Supporting Information for

Relationships Between Poly(ethylene glycol) Modifications on RNA-Spherical Nucleic Acid Conjugates and Cellular Uptake and Circulation Time

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General Information

Table S1. RNA Sequences Used

Name	Sequence (5'-3')
Sense	UGA CAG UCC AAC UAC AAC AGC-$(Sp)_2$-SH
Anti-Sense (AS)	GCU GUU GUA GUU GGA CUG UCA

*Sp denotes a commercially available hexa(ethylene glycol) phosphate spacer.

Table S2. Properties of PEG 2K Used

Backfill Molecule	Structure	n=	MW (Da)	Extended Length (nm)
PEG 2K	CH_3O-$(CH_2CH_2O)_n$-C_2H_4-SH	~45	2,000	16

Materials and Methods

RNA Synthesis and Duplex Formation

RNA oligonucleotides were synthesized on an OligoPilot system (GE Healthcare Life Sciences) using TOM-RNA reagents (ChemGenes) following manufacturer-recommended cleavage and deprotection protocols. Oligonucleotides were purified via reverse-phase high performance liquid chromatography (RP-HPLC) on a Varian Microsorb C18 column (10 μm, 300 × 10 mm^2) with 0.1 M triethylammonium acetate (TEAA) at pH 7 with a 1%/min gradient of 100% CH_3CN at a flow rate of 3 ml/min, while monitoring the UV signal of the nucleic acids at 254 nm. The TEAA buffer was treated with 0.1% diethylpyrocarbonate (DEPC, Sigma) and autoclaved. Following purification, the oligonucleotides were lyophilized and stored at -80°C. RNA duplexes were formed prior to gold nanoparticle functionalization by heating for 5 minutes at 95°C and annealing at 45°C for 1 hour in duplex buffer (IDT). Before functionalization, duplexes were cooled to room temperature.

Determination of RNA and PEG Lengths

In order to approximate the length of the PEG 2K used in this work, previously published methods for approximating the fully extended length of PEG were used.[1] The extended length of the RNA duplex used in this work was approximated based upon published values of the rise per base pair of RNA duplexes,[2] as well as previously reported experimentally determined values for the increase in SNA size that correlates to the addition of a hexa(ethylene glycol) spacer.[3]

Gold Nanoparticle Synthesis and Functionalization

Citrate-stabilized gold nanoparticles (AuNPs, 13 nm ± 1 nm) were synthesized via the Frens method. AuNPs were treated with 0.1% DEPC and autoclaved to inactivate any RNases present.

To synthesize RNA-SNAs using the backfill method, duplexed siRNA (to a final concentration of 2 uM) was added to 10 nM 13 nm AuNPs with 0.2% TWEEN-20 and 150 mM NaCl. The mixture was incubated at room temperature for 4 hours and NaCl was added to a final concentration of 350 mM. Following a 16 hour incubation, PEG 2K (CH_3O-$(CH_2CH_2O)_n$-C_2H_4-SH, n~45, NanoCS) was added to the mixture at a concentration of 20 μM. The resulting SNAs were then purified by centrifugation 3 times in 1X PBS after 12-72 hours incubation with PEG.

RNA-SNAs synthesized by co-adsorbing RNA and PEG to AuNPs were prepared as follows. TWEEN-20 was added to AuNPs to 0.2%, and NaCl was added to 150 mM. siRNA duplex was added to 10 nM AuNPs at 2 uM and PEG 2K was added to AuNPs from 0.02-20 μM. The solution was sonicated for 30 seconds and incubated for 4 hours at room temperature. NaCl was added to a final concentration of 350 mM, the solution was sonicated again, and the AuNPs we incubated for an additional 16 hours. SNAs were then purified by centrifugation three times in 1X PBS.

Method for the Quantification of Oligonucleotides Loaded on SNAs

To determine the number of anti-sense and sense oligonucleotides per SNA, the concentration of AuNPs and the concentration of anti-sense and sense oligonucleotides were measured. The concentration of the SNA was determined through UV-visible spectroscopy and Beer's Law ($A=\varepsilon cb$), using the extinction coefficient of 13 nm AuNPs as $2.7X10^8$ L/mol•cm at 524 nm.

Oligonucleotides were quantified from SNAs using an OliGreen assay (Quant-iT OliGreen Assay Kit, Life Technologies). Anti-sense siRNA oligonucleotides were released from SNAs by incubating 6 nM SNA in 8 M urea for 20 minutes at 45°C to denature the siRNA duplex. TWEEN-20 was added to a final concentration of 0.05% and SNAs were centrifuged for 1 hour at 13,000 rpm. The supernatant, containing the anti-sense oligonucleotides was collected for further analysis. The pellet, containing AuNPs with sense RNA, which remains bound to the AuNP, was collected and washed once with PBS containing 0.1% TWEEN-20 to remove excess anti-sense oligonucleotides that were not collected in the supernatant. AuNPs from the pellet, with sense RNA bound through gold-thiolate bonds, were treated with 0.05 M KCN and 0.05 M DTT to dissolve the gold core, and sense RNA was collected for quantification. The isolated anti-sense and sense RNA strands were quantified using the OliGreen kit (ThermoFisher), which consists of an intercalating dye that fluoresces in the presence of single-stranded RNA. Anti-sense and sense RNA samples, as well as standard curves prepared from known concentrations of either anti-sense or sense oligonucleotide were analyzed following the OliGreen kit protocol, and the amount of anti-sense or sense RNA obtained from SNAs was used to calculate the number of each RNA strands per AuNP.

Quantification of PEG from SNA Surface

To determine the number of PEG molecules per SNA, the concentration of AuNPs and the concentration of anti-sense and sense oligonucleotides were measured. The concentration of the SNA was determined through UV-visible spectroscopy and Beer's Law ($A=\varepsilon cb$), using the extinction coefficient of 13 nm AuNPs as $2.7X10^8$ L/mol•cm at 524 nm.

PEG 2K was quantified using a thiol quantification kit (MeasureIT, ThermoFisher) that generates a fluorescent signal in the presence of thiol. To isolate PEG 2K from SNAs, known amounts of SNA were treated with 0.05 M KCN and 0.05 M DTT to dissolve the AuNP core. To remove DTT and KCN, samples were purified by size exclusion using NAP columns (GE Healthcare Life Sciences), leaving RNA and PEG 2K in solution. To remove RNA, ion exchange was performed by incubating the mixture in ion exchange resin (Dowex 1X2 chloride form, 50-100 mesh, Sigma –Aldrich), generating samples of isolated PEG 2K from SNAs. The number of PEG molecules/SNA were then determined by calculating the number of PEG molecules obtained from the SNA sample.

DLS and Zeta-Potential Measurements

Dynamic light scattering (DLS) and zeta potential measurements were taken using a Malvern Zetasizer NanoZS. Samples were prepared in PBS at a concentration of 0.5 nM SNA and measurements were taken at 25 °C. For diameter, the average and standard deviation of 5 measurements are reported, and for zeta-potential, the average and standard deviation of 3 measurements are reported.

Protein Adsorption Quantification

The BCA assay was used to quantify protein bound to SNAs following incubation in 10% fetal bovine serum. Protein samples were isolated from 1 pmol SNA following 1-4 hours incubation in serum by centrifugation 3X to remove the non-specifically bound proteins. Next, to release the bound proteins from the SNA surface, SNAs were treated with 0.1% SDS and heated to 95 °C. The SNAs were then centrifuged and the supernatant, containing only the specifically bound proteins, was analyzed using the BCA Protein Assay Kit (Pierce), and using bovine serum albumin to generate a standard curve from 0-250 μg protein, to quantify the amount of protein bound to the SNAs.

Modified ELISA Assay to Determine Relative Affinity to Scavenger Receptor A

To analyze the binding affinity of RNA-SNAs for scavenger receptor A, a modified ELISA assay was used, following a previously published procedure.[4] Specifically, 100 μL of 10 μg/ml scavenger receptor A capture antibody in 50 mM sodium carbonate, pH=9.5 was added per well of 96-well plate High Bind ELISA plates (Corning) and incubated overnight at 4°C while shaking. Each well was rinsed three times with 100 μL of PBS containing 0.05% TWEEN-20 (PBS-T). The plates were then blocked with 0.01 mg/mL BSA in PBS for two hours at room temperature while shaking. The plates were rinsed again three times with 100 μL PBS-T. 100 μL of 5 μg/mL scavenger receptor A protein in 20 mM Tris, 140 mM NaCl, 10% glycerol and 2 mM EDTA (Sigma) was then added to half of the wells. The other half served as control wells which were not coated with scavenger receptor A protein, but were treated with the same coating buffer to account for any non-specific binding. The plates were incubated with scavenger receptor A protein for two hours at room temperature with shaking. The plates were washed again three times with 100 μL of PBS-T. Samples were added to the wells (one set of wells coated with scavenger receptor A protein and one set of control wells that were coated with capture antibody but not scavenger receptor A). SNAs were added from 0-100 nM in PBS with 0.05% TWEEN-20 and were incubated with the coated plates for two hours at room temperature. To remove

unbound oligonucleotides, the wells were washed three times with PBS-T. The bound SNAs were then quantified by ICP-MS, where samples were dissolved in 3% HNO_3 in HCl at 55°C for 2 hours. The solutions were then suspended in 2% HNO_3, 2% HCl in water with 5 ppb In, Ho and Bi as an internal standard. The samples were analyzed using a Thermo Fisher X Series ICP-MS to quantify gold content.

Cell Culture and SNA Uptake Quantification

C166 mouse endothelial cells (ATCC) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For uptake studies, 100,000 cells per well were seeded in 12-well plates. The next day, cells were treated with PEGylated RNA-SNAs in OptiMEM (5 nM SNA) for 4 hours. The media containing SNAs was removed and cells were washed 3 times with warm OptiMEM to remove excess SNAs. 300 μL OptiMEM was added per well, and cells were collected after treating with trypsin. The cells were then counted using a Countess Cell Counter (Life Technologies) using trypan blue stain. Cells were digested in 3% HNO_3 in HCl at 55°C for 2 hours. The solutions were then suspended in 2% HNO_3, 2% HCl in water with 5 ppb In, Ho and Bi as an internal standard. The samples were analyzed using a Thermo Fisher X Series ICP-MS to quantify gold content.

Determination of Blood Circulation Half Life in Mice

All experiments were conducted under an approved protocol of the Institutional Animal Care and Use Committee of Northwestern University. 6-8 week old female CD1 mice were injected with 200 μL of 100 nM SNA via the tail vein (n=3 per SNA type). Blood samples were collected 1, 15, 45, 135, 405, 1440, and 2880 minutes following injection via cardiac puncture. Blood samples were weighed, dried, and digested with acid for quantification of gold content using ICP-MS.

Pharmacokinetic Modeling

To calculate pharmacokinetic parameters, the data were fit to a one-compartment model using non-linear regression analysis in GraphPad Prism. This model assumes that NPs are transported between the central and peripheral compartments with first-order kinetics and that NPs are eliminated from the central compartment with first-order kinetics. The data were fit to equation S1, where Cp is the concentration of the particles in blood; A and B are hybrid coefficients; α and β are rate constants for the distribution and elimination processes, respectively; and t is the time component.

$$Cp = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eqn. S3)}$$

Pharmacokinetic parameters were estimated using standard calculations. An initial data point at t=0 was included representing 100% of the injected dose in the blood.

Results

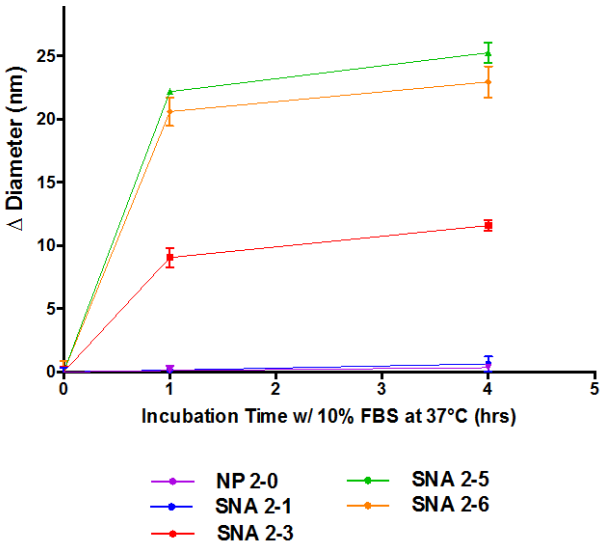

Figure S1. Effect of PEGylation of the biological properties of RNA-SNAs. Increasing PEG content on RNA-SNAs minimizes the adsorption of serum proteins, which correlates to a smaller increase in the hydrodynamic diameter of the NP as measured by DLS.

References

1. Jokerst, J. V.; Lobovkina, T.; Zare, R. N.; Gambhir, S. S. (2011**)** Nanoparticle PEGylation for imaging and therapy. *Nanomedicine-Uk.* *6*, 715-728.
2. Guo, P. (2010**)** The emerging field of RNA nanotechnology. *Nature Nanotech.* *5*, 833-842.
3. Barnaby, S.N.; Perelman, G.S.; Kohlstedt, K.L.; Chinen, A.B.; Schatz, G.C.; Mirkin, C.A. Design Considerations for RNA Spherical Nucleic Acids (SNAs). *Bioconjugate Chem.* DOI: 10.1021/acs.bioconjchem.6b00350
4. Choi, C. H.; Hao, L.; Narayan, S. P.; Auyeung, E.; Mirkin, C. A. (2013**)** Mechanism for the endocytosis of spherical nucleic acid nanoparticle conjugates. *Proc. Natl. Acad. Sci. U.S.A.* *110*, 7625-7630.