

# INCRECENT<sup>®</sup>

**MULTIMERIC OLIGONUCLEOTIDES FOR  
ENHANCED POTENCY AND PRECISION**

**Nonconfidential Data Package**



**December 2020**



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## Introduction

With the success of liver-targeted, monogenic oligonucleotide therapies, the field looks forward to new opportunities focused on targets other than hepatocytes and the delivery of multiplexed therapeutic agents for addressing complex diseases such as cancer.

Developed by MPEG LA, Increscent® is a versatile technology platform that enables drug developers to address hepatocyte or non-hepatocyte targets through the delivery of multimeric oligonucleotides for enhanced bioavailability and bioactivity, along with precision control over dosage ratios when multiplexed therapeutic agents are used.

The platform is based on the delivery of multimeric payloads of the same or different therapeutic agents linked together into a single compound. As demonstrated herein, larger multimers enable dramatically enhanced serum half-life and availability for uptake by target cells and tissue, resulting in enhanced bioactivity with minimal or no increase in typical cytokine markers of cellular toxicity.

The platform is compatible with a variety of therapeutic agents, including small interfering RNA (siRNA), antisense oligonucleotide (ASO), microRNA (miRNA), and others; and is further compatible with a variety of chemical modifications, linkers, and delivery vehicles including ligand-conjugates (e.g., small molecules, aptamers, peptides, and antibodies) and nanoparticles.

Our proprietary synthesis strategies may be used alone or in conjunction with solid phase oligonucleotide synthesis. The strategies involve simple steps carried out in aqueous solution, at room temperature and neutral pH, and produce high yields and high purity.

While we used GalNAc/ASGPR as our test system in work performed to date, we expect the real value of the Increscent® platform to lie in enabling ligand/receptor systems that are not naturally as efficient as GalNAc/ASGPR.

The patent-protected platform is available for licensing and collaborations. For questions and further information, please contact Kristin Neuman, Executive Director, Biotechnology Licensing at [kneuman@mpegla.com](mailto:kneuman@mpegla.com) or +1.914.588.2471.

## Discussion

This package contains data in support of the Increscent® platform for multimeric oligonucleotide therapeutics. While we performed our studies with materials that are well-known and easily assayable, such as siRNA targeting Factor VII mRNA (“siFVII”) in hepatocytes and a tri-antennary GalNAc ligand, the Increscent® platform may be readily translated to other therapeutic oligonucleotides and other cell-targeting moieties.

At the outset, we aimed to determine the bioactivity of a dimeric siRNA as compared to its monomeric counterpart. In the mouse study presented in Section A, we made three isomeric homodimers of siFVII conjugated to GalNAc and tested them against a monomeric siFVII conjugated to GalNAc. In this and all subsequent studies, our siRNA sequences were chemically modified using common strategies to enable the siRNA to withstand degradation without compromising bioactivity. When the knockdown activity was normalized to dose (per unit of siRNA), the activity of the homodimers and monomer control were equivalent. But, when activity was normalized per mole of GalNAc ligand, the homodimers outperformed the monomeric control by approximately two-fold.

Next, we set out to demonstrate that a multi-conjugate of three units of siRNA, each targeting a different mRNA, would be active *in vivo*. In the mouse study presented in Section B, we made two isomeric GalNAc-conjugated heterotrimers of siFVII, siApoB, and siTTR and tested them against a pool of siFVII, siApoB, and siTTR monomers, each conjugated to GalNAc. The potencies of the heterotrimers were on par with the monomeric pool on a per dose basis.

As part of the study presented in Section B, we also made a heterotetramer consisting of two units of siFVII and one unit each of siApoB and siTTR; but we did not test the heterotetramer for bioactivity. A description of the synthesis is provided in Section C.

Working on a hypothesis that larger multimers will have decreased kidney filtration due to their size, leading to enhanced longevity in circulation and greater uptake by target cells, we designed a set of experiments to demonstrate whether large multimers would produce a significant increase in serum half-life. In Section D, we describe the synthesis of a homohexamer of siFVII and the *in vivo* determination of its half-life versus the half-life of a monomer having the same siFVII sequence in a mouse study. The half-life of the homohexamer was upwards of 20 times the serum half-life of the monomer. The same hexamer and monomer were analyzed for cytokine levels, with the hexamer showing minimal to no increase in cytokine markers for cellular toxicity.

Next, we set out to determine where, in a range of siFVII multimers, the increased serum half-life would be most pronounced. In the *in vivo* study of Section E, we synthesized a series of siFVII multimers ranging from monomer to octamer and measured their half-lives in a mouse study. We discovered that a dramatic increase in serum half-life kicks in with the tetramer and maxes out at the hexamer.

In our most recent round of experiments, we compared the knockdown potency of a GalNAc-conjugated heterohexamer containing four units of siFVII, one unit of siApoB, and one unit of siTTR administered via subcutaneous (sc) and intravenous (iv) routes (Section F). The material was essentially inactive when delivered via the sc route, suggesting it was perhaps too large to permeate the relevant tissues. By contrast the material was highly active via iv administration, the activity of the siTTR unit within the hexamer more than equaling the reported activity of a

monomeric GalNAc-siTTR with virtually the same sequence delivered via sc.

In summary, the Increscent® platform provides opportunities to (1) deliver multiple therapeutic agents in controlled ratios in a single, cell-targeting oligonucleotide conjugate; (2) boost activity in cell-targeting oligonucleotide conjugate systems that to-date have not achieved acceptable levels of therapeutic efficacy; and/or (3) lower dosage levels of existing therapeutic oligonucleotide conjugates currently delivered in monomeric form by converting to a multimeric format. The potential increase in the relative dose effectiveness of large multimers versus monomers will likely be even greater in delivery systems other than GalNAc/ASGPR due to likely lower potencies of such ligand/receptor pairs, and this will be particularly important with diseases, such as cancer, that utilize iv administration and require simultaneous knockdown of multiple gene targets, potentially in carefully controlled stoichiometric ratios.

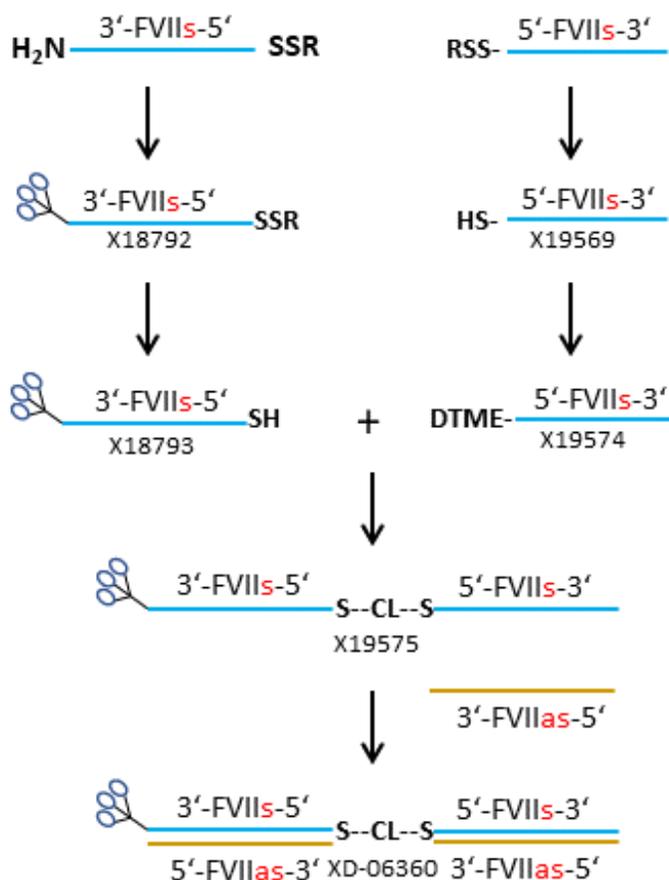
## A. Demonstration of Increased Knockdown Per Mole Ligand

### Synthesis of Homodimers

Three isomeric homodimers of FVII siRNA, each carrying a tri-antennary GalNAc ligand, were synthesized via a mono-DTME derivative prepared as described in General Procedures. Thus, positional isomer XD-06360 was prepared according to Scheme 1 from two FVII sense strands with the sequences:

(C6SSC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C6NH<sub>2</sub>)

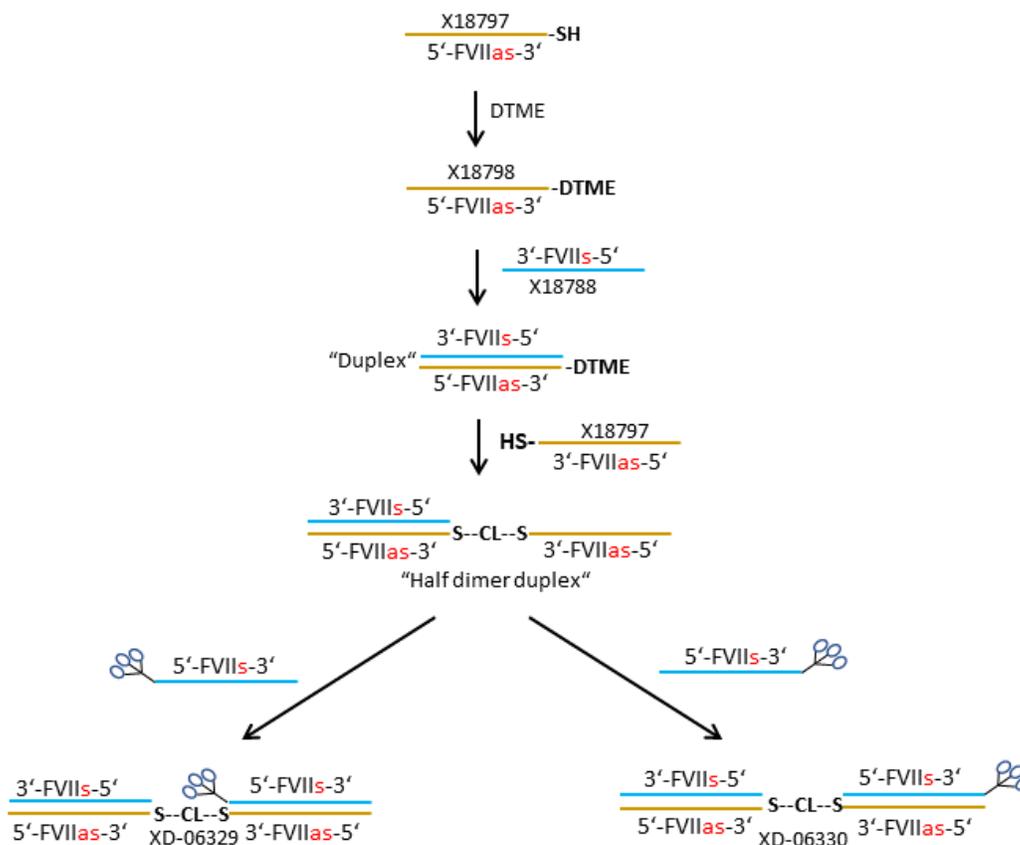
(C6SSC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)



Scheme 1: Synthesis of GalNAc-siFVII dimer XD-06360. The symbol “-CL-” represents the cleavable linkage introduced via the DTME, which contains an internal disulfide group. DTME is dithiobismaleimidoethane.

Positional isomers XD-06329 and XD-06330 were prepared according to Scheme 2, using similar procedures and starting from the siFVII antisense strand X-18797:

### UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C6SH)



Scheme 2: Syntheses of GalNAc-FVII-dimers XD-06329 and XD-06330.

## Determination of Homodimer Bioactivities

The three GalNAc-conjugated homodimeric siFVII isomers (XD-06329, XD-06330 and XD-06360) and a GalNAc-conjugated monomeric siFVII control (XD-06328) were administered to mice subcutaneously at 25 mg/kg or 50 mg/kg in PBS (0.2ml). Group sizes were n=4 mice/group for treated animals and n=5 for saline controls. Blood was collected 1 day prior to treatment and at 1, 3, and 7 days post-treatment, and analyzed for FVII enzyme activity. When the activity data is normalized to dose (per unit of siRNA), the activities of the homodimers and monomeric control are equivalent; however, when the activity data is normalized to ligand per unit of GalNAc, then the homodimers outperform the monomeric control. See FIGS. 1 and 2, respectively:

Legend for FIGS. 1 and 2:

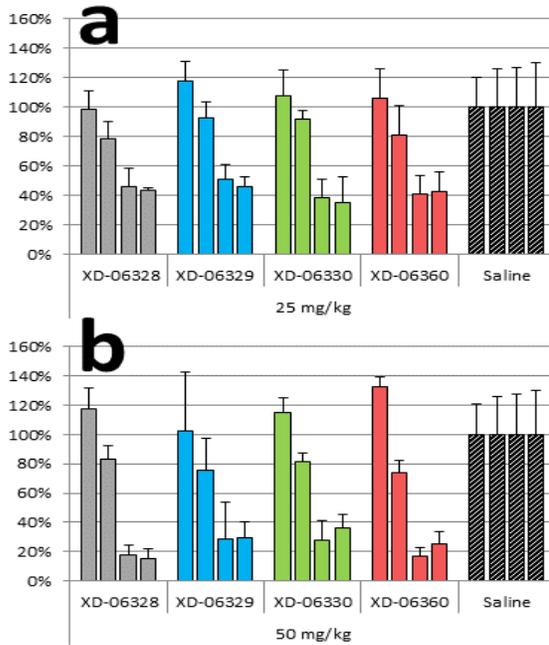


FIG. 1: FVII activity knockdown by homodimers and monomers normalized per unit siRNA at a) 25 mg/kg; and b) 50 mg/kg.

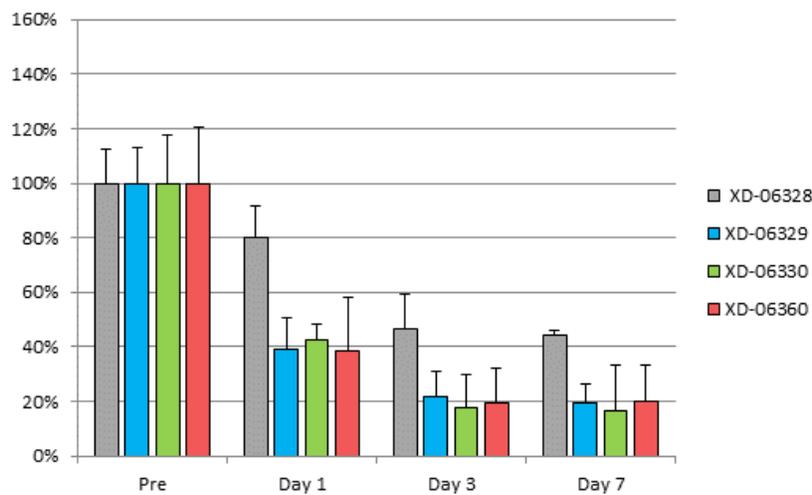


FIG. 2: FVII activity knockdown by homodimers and monomers at 25 mg/kg normalized per unit GalNAc.

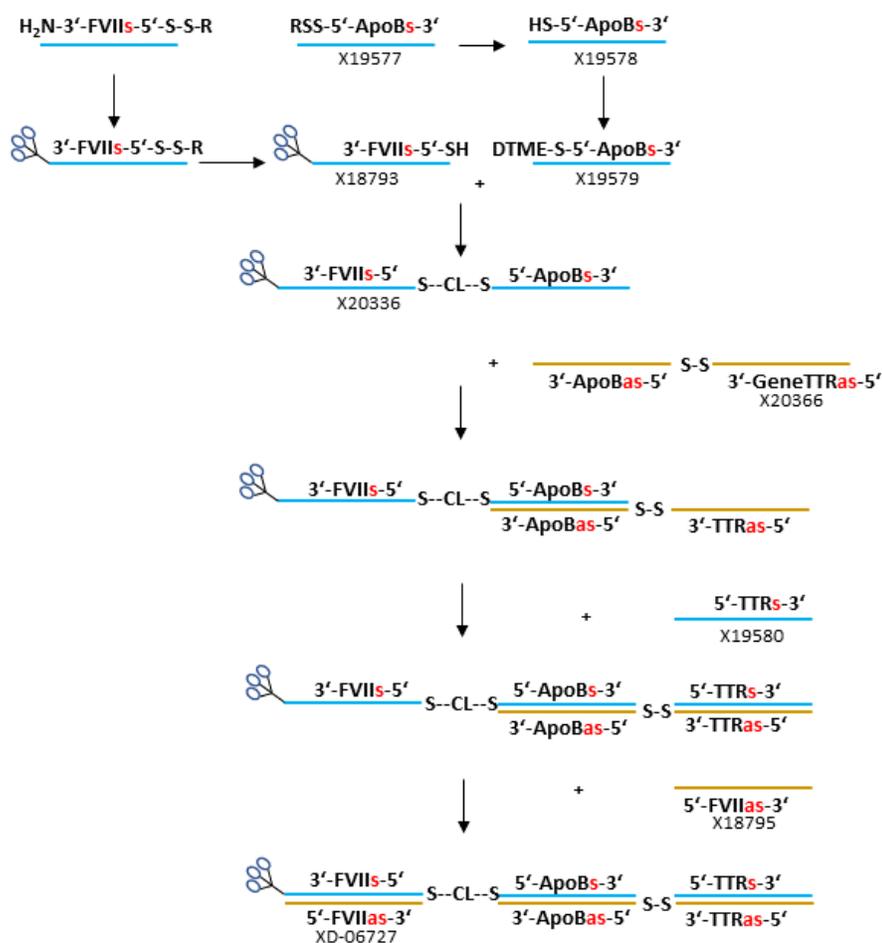
## B. Demonstration of Heterotrimer Bioactivities

### Synthesis of Heterotrimers

GalNAc-conjugated, heterotrimeric positional isomers of siRNA, each containing siFVII, siApoB, and siTTR, were synthesized and tested for biological activity.

#### Preparation of GalNAc-siFVII-siApoB-siTTR Heterotrimer with Cleavable Linkages on Alternating Strands (XD-06727)

9 mg (192 nmol) of GalNAc-conjugated, heterotrimeric siRNA XD-06727 containing siFVII, siApoB, and siTTR, was prepared in high yield and purity by combining single strands stepwise as depicted in Scheme 3 using the methodology described in General Procedures in the proportions listed in Table 1.

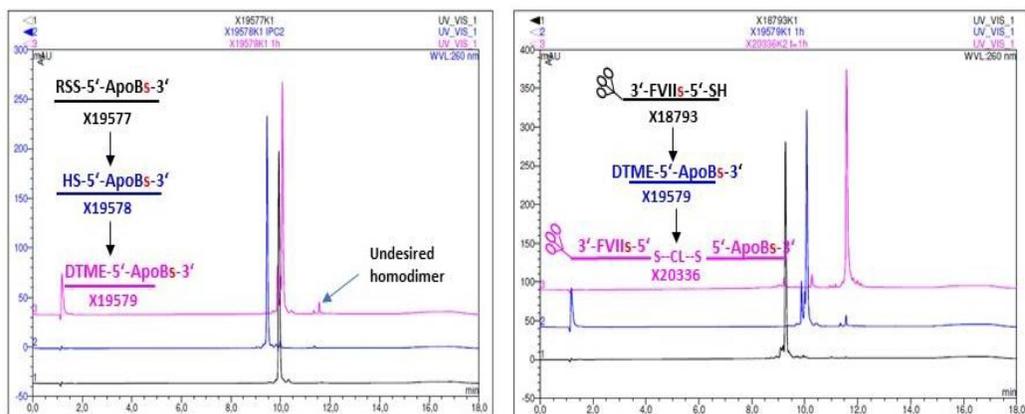


Scheme 3: Synthesis of XD-06727.

Each reaction step was monitored by HPLC, which revealed that nearly 100% conversion of the reactants was achieved at each step as shown in FIG. 3.

FIG. 3: a) Reverse Phase and b) Ion exchange chromatograms of the reactions depicted in Scheme 3.

a)



b)

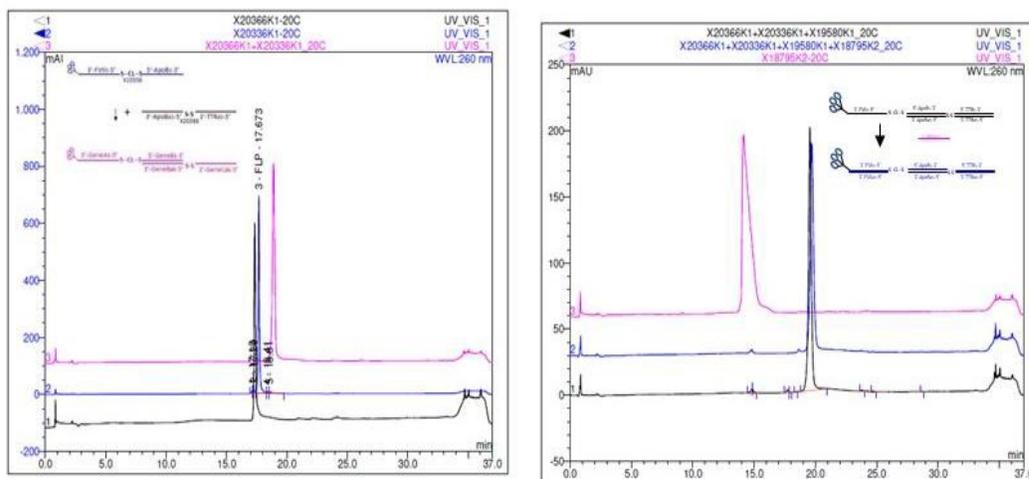
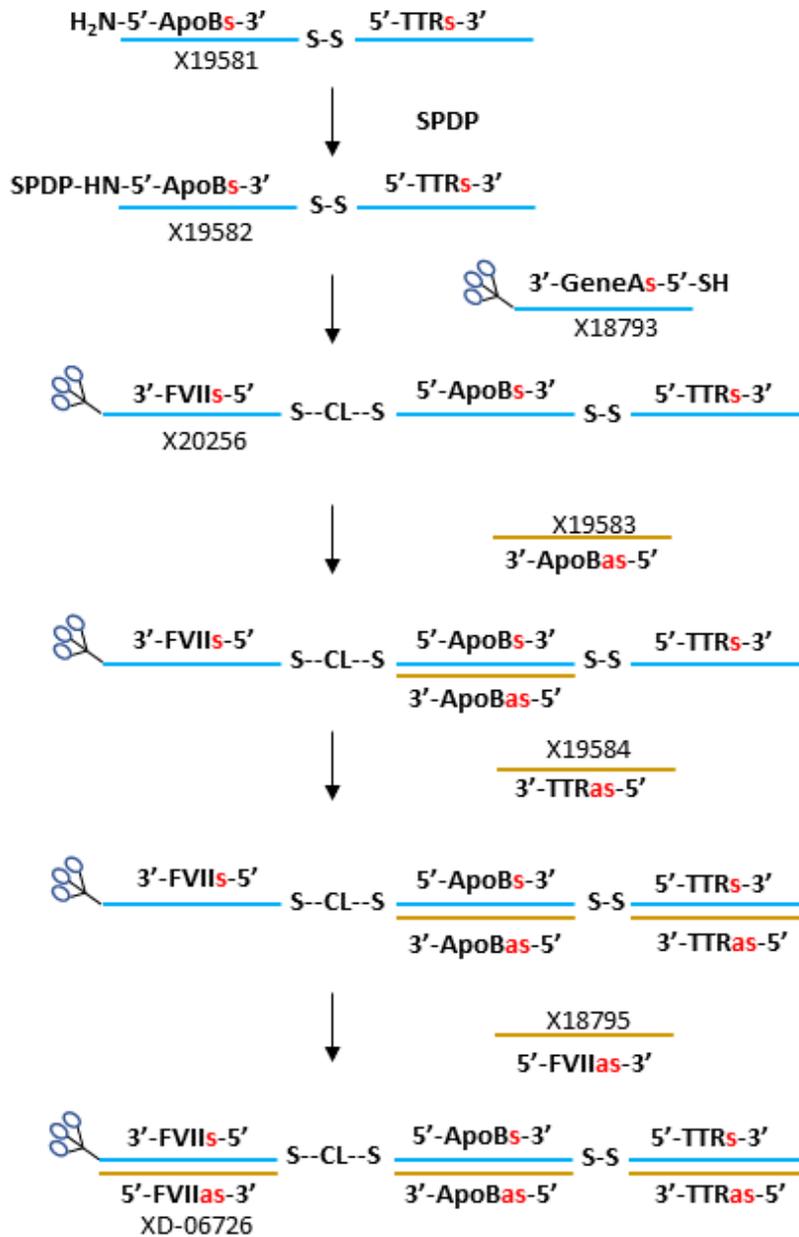


Table 1. Stoichiometries of Oligomers Used in Synthesis of GalNAc-siFVII-siApoB-siTTR Heterotrimer (XD-06727).

ID	Strand	E (L/mol*cm)	1 OD	MW (free Acid)	MW Na salt	Req OD
X20336	FVIIIs-ApoBs	404300	2.47 nmol	15440.1	16341.4	78
X20366	ApoBas-TTRas	446700	2.24 nmol	14748.9	15716.1	86
X19580	TTRs	220300	4.54 nmol	7105.6	7567.2	42
X18795	FVIIIs	194800	5.13 nmol	6849.4	7289.1	37
XD-06727				44144	46913.8	

## Preparation of GalNAc-siFVII-siApoB-siTTR Heterotrimer with Cleavable Linkages on Sense Strands (XD-06726)

7 mg (150 nm) of GalNAc-conjugated, heterotrimeric siRNA containing siFVII, siApoB and siTTR (XD-06726) was prepared in high yield and purity by the procedure depicted in Scheme 4 using the methodology described in General Procedures in the amounts listed in Table 3.



Scheme 4: Synthesis of XD-06726.

In the opening steps of the reaction, the single-stranded heterodimer X19581 with a 5'-amino terminus was prepared using solid phase chemistry and was reacted with 5 equivalents of SPDP (succinimidyl 3-(2-pyridyldithio)propionate), and after purification by reverse phase HPLC was further reacted with the thiol modified oligonucleotide X18793 (1.2 eq) to yield the single-stranded heterotrimer X20256 (containing linked sense strands of siFVII, siApoB, and siTTR) in high purity. The sequence of X20256 is shown in Table 2.

Table 2. Sequence for Single-stranded Heterotrimer (X20256)

ID	Sequence: 5'-3' for each component monomer	Target/Strand
X20256	(SHC <sub>6</sub> )gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdt)(C <sub>6</sub> NH)(GalNAc <sub>3</sub> )(SPDP)(NH C <sub>6</sub> )cuAfuUfuGfgAfgAfgAfaAfuCfGf(invdt)(C <sub>6</sub> SSC <sub>6</sub> )AfsasCfaGfuGfuUfcUfuGfcUfcUfaUfaAf(invdt)	FVIIIs/ApoBs/TTRs

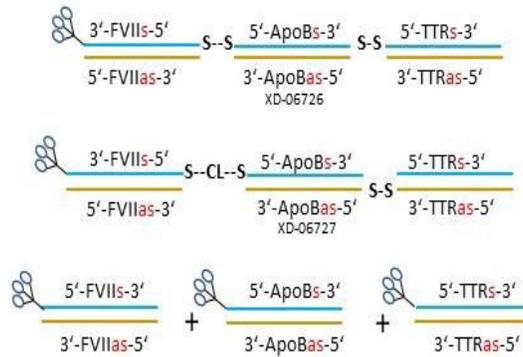
Thereafter, the double-stranded heterotrimer (XD-06726) was prepared by sequentially adding the individual antisense strands stepwise to the heterotrimeric sense-strand intermediate (X20256) according to the duplex titration method described in General Procedures.

Table 3. Stoichiometries of Oligomers Used in Synthesis of GalNAc-siFVII-siApoB-siTTR Heterotrimer (XD-06726).

ID	Strand	E (L/mol*cm)	Nmol/OD	MW (free Acid)	MW Na salt	Req OD
X20256	FVIIIs-ApoBs-TTRs	623900	1.60	22690.8	24075.7	94
X19583	ApoBas	206500	4.84	6762.4	7202.1	31
X19584	TTRas	240400	4.16	7596.1	8079.7	36
X18795	FVIIIs	194800	5.13	6849.4	7289.1	29
XD-06726				43898.7	46646.6	

## Determination of Heterotrimer Bioactivities

Heterotrimers XD-06726 and XD-06727 and, as a positive control, a pool of three GalNAc-conjugated monomeric siRNAs separately targeting FVII, ApoB, and TTR, were each separately administered to mice subcutaneously at a dosage of 50 mg/kg total RNA for the heterotrimers and 17 mg/kg for each of the monomers in PBS (0.1 ml).



Group sizes were n=4 mice/treatment group and n=5 for saline controls. Blood was collected 1 day prior to treatment and at 1, 3, and 7 days post-treatment, and serum levels of FVII, ApoB and TTR were measured. mRNA levels from liver lysates at day 7 were also determined.

Silencing data is plotted in FIG. 4:

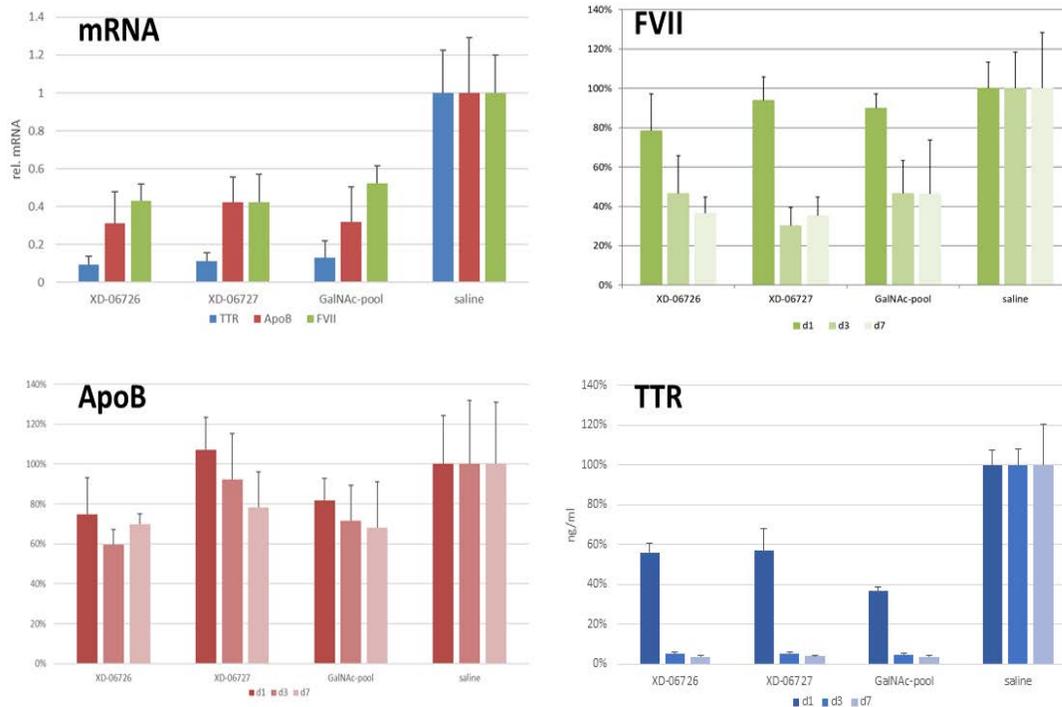
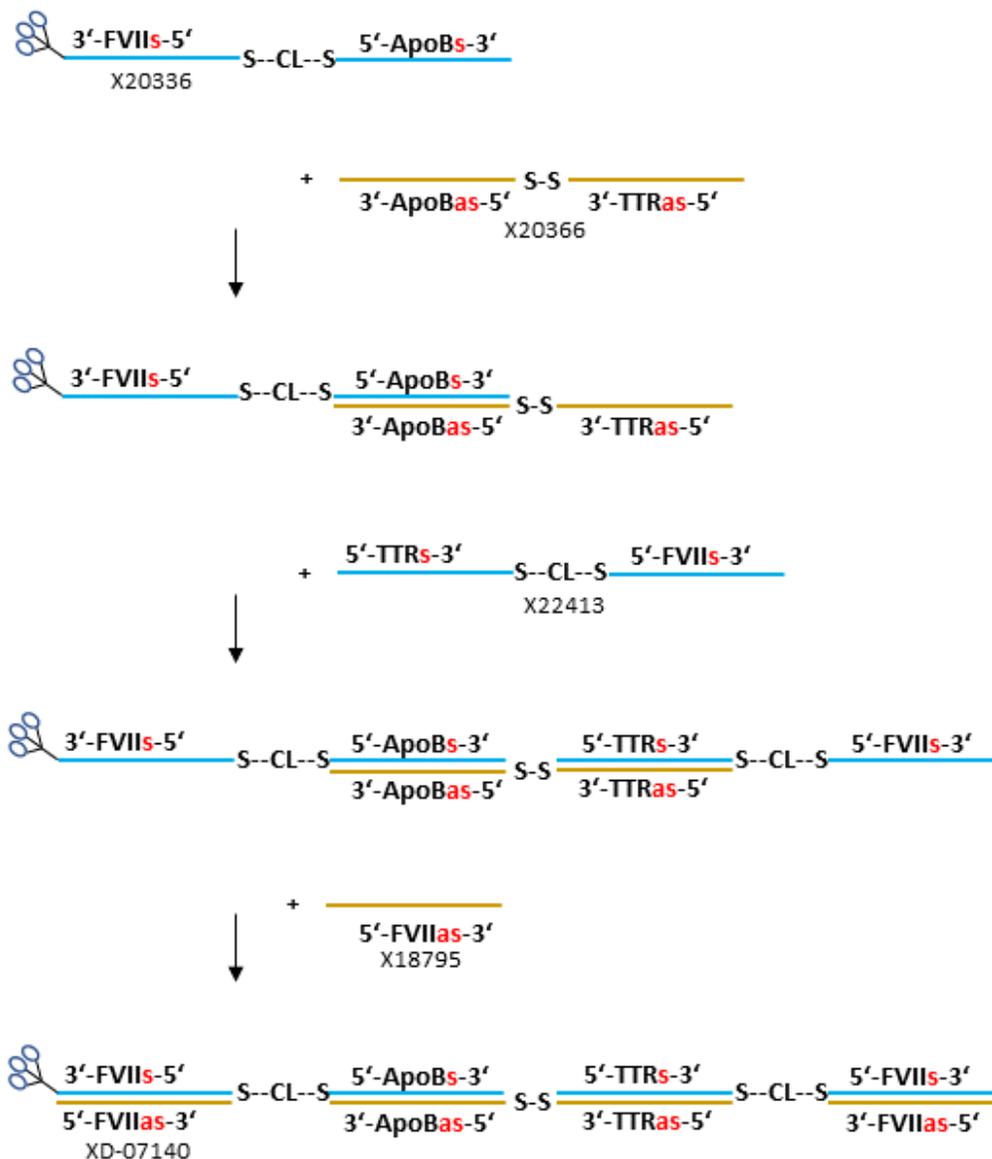


FIG. 4: Serum and mRNA knockdown for FVII, ApoB, and TTR achieved by heterotrimers XD-06726 and XD-06727, and a pool of monomeric GalNAc controls.

## C. Synthesis of a Heterotetramer

A GalNAC-conjugated heterotetramer of siRNA containing two units of siFVII, one unit of siApoB, and one unit of siTTR was prepared in high yield and purity by the procedure depicted in Scheme 5 using the methodology described in General Procedures in the amounts listed in Table 4. The heterotetramer was not tested for biological activity.

### Preparation of GalNAC-siFVII-siApoB-siTTR-siFVII Heterotetramer with Cleavable Linkages on Alternating Strands (XD-07140)



Scheme 5: Synthesis of XD-07140.

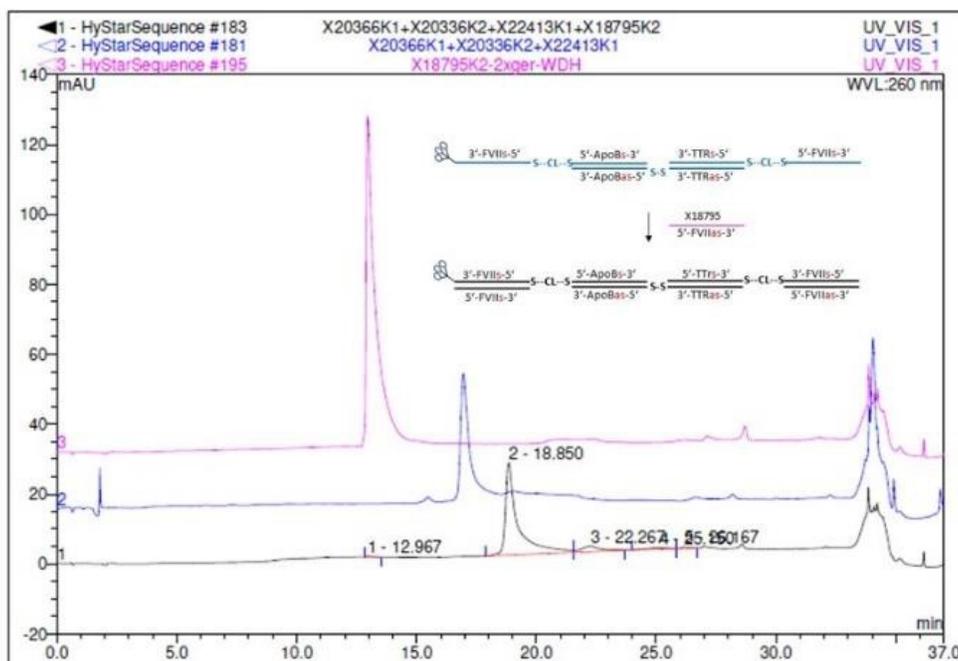


FIG. 5: Overlay of Reverse Phase HPLC traces of the formation of GalNAc-siFVII-siApoB-siTTR-siFVII heterotetramer XD-07140 by the annealing of two equivalents of FVII antisense strand to 1 equivalent of asymmetric tetramer.

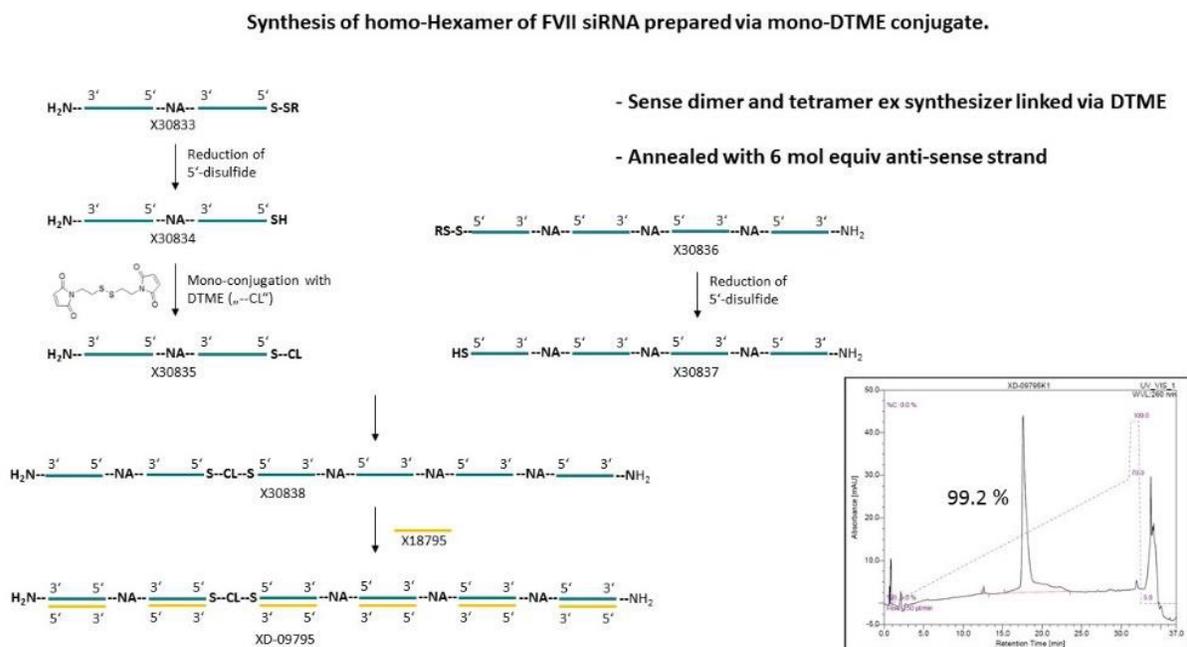
Table 4. Stoichiometries of Oligomers Used in Synthesis of GalNAc-siFVII-siApoB-siTTR Heterotetramer (XD-07140).

ID	Strand	E (L/mol*cm)	1 OD	MW (free Acid)	MW Na Salt	Req OD
X20336	FVIIIs-ApoBs	404300	2.47 nmol	15440.1	16341.4	5
X20366	ApoBas-TTRas	446700	2.24 nmol	14748.9	15716.1	5.5
X22413	TTRs-FVIIIs	412100	2.52 nmol	14041.3	14964.5	4.9
X18795	FVIIIs	194800	5.13 nmol	6849.4 x2	7289.1 x2	4.8
XD-07140				57929.1	61600.2	

## D. Demonstration of Increased Serum Half-life of Hexamer vs Monomer with Minimal to No Increase in Toxicity

### Synthesis of a Homohexamer

To test the hypothesis that a large multimeric siRNA would have an increased half-life in serum relative to a monomeric equivalent, a homohexamer of FVII siRNA (XD-09795) was prepared as described in Scheme 6:



Scheme 6: Preparation of XD-09795.

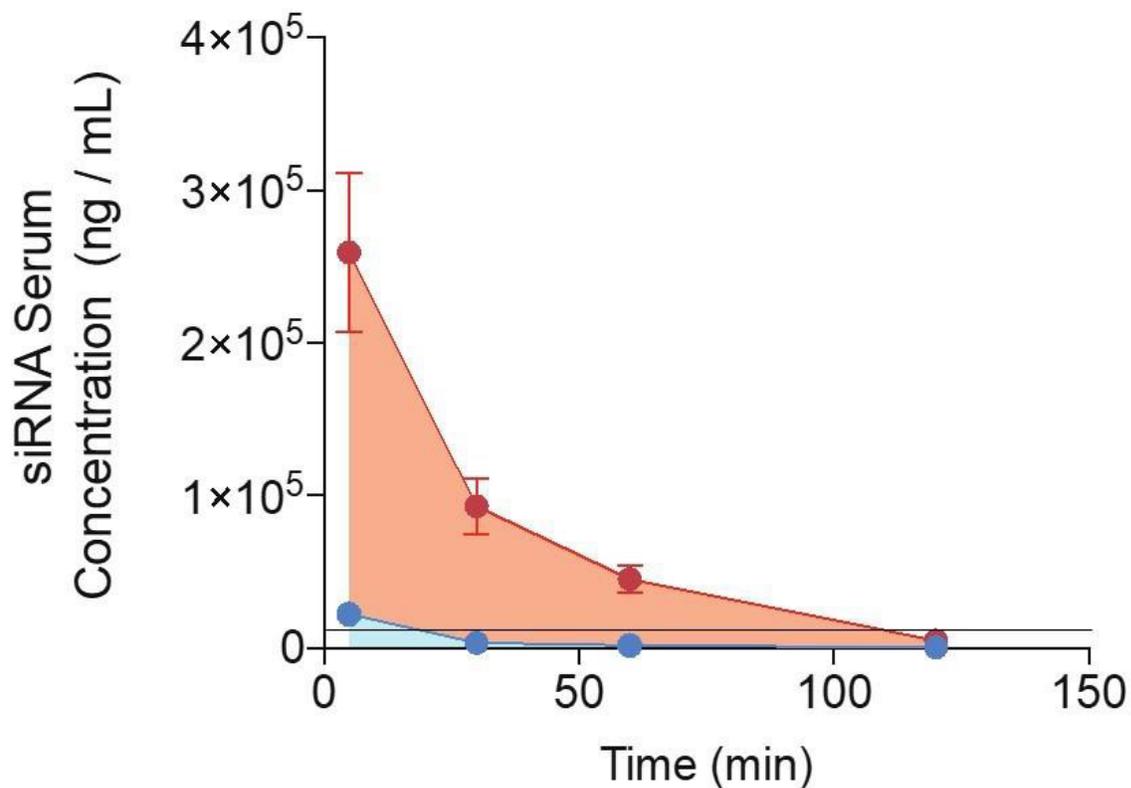
In the opening steps of Scheme 6, disulfide group (“S-SR”) was cleaved from both X30833 (dimer) and X30836 (tetramer) using DTT to give the corresponding 5-thiol derivatives X30834 (dimer) and X30837 (tetramer) in 97.6% and 91.9% yield, respectively. The dimer X30834 (14.9 mg, 986.7 nmol) was then converted to 10.6 mg (700.5 nmol, 71.0%) of the corresponding mono-DTME (“S—CL”) derivative X30835, which was reacted with one equivalent of X30837 to give 4.2 mg (90.7 nmol, 64%) of the single-stranded homohexamer X30838. 3.8 mg (83 nmol) of the single-stranded homohexamer X30838 and 3.7 mg (502 nmol, 6 mol. equiv.) of antisense strand X18795 were then annealed to yield 7.5 mg (83.7 nmol) of the corresponding double-stranded siFVII homohexamer (XD-09795).

## Comparison of Circulation Half-lives of Homohexamer versus Monomer

The serum half-lives of the homohexamer (XD-09795) and a matching monomeric control (XD-09794) were determined by iv bolus injection of the test materials at a concentration of 1 ng/ml in x1 PBS via tail vein into 3 cohorts of 4 C57/BL6N female mice aged approximately 11 weeks per cohort. Dosage was 20 mg/kg for both siFVII monomer and siFVII homohexamer. Blood samples were drawn at 5, 30, 60, and 120 minutes. The concentration of siFVII at these time points was determined as described in General Procedures.

As shown in FIG. 6, the hexamer was retained in serum significantly longer than the monomer as indicated by the relative areas under the curve for monomer [blue] and hexamer [red].

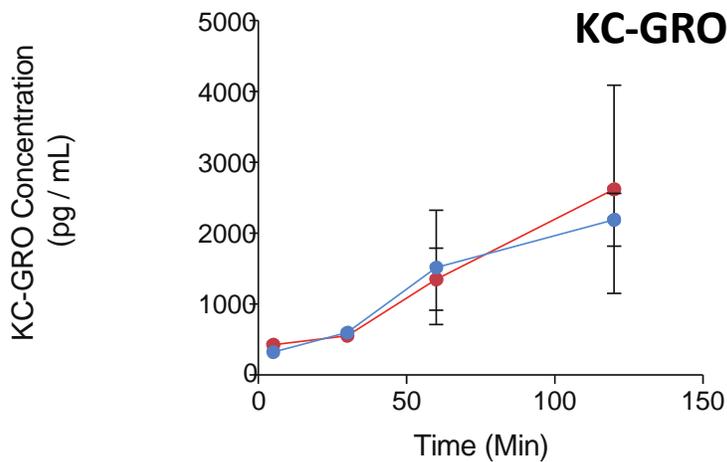
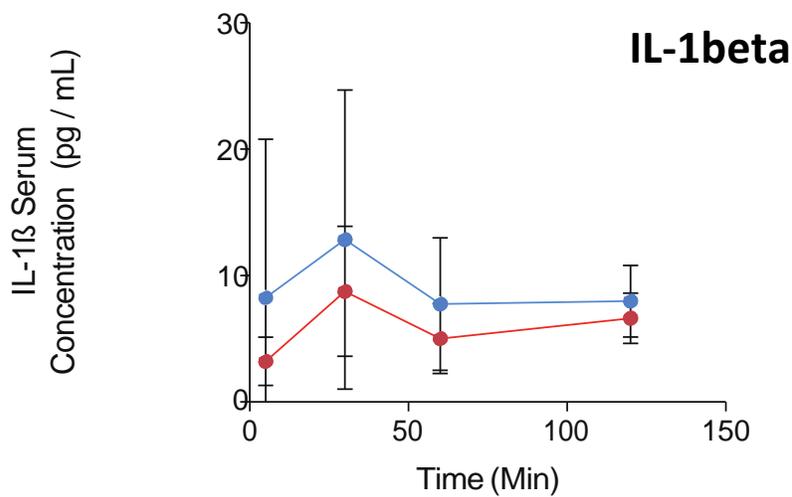
FIG. 6 : Plot of siRNA serum concentration (ng/mL) over time (5, 30, 60 and 120 mins.) for monomer (—●— blue) and hexamer (—●— red).

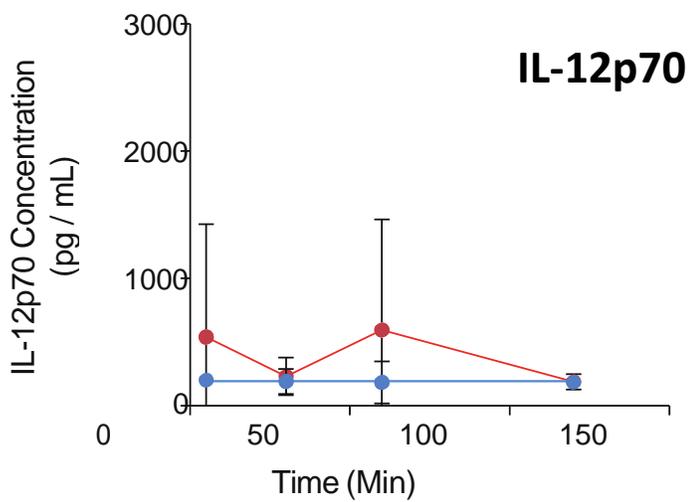
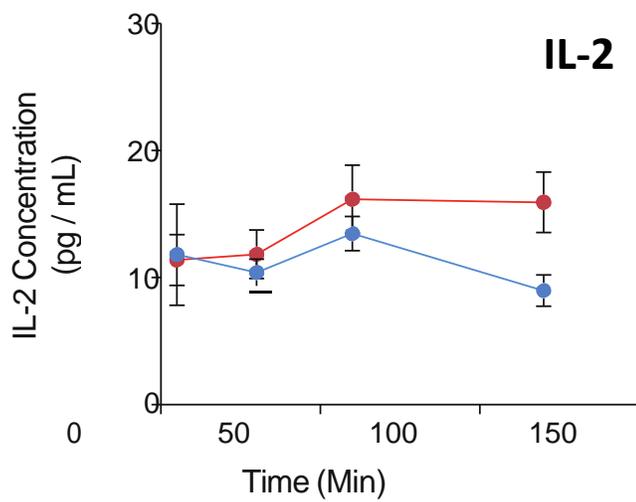
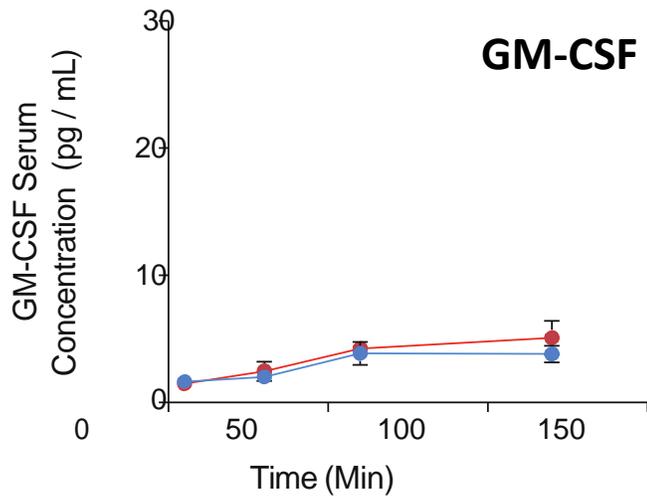


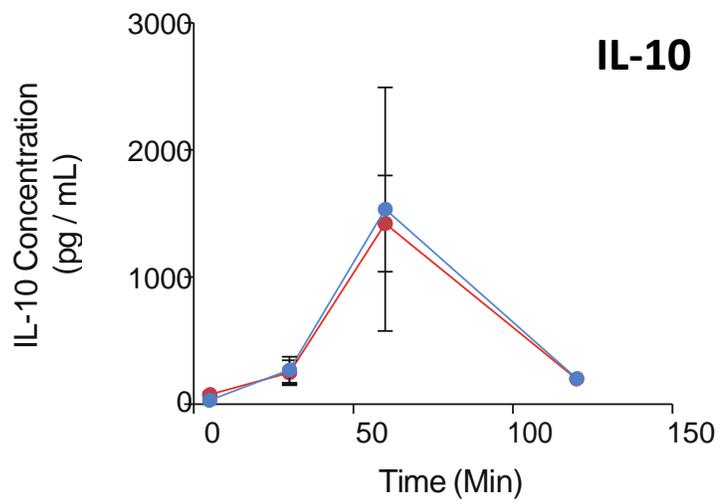
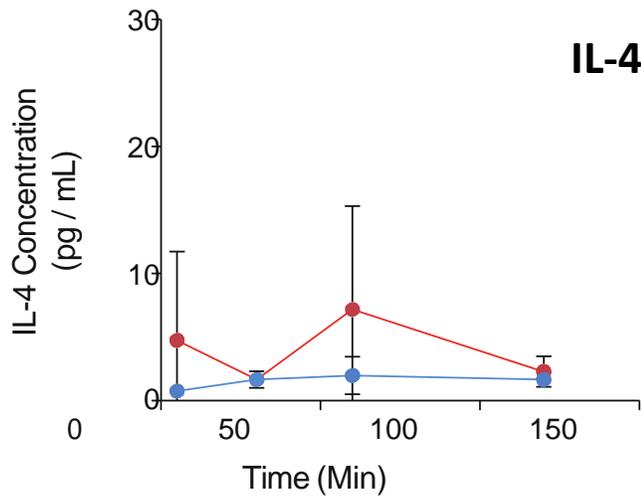
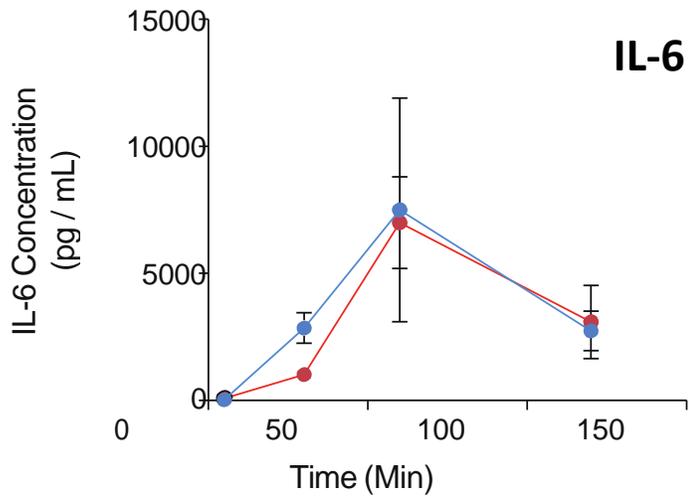
## Demonstration of Minimal to No Increase in Toxicity of Homohexamer versus Monomer

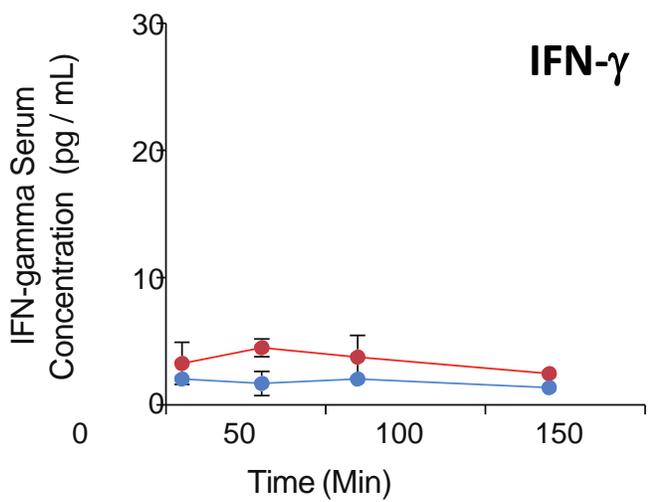
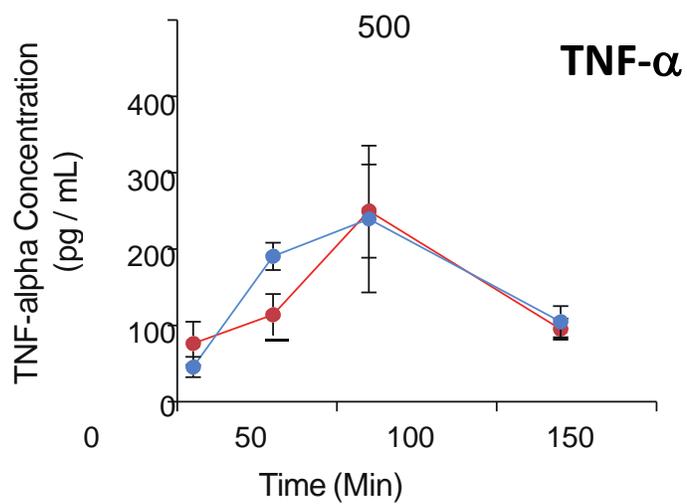
Separately, blood samples from cohorts treated with the monomer XD-09794 and the homohexamer XD-09795 were analyzed for cytokine levels at various time points. The analysis was performed on an MSD U-Plex platform and levels of GM-CSF, IFN-gamma, IL-1beta, IL-2, IL-4, IL-6, IL-10, IL-12p70, KC-GRO, TNF-alpha determined. No significant difference in toxicity between monomer and hexamer was observed.

Key: **Monomer XD-09794** **Hexamer XD-09795**









## E. Determination of Serum Half-life for a Size Range of Homomultimers

### Synthesis of a Size Range of Homomultimers

To determine the size at which the increase in serum half-life commenced and ended, a series of homomultimers of siFVII were prepared via the methodologies described in General Procedures.

The sequence of the siFVII was as follows:

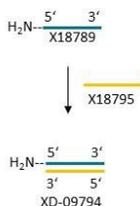
**Sense strand: 5'-gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdt)-3'**

**Antisense strand: 5'-UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu-3'**

The monomeric units of siFVII were linked together using the endonuclease cleavable linkers dCdA and the reductively cleavable linker DTME in a series of reactions (as follows) to form 2-, 3-, 4-, 5-, 6-, 7- and 8-mers.

### Synthesis of siFVII Monomer XD-09794

#### Synthesis of monomer of FVII siRNA

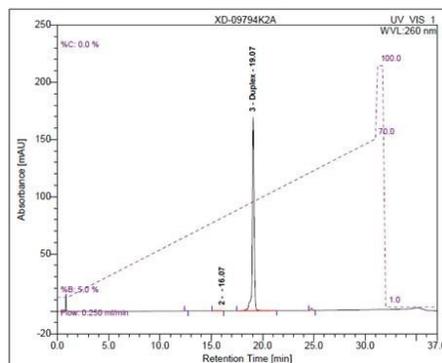


**X18789 =**  
5'-(NH<sub>2</sub>C<sub>6</sub>)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdt)-3'

**X18795 =**  
5'-UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu-3'

- Sense strand ex synthesizer

- Annealed with 1 mol equiv anti-sense strand



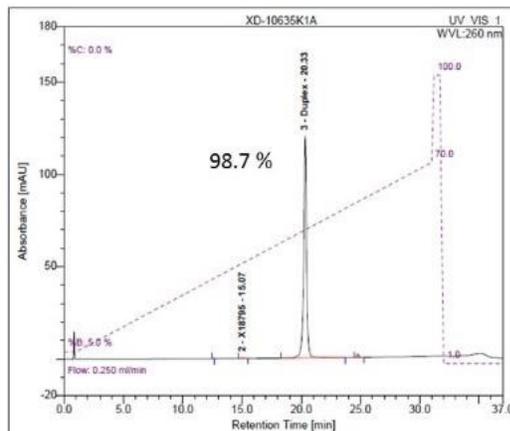
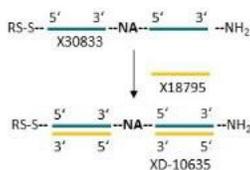
The sense strand (X18789) was synthesized and purified. Yield, 48.3 mg, 6.694 mmol, 18.6%. The corresponding antisense strand (X18795) was likewise synthesized to yield 46.3 mg, 6.35 mmol, 31.9%. Then, 5.35 mg (747.3 nmol) of sense strand (X18789) and 5.45 mg (747.3 nmol) of antisense strand (X18795) were annealed to yield 10.8 mg (747.4 nmol) of the corresponding double-stranded siFVII monomer (XD-09794).

## Synthesis of siFVII Dimer XD-10635

### Synthesis of homo-Dimer of FVII siRNA

- Sense strand ex synthesizer

- Annealed with 2 mol equiv anti-sense strand



X30833 = 5'-(C<sub>6</sub>SSC<sub>6</sub>)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C<sub>6</sub>NH<sub>2</sub>)-3'

X18795 = 5'-UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu-3'

The homodimeric sense strand of siFVII (X30833) with amino and disulfide groups at the 3'- and 5'- termini, respectively, and containing a dCdA cleavable linker (--NA--) was synthesized and purified. Yield, 35.8 mg, 6.694 mmol, 18.6%.

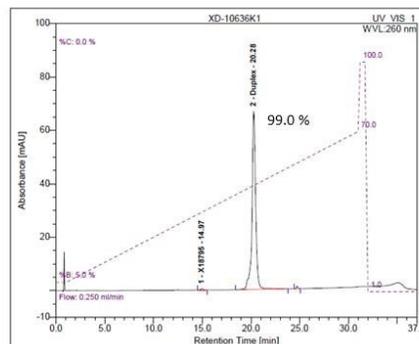
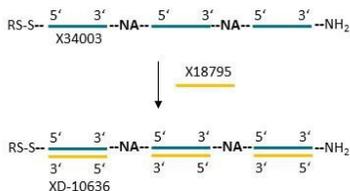
Then, 5.51 mg (362.6 nmol) of the homodimeric sense strand (X30833) and 5.29 mg (725.2 nmol) of the antisense strand (X18795) were annealed to yield 10.8 mg (362.6 nmol) of the corresponding double-stranded siFVII homodimer (XD-10635).

# Synthesis of siFVII Trimer XD-10636

## Synthesis of homo-Trimer of FVII siRNA

- Sense strand ex synthesizer

- Annealed with 3 mol equiv anti-sense strand



**X34003**

= 5'-(C<sub>6</sub>SSC<sub>6</sub>)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dC  
dAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C<sub>6</sub>NH<sub>2</sub>)-3'

**X18795** = 5'-UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu-3'

4

The homotrimeric sense strand of siFVII (X34003) with amino and disulfide groups at the 3'- and 5'- termini, respectively, and containing two dCdA cleavable linkers (--NA--) was synthesized and purified. Yield, 19.6 mg (857.9 nmol, 19.3%).

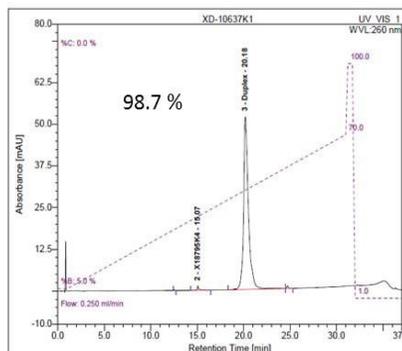
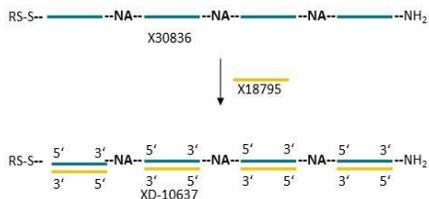
Then, 5.16 mg (225.5 nmol) of the homotrimeric sense strand (X34003) and 4.93 mg (676.5 nmol) of the antisense strand (X18795) were annealed to yield 10.1 mg (225.5 nmol) of the corresponding double-stranded siFVII homotrimer (XD-10636).

## Synthesis of siFVII Tetramer XD-10637

### Synthesis of homo-Tetramer of FVII siRNA

- Sense strand ex synthesizer

- Annealed with 4 mol equiv anti-sense strand



X30836 = 5'-

(C<sub>6</sub>SSC<sub>6</sub>)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C<sub>6</sub>NH<sub>2</sub>)-3'

X18795 = 5'-UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu-3'

Homotetrameric sense strand of FVII siRNA X30836 with amino and disulfide groups at the 3'- and 5'-termini, respectively, and containing three dCdA cleavable linkers (--NA--) was synthesized and purified. Yield, 53.1 mg (1734.5 nmol, 13%).

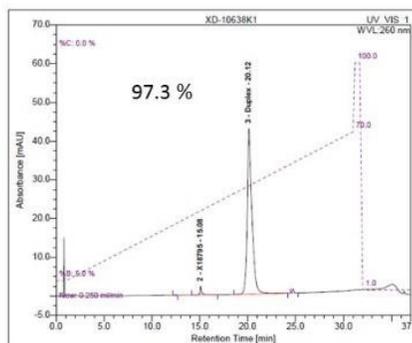
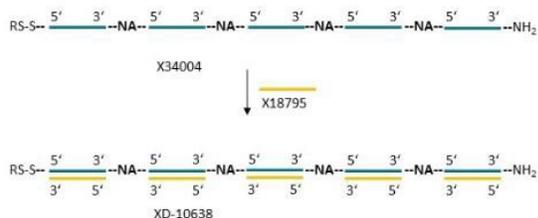
Then, 5.53mg (180.8 nmol) of the homotetrameric sense strand (X30836) and 5.27 mg (723.2 nmol) of the antisense strand (X18795) were annealed to yield 10.8 mg (180.8 nmol) of the corresponding double-stranded FVII homotetramer (XD-10637).

## Synthesis of siFVII Pentamer XD-10638

### Synthesis of homo-Pentamer of FVII siRNA

- Sense strand ex synthesizer

- Annealed with 5 mol equiv anti-sense strand



**X34004** = 5'-(C<sub>6</sub>SSC<sub>6</sub>)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C<sub>6</sub>NH<sub>2</sub>)-3'

**X18795** = 5'-UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu-3'

The homopentameric sense strand of siFVII (X34004) with amino and disulfide groups at the 3'- and 5'- termini, respectively, and containing four dCdA cleavable linkers (--NA--) was synthesized and purified. Yield, 35.9 mg (938 nmol, 10.6%).

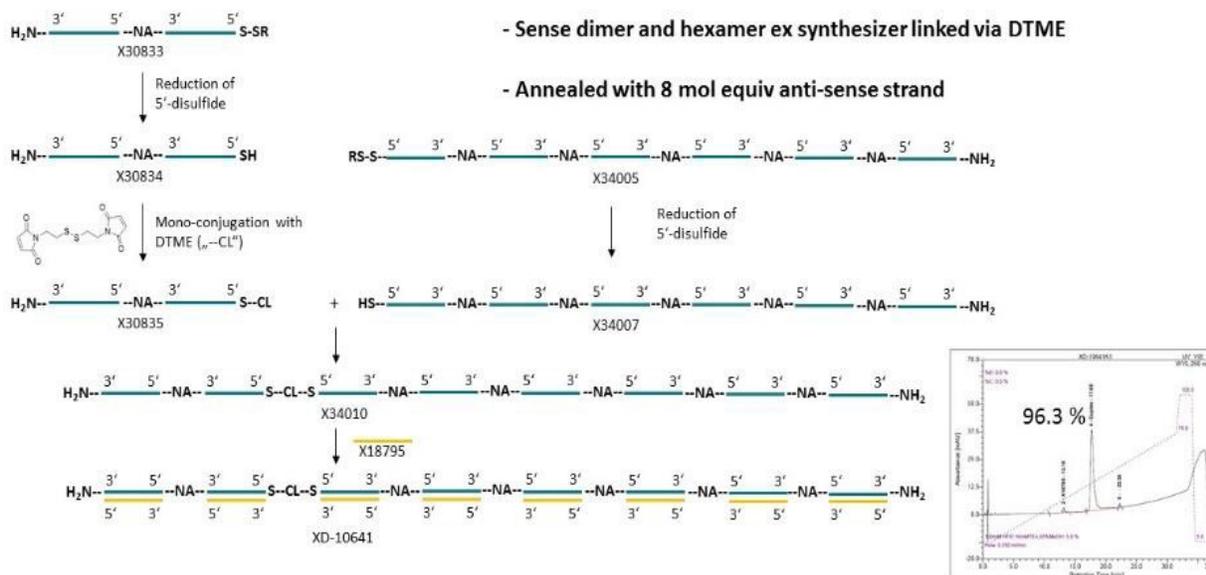
Then, 5.53 mg (144.5 nmol) of the homopentameric sense strand (X34004) and 5.27 mg (723.2 nmol) of the antisense strand (X18795) were annealed to yield 10.8 mg (144.5 nmol) of the corresponding double-stranded siFVII homopentamer (XD-10638).





## Synthesis of siFVII Octamer XD-10641

Synthesis of homo-Octamer of FVII siRNA prepared via mono-DTME conjugate.



The homooctameric sense strand of siFVII (X34010) with amino groups at both the 3'- and 5'-termini and containing six dCdA cleavable linkers (--NA--) and one reductively cleavable DTME linker (S—CL—S) was synthesized and purified via the single-stranded homodimer X30833 and homohexamer X34005, as prepared above. The disulfide group was cleaved from X-34005 using DTT to give the corresponding 5-thiol derivative (X34007) (11.5mg, 251nmol, 99.7%), which was reacted with the previously obtained mono-DTME homodimer derivative (X30835) (3.85mg, 250.2 nmol) to give the single-stranded homooctamer (X34010) (5.2 mg, 85.0 nmol, 34.0%). Then, 4.92 mg (80.33 nmol) of the homooctameric sense strand (X-34010) and 4.68 mg (642.4 nmol) of the antisense strand (X18795) were annealed to yield 9.6 mg (80.3 nmol) of the corresponding double-stranded siFVII homooctamer (XD-10641).

## Determination of Serum Half-lives of the Homomultimers

The serum half-lives of the homomultimers XD-10635, XD-10636, XD-10637, XD-10638, XD-10639, XD-10640, and XD-10641 and the corresponding monomer XD-09794 were determined by iv bolus injection of test material at a concentration of 1 ng/ml in x1 PBS via tail vein into 3 cohorts of 4 C57/BL6N female mice aged approximately 11 weeks per cohort. Dosage was 20 mg/kg for both siFVII monomer and siFVII multimers, and blood samples were drawn at 5, 30, 60, 120 and 360 minutes.

The serum samples were digested with proteinase K and a specific complementary Atto425-Peptide Nucleic Acid-fluorescent probe was hybridized to the antisense strand. Subsequent AEX-HPLC analysis enabled discrimination of intact antisense strand from metabolites leading to high specificity of the method. Only values for the intact parent compound are illustrated in FIG. 7.

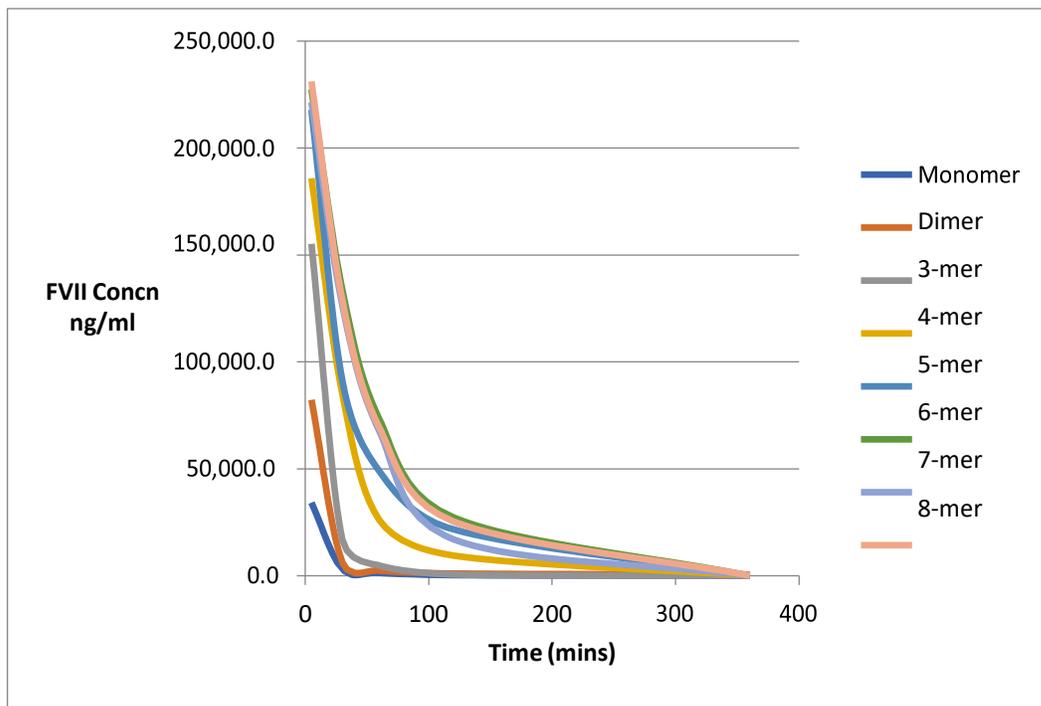


FIG. 7: Smooth line scatter plot of FVII siRNA levels in serum for siFVII monomer and siFVII multimers over time.

From these data, the concentrations of the siFVII multimers at each of the specific timepoints can be represented and compared. FIGS. 8, 9, 10 and 11 show the FVII siRNA levels in serum for the siFVII monomer (1) and each of the siFVII multimeric species (2, 3, 4, 5, 6, 7 and 8) at 5, 30, 60 and 120 minutes, respectively. As is apparent from FIG. 9, the monomer is virtually gone at 30 minutes, whereas the larger multimers (4-mer and higher) are still present in significant concentrations.

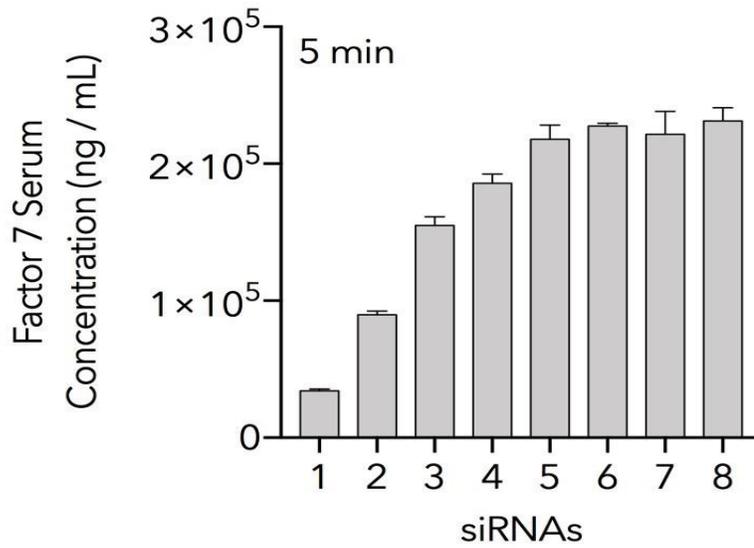


FIG. 8: Bar chart of FVII siRNA levels in serum for siFVII monomer and siFVII multimers at 5 mins.

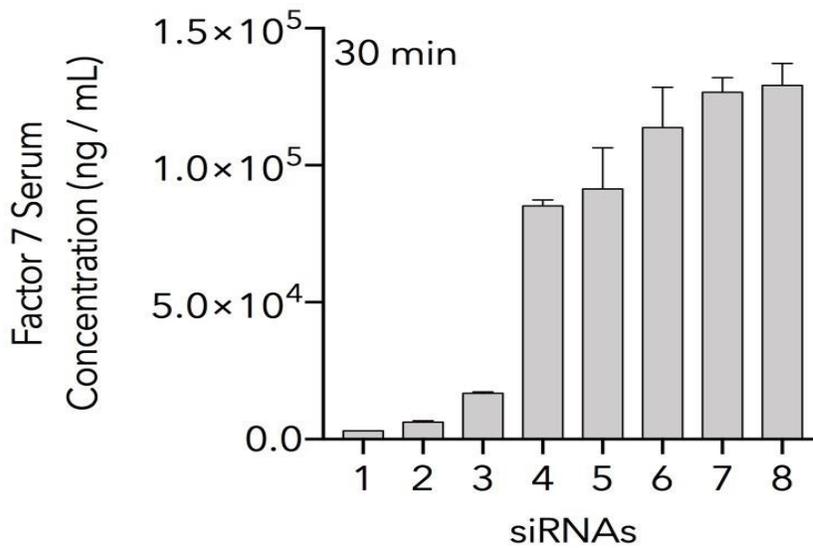


FIG. 9: Bar chart of FVII siRNA levels in serum for FVII monomer and multimers at 30 mins.

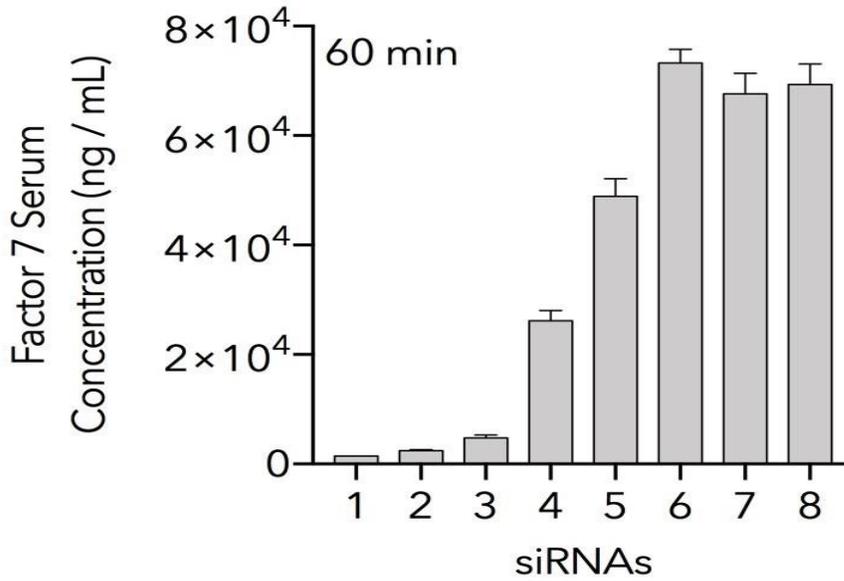


FIG. 10: Bar chart of siFVII levels in serum for siFVII monomer and siFVII multimers at 60 mins.

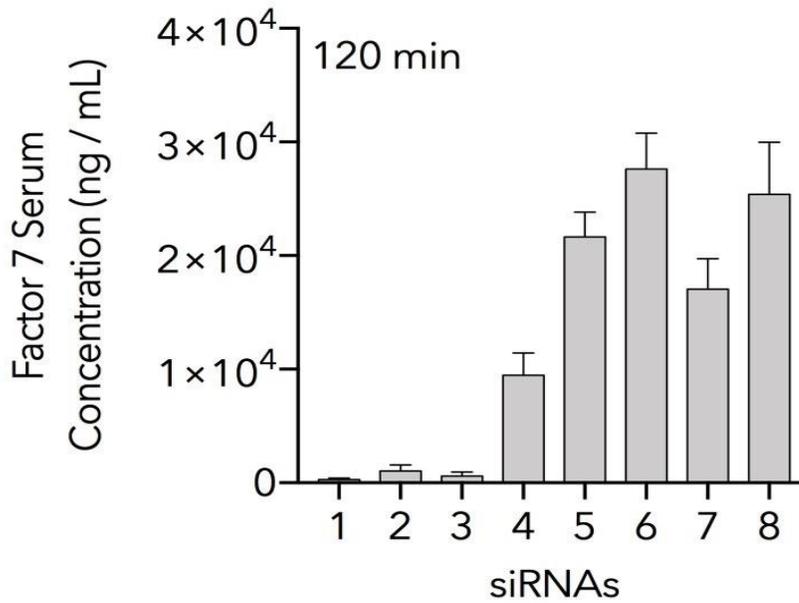


FIG. 11: Bar chart of siFVII levels in serum for siFVII monomer and siFVII multimers at 120 mins.

The area under each of the half-life curves in FIG. 7 serves as a proxy for bioavailability, both in absolute units (FIG. 12) and normalized to monomer (Table 5 and FIG. 13).

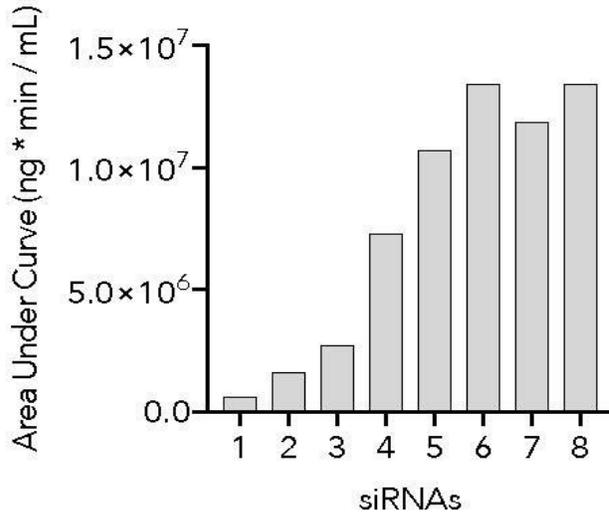


FIG. 12: Bar Chart of total siFVII levels in serum (Area Under the Curve) for siFVII monomer and siFVII multimers.

TABLE 5: Area Under the Curve – i) Total AUC - absolute numbers in minutes \* ng / mL; and ii) AUC normalized to monomer.

	Monomer	Dimer	3-mer	4-mer	5-mer	6-mer	7-mer	8-mer
5	34,245.0	82272	155182	185882	217903	227496	221433	231173
30	3,165.0	6302	16889	85180	91357	133832	126609	129128
60	1,364.0	2460	4779	26102	48935	73257	67669	69315
120	307.0	1043	612	9471	21656	27268	17058	25407
360	6.6	625	20	86	23.9	50.6	77	115
<b>Total AUC (minute * ng / mL)</b>	<b>621727</b>	<b>1604630</b>	<b>2713490</b>	<b>7271583</b>	<b>10689448</b>	<b>13420917</b>	<b>11862813</b>	<b>13384588</b>
<b>AUC, Normalized to Monomer</b>	<b>1.0</b>	<b>2.6</b>	<b>4.4</b>	<b>11.7</b>	<b>17.2</b>	<b>21.6</b>	<b>19.1</b>	<b>21.5</b>

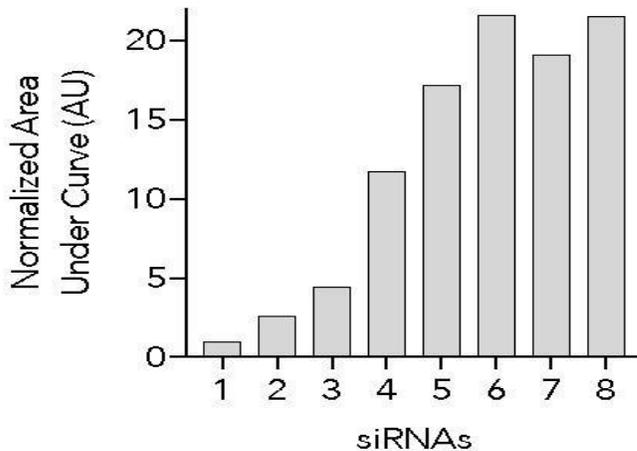


FIG. 13: Bar Chart of Total siFVII levels in serum (Area Under the Curve) for siFVII multimers normalized to monomer.

## Calculation of Time Taken for Multimers to Reach Same FVII siRNA Concentration as Monomer at 5 minutes

Because the FVII concentration of the monomer was already significantly less than 50% of that injected at the first sample time (5 minutes), the times taken for the serum FVII levels of the multimers to equal that of the monomer at 5 minutes were also calculated using the following equation:

$$Y = (Y_0 - \text{Plateau}) * \exp(-K * X) + \text{Plateau}$$

wherein plateau was set at the concentration of monomer at 5 minutes (34245 ng/ml) (FIG. 14 and Table 6).

TABLE 6: Calculated times for homomultimers to reach concentration of FVII monomer at 5 minutes.

34245 = (231173 - 0) * e <sup>-kx</sup> + 0, where x is minutes										
34245 = 231173 * e <sup>-kx</sup>	Monomer	Dimer	3-mer	4-mer	5-mer	6-mer	7-mer	8-mer		
0.14813453 = e <sup>-kx</sup>	K values for different constructs									
ln(0.14813453) = -kx	0.3819	0.1882	0.08203	0.03487	0.02683	0.01996	0.02015	0.019		
-1.909625779	5.0	10.1	23.3	54.8	71.2	95.7	94.8	100.5	Time (min)	

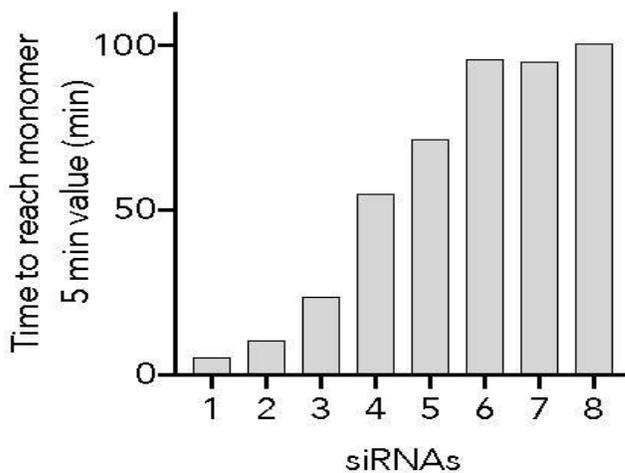


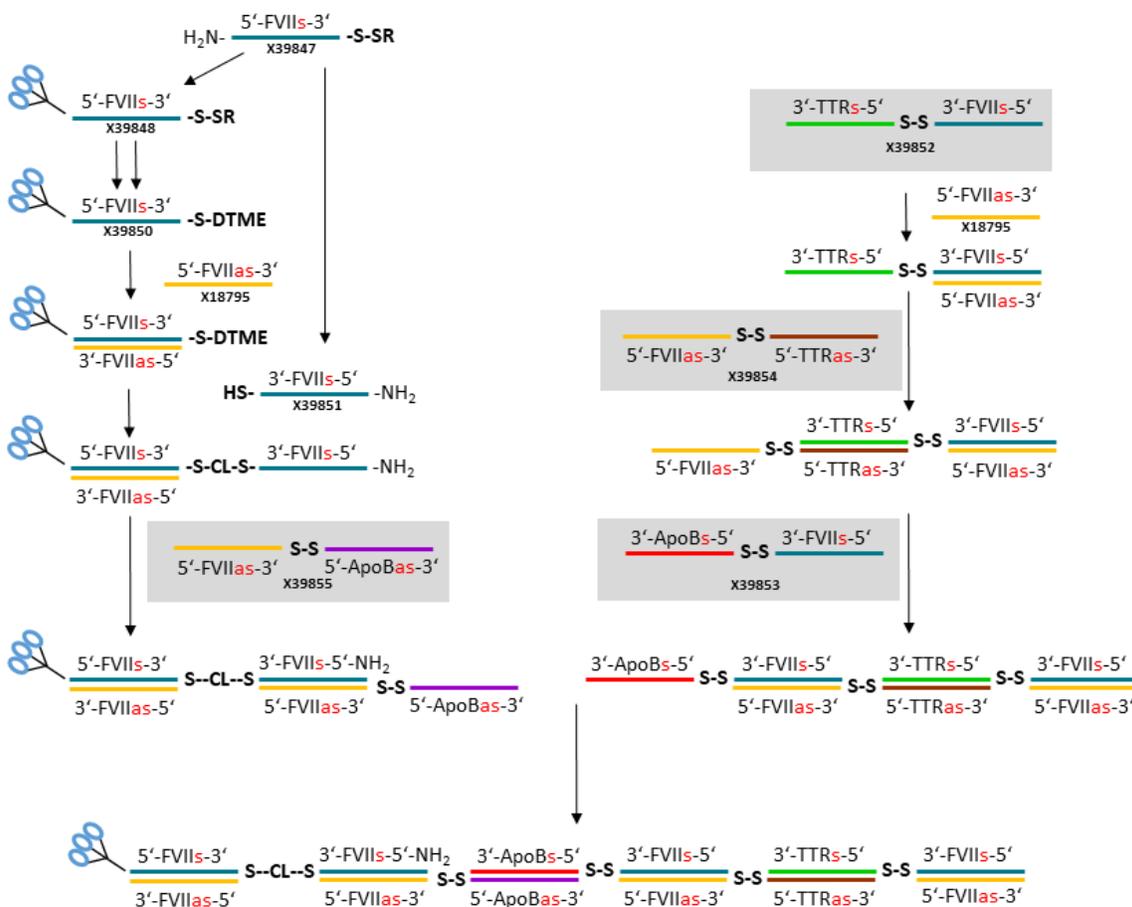
FIG. 14: Bar Chart of time taken for multimers to reach same FVII siRNA concentration as monomer at 5 minutes

## F. Demonstration of Increased Bioactivity of Hexameric vs Monomeric siRNA

To test the hypothesis that individual siRNA units in a large multimeric siRNA would have an effective bioactivity greater than a monomeric equivalent, a GalNAc-conjugated heterohexamer containing four copies of siFVII, one copy of siApoB, and one copy of siTTR was prepared and its activity determined in mice.

### Preparation of a GalNAc-conjugated 4:1:1 Heterohexamer

4.4 mg of a GalNAc-conjugated heterohexameric siRNA containing 4 units of siFVII, 1 unit of siApoB, and 1 unit of siTTR, was prepared in high yield and purity by the procedure depicted in Scheme 7 using materials prepared on the synthesizer and linked via the methods described in General Procedures.



Scheme 7: Synthesis of GalNAc-4:1:1-siFVII-siApoB-siTTR Heterohexamer.

Monomeric siFVII sense strand (X39847) with disulfide and amino groups at the 3'- and 5'-termini, respectively, and monomeric siFVII antisense strand (X18795) were prepared on the synthesizer. Half of the quantity of X39847 was then derivatized with GalNAc ligand to form X39848, treated sequentially with DTT and DTME to form X39850, which was then annealed with the siFVII antisense strand (X18795). The other half of the quantity of X39847 was treated solely with DTT and the resulting thiol derivative (X39851) was reacted with the GalNAc siFVII-mono-DTME derivative previously obtained to yield a partially double-stranded and partially single-stranded dimerical siFVII GalNAc conjugate.

Separately, four single-stranded asymmetric heterodimers of various combinations of siFVII, siApoB and siTTR sense and antisense strands, linked by disulfide groups, were prepared on the synthesizer in high purity (X39852, X39853, X39854 and X39855 – indicated in the gray boxes in Scheme 7, above), as shown in FIG. 15.

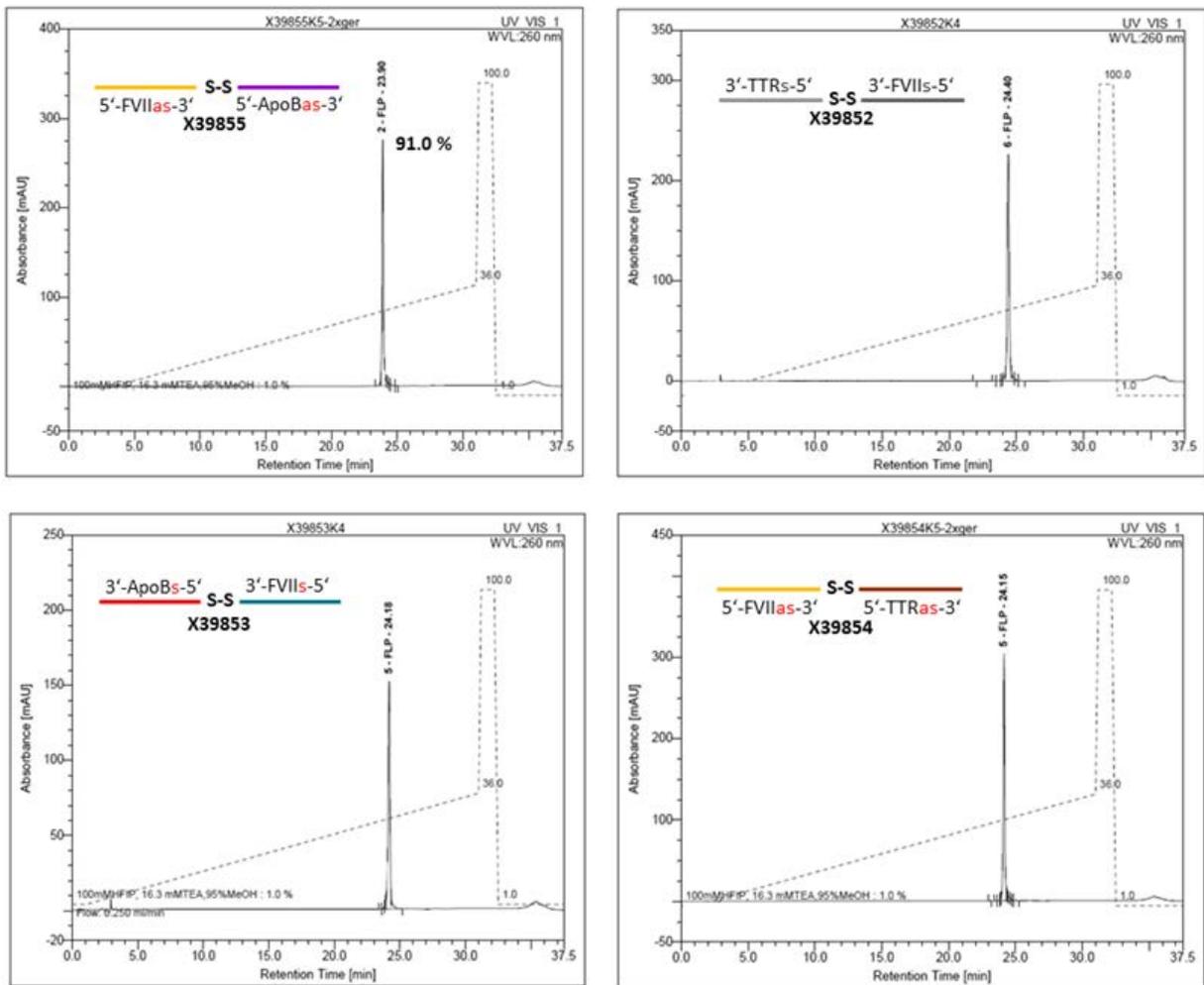


FIG. 15: Reverse phase HPLC traces of asymmetric single-stranded heterodimers of siFVII, siApoB and siTTR (X39852, X39853, X39854 and X39855).

These materials were annealed together with the previously obtained partially double-stranded and partially single-stranded dimerical siFVII GalNAc conjugate to form the GalNAc-conjugated 4:1:1 heterohexamer according to Scheme 7 via the methods described in General Procedures. Each annealing was performed using essentially equimolar proportions of reactants and the products were obtained in near quantitative yields in all cases, as shown in FIG. 16.

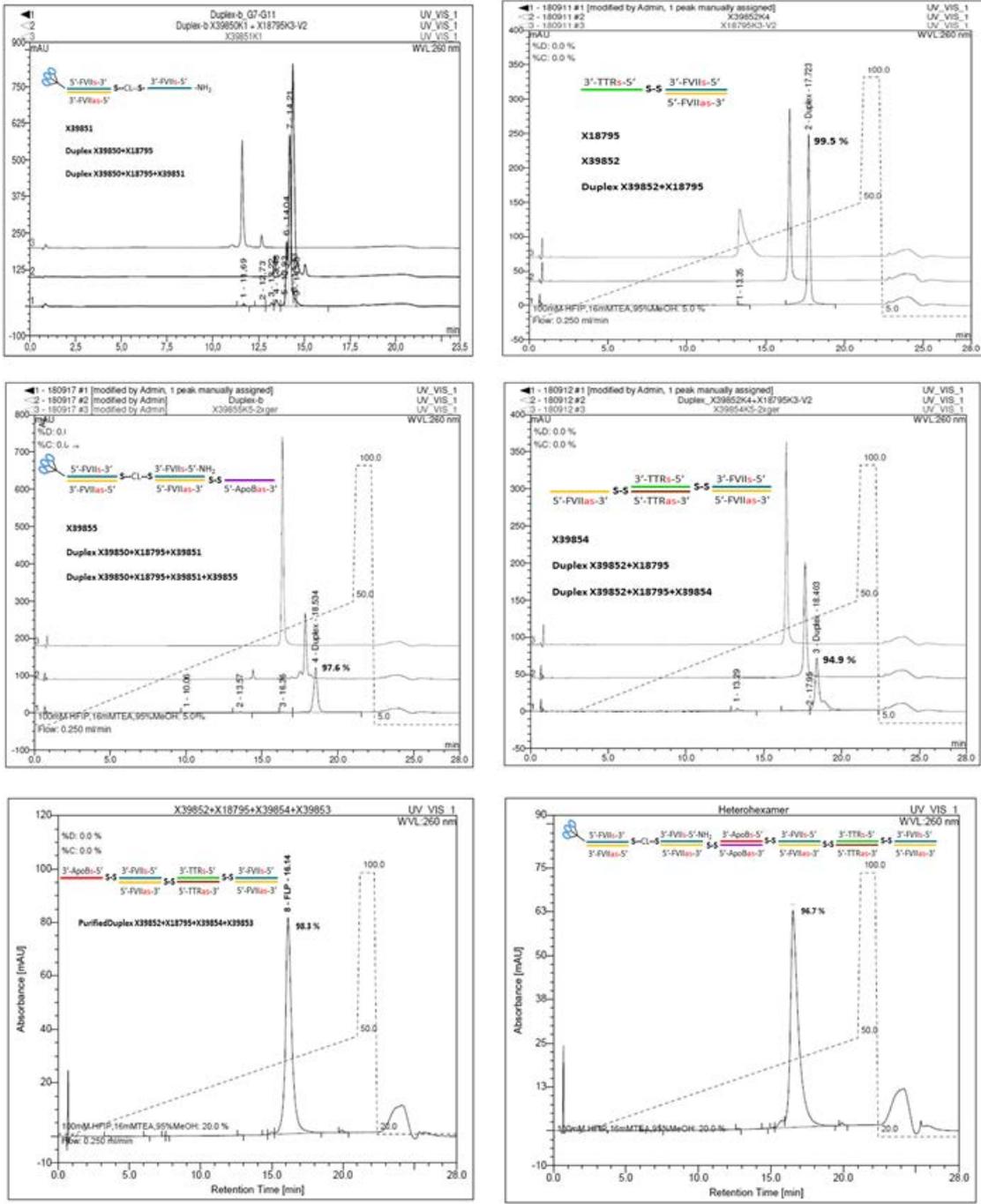


FIG. 16: Reverse Phase HPLC traces of annealing steps in the assembly of the GalNAc-conjugated 4:1:1 siFVII:siApoB:siTTR heterohexamer.

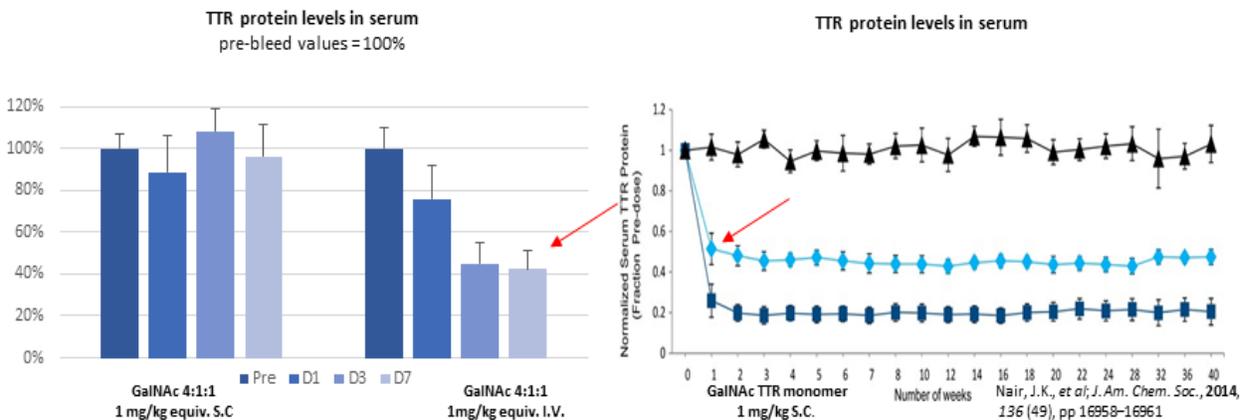
## Determination of Bioactivity of the GalNAc-conjugated 4:1:1 Heterohexamer

The GalNAc-conjugated 4:1:1 siFVII:siApoB:siTTR heterohexamer was administered to mice at 6 mg/kg in PBS via both iv and sc administration routes (each at 0.2 ml). This overall dosage of 6mg/kg provided effective doses of 4, 1, and 1 mg/kg of FVII, ApoB, and TTR siRNAs, respectively. Group sizes were n=5 mice. Blood was collected 1 day prior to treatment and at 1, 3, and 7 days post-treatment, and serum levels of FVII, ApoB, and TTR were measured. mRNA levels from liver lysates at day 7 were also determined. Silencing results for TTR and FVII are plotted in FIG. 17 (left-hand chart) and FIG. 19 (left-hand and middle charts), respectively.

The TTR silencing data shows that the GalNAc-conjugated 4:1:1 heterohexamer was essentially inactive when administered via the sc route (FIG. 17, left-hand chart, left-hand set of bars), suggesting it was perhaps too large to permeate the relevant biological tissues.

By contrast, the GalNAc-conjugated heterohexamer was highly active when administered via iv – producing approximately 60% knockdown of TTR at day 7 (with an effective dose of only 1 mg/kg siTTR) (FIG. 17, left-hand chart, right-hand set of bars).

Further, we observed that TTR knockdown by the single siTTR unit within our heterohexamer (effective dose of 1 mg/kg, delivered iv) more than equaled the reported TTR knockdown<sup>1</sup> by a GalNAc-conjugated monomeric siTTR with virtually the same sequence<sup>2</sup> delivered sc at 1 mg/kg. Compare our silencing data in FIG. 17, left-hand chart, right-hand set of bars with the Nair et al. silencing data in FIG. 17, right-hand chart – [aqua data points](#). (Red arrows added for emphasis.)



<sup>1</sup> Nair, J.K., et al., J. AM. CHEM. SOC., 2014, 136 (49), pp 16958-16961.

<sup>2</sup> Our siTTR sequence and the Nair et al. siTTR sequence are virtually identical in terms of RNA sequence and chemical modifications; the only difference being that the Increscent® sequence has an inverted thymidine on the 3' end of the sense strand, whereas the Nair et al. sequence does not. While likely of no consequence, the Nair et al. siTTR is conjugated to GalNAc on the 3' end of the sense strand; whereas the Increscent® siTTR is conjugated to GalNAc via the 5' end of the sense strand. Finally, the GalNAc sequences are identical in the active sugar regions, but differ in the spacer and linker portions of the sequences.

FIG. 17: Left-hand chart: knockdowns of TTR protein in serum at days 1,3 and 7 achieved with 6 mg/kg GalNAc-heterohexamer (equivalent to 1 mg/kg siTTR) via sc (left) and iv (right) (day 7 iv result indicated by red arrow). Right-hand chart: reported knockdown by 1 mg/kg GalNAc-siTTR monomer via sc at day 7 (indicated by red arrow). Each of the charts show protein levels normalized to pre-bleed levels (100% for the left-hand chart; 1 for the right-hand chart).

Knockdown of TTR mRNA via iv administration of the single unit of siTTR in our GalNAc-conjugated heterohexamer (at the effective dose of 1 mg/kg) was approximately 30% (see FIG. 18).

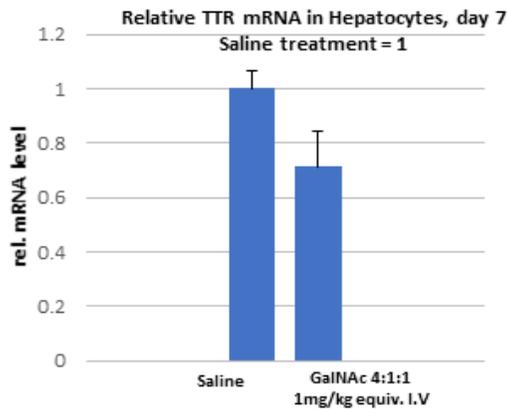
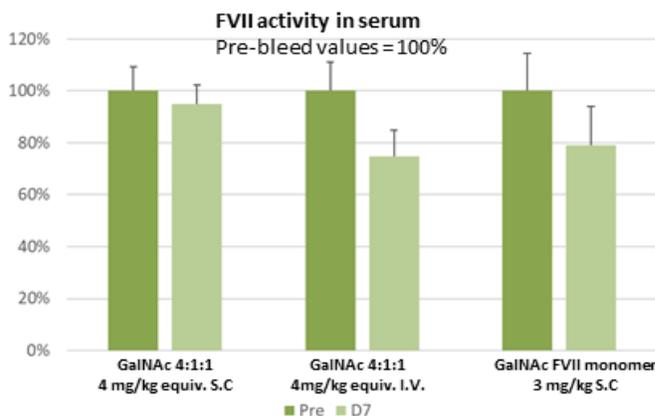


FIG. 18: Relative levels of TTR mRNA from liver lysates obtained on day 7 from the saline control group and from the iv-treatment group.

FVII levels measured in blood samples taken pre-treatment and on day 7 of the mouse study from the iv and sc heterohexamer treatment groups (each receiving 6 mg/kg GalNAc-conjugated heterohexamer, equivalent to 4 mg/kg siFVII) are presented in FIG 19, left-hand and middle sets of bars. The FVII levels from an earlier mouse study in which GalNAc-conjugated FVII monomer (identical sequence) was administered sc at 3 mg/kg are presented for comparison in FIG. 19, right-hand set of bars.



*FIG. 19: Knockdowns of FVII activity in serum at days -1 (“Pre”) and 7 achieved with 6 mg/kg GalNAc-conjugated heterohexamer (equivalent to 4 mg/kg siFVII) via sc (left-hand set of bars) and iv (middle set of bars); knockdown data represented by the right-hand set of bars is from a previous study in which 3 mg/kg of a GalNAc-conjugated siFVII monomer was delivered via sc.*

As demonstrated in FIG. 19, the GalNAc-conjugated heterohexamer (having an effective siFVII dose of 4 mg/kg siFVII) was relatively inactive when delivered via sc. When the same material was delivered via iv, however, it produced approximately 25% silencing of FVII – demonstrating approximately the same potency as a GalNAc-conjugated siFVII monomer (of the same sequence) which produced approximately 20% silencing at a dose of 3 mg/kg via sc in a previous study.

Together with the previous results, the heterohexamer data suggest that the effect (per dose) of an oligonucleotide delivered via intravenous administration may be greatly increased by administering the oligonucleotide in hexameric or larger form to maximize serum half-life and hence bioavailability with minimal to no increase in associated toxicity.

While our results were produced with the highly efficient GalNAc-ASGPR system, the potential increase in the relative dose effectiveness of large multimers versus monomers likely will be even greater in ligand-receptor systems other than GalNAc-ASGPR, due to the likely lower potencies of such ligand-receptor systems due to lower cell count, lower receptor copy number and/or suboptimal internalization rates. One or more of these adverse factors likely will make administration of monomeric siRNAs and other oligonucleotide therapeutics ineffective, as diminished uptake by a target receptor will be compounded by rapid loss of therapeutic agent through the kidney and/or other elimination organs or systems. This problem may be overcome by administration of multimeric oligonucleotides, which, by virtue of their increased bioavailability will enable receptors present on a suboptimal number of cells, or present on cells in suboptimal copy number, to have more opportunity to bind the targeting ligand and internalize the payload. Likewise, the increased bioavailability of multimeric oligonucleotides will allow for inefficient receptors to have more time to bind the targeting ligand, internalize the payload, and “reload” for another round of delivery. In all of these scenarios, the improved delivery conditions brought about by administration of multimeric oligonucleotides are further enhanced by virtue of the multimeric payload, which, if configured as a homomultimer (at least in part), delivers multiple active copies of a single therapeutic agent across the cell membrane in a single binding event. Thus, the approach that we have demonstrated may be designed to be “double-barreled” in the sense that both PK and PD enhancement (stemming from increased bioavailability and a larger payload) are obtained simultaneously through multimerization of the therapeutic agent without the use of added agents or materials.

## General Procedures

### Single Chain Oligonucleotide Synthesis

Oligoribonucleotides were assembled on ABI 394 and 3900 synthesizers (Applied Biosystems) at the 10  $\mu\text{mol}$  scale, or on an Oligopilot 10 synthesizer at 28  $\mu\text{mol}$  scale, using phosphoramidite chemistry. Solid supports were polystyrene loaded with 2'-deoxythymidine (Glen Research, Sterling, Virginia, USA), or controlled pore glass (CPG, 520 $\text{\AA}$ , with a loading of 75  $\mu\text{mol/g}$ , obtained from Prime Synthesis, Aston, PA, USA). Ancillary synthesis reagents, DNA-, 2'-O-methyl RNA-, and 2'-deoxy-2'-fluoro-RNA phosphoramidites were obtained from SAFC Proligo (Hamburg, Germany). Specifically, 5'-O-(4,4'-dimethoxytrityl)-3'-O-(2-cyanoethyl-N,N-diisopropyl) phosphoramidite monomers of 2'-O-methyl-uridine (2'-OMe-U), 4-N-acetyl-2'-O-methyl-cytidine (2'-OMe-C<sup>Ac</sup>), 6-N-benzoyl-2'-O-methyl-adenosine (2'-OMe-A<sup>bz</sup>) and 2-N-isobutylguanosine (2'-OMe-G<sup>ibu</sup>) were used to build the oligomer sequences. 2'-Fluoro modifications were introduced employing the corresponding phosphoramidites carrying the same nucleobase protecting groups as the 2'-OMe RNA building blocks. Coupling time for all phosphoramidites (70 mM in Acetonitrile) was 3 min employing 5-Ethylthio-1H-tetrazole (ETT, 0.5 M in Acetonitrile) as activator. Phosphorothioate linkages were introduced using 50 mM 3-((Dimethylamino-methylidene)amino)-3H-1,2,4-dithiazole-3-thione (DDTT, AM Chemicals, Oceanside, California, USA) in a 1:1 (v/v) mixture of pyridine and acetonitrile.

Upon completion of the solid phase synthesis, including removal of the DMT group ("DMT off synthesis"), oligonucleotides were cleaved from the solid support and deprotected using a 1:1 mixture consisting of aqueous methylamine (41%) and concentrated aqueous ammonia (32%) for 3 hours at 25°C according to published methods. Wincott, F., *et al*, Synthesis, deprotection, analysis and purification of RNA and ribozymes, NUCLEIC ACIDS RES, 23: 2677-2684 (1995).

Subsequently, crude oligomers were purified by anionic exchange HPLC using a column packed with Source Q15 (GE Healthcare) and an AKTA Explorer system (GE Healthcare). Buffer A was 10 mM sodium perchlorate, 20 mM Tris, 1 mM EDTA, pH 7.4 (Fluka, Buchs, Switzerland) in 20% aqueous acetonitrile and buffer B was the same as buffer A with 500 mM sodium perchlorate. A gradient of 22% B to 42% B within 32 column volumes (CV) was employed. UV traces at 280 nm were recorded. Appropriate fractions were pooled and precipitated with 3M NaOAc, pH=5.2 and 70% ethanol. Pellets were collected by centrifugation. Alternatively, desalting was carried out using Sephadex HiPrep columns (GE Healthcare) according to the manufacturer's recommendations.

Oligonucleotides were reconstituted in water and identity of the oligonucleotides was confirmed by electrospray ionization mass spectrometry (ESI-MS). Purity was assessed by analytical anion-exchange HPLC.

5'-aminohexyl linkers were introduced employing the TFA-protected hexylamino-linker phosphoramidite (Sigma-Aldrich, SAFC, Hamburg, Germany). 3'-hexylamino-linkers were introduced using a phthalimido protected hexylamino-linker immobilized on CPG (Prime

Synthesis, Aston, PA, USA). Deprotection and purification were performed as above.

## Generation of Thiol-terminated Oligomer

3'- or 5'-terminal thiol groups were introduced to the oligomers via 1-O-Dimethoxytrityl-hexyl-disulfide,1'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite linker (NucleoSyn, Olivet Cedex, France). After deprotection and purification as above, each disulfide containing oligomer was reduced using dithiothreitol (DTT) (0.1 M DTT stock solution) (Sigma-Aldrich Chemie GmbH, Munich, Germany, #646563) in triethylammonium bicarbonate buffer (TEABc, 0.1M, pH 8.5, Sigma, #90360). The oligonucleotide was dissolved in TEABc buffer (100mM, pH 8.5) to yield a 1 mM solution. To accomplish the disulfide reduction a 50- to 100-fold molar excess of DTT was added to the oligonucleotide solution. The progress of the reduction was monitored by analytical AEX HPLC on a Dionex DNA Pac 200 column (4x 250 mm) obtained from Thermo Fisher. The reduced material, i.e., the corresponding thiol (C6SH), elutes prior to the starting material. After completion of the reaction, excess reagent is removed by size exclusion chromatography using a HiPrep column from GE Healthcare and water as eluent. Subsequently, the oligonucleotide is precipitated using 3 M NaOAc (pH 5.2) and ethanol and stored at minus 20 °C.

## Preparation of Mono-DTME Oligomer

Thiol-modified oligonucleotide was dissolved in 300 mM NaOAc (pH 5.2) containing 25% acetonitrile to give a 20 OD/mL solution. 40 equivalents dithiobismaleimidoethane (DTME, Thermo Fisher, # 22335) were dissolved in acetonitrile to furnish a 15.6 mM solution. The DTME solution was added to the oligonucleotide-containing solution and agitated at 25 °C on a Thermomixer (Eppendorf, Hamburg, Germany). Progress of the reaction was monitored by analytical AEX HPLC using a Dionex DNA Pac200 column (4x 250 mm). Depending on the required purity level, excess DTME is either removed by size exclusion HPLC using a HiPrep column (GE Healthcare) or the crude reaction mixture is purified by preparative AEX HPLC using a column packed with Source 15 Q resin commercially available from GE Healthcare.

## Preparation of Dimer via DTME Functionality

The DTME-modified oligonucleotide prepared according to the above procedure was reacted with another oligonucleotide equipped with a thiol linker. This reaction could either be carried out on the single-stranded sequence, or after prior annealing of the complementary oligonucleotide of one of the reaction partners. Consequently, if desired, the DTME-modified oligonucleotide was reacted with the thiol modified oligonucleotide directly, or was annealed with its complementary strand, and the resulting duplex reacted with the thiol-modified oligonucleotide. Alternatively, the thiol-modified oligonucleotide was annealed with its complementary strand and this duplex reacted with the DTME-modified single strand. In all cases, the reaction was carried out in aqueous solution in the presence of 300 mM NaOAc (pH 5.2).

## **Annealing of Single-Stranded RNAs (ssRNAs) to Form Double-Stranded RNA (dsRNA)**

dsRNAs were generated from RNA single strands by mixing a slight excess of the required complementary antisense strand(s) relative to sense strand and annealing in 20 mM NaCl/4 mM sodium phosphate pH 6.8 buffer. Successful duplex formation was confirmed by native size exclusion HPLC using a Superdex 75 column (10 x 300 mm) from GE Healthcare. Samples were stored frozen until use.

In the sequences described herein, upper case letters “A”, “C”, “G” and “U” represent RNA nucleotides; lower case letters “c”, “g”, “a”, and “u” represent 2'-O-methyl-modified nucleotides; “s” represents phosphorothioate; “dT” represents deoxythymidine residues; upper case letters A, C, G, U followed by “f” indicate 2'-fluoro nucleotides; “(SHC6)” represents a thiohexyl linker; “(DTME)” represents the cleavable homobifunctional crosslinker dithiobismaleimidoethane; “C6NH<sub>2</sub>” and “C6NH” are used interchangeably to represent the aminohexyl linker; “C6SSC6” represents the dihexyldisulfide linker; and “InvdT” means inverted deoxythymidine.

## **Generation of Multimeric siRNAs by Sequential Annealing**

Preparation of multimeric siRNAs via stepwise annealing was performed in water and utilized stepwise addition of complementary strands. No heating/cooling of the solution was required. After each addition, an aliquot of the annealing solution was removed and monitored for duplex formation using analytical RP HPLC under native conditions (20°C). The required amounts to combine equimolar amounts of complementary single strands were calculated based on the extinction coefficients for the individual single strands computed by the nearest neighbor method. If the analytical RP HPLC trace showed excess single strand, additional amounts of the corresponding complementary strand were added to force duplex formation (“duplex titration”).

Duplex titration was monitored using a Dionex Ultimate 3000 HPLC system equipped with a XBride C18 Oligo BEH (2.5 μm; 2.1x50 mm, Waters) column equilibrated to 20°C. The diagnostic wavelength was 260 nm. Buffer A was 100 mM hexafluoro-isopropanol (HFIP), 16.3 mM triethylamine (TEA) containing 1 % methanol. Buffer B had the same composition except MeOH was 95 %. A gradient from 5 % to 70 % buffer B in 30 minutes was applied at a flow rate of 250 μL/min. The two complementary strands were run independently to establish retention times. Then, the aliquot containing the duplex solution was analyzed and compared to the retention times of the constituent single strands. In case the duplex solution showed a significant amount of single strand, the corresponding complementary strand was added to the duplex solution.