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Supplemental Information

Ligand conjugate SAR and enhanced delivery in NHP

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Supplemental Information

Figure S1
Trivalent GalNAC ligand (compound 1) utilized in linker SAR studies (Table 1)

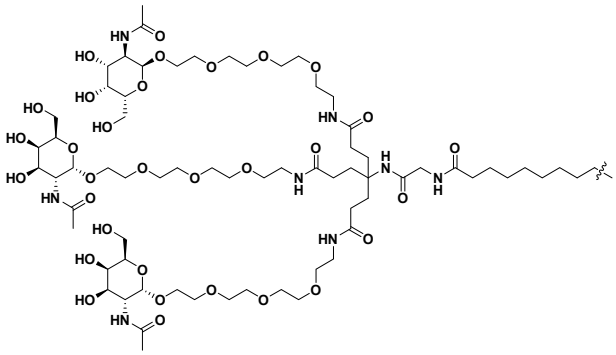
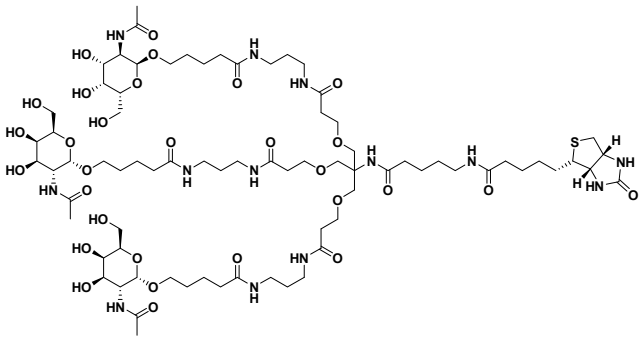


Figure S2
In vitro binding studies of trivalent GalNAc on HepG2 cultures: A. Compound 2- biotinylated trivalent ligand based on previously reported GalNAc cluster. B. Dose response binding curve of compound 2/streptavidin AF488 complex in HepG2 cells (T=1.5 h). C. Competitive inhibition of different valency GalNAc ligands and asialofetuin with compound 2/streptavidin AF488 complex in HepG2 cells.

A



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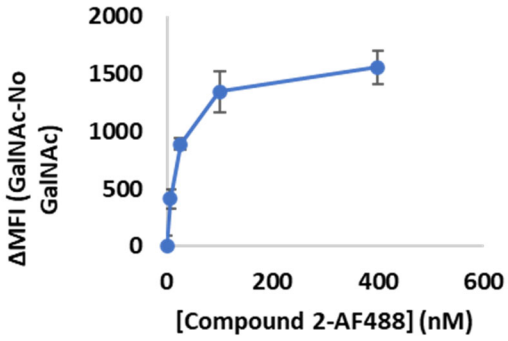

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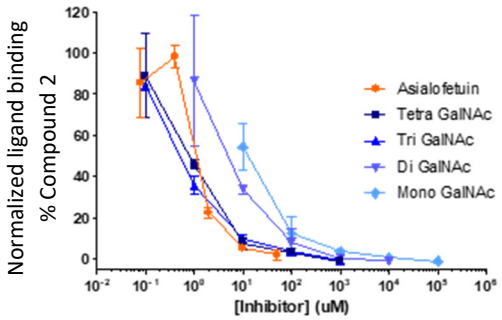
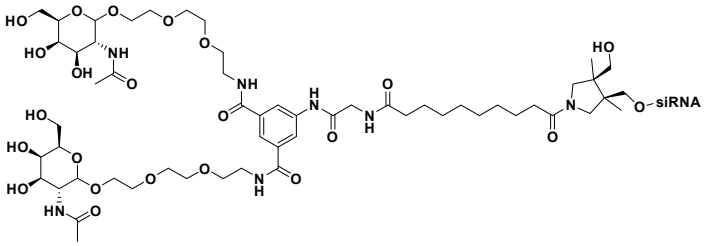


Figure S3

Pharmacokinetic profile of bivalent and tetravalent conjugate TTR siRNA in mice. C57BL/6 female mice aged 6-8 weeks (n=4 per group) were subcutaneously injected with a single dose of 2 mg/kg tetravalent GalNAc ligand (compound 14 in Table 4) or bivalent GalNAc ligand (compound 15, structure shown in panel A) conjugated TTR siRNA. A compound 14 conjugated siRNA targeting luciferase gene was used as a negative control. The pharmacokinetics in the plasma (0.5, 1, 3, 6 and 24 h post dosing) were characterized by measuring siRNA concentration using oligoELISA (B). The activity of the conjugates was assessed by quantifying liver TTR mRNA using QuantiGene assay (C) and plasma TTR protein level with quantitative ELISA assay at 24 h post dosing (D).

A



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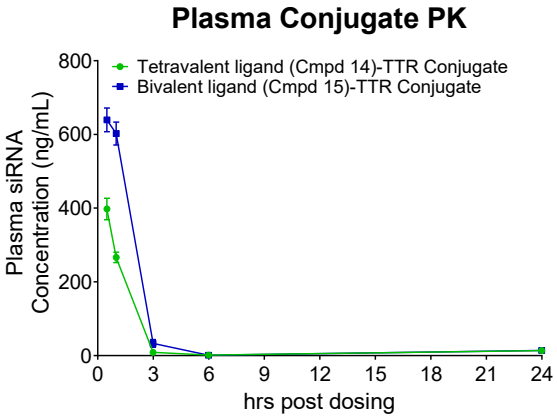

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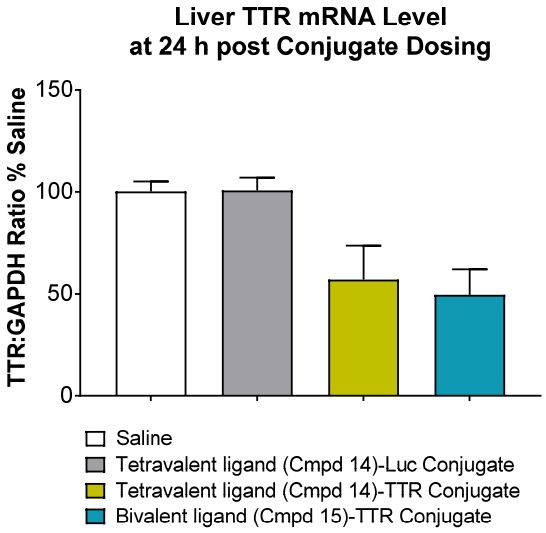

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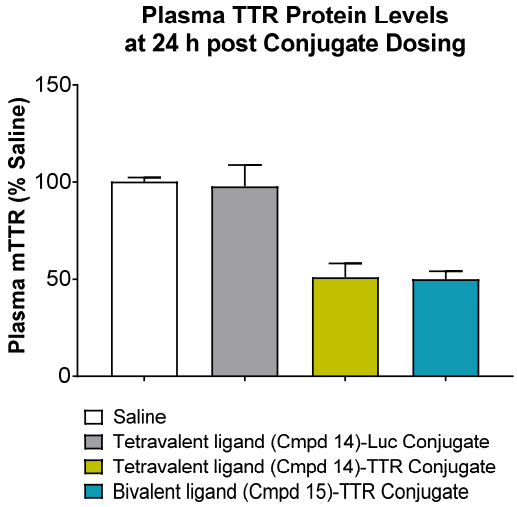


Figure S4

Bivalent scaffold f or evaluation of sugar modifications (Table 3)

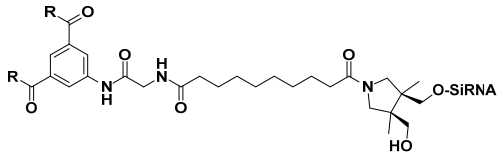


Figure S5

Hemolysis assay for the polymer micelle. The polymer micelle was incubated with isolated RBCs at pH 7.4, 6.6 or 5.8 to mimic the extracellular, early endosome and late endosome pH environment respectively. The released hemoglobin was measured by absorbance at 400 nm and normalized to the positive control (RBC incubated with 20% Triton X-100). The data presented here represents the average signal from 3 replicates with standard deviations.

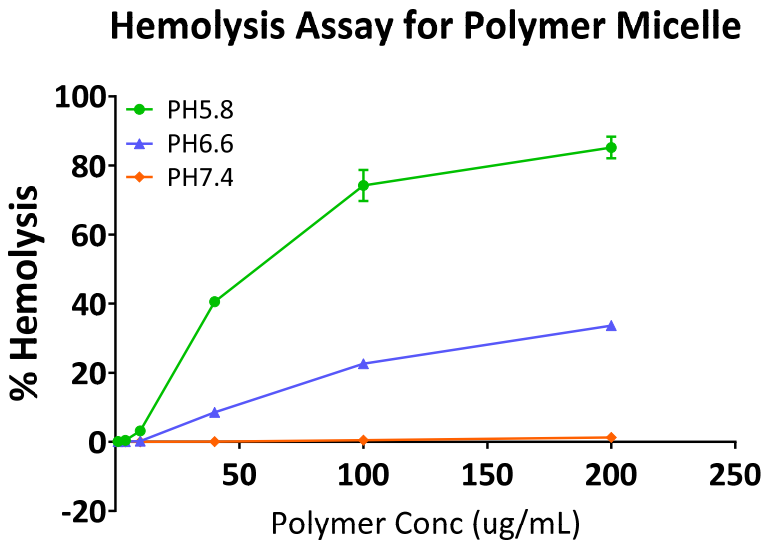
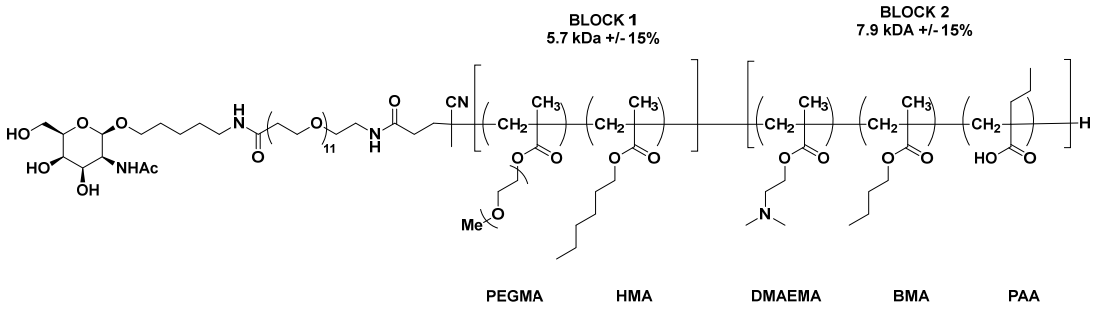


Figure S6

GalNAc targeted "co-block" endosomal release polymer structure with percentage monomer incorporation and description of functionality.



Block 1 Monomer incorporation	Monomer manufacturing release specification
	Polyethylene Glycol MethAcrylate 4-5 (PEGMA 4-5) – (71-79%)
	Hexyl MethAcrylate (HMA) – (21-29%)
Block 2 Monomer incorporation	DiMethylAminoEthyl Acrylate (DMAEA) – (30-40%)
	ButylMethAcrylate (BMA) – (47-57%)
	PropylAcrylic Acid (PAA) – (9-16%)

The polymer is comprised of three distinct regions:

At the alpha end, N-Acetylgalactoseamine monosaccharide mediates hepatocyte targeting via interaction with the ASGPr receptor.

Block 1 is a solubilizing hydrophilic region comprised of polyethyleneglycol methacrylate 4-5 (PEGMA 4-5) and hydroxyethyl methacrylate (HMA).

Block 2 is a hydrophobic, pH sensitive region comprised of dimethylaminoethyl acrylate (DMAEA), butyl methyacrylate (BMA) and propylacrylic acid (PAA).

The combination of the two polymeric regions are important for maximizing efficacy. At physiological or neutral pH the polymer is neutral. Moreover at neutral pH the block 2 region displays hydrophobic character. In combination with the hydrophilic domain, if the polymer is above the critical micelle concentration (CMC) in aqueous media, small micelle structures spontaneously form. These have been shown to have pH responsive membrane destabilizing activity in red blood cell hemolysis assays (see figure S5). Below the CMC, hemolysis drops off precipitously.

During endocytosis and subsequent decrease in pH, the polymer becomes positively charged and consequently promotes endosomal release through membrane disruption.

Descriptions of the synthesis of the polymer can be found in the supplemental section of the recent Molecular Therapy paper Prieve et al Mol Ther 26, 3, 2018

Table S1

Evaluation of Inflammatory Biomarkers in Non-Human Primates

		CRP (µg/mL)		Complement C3 (mg/mL)		Fibrinogen (mg/mL)		MMP-3 (ng/mL)		MMP-9 (ng/mL)	
Treatment	Animal	Pre	6h	Pre	6h	Pre	6h	Pre	6h	Pre	6h
Saline	1	0.15	0.21	0.76	0.49	0.01	0.13	4.2	3.0	78	64
	2	0.51	0.36	0.53	0.53	0.25	0.22	4.4	3.2	116	101
	3	0.05	0.07	0.49	0.46	0.17	0.14	4.1	4.0	106	78
	AVG	0.24	0.21	0.59	0.49	0.14	0.16	4.2	3.4	100	81
	SEM	0.14	0.08	0.08	0.02	0.07	0.03	0.1	0.3	11	11
Tetravalent + polymer	1	0.28	0.69	0.75	0.69	0.24	0.35	3.0	2.8	130	101
	2	0.12	1.10	0.56	0.42	0.11	0.18	7.4	5.7	23	22.5
	3	0.24	0.40	0.59	0.67	0.24	0.24	1.3	1.1	64	167
	AVG	0.21	0.73	0.63	0.59	0.20	0.26	3.9	3.2	72	97
	SEM	0.05	0.20	0.06	0.09	0.04	0.05	1.8	1.3	31	42
Tetravalent	1	1.50	1.30	0.66	0.73	0.28	0.32	1.9	1.6	84	93
	2	0.05	0.22	0.7	0.57	0.03	0.26	2.6	5.3	143	128
	3	0.17	0.93	0.54	0.58	0.20	0.36	2.1	2.2	95	121
	AVG	0.57	0.82	0.63	0.63	0.17	0.31	2.2	3.0	107	114
	SEM	0.46	0.32	0.05	0.05	0.08	0.03	0.2	1.1	18	11
Polymer	1	0.16	0.59	0.73	0.63	0.23	0.22	2.1	2.1	126	22.5
	2	0.11	0.15	0.67	0.57	0.01	0.07	6.9	2.5	111	155
	3	0.02	0.33	0.53	0.6	0.03	0.22	5.0	4.4	84	87
	AVG	0.10	0.36	0.64	0.60	0.09	0.17	4.7	3.0	107	88
	SEM	0.04	0.13	0.06	0.02	0.07	0.05	1.4	0.7	12	38

Additional markers assessed that are below the lower limit of quantitation (data not shown): Interferon gamma (IFN-gamma), Interleukin-1 alpha (IL-1 alpha), Interleukin-1 beta (IL-1 beta), Interleukin-2 (IL-2), Interleukin-3 (IL-3), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Macrophage Inflammatory Protein-1 alpha (MIP-1 alpha), Tumor Necrosis Factor alpha (TNF-alpha), and Tumor Necrosis Factor beta (TNF-beta).

A single subcutaneous injection of saline, 3 mg/kg tetravalent GalNAc-siRNA, 0.6 mg/kg tetravalent GalNAc-siRNA + 8.8 mg/kg polymer, or 8.8 mg/kg polymer alone was administered to male cynomolgus monkeys (Macaca fascicularis). Blood samples were collected at 6 h post-dose, processed to plasma, and analyzed with a quantitative, multiplexed inflammatory biomarker assay. Data are presented as mean ± SEM (n=3 per group).

Figure S7

Comparison of activity when GalNAc-siRNA and polymer are co-administered from the same vial vs separate injections of each component in mice. C57BL/6 female mice aged 6-8 weeks (n=4 per group) were dosed subcutaneously with 0.3 mg/kg bivalent GalNAc-siRNA alone, or with 10 mg/kg polymer. In one instance, the GalNAc-siRNA and polymer test articles were provided in separate vials and two subcutaneous injections were administered to mice. In another treatment group, GalNAc-siRNA and polymer were provided in the same vial and a single subcutaneous injection was administered to mice. TTR protein levels were analyzed by a quantitative ELISA assay (Abnova, Taipei, TW), as per the manufacturer's instructions. Data represented as mean ± SEM. "n.s" = $p > 0.05$, "***" = $p < 0.001$, two-way ANOVA analysis.

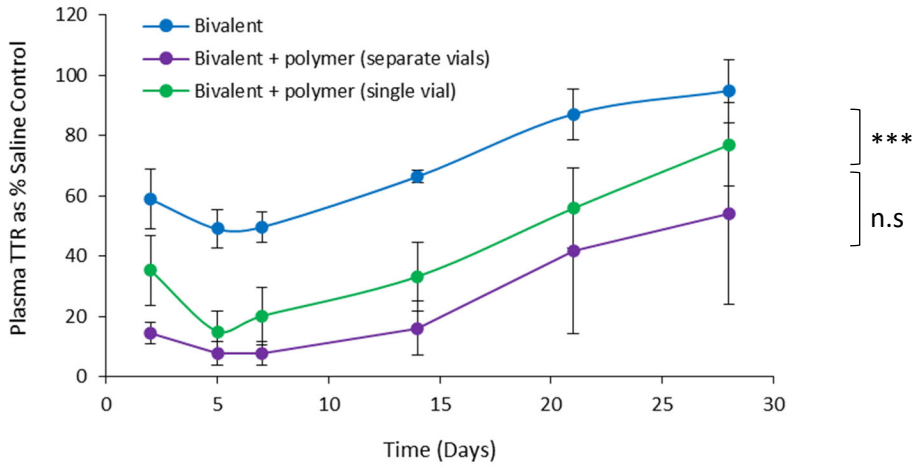


Figure S8

Saline or polymer micelle was subcutaneously administered to male cynomolgus monkeys (Macaca fascicularis) at 8.8 mg/kg. Blood samples were processed to serum and analyzed with a quantitative biomarker immunoassay. Serum TTR levels of each animal were normalized to its respective baseline level at pre-dose. Group average at each timepoint is represented as a percentage relative to its pre-dose group average. Data are presented as mean ± SEM (n=3 per group). "n.s" = p > 0.05, two-way ANOVA analysis.

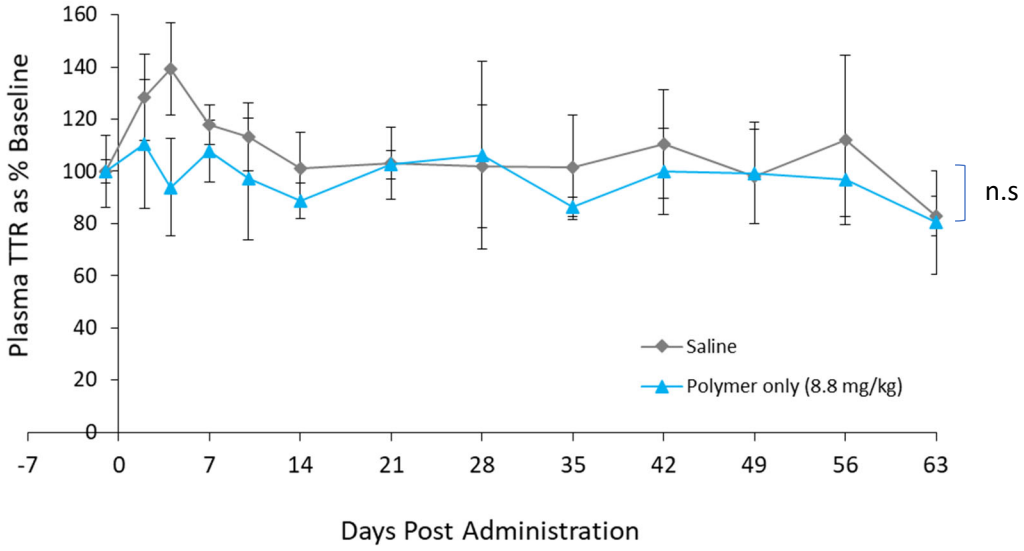


Table S2

Dynamic light scattering analysis on the interaction between polymer micelle and GalNAc-siRNA conjugate.

Test Sample	size	PDI
Polymer micelle alone (1.25 mg/mL)	14.3 nm	0.16
Polymer micelle (1.25 mg/mL) + Tetravalent GalNAc ligand (cmpd 14)-TTR siRNA conjugate (0.025mg/mL)	14.5 nm	0.17
Tetravalent GalNAc ligand (cmpd 14)-TTR siRNA conjugate alone (0.025mg/mL)	Unable to measure (out of detection range)	NA

Supplemental Methods

pH-dependent Hemolysis Assay

The endosomolytic capability of the polymer micelle was determined using hemolysis essay as previously described (Journal of Controlled Release 133 (2009) 221–229; J Vis Exp. 2013; (73): 50166). In brief, whole human blood containing EDTA as anticoagulant was purchased from BioIVT. Blood was centrifuged and plasma was removed. The isolated red blood cells (RBCs) were washed three times with 150 mM NaCl and resuspended in phosphate buffer at pH 7.4, pH 6.6 and pH 5.8 to mimic the extracellular, early endosome and late endosome environment, respectively. Polymer (1-200 μg/mL) was incubated with the RBC at the three pH values for 1 h at 37 ˚C. Intact RBCs were then spined down by centrifugation and the released hemoglobin in supernatant due to hemolysis was measured by absorbance at 400 nm. 20% Triton X-100 was used as a positive control and the absorbance from RBCs incubated with this positive control was defined as 100% hemolysis. The absorbance from the polymer samples were all normalized to the positive control.

OligoELISA Analysis for Plasma Samples

Conjugates with known concentrations (100 ng/mL to 0.78 ng/mL) were used as standards and prepared by 2 fold serial dilution in naïve mouse plasma. Collected mouse plasma samples post conjugate dosing and diluted standards were further diluted 1:5 to 1:10 with 2% Triton X-100 in Dulbecco's PBS. The diluted plasma samples and standards were then hybridized with LNA capture and detection probes (Qiagen), which contain sequences complementary to the sense strand of the TTR siRNA as well as a biotin moiety (capture probe) or a Digoxigenin moiety (detection probe). A working probe solution containing 5 nM of each probe in hybridization buffer (6xSSC/1% N-Lauroylsarcosine sodium salt) was prepared and 125 μL of this solution was mixed with 25 μL of the above diluted plasma (samples and standards) in a 96-well PCR plate. Hybridization was performed on a thermocycler using the following cycles: 1 cycle at 90°C for 2 min, 1 cycle at 53°C for 30 min, and 1 cycle at 23°C for 2 min.

For the ELISA, a Neutravidin Coated High Capacity black 96-well plate (Thermo Fisher) was washed twice with wash buffer (1xTBS/600 mM NaCl) and 100 μL of the hybridization products was applied to the plate and it was incubated at room temperature for 1 h with mixing at 100 RPM. Following this binding step, the plate was washed 4 times with the wash buffer and 100 μL of an anti-digoxigenin antibody-AP (Roche) working solution (1:2000 dilution in Superblock T20 Blocking Solution in TBS with 1.5 M NaCl) was added to the wells. The plate was incubated at room temperature for 30 min with mixing at 100 RPM. It was then washed 4 times with wash buffer and the plate was developed by addition of 100 μL of an Attophos working solution (Promega) followed by incubation at room temperature for 30 min with mixing at 100 RPM. Fluorescence in the wells was read on a Tecan Saffire2 fluorescence plate reader using excitation and emission wavelengths of 435 nm and 555 nm, respectively, slit widths of 20 nm, and gain of 40. Concentration of the appropriate siRNA in plasma was determined by comparing the fluorescence intensity of the samples to the appropriate standard curve.

QuantiGene Assay

TTR mRNA in conjugate treated mouse livers were quantified using bDNA assay (QuantiGene, Thermofisher). In brief, liver samples from mice were minced, incubated in RNAlater (Thermofisher) for 24 h at 4°C. Between 20 and 25 mg of RNAlater fixed liver tissue was homogenized in Epicentre lysis buffer containing 1% proteinase K using the

FastPrep®-24 homogenization instrument (4.0 m/s, 3×15 second bursts, MP Biomedicals). Samples were kept on ice both before and after the homogenization to prevent degradation. The lysates were cleared by centrifugation at 16,000×g at 16°C for 5 min and diluted in lysis working buffer to ensure all values would be in the assay linear range of detection before being subjected to bDNA assay using the QuantiGene 2.0 assay kit, according to the manufacturer's protocol. The bDNA probes were specifically designed to target: mouse TTR and mouse GAPDH (endogenous control). The mTTR levels were normalized to the mGAPDH levels when comparing different animals and treatment groups.

Dynamic Light Scattering Analysis

Using a Malvern Zetasizer Nano S (Malvern Instruments, Malvern UK), the Z average & PDI (polydispersity index) were measured for the following solutions in PBS: 1) 1.25 mg/mL PRX-847 ; 2)1.25 mg/mL PRX-847 & 0.025 mg/mL Ligand AR-siTTR (50:1 wt ratio); 3) 0.025 mg/mL Ligand AR-siTTR. Briefly, 1.5mL of the solutions were placed in 10mm x 10mm x 47mm disposable cuvettes (Sarstedt, Germany). The instrument utilizes a 632.8 nm helium–neon laser and analyzes scattered light at an angle of 173° by utilizing a noninvasive backscatter technique.