1 μmol synthesis was loaded per gel) at constant current of 30 mA for 1.5 hours (30 min at 250 V followed by 1 hr at 500 V), in 1xTBE buffer. The product bands were excised under UV shadowing on silica plates, and the gel pieces were crushed and incubated in 12 mL of sterile water at 60 °C for 12-16 hours. Samples were then dried to ca. 1.5 mL, desalted using size exclusion chromatography (Sephadex G-25), and quantified ($OD_{260}$) using UV-Vis spectroscopy.

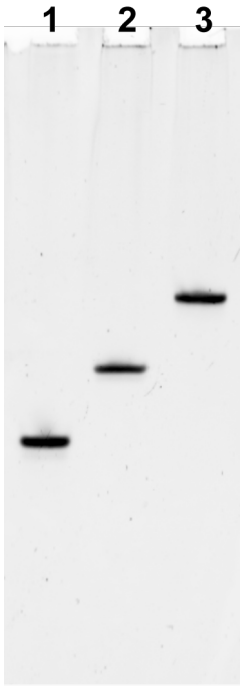


**Figure SF1 - Analytical PAGE on purified oligonucleotides.** Gel: 20% polyacrylamide/8 M urea analytical PAGE. Run at constant current of 30 mA for 1.5 hours (30 min at 250 V followed by 1 hr at 500 V). Loading is 10 pmol of DNA per lane and bands were visualized using GelRed™. Lane 1 – **Luc-ASO**, Lane 2 - **$HE_{12}$-Luc-ASO**, Lane 3 – **$(HEG\text{-}HE)_6$-Luc-ASO**.

## IIIc. HPLC Analysis

Solvent system; A:TEAA buffer, B:Acetonitrile. TEAA buffer (50mM Triethylammonium acetate, pH 8.0) was filtered through 0.22mm cellulose membrane before use. The elution gradient was 3-50% solvent B over 30 minutes. Column: Hamilton PRP-C18 5 µm 100 Å 2.1 x 150 mm. For each separation approximately 0.5 $OD_{260}$ of crude DNA in Millipore water was injected and the absorbance monitored at 260nm. The retention times for the **$HE_{12}$-Luc-ASO** and **$(HE\text{-}HEG)_6$-Luc-ASO** products were 29 and 22 minutes respectively, in contrast to control **Luc-ASO** which eluted at 14 minutes.

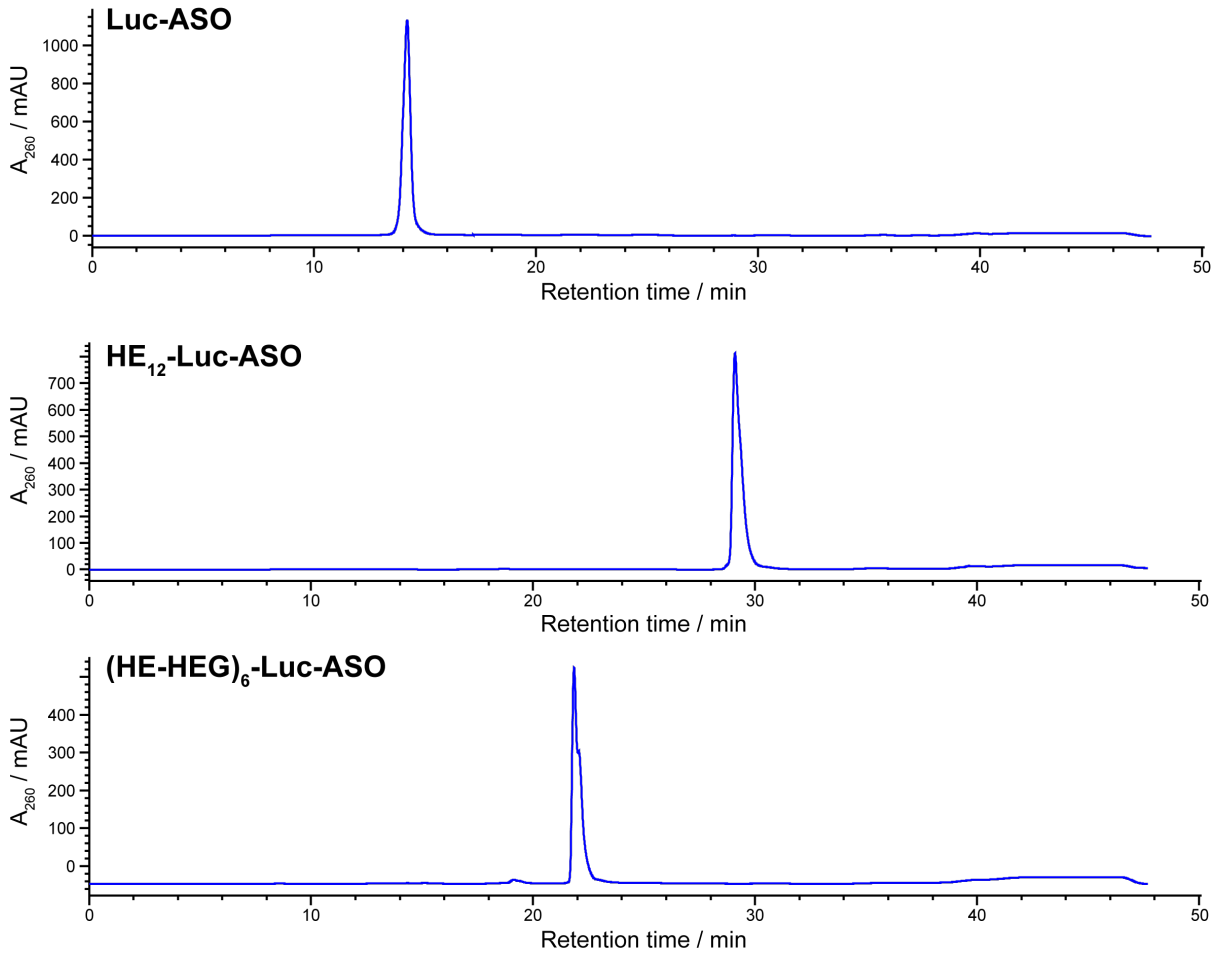


**Figure SF2 – HPLC traces of purified products.**

## IIId. LC-ESI-MS characterization

The oligonucleotides were analyzed by LC-ESI-MS in negative ESI mode. Samples were run through an Acclaim RSLC 120 C18 column (2.2 mM 120 Å 2.1 x 50 mm) using a gradient of 98% mobile phase A (100 mM 1,1,1,3,3,3-hexafluoro-2-propanol and 5 mM triethylamine in water) and 2 % mobile phase B (Methanol) to 40 % mobile phase A and 60% mobile phase B in 8 minutes. The data was processed and de-convoluted using the Bruker Data Analysis software version 4.1.

**Table ST2 - LC-ESI-MS data for oligonucleotides.**

| Molecule | Calculated (exact mass/Da) | Found (exact mass/Da) |
|---|---|---|
| **Luc-ASO** | 5669.26 | 5669.40 |
| **$HE_{12}$-Luc-ASO** | 8844.25 | 8844.27 |
| **$(HE\text{-}HEG)_6$-Luc-ASO** | 9335.88 | 9335.90 |

## S-IV Structural investigation of PEI-ASO complexes

### IVa. Dynamic light scattering

Samples, water and buffer were filtered using a 0.22 µm nylon syringe filter prior to use in DLS sample preparation. Scattering measurements were carried out at 25 °C, and a cumulants fit model was used to determine the size and distribution of spherical particles. For the PEI-ASO complexes the PEI and oligo solutions were mixed and incubated at room temperature for 10 minutes prior to measurement.

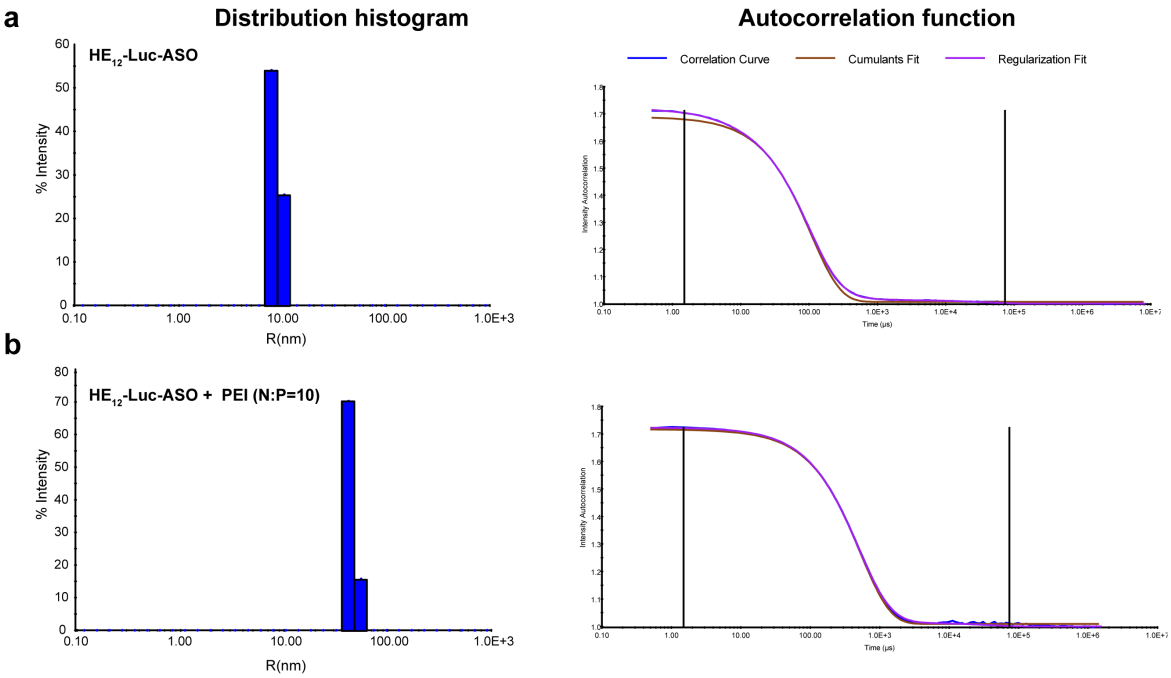

**Figure SF3 – Representative DLS data.** Shown here are DLS regularization distribution histograms and the related correlation functions. a) **$HE_{12}$-Luc-ASO**, b) **$HE_{12}$-Luc-ASO (N:P = 10)**. The large increase in hydrodynamic radii observed upon addition of PEI (25kDa) is consistent with the TEM and AFM data.

**Table ST3 - Summary of DLS measurements.** At least quadruplicate measurements were obtained for each sample, mean values for hydrodynamic radii and polydispersity of each are shown below. Error margins are derived from the standard deviation of all measurements.

| Sample | N:P ratio | Mean hydrodynamic radius (nm) | Mean polydispersity (%) |
|---|---|---|---|
| **$HE_{12}$-Luc-ASO** | n/a | 8.6 ± 0.1 | 13.8 ± 0.1 |
| **$HE_{12}$-Luc-ASO** | 10 | 41.3 ± 2.6 | 11.9 ± 1.4 |

## IVb. Atomic force microscopy

All images were obtained using tapping mode in air with Tap300Al-G cantilevers (Nominal values: Tip radius - <10nm, Resonant frequency – 300kHz, Force constant – 40 N/m) from Asylum Research. Samples were diluted to 1-4μM in 1xTAMg buffer and 5 μL of this solution was deposited on a freshly cleaved mica surface (ca. 7 x 7 mm) and allowed to adsorb for 1-2 seconds. Next, 50μL of 0.22 μm filtered Millipore water was dropped on the surface and instantly removed with filter paper. The surface was then washed with a further 4x50 μL of water and the excess removed with a strong flow of nitrogen. Samples were dried under vacuum for 10-20 minutes prior to imaging.

## IVc. Transmission electron microscopy

TEM samples were simply prepared by depositing 3 μL of sample solution (1 μM, 1xTAMg) onto the carbon-coated grid. After 90 seconds, the droplet was removed using filter paper and the grid was held under vacuum for 4 hours before microscopy.

## IVd. Additional microscopy images

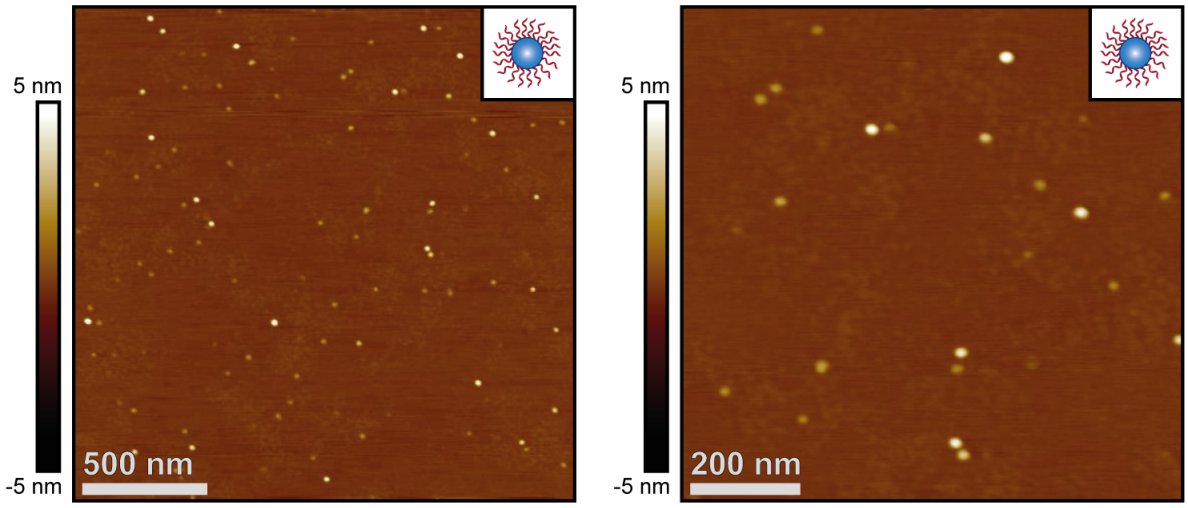


**Figure SF4 – Additional AFM images of $HE_{12}$-Luc-ASO.**

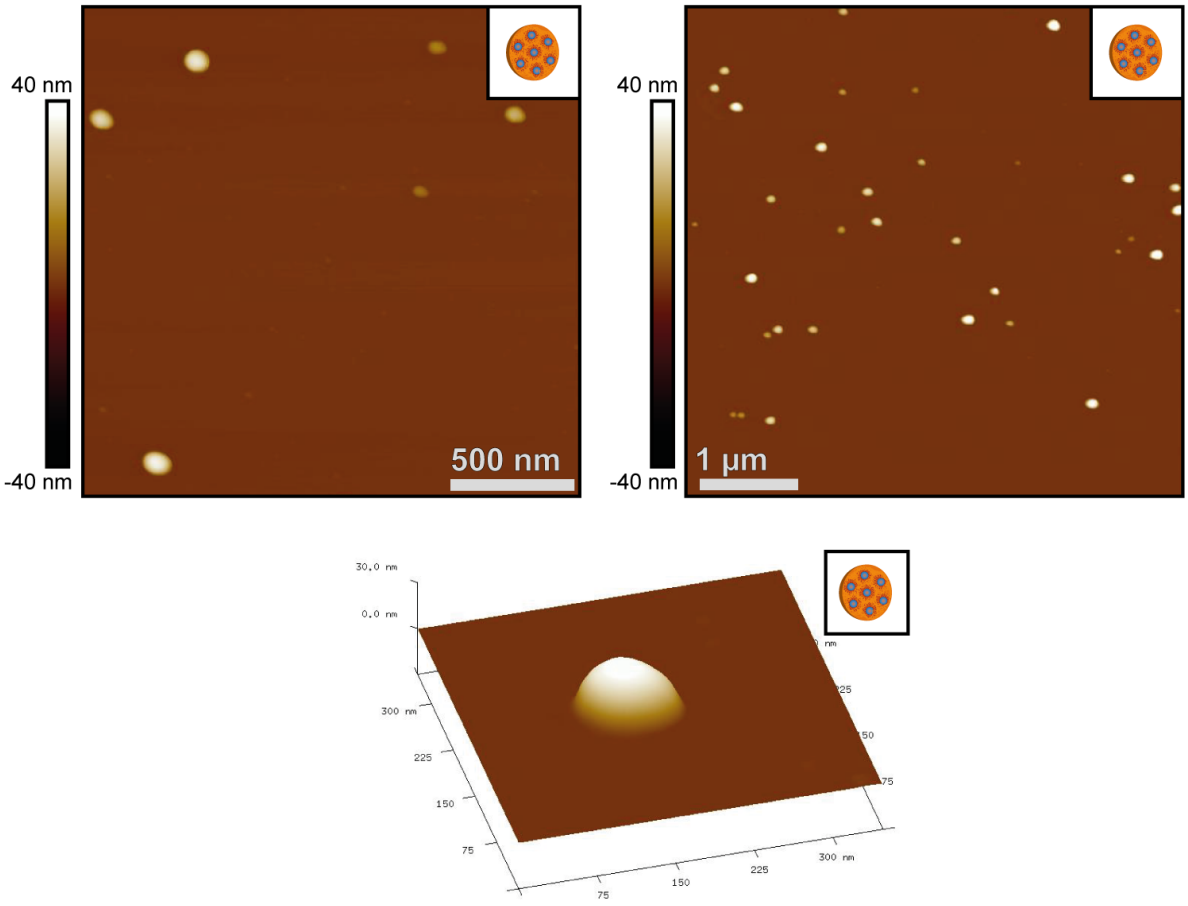


**Figure SF5 – Additional AFM images of $HE_{12}$-Luc-ASO: PEI complexes (25kDa, N:P=10).**

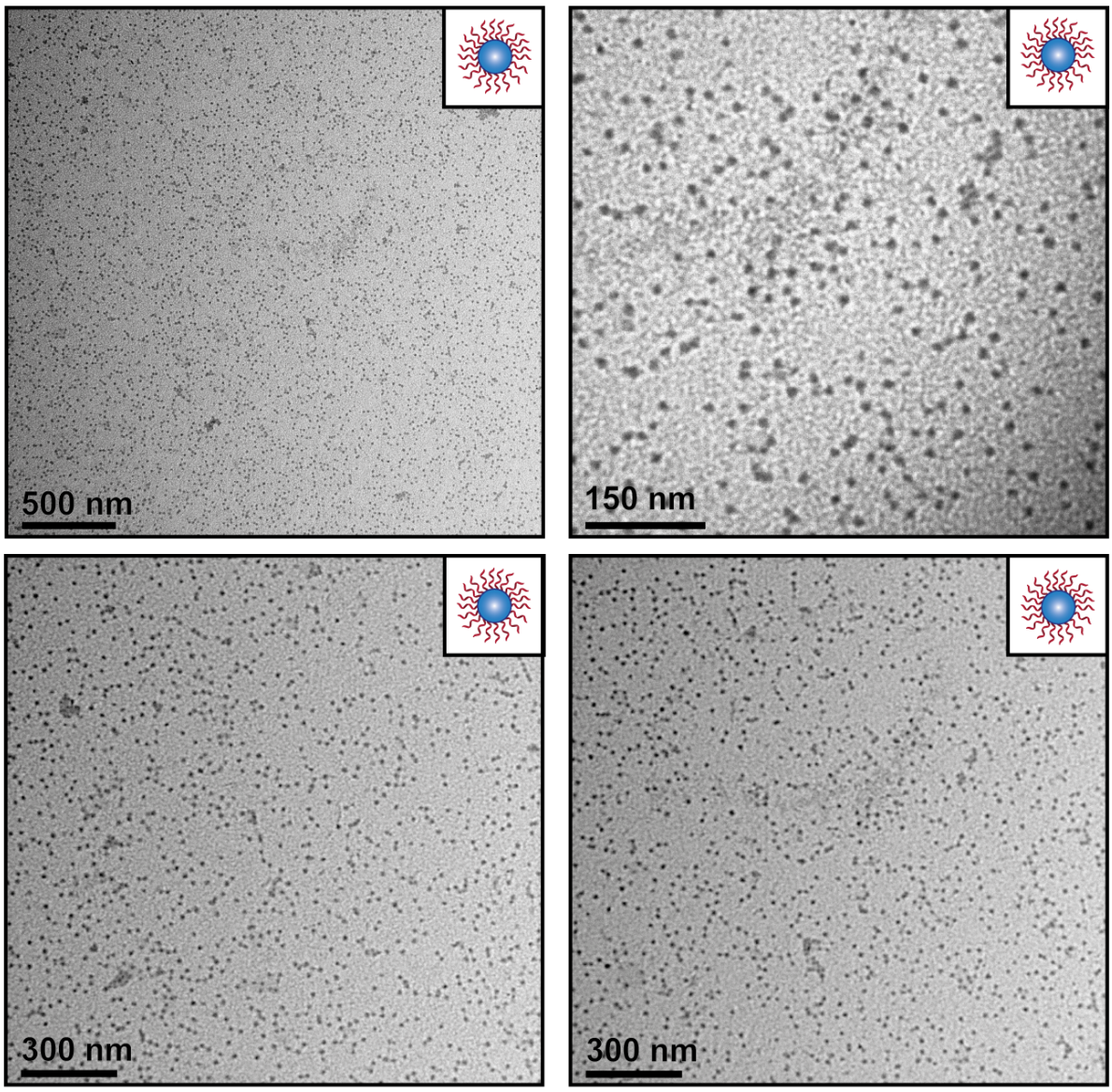


**Figure SF6 – Additional TEM images of $HE_{12}$-Luc-ASO.**

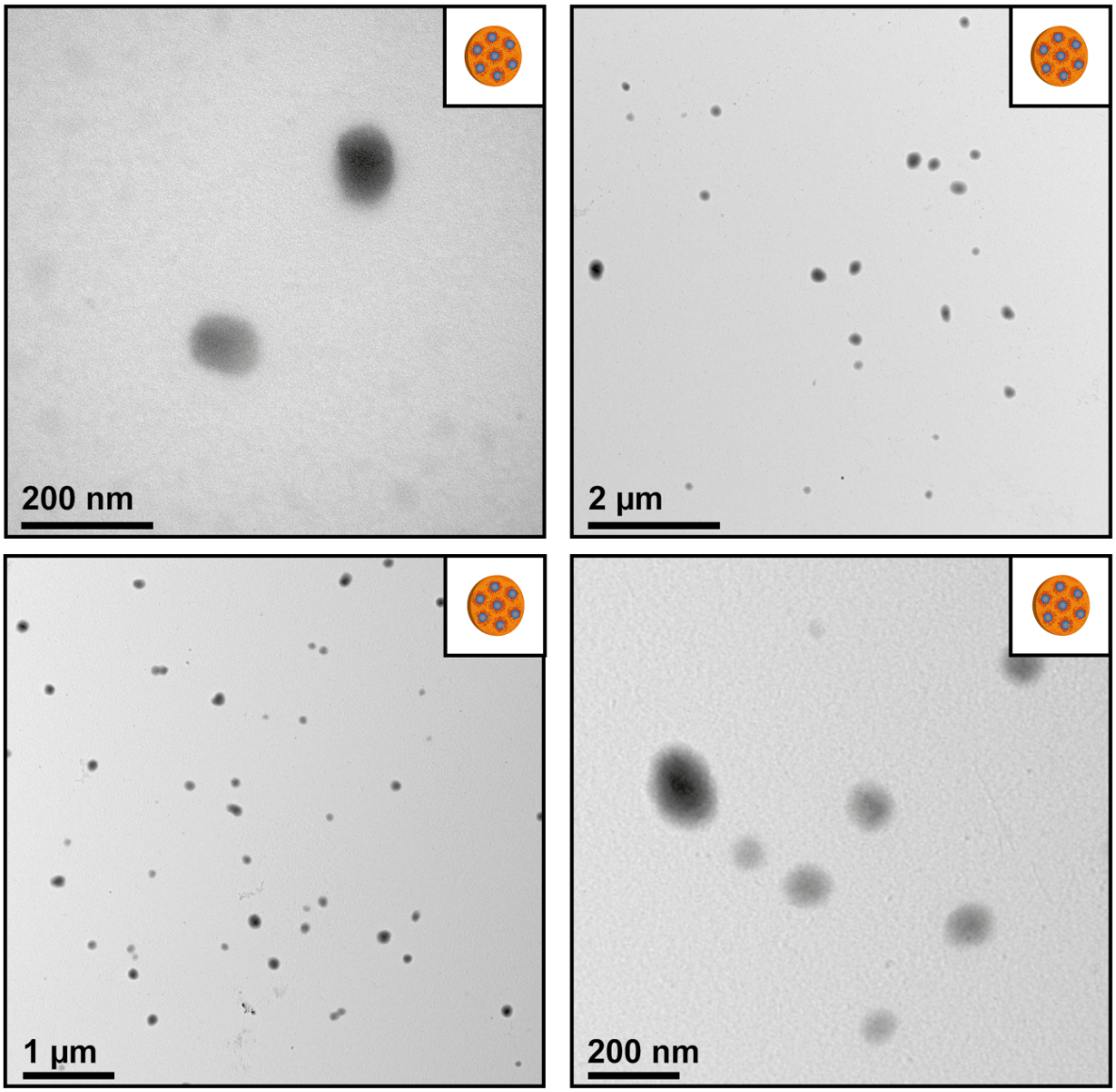


**Figure SF7 – Additional TEM images of $HE_{12}$-Luc-ASO: PEI complexes (25kDa, N:P=10).**

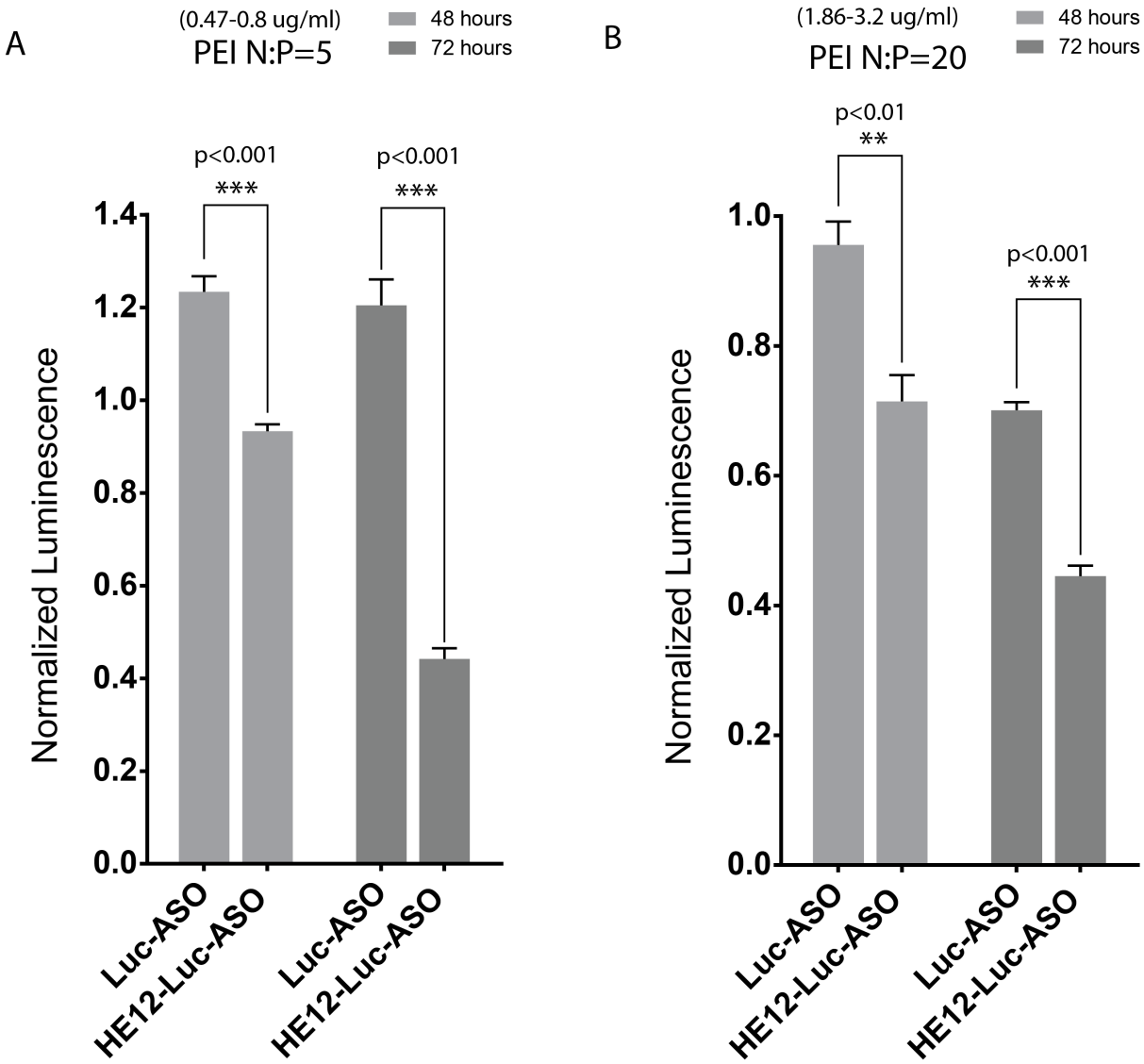


**Figure SF8. Firefly Luciferase Knockdown Activity of ASO-Polymer/PEI Complexes at N:P=5 and 20.** Firefly luciferase activity was measured after treatment with ASO alone and ASO-polymer complexes. Increasing concentrations of PEI were used for transfection over 24 hours. Fluorescence was normalized to PEI only-treated samples.

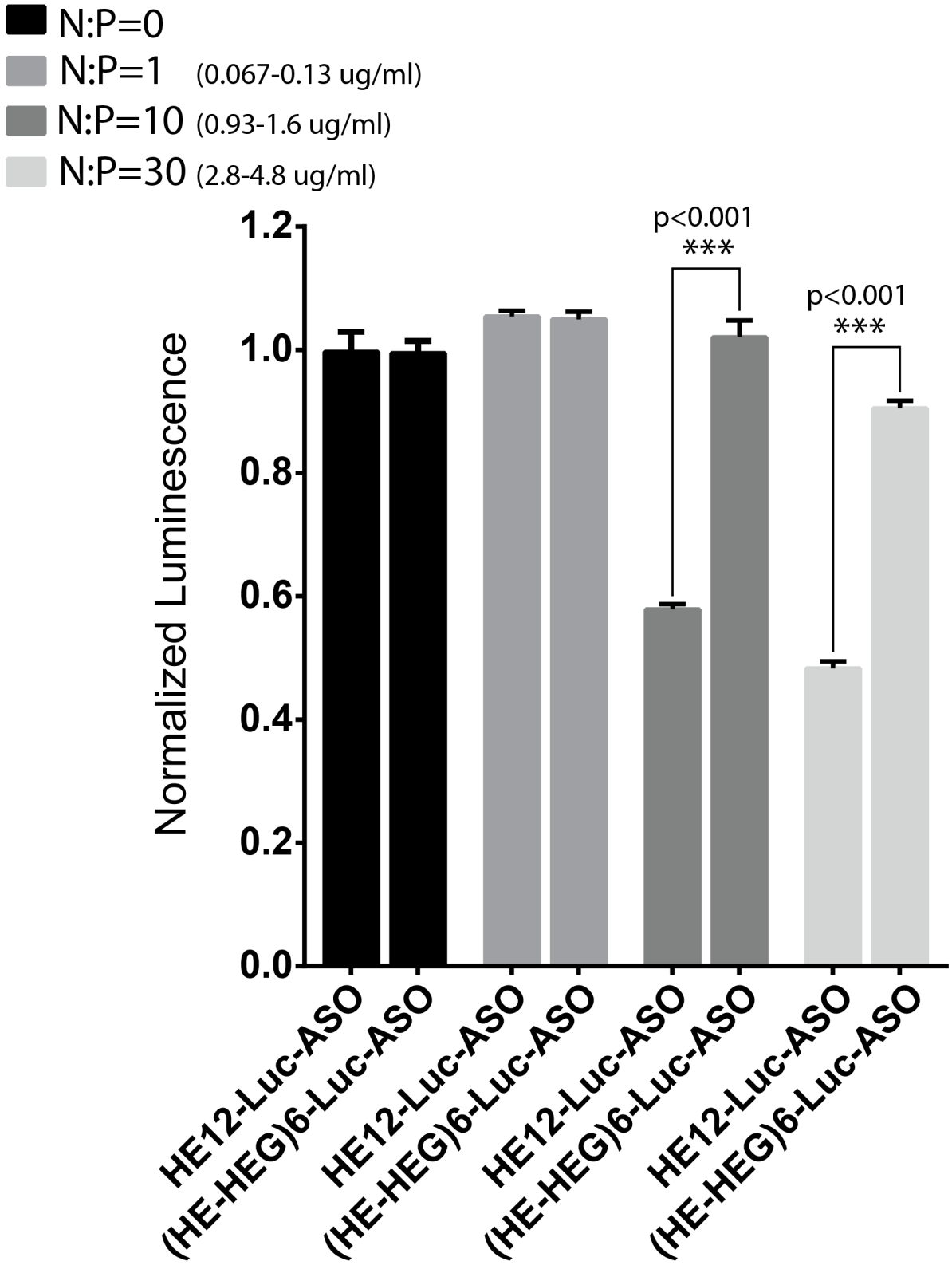


**Figure SF9. Firefly Luciferase Knockdown Activity of Micelle-forming and Non-micelle-Forming DPs.** Firefly luciferase activity was measured after treatment with ASO-

polymer complexes. Increasing concentrations of PEI were used for transfection over 24 hours. Fluorescence was normalized to PEI only-treated samples.

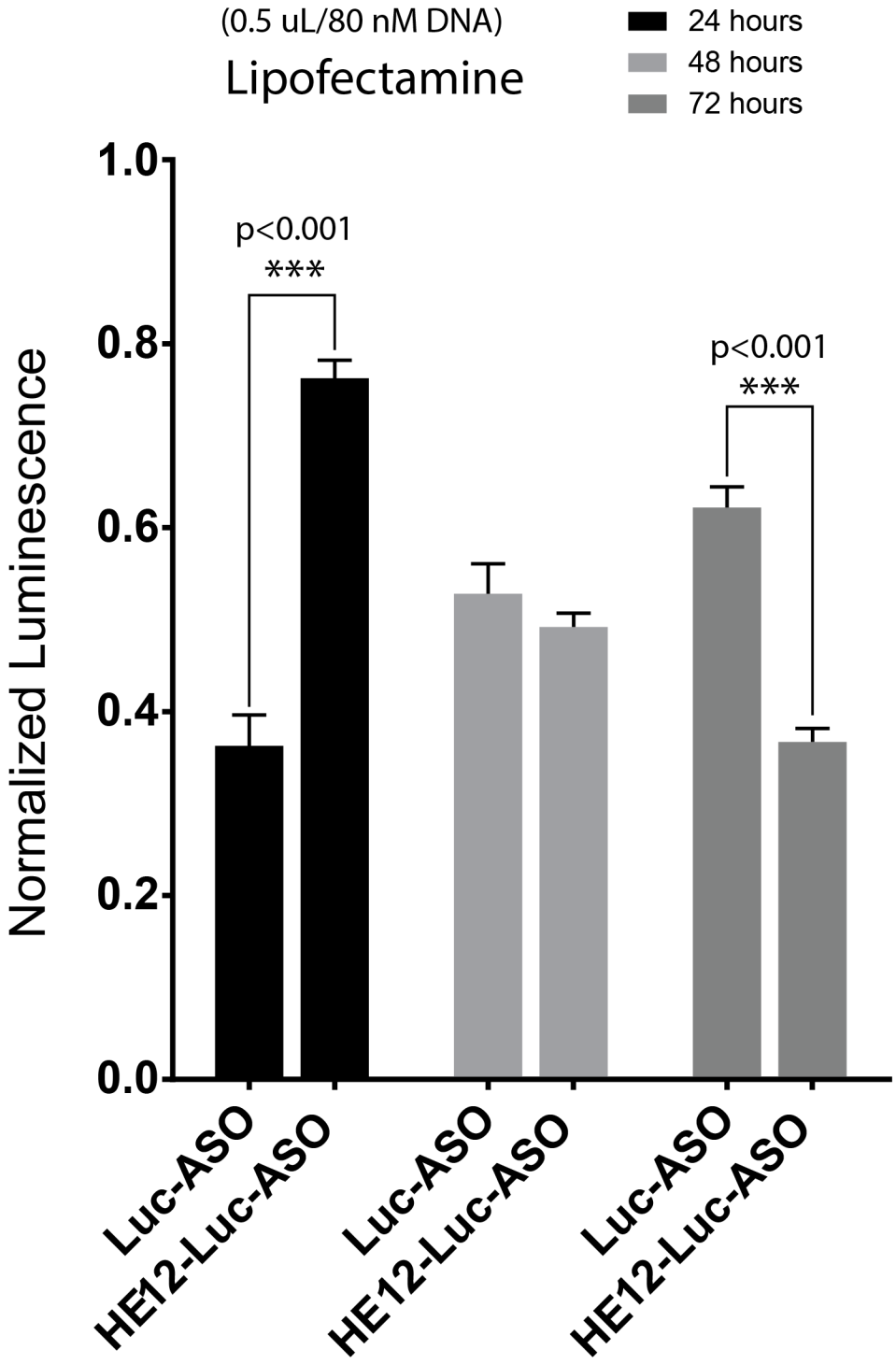

**Figure SF10. Transfection and knockdown of DNA-polymers with commercial reagents.** Knockdown was validated Lipofectamine 3000 (Invitrogen). Transfection was performed was performed as described earlier, but samples were left in FBS-free media for 4 hours (as per manufacturer's instructions) and then the media was supplemented with FBS for a final concentration of 10%. Samples were then incubated for 24 hours and luciferase activity was measured as previously described. Samples were normalized to transfection control wells (only containing reagent without DNA).

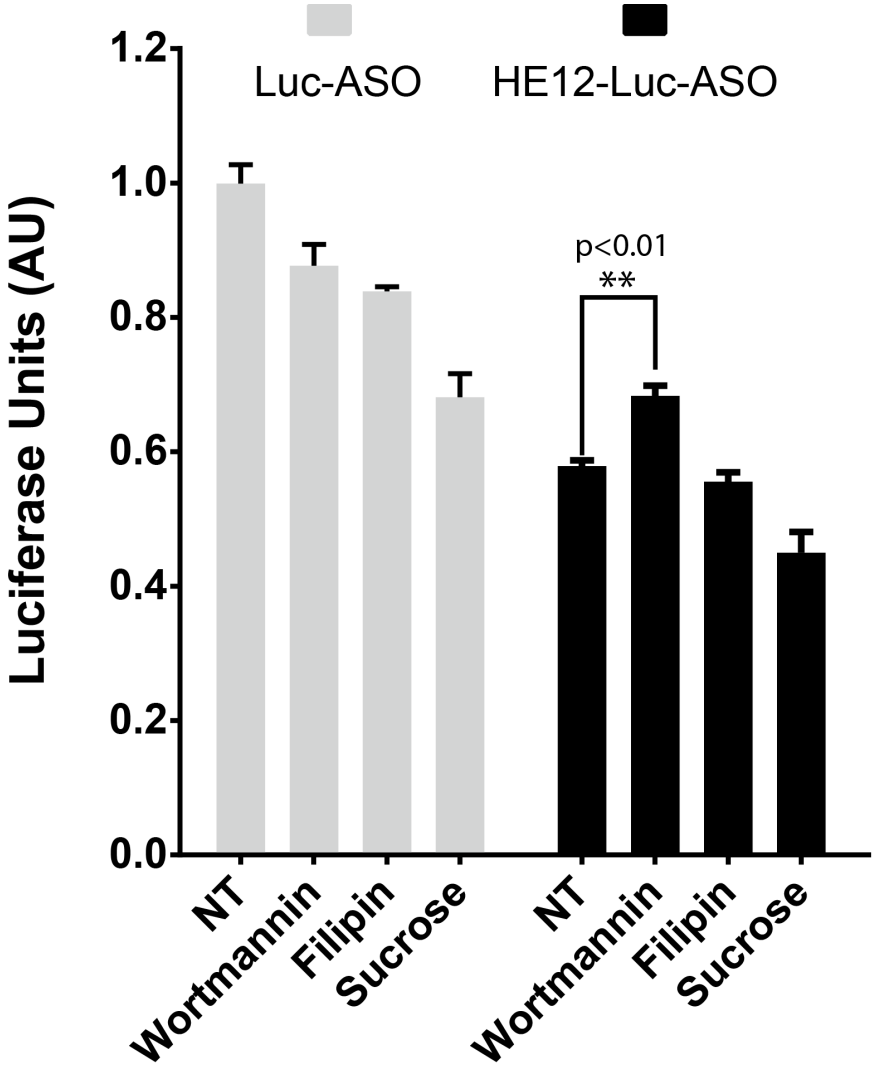


**Figure SF11. Figure 6 - The effect of uptake inhibitors on the knockdown activity of ASO-Polymer/PEI Complexes.** Knockdown activity was measured after treatment with

uptake pathway inhibitors, wortmannin, filipin, and sucrose. Treatment with inhibitors was performed for 1 hour at 37°C. Subsequently, fresh media was added that contained PEI/ASO complexes and luciferase activity was measured as described earlier. Samples were normalized to drug-treated wells without any PEI.

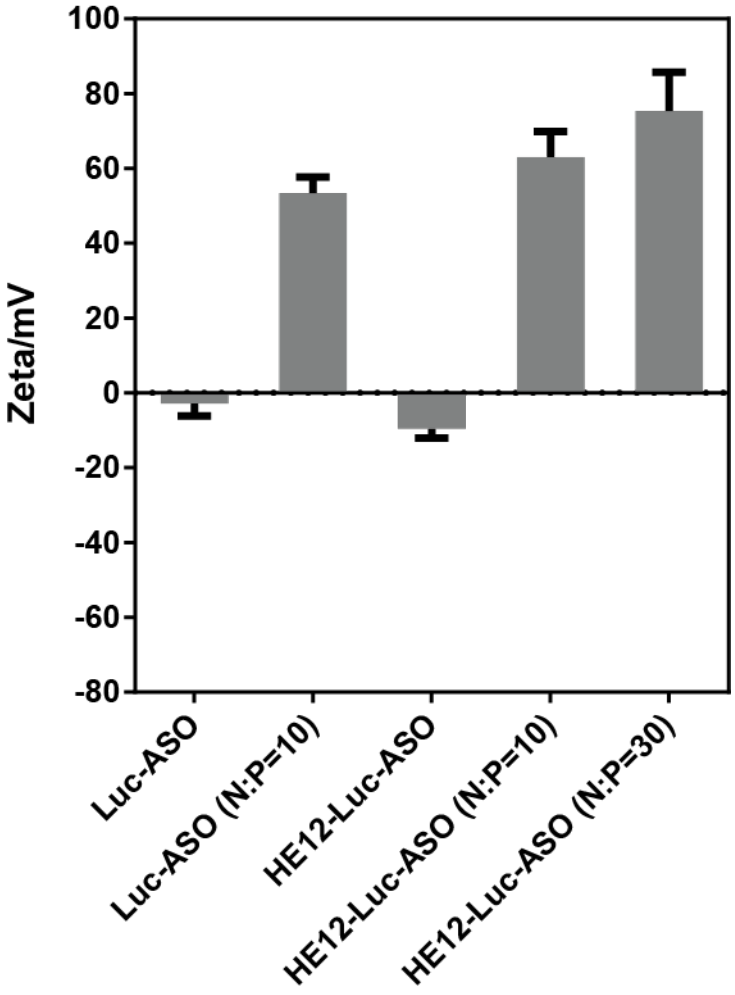


**Figure SF12. Effects of N:P ratio on ASO and DP/PEI Complexes.**

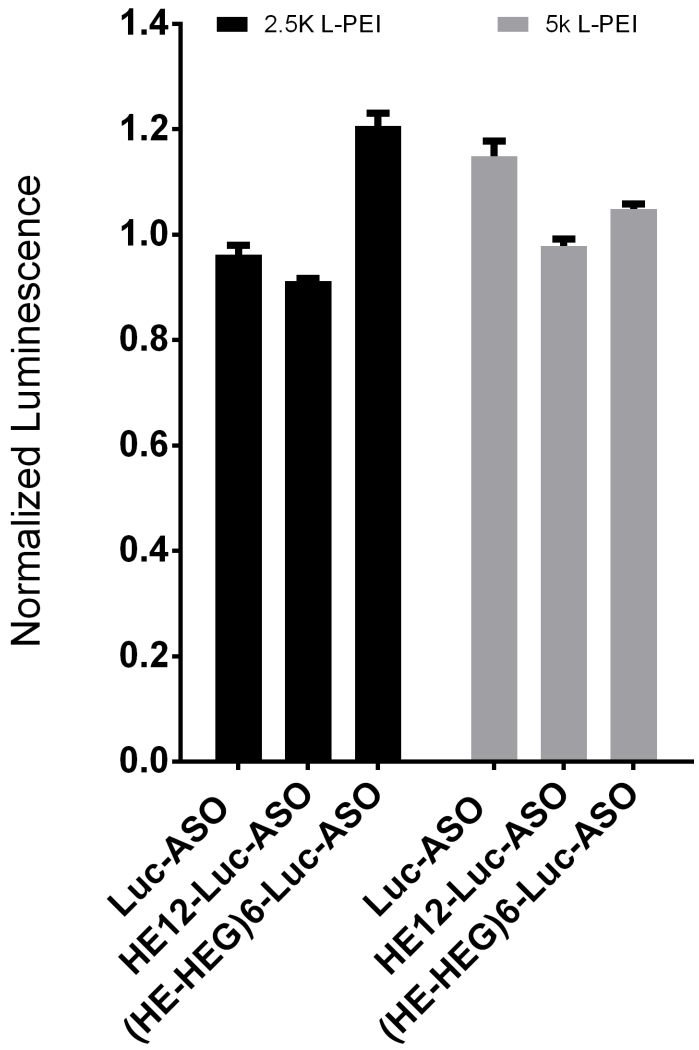


**Figure SF13. Comparison of knockdown of DP conjugates in the presence of LMW L-PEI. N:P=10, DNA concentration 80 nM, and time of incubation 24 hours.**