DEFINED MULTI-CONJUGATE OLIGONUCLEOTIDES

FIELD OF THE INVENTION

[0001] The present invention relates to defined multi-conjugate oligonucleotides having predetermined sizes and compositions. For example, in various embodiment, the present invention relates to defined multi-conjugate oligonucleotides having advantageous properties, for example in the form of defined multi-conjugate siRNA (i.e., including two, three or more siRNA) having enhanced intracellular delivery and/or multi-gene silencing effects. In various embodiment, the present invention also relates to new synthetic intermediates and methods of synthesizing the defined multi-conjugate oligonucleotides. The present invention also related to methods of using the defined multi-conjugate oligonucleotides, for example in reducing gene expression, biological research, treating or preventing medical conditions, or to produce new or altered phenotypes in cells or organisms.

BACKGROUND

[0002] Currently there are a number of new therapeutic and bioengineering modalities involving the delivery of biologically active molecules such as small interfering RNA (siRNA) and microRNA (miRNA), to name a few, across cell membranes and cell walls to produce biological effects within the cell, such as, in the case of siRNA and miRNA, to suppress protein production. Other techniques and/or biologically active molecules delivered into the cell have the effect of enhancing gene expression and protein production.

[0003] However, RNA and other oligonucleotides in their native state are labile *in vivo* and easily decomposed within a short period of time. Furthermore, many of them, like RNA, are anionic which makes cell membrane transmission difficult, resulting in low intracellular delivery efficiency.

[0004] Taking siRNA as an example, efforts to increase its delivery efficiency include preparation of a nano-sized ionic complex through ionic bonding of siRNA and diverse cationic carrier materials such as cationic polymers, lipids or peptides. Jeong et al., Bioconjugate Chem, 20(1): 5-14 (2009). However, there are challenges associated with the preparation of a stable siRNA/cationic carrier complex.

[0005] Other efforts to increase delivery efficiency of oligonucleotides such as siRNA include conjugation of the oligonucleotide to a specific cell targeting moiety. E.g., Nair et al., "Mulivalent *N*-Acetylgalactosamine-Conjugated siRNA Localizes in Hepatocytes and Elicits Robust RNAi-Mediated Gene Silencing," J Am Chem Soc, 136 (49): 16958-16961 (2014).

[0006] However, these and other prior art approaches do not solve the problems of oligonucleotide delivery. Accordingly, there remains a need for improved oligonucleotide compositions.

SUMMARY OF THE INVENTION

[0007] The present invention relates to defined multi-conjugate oligonucleotides having predetermined sizes and compositions. The present invention also relates to methods of using the defined multi-conjugate oligonucleotides. The present invention also relates to methods of synthesizing the defined multi-conjugate oligonucleotides, as well as new intermediate compounds used in the synthesis of the defined multi-conjugate oligonucleotides.

[0008] Accordingly, the present invention provides RNA and/or DNA multi-conjugates having predetermined sizes and compositions, improved charge density, improved delivery, and/or improved efficacy (e.g., as compared to the same moieties in their unconjugated state). When the multi-conjugates are complexed with a suitable carrier and/or conjugated to another chemical or biological moiety such as a cell-targeting ligand, they can be delivered with greater efficiency and safety across a cell membrane or cell wall for enhanced biological or therapeutic effects.

[0009] Accordingly, advantages of the defined multi-conjugate oligonucleotides of the present invention can include: increasing oligonucleotide delivery to a cell (e.g., delivering more oligonucleotide per cell targeting ligand binding event), the ability to deliver a predetermined stoichiometric ratio of different oligonucleotides to a cell (e.g., 1:1:1 in the case of a trimeric multi-conjugate comprising three different oligonucleotides), and/or the ability to deliver a combination of therapeutic oligonucleotides as a single chemical entity (e.g., a trimeric multi-conjugate comprising three different oligonucleotides is one molecule) thus simplifying their use and regulatory review.

[0010] The invention is also based, at least in part, upon the development of new synthetic methodology and intermediates, which allow the preparation of the defined multi-conjugate oligonucleotides having predetermined sizes and compositions.

[0011] In various aspects, the invention provides an oligonucleotide coupled to a covalent linker, which can be used, for example, in the synthesis of defined multi-conjugate oligonucleotides having predetermined sizes and compositions.

[0012] In one aspect, the invention provides a compound according to Structure 1:

X - R1 - R2 - A - R3 - B (Structure 1)

wherein:

X is a nucleic acid bonded to R1 through its 3' or 5' terminus;

R1 a phosphodiester, thiophosphodiester, sulfate, amide, glycol, or is absent;

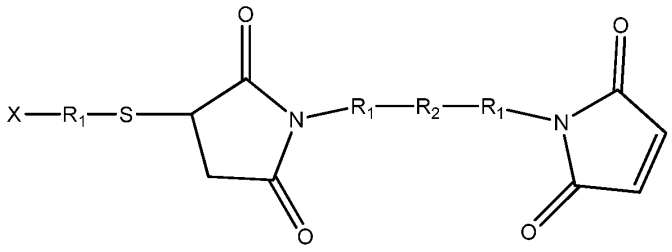
R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

A is the reaction product of a nucleophile and an electrophile;

R3 is a C2-C10 alkyl, alkoxy, aryl, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and

B is a nucleophile or electrophile.

[0013] In one aspect, the invention provides a compound according to Structure 2:



(Structure 2)

wherein:

X is a nucleic acid bonded to R1 via a phosphate or thiophosphate at its 3' or 5' terminus;

each R1 is independently a C2-C10 alkyl, alkoxy, or aryl group; and

R2 is a thiopropionate or disulfide group.

[0014] In one aspect, the invention provides a compound according to Structure 3:

X - R1 - R2 - A - R3 - B (Structure 3)

wherein:

X is a nucleic acid bonded to R1 through its 3' or 5' terminus;

R1 a phosphate, thiophosphate, sulfate, amide, glycol, or is absent;

R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

A is the reaction product of a first and a second reactive moiety;

R3 is an C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and

B is a third reactive moiety.

[0015] In various aspects, the invention provides methods for synthesizing an oligonucleotide coupled to a covalent linker.

[0016] In one aspect, the invention provides a method for synthesizing a compound according to Structure 1 (or adapted for synthesizing a compounds according to Structure 2 or 3), the method comprising:

reacting a functionalized nucleic acid X - R1 - R2 - A' and a covalent linker A'' - R3 - B, wherein A' and A'' comprise a nucleophile and an electrophile, in a dilute solution of X - R1 - R2 - A' and with a stoichiometric excess of A'' - R3 – B, thereby forming the compound X - R1 - R2 - A - R3 - B (Structure 1)

wherein:

X is a nucleic acid bonded to R1 through its 3' or 5' terminus;

R1 a phosphodiester, thiophosphodiester, sulfate, amide, glycol, or is absent;

R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

A is the reaction product of a nucleophile and an electrophile;

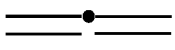
R3 is a C2-C10 alkyl, alkoxy, aryl, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and

B is a nucleophile or electrophile.

[0017] The method can further comprise the step of synthesizing the functionalized nucleic acid X - R1 - R2 - A', wherein A' comprises a thiol (-SH) by (i) introducing a the thiol during solid phase synthesis of the nucleic acid using phosphoramidite oligomerization chemistry or (ii) reduction of a disulfide introduced during the solid phase synthesis.

[0018] In various aspects, the invention provides dimeric defined multi-conjugate oligonucleotides.

[0019] In one aspect, the invention provides an isolated compound according to Structure 4:

 (Structure 4)

wherein:

each ——— is a double stranded oligonucleotide designed to react with the same molecular target *in vivo*, and

• is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, and having the structure - R1 - R2 - A - R3 - A - R2 - R1 - wherein:

each R1 is independently a phosphodiester, thiophosphodiester, sulfate, amide, glycol, or is absent;

each R2 is independently a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

each A is independently the reaction product of a nucleophile and an electrophile, and

R3 is a C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

[0020] In one aspect, the invention provides an isolated compound according to Structure 5:

 (Structure 5)

wherein:

——— is a first single stranded oligonucleotide

 is a second single stranded oligonucleotide having a different sequence from the first, and

● is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, and having the structure - R1 - R2 - A - R3 - A - R2 - R1 - wherein:

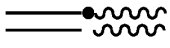
each R1 is independently a phosphate, sulfate, amide, glycol, or is absent;

each R2 is independently a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

each A is independently the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group, and

R3 is an C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

[0021] In one aspect, the invention provides an isolated compound according to Structure 6:

 (Structure 6)

wherein:

 is a first double stranded oligonucleotide

 is a second double stranded oligonucleotide having a different sequence from the first, and

● is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, and having the structure - R1 - R2 - A - R3 - A - R2 - R1 - wherein:

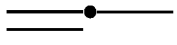
each R1 is independently a phosphate, sulfate, amide, glycol, or is absent;

each R2 is independently a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

each A is independently the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group, and

R3 is an C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

[0022] In one aspect, the invention provides an isolated compound according to Structure 11:

 (Structure 11)

wherein:

 is a double stranded oligonucleotide,

 is a single stranded oligonucleotide, and

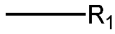
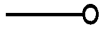
• is a covalent linker joining single strands of adjacent single stranded oligonucleotides.

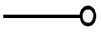
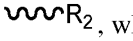
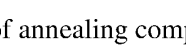
[0023] In various aspects, the invention provides methods for synthesizing dimeric defined multi-conjugate oligonucleotides.

[0024] In one aspect, the invention provides a method for synthesizing a compound of Structure 5:

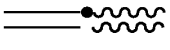
 (Structure 5)

wherein  is a first single stranded oligonucleotide, is a second single stranded oligonucleotide having a different sequence from the first, and • is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

(i) reacting a first single stranded oligonucleotide  with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○ under conditions that produce the mono-substituted product ;

(ii) reacting  with a second single stranded oligonucleotide , wherein R2 is a chemical group capable of reacting with ○, thereby forming .

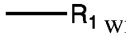
[0025] The method can further comprise the step of annealing complementary  and  to yield Structure 6:

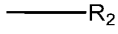
 (Structure 6).

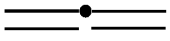
[0026] In one aspect, the invention provides a method for synthesizing an isolated compound of Structure 4:

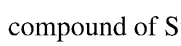
 (Structure 4)

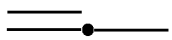
wherein each is a double stranded oligonucleotide and • is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

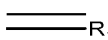
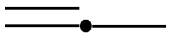
(i) reacting a first single stranded oligonucleotide  with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○, thereby forming a mono-substituted product ;

(ii) reacting  with a second single stranded oligonucleotide , wherein R2 is a chemical group capable of reacting with ○, thereby forming a single stranded dimer ;

(iii) annealing single stranded oligonucleotides, at the same time or sequentially, thereby forming .

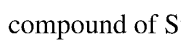
[0027] In one aspect, the invention provides a method for synthesizing an isolated compound of Structure 4:  (Structure 4) wherein each is a double stranded oligonucleotide and • is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

(i) forming  by:

(a) annealing a first single stranded oligonucleotide and a second single stranded oligonucleotide , thereby forming , and reacting  with a third single stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ; or

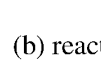
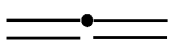
(b) reacting the second single stranded oligonucleotide and the third single stranded oligonucleotide , thereby forming , and annealing the first single stranded oligonucleotide and , thereby forming ;

(ii) annealing  and a fourth single stranded oligonucleotide , thereby forming .

[0028] In one aspect, the invention provides a method for synthesizing an isolated compound of Structure 4:  (Structure 4) wherein each is a double stranded oligonucleotide and • is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

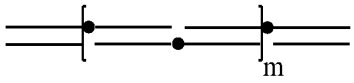
(a) annealing a first single stranded oligonucleotide and a second single stranded oligonucleotide , thereby forming ;

(b) annealing a third single stranded oligonucleotide  and a fourth single stranded oligonucleotide , thereby forming ;

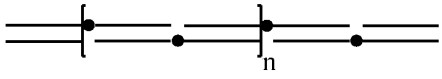
(b) reacting  and with wherein R1 and R2 , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming .

[0029] In various aspects, the invention provides multimeric (n>2) defined multi-conjugate oligonucleotides, including defined tri-conjugates and defined tetraconjugates.

[0030] In one aspect, the invention provides a compound according to Structure 7 or 8:



(Structure 7)



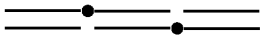
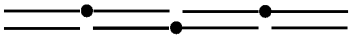
(Structure 8)

wherein:

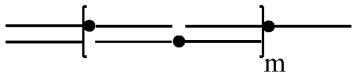
each ═══ is a double stranded oligonucleotide,

each ● is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and

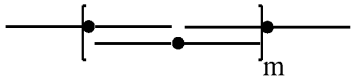
m is an integer ≥ 1 and n is an integer ≥ 0.

[0031] In one aspect, the invention provides a compound according to Structure 9 and wherein n = 0:  (Structure 9). In one aspect, the invention provides a compound according to Structure 10 and wherein m = 1:  (Structure 10).

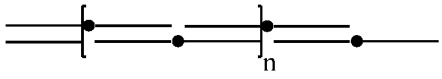
[0032] In one aspect, the invention provides a compound according to Structure 12, 13, 14, or 15:



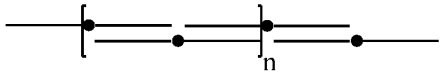
(Structure 12)



(Structure 13)



(Structure 14)



(Structure 15)

wherein:

each ═══ is a double stranded oligonucleotide,

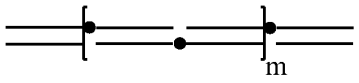
each ——— is a single stranded oligonucleotide,

each ● is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and

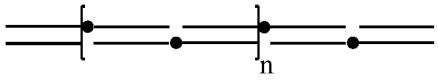
m is an integer ≥ 1 and n is an integer ≥ 0.

[0033] In various aspects, the invention provides methods for synthesizing multimeric (n>2) defined multi-conjugate oligonucleotides, including defined tri-conjugates and defined tetraconjugates.

[0034] In one aspect, the invention provides a method for synthesizing a compound according to Structure 7 or 8:

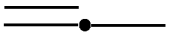


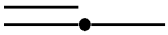
(Structure 7)

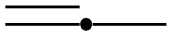


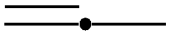
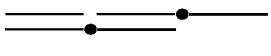
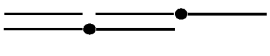
(Structure 8)

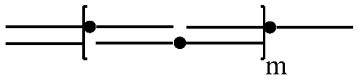
wherein: each ═══ is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and m is an integer ≥ 1 and n is an integer ≥ 0, the method comprising the steps of:

(i) forming  by:

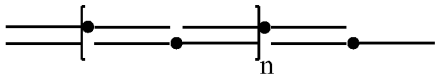
(a) annealing a first single stranded oligonucleotide ——— and a second single stranded oligonucleotide ———R_1, thereby forming ═══R_1, and reacting ═══R_1 with a third single stranded oligonucleotide ———R_2, wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ; or

(b) reacting the second single stranded oligonucleotide ———R_1 and the third single stranded oligonucleotide ———R_2, thereby forming , and annealing the first single stranded oligonucleotide ——— and , thereby forming ;

(ii) annealing  and a second single stranded dimer , thereby forming  and, optionally, annealing one or more additional single stranded dimers to  thereby forming,



or



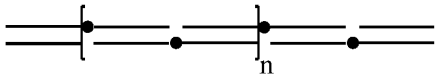
wherein m is an integer ≥ 1 and n is an integer ≥ 0; and

(iii) annealing a fourth single stranded oligonucleotide ——— to the product of step (ii), thereby forming structure 7 or 8.

[0035] In one aspect, the invention provides a method for synthesizing a compound according to Structure 7 or 8:



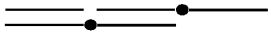
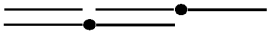
(Structure 7)



(Structure 8)

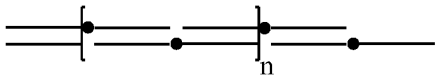
wherein: each ═══ is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and m is an integer ≥ 1 and n is an integer ≥ 0, the method comprising the steps of:

(i) annealing a first single stranded oligonucleotide ——— and a first single stranded dimer ———•———, thereby forming ═══•———;

(ii) annealing ═══•——— and a second single stranded dimer ———•———, thereby forming  and, optionally, annealing one or more additional single stranded dimers ———•——— to  thereby forming,

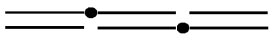


or



wherein m is an integer ≥ 1 and n is an integer ≥ 0; and

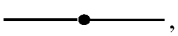
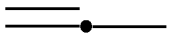
(iii) annealing a second single stranded oligonucleotide ——— to the product of step (ii), thereby forming structure 7 or 8.

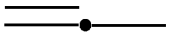
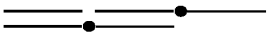
[0036] In one aspect, the invention provides a method for synthesizing a compound of Structure 9:  (Structure 9), wherein each ═══ is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, the method comprising the steps of:

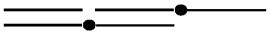
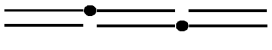
(i) forming ═══•——— by:

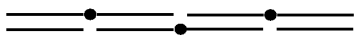
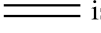
(a) annealing a first single stranded oligonucleotide ——— and a second single stranded oligonucleotide ———R_1, thereby forming ═══R_1, and reacting ═══R_1 with a third single stranded oligonucleotide ———R_2, wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ═══•———; or

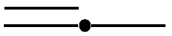
(b) reacting the second single stranded oligonucleotide ———R_1 and the third single stranded oligonucleotide ———R_2, thereby forming ———•———, and

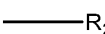
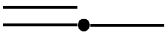
annealing the first single stranded oligonucleotide and , thereby forming ;

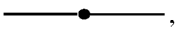
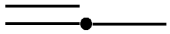
(ii) annealing  and a single stranded dimer , thereby forming ; and

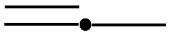
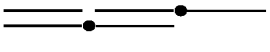
(iii) annealing  and a fourth single stranded oligonucleotide , thereby forming .

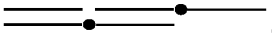
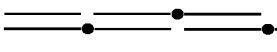
[0037] In one aspect, the invention provides a method for synthesizing a compound of Structure 10:  (Structure 10), wherein each  is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, the method comprising the steps of:

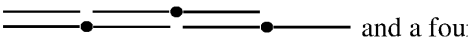
(i) forming  by:

(a) annealing a first single stranded oligonucleotide and a second single stranded oligonucleotide , thereby forming , and reacting  with a third single stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ; or

(b) reacting the second single stranded oligonucleotide and the third single stranded oligonucleotide , thereby forming , and annealing the first single stranded oligonucleotide and , thereby forming ;

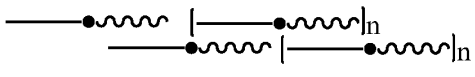
(ii) annealing  and a single stranded dimer , thereby forming ;

(iii) annealing  and a second single stranded dimer , thereby forming ; and

(iv) annealing  and a fourth single stranded oligonucleotide , thereby forming .

[0038] In various aspects, the invention provides sense-antisense multi-conjugate oligonucleotides, as well as methods for their synthesis.

[0039] In one aspect, the invention provides a composition comprising a plurality of molecules, each molecule having Structure 16:

 (Structure 16), wherein n is an integer ≥ 1;

each ——— is a single stranded oligonucleotide;

each ∿∿∿ is a single stranded oligonucleotide that hybridizes with a ———;

∿∿∿
——— is a double stranded oligonucleotide; and

each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides.

[0040] In one aspect, the invention provides a method for synthesizing composition comprising a plurality of molecules, each molecule having Structure 16, the methods comprising:

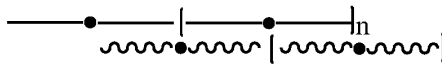
(i) reacting a first single stranded oligonucleotide ———R_1 with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○ under conditions that produce the mono-substituted product ———○;

(ii) reacting ———○ with a second single stranded oligonucleotide ∿∿∿R_2, wherein R2 is a chemical group capable of reacting with ○, thereby forming ———•∿∿∿;

(iii) annealing a plurality of ———•∿∿∿, thereby forming a composition comprising a plurality of molecules, each molecule having Structure 16.

[0041] In various aspects, the invention provides methods for synthesizing multi-conjugate oligonucleotides.

[0042] In one aspect, the invention provides method for synthesizing a composition comprising a plurality of molecule comprising Structure 17:

 (Structure 17), wherein n is an integer ≥ 1; each ——— is a single stranded oligonucleotide; each ∿∿∿ is a single stranded oligonucleotide that hybridizes with a ———; ∿∿∿
——— is a double stranded oligonucleotide; and each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides to form ———•——— and ∿∿∿•∿∿∿, the method comprising the steps of:

annealing a plurality of ———•——— and ∿∿∿•∿∿∿ at:

(i) a total oligonucleotide concentration of about 200-300 μM for ———•——— and ∿∿∿•∿∿∿,

(ii) about 0.1-0.3x phosphate buffered saline (PBS), and

(iii) at a temperature of about 70-80 °C to about 20-30 °C for about 1.5-2.5 hours.

[0043] In various aspects, the invention provides pharmaceutical compositions comprising multi-conjugate oligonucleotides.

[0044] In one aspect, the invention provides a composition (e.g., pharmaceutical composition) comprising (i) a compound or composition according to the invention and (ii) a pharmaceutically acceptable excipient.

[0045] In one aspect, the invention provides a compound or composition according to the invention for use a medicament, or for use in the manufacture of a medicament. The medicament can be for silencing or reducing the expression of at least one overexpressed gene, for example for silencing or reducing the expression of two, three, four, or more overexpressed genes.

[0046] In one aspect, the invention provides a composition (e.g., pharmaceutical composition) comprising a compound or composition according to the invention, formulated in lipid nanoparticles (LNP), exosomes, microvesicles, or viral vectors.

[0047] In various aspects, the invention provides methods for using multi-conjugate oligonucleotides.

[0048] In one aspect, the invention provides a method for reducing gene expression comprising administering an effective amount of a compound or composition according to the invention to a subject in need thereof.

[0049] In one aspect, the invention provides a method for treating a subject comprising administering an effective amount of a compound or composition according to the invention to a subject in need thereof.

[0050] In one aspect, the invention provides a method for silencing two or more genes comprising administering an effective amount of a compound or composition according to the invention to a subject in need thereof, wherein the compound or composition comprises oligonucleotides targeting two or more genes. The compound or composition can comprise oligonucleotides targeting two, three, four, or more genes.

[0051] In one aspect, the invention provides a method for delivering two or more oligonucleotides to a cell per targeting ligand binding event comprising administering an effective amount of a compound or composition according to the invention to a subject in need thereof, wherein the compound or composition comprises a targeting ligand.

[0052] In one aspect, the invention provides a method for delivering a predetermined stoichiometric ratio of two or more oligonucleotides to a cell comprising administering an effective amount of a compound or composition according to the invention to a subject in need

thereof, wherein the compound or composition comprises the predetermined stoichiometric ratio of two or more oligonucleotides.

[0053] In various aspects, the invention provides oligonucleotides having a specific sequence.

[0054] In one aspect, the invention provides an siRNA having SEQ ID NO:106.

[0055] In one aspect, the invention provides an siRNA having SEQ ID NO:115.

[0056] One skilled in the art will recognize that the aspects above can be combined with one or more suitable features described below.

[0057] In various embodiments, a covalent linker (e.g., one or all of •) can comprise the reaction product of a nucleophile and electrophile. For example, a covalent linker (e.g., one or all of •) can comprise the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group. In various embodiments, covalent linkers are not oligonucleotides.

[0058] In various embodiments, the nucleophile and electrophile (e.g., of A in Structure 1 or 4-6) can comprise a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group. Similarly, the reactive moieties in Structure 3 can comprise a nucleophile and electrophile, for example a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group.

[0059] In various embodiments, the nucleophile or electrophile (e.g., of B in Structure 1) can comprise a thiol, maleimide, vinylsulfone, pyridyldisulfide, iodoacetamide, acrylate, azide, alkyne, amine, or carboxyl group.

[0060] In various embodiments, a linker (e.g., • or the linkers shown in Structures 1-3) can comprise the reaction product of a DTME (dithiobismaleimidoethane), BM(PEG)2 (1,8-bis(maleimido)diethylene glycol), BM(PEG)3 (1,11-bismaleimido-triethyleneglycol), BMOE (bismaleimidoethane), BMH (bismaleimidohexane), or BMB (1,4-bismaleimidobutane). For example, the linker • can comprise the reaction product of a thiol and DTME (dithiobismaleimidoethane), BM(PEG)2 (1,8-bis(maleimido)diethylene glycol), BM(PEG)3 (1,11-bismaleimido-triethyleneglycol), BMOE (bismaleimidoethane), BMH (bismaleimidohexane), or BMB (1,4-bismaleimidobutane).

[0061] In various embodiments comprising two or more covalent linkers • (e.g., in Structures 7-16), the linkers are all the same. Alternatively, the compound or composition can comprise two or more different covalent linkers •.

[0062] In various embodiments, in Structure 1,

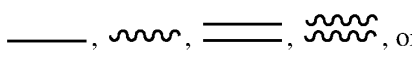
R1 is a phosphodiester or thiophosphodiester;

R2 is a C2-C10 alkyl;

A is the reaction product of a thiol and maleimide;

R3 is a disulfide; and

B is a thiol or maleimide.

[0063] In various embodiments, the nucleic acid (e.g., X) or oligonucleotide (e.g., ) is RNA, DNA, or comprises an artificial or non-natural nucleic acid analog.

[0064] In various embodiments, the nucleic acid or oligonucleotide is DNA, for example an antisense DNA (aDNA) or antisense gapmer.

[0065] In various embodiments, the nucleic acid or oligonucleotide is RNA, for example an antisense RNA (aRNA), CRISPR RNA (crRNA), long noncoding RNA (lncRNA), microRNA (miRNA), piwi-interacting RNA (piRNA), small interfering RNA (siRNA), messenger RNA (mRNA), short hairpin RNA (shRNA), small activating (saRNA), antagomir, or ribozyme. In one embodiment, the RNA is siRNA.

[0066] In various embodiments, the nucleic acid or oligonucleotide is an aptamer.

[0067] In various embodiments, the nucleic acid or oligonucleotide further comprises a chemical modification. The chemical modification can comprise a modified nucleoside, modified backbone, modified sugar, or modified terminus.

[0068] In various embodiments, the nucleic acid or oligonucleotide further comprises a targeting ligand. The targeting ligand can be bound (e.g., directly) to the nucleic acid, for example through its 3' or 5' terminus. In one embodiment, the targeting ligand comprises *N*-Acetylgalactosamine (GalNAc), cholesterol, tocopherol, folate, 2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid (DUPA), or anisamide.

[0069] In various embodiment, the method can include coupling a targeting ligand to the molecule.

[0070] In various embodiments, the nucleic acid or oligonucleotide is single stranded.

[0071] In various embodiments, the nucleic acid or oligonucleotide is double stranded.

[0072] In various embodiments, the nucleic acid or oligonucleotide is 15-30, 17-27, 19-26, 20-25, 40-50, 40-150, 100-300, 1000-2000, or up to 10000 nucleotides in length.

[0073] In various embodiments, the nucleic acid or oligonucleotide is connected to the linker via a phosphodiester or thiophosphodiester (e.g., R1 in Structure 1 is a phosphodiester or thiophosphodiester).

[0074] In various embodiments, the nucleic acid or oligonucleotide is connected to the linker via a C2-C10, C3-C6, or C6 alkyl (e.g., R2 in Structure 1 is a C2-C10, C3-C6, or C6 alkyl).

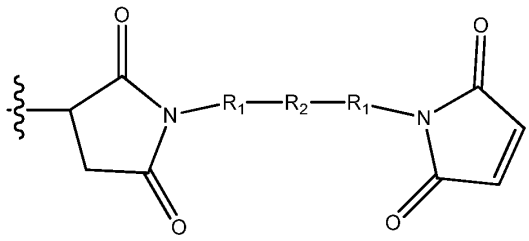
[0075] In various embodiments, the nucleic acid or oligonucleotide is connected to the linker via the reaction product of a thiol and maleimide group. (e.g., A in Structure 1 is the reaction product of a thiol and maleimide group).

[0076] In various embodiments, the linker (e.g., •, R3 in Structure 1, R1-R2-R1 in Structure 2, or A-R3-B in Structure 3) is cleavable. In one embodiment, the cleavable covalent linker comprises an acid cleavable ester bond, hydrazine bond, or acetal bond. In one embodiment, the cleavable covalent linker comprises a reductant cleavable bond. In one embodiment, the reductant cleavable bond is a disulfide bond. In one embodiment, the cleavable covalent linker is cleavable under intracellular conditions. In one embodiment, the cleavable covalent linker comprises a biocleavable bond. In one embodiment, the cleavable covalent linker comprises an enzyme cleavable bond.

[0077] In various embodiments, the linker is not cleavable. In one embodiment, one or more of • comprises a noncleavable covalent linker. In one embodiment, the noncleavable covalent linker comprises an amide bond or urethane bond. A noncleavable covalent linker can be an alkyl, aryl, or similar hydrocarbon group.

[0078] In various embodiments, the linker comprises a thiopropionate or disulfide (e.g., R3 is a thiopropionate or disulfide).

[0079] In various embodiments, the moiety



in Structure 2 comprises the reaction product of a DTME (dithiobismaleimidoethane), BM(PEG)2 (1,8-bis(maleimido)diethylene glycol), BM(PEG)3 (1,11-bismaleimido-triethyleneglycol), BMOE (bismaleimidoethane), BMH (bismaleimidohexane), or BMB (1,4-bismaleimidobutane).

[0080] In various embodiments, the linker is a homo bifunctional linker. For example, in one embodiment B comprises one of the same groups as A in Structure 1 or Structure 3.

[0081] In various embodiments, the linker is a hetero bifunctional linker. For example, in one embodiment B comprises a different group from A in Structure 1 or Structure 3.

[0082] In various embodiments, the compound is isolated or substantially pure. For example, the compound can be at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure. In one embodiment, the compound is about 85-95 % pure. Likewise, the methods for synthesizing the compounds and compositions according to the invention can result in a product that is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure. In one embodiment, the product is about 85-95 % pure.

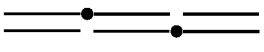
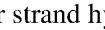
[0083] In various embodiments, each double stranded oligonucleotide is an siRNA and/or has a length of 15-30 base pairs.

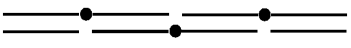
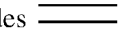
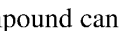
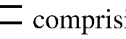
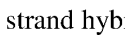
[0084] In various embodiments, each may independently comprise two sense or two antisense oligonucleotides.

[0085] In various embodiments, each may independently comprise one sense and one antisense oligonucleotide.

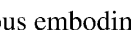
[0086] In various embodiments, the compound or composition comprises a homo-multimer of substantially identical double stranded oligonucleotides. The substantially identical double stranded oligonucleotides can each comprise an siRNA targeting the same molecular target *in vivo*.

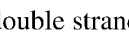
[0087] In various embodiments, the compound or composition comprises a hetero-multimer of two or more substantially different double stranded oligonucleotides. The substantially different double stranded oligonucleotides can each comprise an siRNA targeting different genes.

[0088] In various embodiments, the compound comprises Structure 9 and wherein n = 0:  (Structure 9). The compound can further comprise a targeting ligand. The compound can further comprise 2 or 3 substantially different double stranded oligonucleotides  each comprising an siRNA targeting a different molecular target *in vivo*. The compound can further comprise a targeting ligand, one  comprising a first siRNA guide strand targeting Factor VII and a first passenger strand hybridized to the guide strand, one  comprising a second siRNA guide strand targeting Apolipoprotein B and a second passenger strand hybridized to the second guide strand, and one  comprising a third siRNA guide strand targeting TTR and a third passenger strand hybridized to the third guide strand. The targeting ligand can comprise *N*-Acetylgalactosamine (GalNAc).

[0089] In various embodiments, the compound comprises Structure 10 and wherein m = 1:  (Structure 10). The compound can further comprise a targeting ligand. The compound can further comprise 2, 3, or 4 substantially different double stranded oligonucleotides  each comprising an siRNA targeting a different molecular target *in vivo*. The compound can further comprise a targeting ligand, one  comprising a first siRNA guide strand targeting Factor VII and a first passenger strand hybridized to the guide strand, one  comprising a second siRNA guide strand targeting Apolipoprotein B and a second passenger strand hybridized to the second guide strand, and one  comprising a third siRNA guide strand targeting TTR and a third passenger strand hybridized to the third guide strand. The targeting ligand can comprise *N*-Acetylgalactosamine (GalNAc).

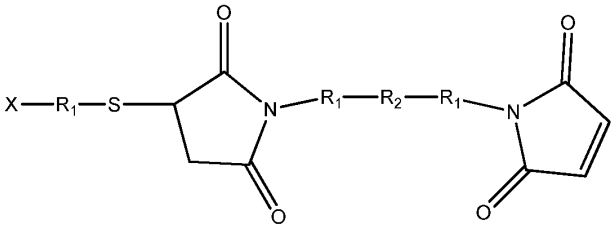
[0090] In various embodiments relating to Structure 16, each has a length of 15-30 base pairs; each is an siRNA; and/or n is an integer from 1 to 100.

[0091] In various embodiments, each double stranded oligonucleotide (e.g., , for example in Structure 4) comprises an siRNA guide strand targeting Factor VII and a passenger strand hybridized to the guide strand.

[0092] In various embodiments (e.g., in Structure 4), the compound further comprises a targeting ligand, each double stranded oligonucleotide (e.g., ) comprises an siRNA guide strand and a passenger strand hybridized to the guide strand, and the compound is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.

[0093] In various embodiments, at least one double stranded oligonucleotide (e.g., , for example in Structure 6) comprises a first siRNA guide strand targeting Factor VII and a first passenger strand hybridized to the guide strand, and at least one double stranded oligonucleotide (e.g., , for example in Structure 6) comprises a second siRNA guide strand targeting Apolipoprotein B and a second passenger strand hybridized the second guide strand.

[0094] In various embodiments, the method for synthesizing the compound of Structure 1 further comprises synthesizing the compound of Structure 2:



(Structure 2).

[0095] In various embodiments, the method for synthesizing the compound of Structure 1 or 2 is carried out under conditions that substantially favor the formation of Structure 1 or 2 and substantially prevent dimerization of X. The conditions can improve the yield of the reaction (e.g., improve the purity of the product).

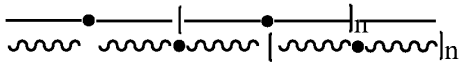
[0096] In various embodiments, the method for synthesizing the compound of Structure 1 or 2, the step of reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out at a X - R1 - R2 - A' concentration of below about 1 mM, 500 μM, 250 μM, 100 μM, or 50 μM. Alternatively, the X - R1 - R2 - A' concentration can be about 1 mM, 500 μM, 250 μM, 100 μM, or 50 μM.

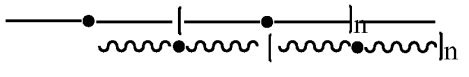
[0097] In various embodiments, the method for synthesizing the compound of Structure 1 or 2, the step of reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out with a molar excess of A'' - R3 – B of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100. Alternatively, the molar excess of A'' - R3 – B can be about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100.

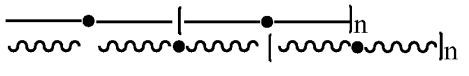
[0098] In various embodiments, the method for synthesizing the compound of Structure 1 or 2, the step of reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out at a pH of below about 7, 6, 5, or 4. Alternatively, the pH can be about 7, 6, 5, or 4.

[0099] In various embodiments, the method for synthesizing the compound of Structure 1 or 2, the step of reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out in a solution comprising water and a water miscible organic co-solvent. The water miscible organic co-solvent can comprise DMF, NMP, DMSO, or acetonitrile. The water miscible organic co-solvent can comprise about 10, 15, 20, 25, 30, 40, or 50 %V (v/v) of the solution.

[00100] In various embodiments (e.g., for synthesizing Structure 17), the method further comprises the step of annealing a plurality of ——— and/or ∿∿∿, thereby forming a plurality of molecules comprising Structure 18:


(Structure 18).


(Structure 19), and/or


(Structure 20).

[00101] In various embodiments (e.g., for synthesizing Structure 17), the method further comprises annealing with the plurality of plurality of and .

[00102] In various embodiments (e.g., for synthesizing Structure 17), the molar ratio of to is about 5:100, 10:100, 20:100, 30:100, 40:100, or 50:100.

[00103] In various embodiments (e.g., for synthesizing Structure 17), the method further comprises annealing with the plurality of plurality of and .

[00104] In various embodiments (e.g., for synthesizing Structure 17), the molar ratio of to is about 5:100, 10:100, 20:100, 30:100, 40:100, or 50:100.

[00105] In various embodiments (e.g., for synthesizing Structure 17), the molar ratio of and is about 1:1.

[00106] In various embodiments (e.g., for synthesizing Structure 17), the molar ratio of to or the molar ratio of to is about 100:90, 100:80, 100:75, 100:70, or 100:60.

[00107] In various embodiments (e.g., for synthesizing Structure 17), each has a length of 15-30 base pairs.

[00108] In various embodiments (e.g., for synthesizing Structure 17), each is an siRNA.

[00109] In various embodiments (e.g., for synthesizing Structure 17), each comprises siRNA guide strand targeting Factor VII and a passenger strand hybridized to the guide strand.

[00110] In various embodiments (e.g., for synthesizing Structure 17), n is an integer from 1 to 100.

[00111] In various embodiments (e.g., for synthesizing Structure 17), • is a cleavable or non-cleavable linker.

[00112] In various embodiments, the method further comprises formulating any of the compounds or compositions in a nanoparticle.

[00113] In various embodiments, the oligonucleotide has a specific sequence, for example any one of the sequences disclosed herein. In one embodiment, the oligonucleotide is an siRNA having SEQ ID NO:106. In one embodiment, the oligonucleotide is an siRNA having SEQ ID NO:115.

[00114] In various embodiments, the subject is a cell, mammal, or human.

[00115] These and other advantages of the present technology will be apparent when reference is made to the accompanying drawings and the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

[00116] Fig. 1 presents the chemical structure of a tri-antennary *N*-actylgalactosamine ligand.

[00117] Fig. 2 presents a schematic of a synthesis of an FVII-ApoB heterodimer (XD-05311), which is discussed in connection with Example 9.

[00118] Fig. 3 presents data showing FVII activity from mouse serum *in vivo*, which is discussed in connection with Example 10.

[00119] Fig. 4 presents data showing FVII and ApoB mRNA level from liver tissue in animal experiment MausRNAi-TV30, which is discussed in connection with Example 10.

[00120] Fig. 5 presents a 5'-GalNAc-FVII canonical control, which is discussed in connection with Example 11.

[00121] Fig. 6 presents a GalNAc-homodimer conjugate (XD-06330), which is discussed in connection with Example 12.

[00122] Fig. 7 presents a schematic of a synthesis of a GalNAc-homodimer conjugate (XD-06360), which is discussed in connection with Example 13.

[00123] Fig. 8 presents a schematic of a synthesis of a GalNAc-homodimer conjugate (XD-06329), which is discussed in connection with Example 14.

[00124] Fig. 9 presents data showing FVII activity in mouse serum (knockdown by FVII homodimeric GalNAc conjugates), which is discussed in connection with Example 15.

[00125] Figs. 10A and 10B and 10C present data showing FVII activity in mouse serum (knockdown by FVII homodimeric GalNAc conjugates normalized for GalNAc content), which is discussed in connection with Example 15.

[00126] Fig. 11 presents canonical GalNAc-siRNAs independently targeting FVII, ApoB and TTR, which are discussed in connection with Example 16.

[00127] Fig. 12 presents a GalNAC-heterotrimer conjugate (XD06726), which is discussed in connection with Example 17. Key: In this example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00128] Fig. 13 presents a schematic of a synthesis strategy for a GalNAc-conjugated heterotrimer (XD06726), which is discussed in connection with Example 17. Key: In this example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00129] Fig. 14 presents a GalNAc-heterotrimer conjugate (XD06727), which is discussed in connection with Example 18. Key: In this example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00130] Fig. 15 presents a schematic of a synthesis strategy for GalNAc-Conjugated Heterotrimer (XD06727), which is discussed in connection with Example 18. Key: In this example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00131] Fig. 16 presents data for an HPLC analysis of reaction of action of X20336 to X20366, which is discussed in connection with Example 18.

[00132] Fig. 17 presents data for an HPLC analysis of addition of X19580 to the reaction, which is discussed in connection with Example 18.

[00133] Fig. 18 presents data for an HPLC analysis of addition of X18795 (5'-siFVIIantisense-3') to reaction mixture to yield XD-06727, which is discussed in connection with Example 18.

[00134] Figs. 19A and 19B present data for TTR protein levels in serum samples (measured by ELISA), which is discussed in connection with Example 20.

[00135] Figs. 20A and 20B present data for FVII enzymatic activity in serum samples, which is discussed in connection with Example 20.

[00136] Figs. 21A and 21B present data for ApoB protein levels in serum samples (measured by ELISA), which is discussed in connection with Example 20.

[00137] Figs. 22A and 22B present target knockdown in liver data, which is discussed in connection with Example 20.

[00138] Fig. 23 presents a GalNAc-heterotetramer conjugate (XD-07140), which is discussed in connection with Example 21. Key: In this example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00139] Fig. 24 presents a schematic of a synthesis of a GalNAc-heterotetramer conjugate (XD-07140), which is discussed in connection with Example 21. Key: In this example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00140] Fig. 25 presents a HPLC Analysis of a GalNAc-siFVII-siApoB-siTTR-siFVII Tetramer(XD-07140), which is discussed in connection with Example 21.

[00141] Fig. 26 presents a synthesis of a homodimer, which is discussed in connection with Example 22.

[00142] Fig. 27 presents an SEC HPLC analysis of XD-05305, which is discussed in connection with Example 22.

[00143] Fig. 28 presents an SEC HPLC Analysis of XD-05305, which is discussed in connection with Example 22.

[00144] Fig. 29 presents an IEX HPLC analysis of XD-05305, which is discussed in connection with Example 22.

[00145] Fig. 30 presents an SEC HPLC analysis of XD-05305, which is discussed in connection with Example 22. Multimeric siRNA is the left-hand peak; dimeric siRNA is the middle peak; and canonical siRNA is the right-hand peak.

[00146] Fig. 31 presents the effect of salt concentration and reaction temperature on a multimeric siRNA mixture, which is discussed in connection with Example 22.

[00147] Fig. 32 presents data showing that the higher the concentration of termination strand (in this case, the antisense strand was used as the terminator), the smaller the multimerized siRNA fraction. The data is discussed in connection with Example 22.

[00148] Fig. 33 presents data showing that the smaller the concentration of sense homodimer, the smaller the multimerized siRNA fraction. The data is discussed in connection with Example 22.

[00149] Fig. 34A presents the gel for sample nos. 1-15, which is discussed in connection with Example 22.

[00150] Fig. 34B presents the gel for sample nos. 1'-10', which is discussed in connection with Example 22.

[00151] Fig. 35 presents data showing FVII activity determined from mouse serum in animal experiment MausRNAi-TV29, which is discussed in connection with Example 23.

[00152] Fig. 36 presents data showing FVII activity determined from mouse serum in animal experiment MausRNAi-TV30, which is discussed in connection with Example 23.

[00153] Fig. 37 presents a FVIIs-FVIIas heterodimer (X12714), which is discussed in connection with Example 24.

[00154] Fig. 38 presents a gel analysis of heterodimer X12714 (Lane 12), which is discussed in connection with Example 24.

[00155] Fig. 39 presents dose-response data for ApoB screening NMuLi cells, which is discussed in connection with Example 26.

[00156] While the invention comprises embodiments in many different forms, there are shown in the drawings and will herein be described in detail several specific embodiments with the understanding that the present disclosure is to be considered as an exemplification of the

principles of the technology and is not intended to limit the invention to the embodiments illustrated.

DETAILED DESCRIPTION

[00157] The various aspects of the invention have in common the manufacture and use of multi-conjugates containing oligonucleotides. The multi-conjugates may contain RNA and/or DNA. The RNA may be any form of bioactive RNA amenable to the multi-conjugation reactions conditions provided herein, such as siRNA, miRNA, and small activating RNA (saRNA).

[00158] The oligonucleotides used in the various aspects of the invention may be any that are of interest or used (A) in the medical arts, for example, to suppress or enhance expression of a target gene or protein in a subject, and in the treatment or prevention of any disease that would benefit from suppression or enhancement of the expression of a target gene or protein; (B) in performing biological research; and (C) to produce new or altered phenotypes in animals and plants. As a nonlimiting example, the oligonucleotides may be any RNA that is being used for RNA interference, RNA activation, or gene therapy, or is expected to be used in near future, such as RNA designed to be active in relation to c-myc, c-myb, c-fos, c-jun, bcl-2 or VEGF, VEGF-B, VEGF-C, VEGF-D, or PIGF.

[00159] The manufacturing methods described herein produce various multi-conjugates at higher levels of purity than have been previously described in the art. This feature of the invention is particularly advantageous for therapeutic applications of the multi-conjugates, and is likely to produce advantages for manufacture and use of the multi-conjugates in other applications such as research.

[00160] One aspect of the invention is oligonucleotide-containing multi-conjugates having a predetermined size and composition, and a method for making such multi-conjugates. The method produces multi-conjugates at higher levels of purity than have been previously described in the art.

[00161] Various features of the invention are discussed, in turn, below.

[00162] ***Nucleic Acids***

[00163] In various embodiments, the nucleic acid or oligonucleotide is RNA, DNA, or comprises an artificial or non-natural nucleic acid analog. In various embodiments, the nucleic acid or oligonucleotide is single stranded. In various embodiments, the nucleic acid or oligonucleotide is double stranded (e.g., antiparallel double stranded).

[00164] In various embodiments, the nucleic acid or oligonucleotide is RNA, for example an antisense RNA (aRNA), CRISPR RNA (crRNA), long noncoding RNA (lncRNA), microRNA (miRNA), piwi-interacting RNA (piRNA), small interfering RNA (siRNA), messenger RNA (mRNA), short hairpin RNA (shRNA), small activating (saRNA), or ribozyme.

[00165] In one embodiment, the RNA is siRNA. For example, each double stranded oligonucleotide is an siRNA and/or has a length of 15-30 base pairs.

[00166] In various embodiments, the nucleic acid or oligonucleotide is an aptamer.

[00167] siRNA (small interfering RNA) is a short double-stranded RNA composed of 19-22 nucleic acids, which targets mRNA (messenger RNA) of a gene whose nucleotide sequence is identical with its sense strand in order to suppress expression of the gene by decomposing the target gene (Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494-8).

[00168] Another class of nucleic acid, useful in the methods of the invention, are miRNAs. MiRNAs are non-coding RNAs that play key roles in post-transcriptional gene regulation. miRNA can regulate the expression of 30% of all mammalian protein-encoding genes. Specific and potent gene silencing by double stranded RNA (RNAi) was discovered, plus additional small noncoding RNA (Canver, M.C. et al., Nature (2015)). Pre-miRNAs are short stem loops ~70 nucleotides in length with a 2-nucleotide 3'-overhang that are exported, into the mature 19-25 nucleotide duplexes. The miRNA strand with lower base pairing stability (the guide strand) can be loaded onto the RNA-induced silencing complex (RISC). The passenger guide strand can be functional but is usually degraded. The mature miRNA tethers RISC to partly complementary sequence motifs in target mRNAs predominantly found within the 3' untranslated regions (UTRs) and induces posttranscriptional gene silencing (Bartel, D.P. Cell, 136: 215-233 (2009); Saj, A. & Lai, E.C. Curr Opin Genet Dev, 21: 504-510 (2011)). MiRNAs mimics are described for example, in US Patent No. 8,765,709.

[00169] In some embodiments, the RNA can be short hairpin RNA (shRNA), for example, as described in US Patent Nos. 8,202,846 and 8,383,599.

[00170] In some embodiments, the RNA can CRISPR RNA (crRNA), for example, CRISPR array of Type V can be processed into short mature crRNAs of 42-44 nucleotides in length, with each mature crRNA beginning with 19 nucleotides of direct repeat followed by 23-25 nucleotides of spacer sequence. Alternatively, mature crRNAs in Type II systems can start with 20-24 nucleotides of spacer sequence followed by about 22 nucleotides of direct repeat.

CRISPR systems are described for example, in US Patent No. 8,771,945, Jinek et al., Science, 337(6096): 816-821 (2012), and International Patent Application Publication No. WO 2013/176772.

[00171] In various embodiments, the nucleic acid or oligonucleotide is 15-30, 17-27, 19-26, 20-25, 40-50, 40-150, 100-300, 1000-2000, or up to 10000 nucleotides in length.

[00172] In various embodiments, the oligonucleotide is double stranded and complementary. Complementary can be 100% complementary, or less than 100% complementary where the oligonucleotide nevertheless hybridizes and remains double stranded under relevant conditions (e.g., physiologically relevant conditions). For example, a double stranded oligonucleotide can be at least about 80, 85, 90, or 95 % complementary.

[00173] In some embodiments, RNA is long noncoding RNA (lncRNA), LncRNAs are a large and diverse class of transcribed RNA molecules with a length of more than 200 nucleotides that do not encode proteins (or lack > 100 amino acid open reading frame). lncRNAs are thought to encompass nearly 30,000 different transcripts in humans, hence lncRNA transcripts account for the major part of the non-coding transcriptome (see, e.g., Derrien et al., The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res, 22(9): 1775-89 (2012)).

[00174] In yet other embodiments, RNA is messenger RNA (mRNA). mRNA and its application as a delivery method for in-vivo production of proteins, is described, for example, in International Patent Application Publication No. WO 2013/151736.

[00175] In other embodiments, RNA can be small activating (saRNA) (e.g., as described in Chappell et al., Nature Chemical Biology, 11: 214-220 (2015)), or ribozyme (Doherty et al., Ann Rev Biophys Biomo Struct, 30: 457-475 (2001)).

[00176] In some embodiments, the nucleic acid or oligonucleotide is DNA, for example an antisense DNA (aDNA) (e.g., antagomir) or antisense gapmer. Examples of aDNA, including gapmers and multimers, are described for example in Subramanian et al., Nucleic Acids Res, 43(19): 9123–9132 (2015) and International Patent Application Publication No. WO 2013/040429. Examples of antagamirs are described for example, in US Patent No. 7,232,806.

[00177] In various embodiments, the oligonucleotide has a specific sequence, for example any one of the sequences disclosed herein. In one embodiment, the oligonucleotide is an siRNA having SEQ ID NO:106. In one embodiment, the oligonucleotide is an siRNA having SEQ ID NO:115.

[00178] A general procedure for oligonucleotide synthesis is provided in the Examples below. Other methods that can be adapted for use with the invention are known in the art.

[00179] ***Modifications to Nucleic Acids***

[00180] In various embodiments, the nucleic acid or oligonucleotide further comprises a chemical modification. The chemical modification can comprise a modified nucleoside, modified backbone, modified sugar, or modified terminus.

[00181] Phosphorus-containing linkages include, but are not limited to, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates comprising 3'alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates comprising 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'.

[00182] The oligonucleotides contained in the multi-conjugates of this invention may be modified using various strategies known in the art to produce a variety of effects, including, e.g., improved potency and stability *in vitro* and *in vivo*. Among these strategies are: artificial nucleic acids, e.g., 2'-O-methyl-substituted RNA; 2'-fluro-2'deoxy RNA, peptide nucleic acid (PNA); morpholinos; locked nucleic acid (LNA); Unlocked nucleic acids (UNA); bridged nucleic acid (BNA); glycol nucleic acid (GNA) ; and threose nucleic acid (TNA); or more generally, nucleic acid analogs, e.g., bicyclic and tricyclic nucleoside analogs, which are structurally similar to naturally occurring RNA and DNA but have alterations in one or more of the phosphate backbone, sugar, or nucleobase portions of the naturally-occurring molecule. Typically, analogue nucleobases confer, among other things, different base pairing and base stacking properties. Examples include universal bases, which can pair with all four canon bases. Examples of phosphate-sugar backbone analogues include PNA. Morpholino-based oligomeric compounds are described in Braasch et al., Biochemistry, 41(14): 4503-4510 (2002) and US Patent Nos. 5,539,082; 5,714,331; 5,719,262; and 5,034,506.

[00183] In the manufacturing methods described herein, some of the oligonucleotides are modified at a terminal end by substitution with a chemical functional group. The substitution can be performed at the 3' or 5' end of the oligonucleotide, and is preferably performed at the 3' ends of both the sense and antisense strands of the monomer, but is not always limited thereto. The chemical functional groups may include, e.g., a sulfhydryl group (-SH), a carboxyl group (-COOH), an amine group (-NH2), a hydroxy group (-OH), a formyl

group (-CHO), a carbonyl group (-CO-), an ether group (-O-), an ester group (-COO-), a nitro group ($-NO_2$), an azide group ($-N_3$), or a sulfonic acid group ($-SO_3H$).

[00184] The oligonucleotides contained in the multi-conjugates of this invention may be modified can also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, *e.g.*, hypoxanthine, 6-methyladenine, 5-Me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2' deoxycytosine and often referred to in the art as 5-Me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well as synthetic nucleobases, *e.g.*, 2-aminoadenine, 2-(methylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalklyamino)adenine or other heterosubstituted alkyladenines, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N6 (6-aminohexyl)adenine, and 2,6-diaminopurine. Kornberg, A., DNA Replication, W. H. Freeman & Co., San Francisco, pp 75-77 (1980); Gebeyehu *et al.*, Nucl. Acids Res, 15: 4513 (1997). A "universal" base known in the art, *e.g.*, inosine, can also be included. 5-Me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 ºC. (Sanghvi, Y. S., in Crooke, S. T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, pp 276-278 (1993) and are aspects of base substitutions. Modified nucleobases can include other synthetic and natural nucleobases, such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudo-uracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylquanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine, and 3-deazaguanine and 3-deazaadenine. Hydroxy group (—OH) at a terminus of the nucleic acid can be substituted with a functional group such as sulfhydryl group (—SH), carboxyl group (—COOH) or amine group ($—NH_2$).The substitution can be performed at 3' end or 5' end, and is preferably occurred at 3' ends of both sense and antisense are substituted with such functional group, but not always limited thereto.

[00185] ***Linkers***

[00186] In various aspects and embodiments of the invention, oligonucleotides are linked covalently. Linkers may be cleavable (e.g., under intracellular conditions, to facilitate

oligonucleotide delivery and/or action) or non-cleavable. Although generally described below and in the Examples in context of linkers using nucleophile-electrophile chemistry, other chemistries and configurations are possible. And, as will be understood by those having ordinary skill, various linkers, including their composition, synthesis, and use are known in the art and may be adapted for use with the invention.

[00187] In various embodiments, a covalent linker can comprise the reaction product of nucleophilic and electrophilic group. For example, a covalent linker can comprise the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group. As described herein, one of these groups is connected to an oligonucleotide (e.g., thiol (-SH) functionalization at the 3' or 5' end) and the other groups is encompassed by a second molecule (e.g., linking agent) that ultimately links two oligonucleotides (e.g., maleimide in DTME).

[00188] In various embodiments, the nucleic acid or oligonucleotide is connected to the linker via a phosphodiester or thiophosphodiester (e.g., R1 in Structure 1 is a phosphodiester or thiophosphodiester). In various embodiments, the nucleic acid or oligonucleotide is connected to the linker via a C2-C10, C3-C6, or C6 alkyl (e.g., R2 in Structure 1 is a C2-C10, C3-C6, or C6 alkyl). Alternatively, these moieties (e.g., R1 and/or R2 in Structure 1) are optional and a direct linkage is possible.

[00189] In various embodiments, the nucleic acid or oligonucleotide is connected to the linker via the reaction product of a thiol and maleimide group. (e.g., A in Structure 1 is the reaction product of a thiol and maleimide group). Preferred linking agents utilizing such chemistry include DTME (dithiobismaleimidoethane), BM(PEG)2 (1,8-bis(maleimido)diethylene glycol), BM(PEG)3 (1,11-bismaleimido-triethyleneglycol), BMOE (bismaleimidoethane), BMH (bismaleimidohexane), or BMB (1,4-bismaleimidobutane).

[00190] Again, the examples are illustrative and not limiting. In various embodiments, oligonucleotides can be linked together directly, via functional end-substitutions, or indirectly by way of a linking agent. In various embodiments, the oligonucleotide can be bound directly to a linker (e.g., R1 and R2 of Structure 1 are absent). Such bonding can be achieved, for example, through use of 3'-thionucleosides, which can be prepared according to the ordinary skill in the art. See, e.g., Sun et al. "Synthesis of 3'-thioribonucleosides and their incorporation into oligoribonucleotides via phosphoramidite chemistry" RNA. 1997 Nov;3(11):1352-63. In various embodiments, the linking agent may be a non-ionic hydrophilic polymer such as polyethyleneglycol (PEG), polyvinylpyrolidone and polyoxazoline, or a hydrophobic polymer such as PLGA and PLA.

[00191] A polymer linking agent used as a mediator for a covalent bond may be non-ionic hydrophilic polymers including PEG, Pluronic, polyvinylpyrolidone, polyoxazoline, or copolymers thereof; or one or more biocleavable polyester polymers including poly-L-lactic acid, poly-D-lactic acid, poly-D,L-lactic acid, poly-glycolic acid, poly-D-lactic-co-glycolic acid, poly-L-lactic-co-glycolic acid, poly-D,L-lactic-co-glycolic acid, polycaprolactone, polyvalerolactone, polyhydroxybutyrate, polyhydroxyvalerate, or copolymers thereof, but is not always limited thereto.

[00192] The linking agent may have a molecular weight of 100-10,000 Daltons. Examples of such linking agent include dithio-bis-maleimidoethane (DIME), 1,8-bis-maleimidodiethyleneglycol (BM(PEG)2), tris- (2-maleimidoethyl)-amine (TMEA), tri-succinimidyl aminotriacetate (TSAT), 3-arm-poly(ethylene glycol) (3-arm PEG), maleimide, N-hydroxysuccinimide (NHS), vinylsulfone, iodoacetyl, nitrophenyl azide, isocyanate, pyridyldisulfide, hydrazide, and hydroxyphenyl azide.

[00193] A linking agent having cleavable bonds (such as a reductant bond that is cleaved by the chemical environment of the cytosol) or a linking agent having non-cleavable bonds can be used herein. For example, the linking agent of the foregoing aspects of present invention can have non-cleavable bonds such as an amide bond or a urethane bond. Alternatively, the linking agent of the foregoing aspects of the present invention can have cleavable bonds such as an acid cleavable bond (e.g., a covalent bond of ester, hydrazone, or acetal), a reductant cleavable bond (e.g., a disulfide bond), a bio-cleavable bond, or an enzyme cleavable bond. In one embodiment, the cleavable covalent linker is cleavable under intracellular conditions. Additionally, any linking agent available for drug modification can be used in the foregoing aspects of the invention without limitation.

[00194] Further, combinations of functional groups and linking agents may include: (a) where the functional groups are amino and thiol, the linking agent may be Succinimidyl 3-(2-pyridyldithio)propionate, or Succinimydyl 6-([3(2-pyridyldithio)propioamido]hexanoate; (b) where the functional group is amino, the linking agent may be 3,3'dithiodipropionic acid di-(N-succinimidyl ester), Dithio-bis(ethyl 1H-imidazole-1-carboxylate), or Dithio-bis(ethyl 1H-imidazole-1-carboxylate); (c) where the functional groups are amino and alkyne, the linking agent may be Sulfo-N-succinimidyl3-[[2-(p-azidosalicylamido)ethyl]-1,3'-dithio]propionate; and (d) where the functional group y is thiol, the linking agent is dithio-bis-maleimidoethan (DTME); 1,8-Bis-maleimidodiethyleneglycol (BM(PEG)2); or dithiobis(sulfosuccinimidyl propionate) (DTSSP).

[00195] In the foregoing methods of preparing compounds, an additional step of activating the functional groups can be included. Compounds that can be used in the activation of the functional groups include but are not limited to 1-ethyl-3,3-dimethylaminopropyl carbodiimide, imidazole, N-hydroxysuccinimide, dichlorohexylcarbodiimide, N-beta-Maleimidopropionic acid, N-beta-maleimidopropyl succinimide ester or N-Succinimidyl 3-(2-pyridyldithio)propionate.

[00196] ***Monomeric Intermediate Compounds***

[00197] In various aspects, the invention provides an oligonucleotide coupled to a covalent linker, which can be used, for example, in the synthesis of defined multi-conjugate oligonucleotides having predetermined sizes and compositions.

[00198] In one aspect, the invention provides a compound according to Structure 1:

X - R1 - R2 - A - R3 - B (Structure 1)

wherein:

X is a nucleic acid bonded to R1 through its 3' or 5' terminus;

R1 a phosphodiester, thiophosphodiester, sulfate, amide, glycol, or is absent;

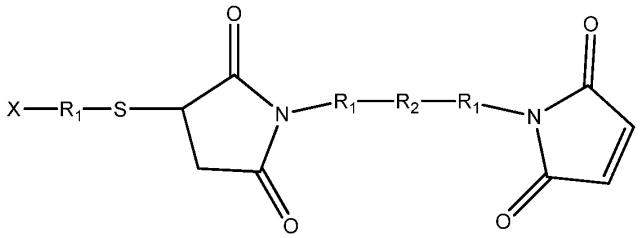
R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

A is the reaction product of a nucleophile and an electrophile;

R3 is a C2-C10 alkyl, alkoxy, aryl, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and

B is a nucleophile or electrophile (e.g., a thiol, maleimide, vinylsulfone, pyridyldisulfide, iodoacetamide, acrylate, azide, alkyne, amine, or carboxyl group).

[00199] In one aspect, the invention provides a compound according to Structure 2:



(Structure 2)

wherein:

X is a nucleic acid bonded to R1 via a phosphate or thiophosphate at its 3' or 5' terminus;

each R1 is independently a C2-C10 alkyl, alkoxy, or aryl group; and

R2 is a thiopropionate or disulfide group.

[00200] In one aspect, the invention provides a compound according to Structure 3:

X - R1 - R2 - A - R3 - B (Structure 3)

wherein:

X is a nucleic acid bonded to R1 through its 3' or 5' terminus;

R1 a phosphate, thiophosphate, sulfate, amide, glycol, or is absent;

R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

A is the reaction product of a first and a second reactive moiety;

R3 is an C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and

B is a third reactive moiety.

[00201] In various aspects, the invention also provides methods for synthesizing an oligonucleotide coupled to a covalent linker.

[00202] In one aspect, the invention provides a method for synthesizing a compound according to Structure 1 (or adapted for synthesizing a compounds according to Structure 2 or 3), the method comprising:

reacting a functionalized nucleic acid X - R1 - R2 - A' and a covalent linker A" - R3 - B, wherein A' and A" comprise a nucleophile and an electrophile, in a dilute solution of X - R1 - R2 - A' and with a stoichiometric excess of A" - R3 – B, thereby forming the compound X - R1 - R2 - A - R3 - B (Structure 1), wherein:

X is a nucleic acid bonded to R1 through its 3' or 5' terminus;

R1 a phosphodiester, thiophosphodiester, sulfate, amide, glycol, or is absent;

R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

A is the reaction product of a nucleophile and an electrophile;

R3 is a C2-C10 alkyl, alkoxy, aryl, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and

B is a nucleophile or electrophile (e.g., a thiol, maleimide, vinylsulfone, pyridyldisulfide, iodoacetamide, acrylate, azide, alkyne, amine, or carboxyl group).

[00203] The method can further comprise the step of synthesizing the functionalized nucleic acid X - R1 - R2 - A', wherein A' comprises a thiol (-SH) by (i) introducing a the thiol during solid phase synthesis of the nucleic acid using phosphoramidite oligomerization chemistry or (ii) reduction of a disulfide introduced during the solid phase synthesis.

[00204] In various embodiments, the method for synthesizing the compound of Structure 1 further comprises synthesizing the compound of Structure 2.

[00205] The oligonucleotide coupled to a covalent linker can include any one or more of the features described herein, including in the Examples. For example, the compounds can include any one or more of the nucleic acids (with or without modifications), targeting ligands,

and/or linkers described above, or any of the specific structures or chemistries shown in the summary or Examples. Example 1 provides an example methodology for generating thiol terminated oligonucleotides. Example 2 provides an example methodology for preparing an oligonucleotide coupled to a linker.

[00206] In various embodiments, the method for synthesizing the compound of Structure 1 or 2 is carried out under conditions that substantially favor the formation of Structure 1 or 2 and substantially prevent dimerization of X. The conditions can improve the yield of the reaction (e.g., improve the purity of the product).

[00207] In various embodiments, the method for synthesizing the compound of Structure 1 or 2, the step of reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A" - R3 - B is carried out at a X - R1 - R2 - A' concentration of below about 1 mM, 500 μM, 250 μM, 100 μM, or 50 μM. Alternatively, the X - R1 - R2 - A' concentration can be about 1 mM, 500 μM, 250 μM, 100 μM, or 50 μM.

[00208] In various embodiments, the method for synthesizing the compound of Structure 1 or 2, the step of reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A" - R3 - B is carried out with a molar excess of A" - R3 - B of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100. Alternatively, the molar excess of A" - R3 - B can be about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100.

[00209] In various embodiments, the method for synthesizing the compound of Structure 1 or 2, the step of reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A" - R3 - B is carried out at a pH of below about 7, 6, 5, or 4. Alternatively, the pH can be about 7, 6, 5, or 4.

[00210] In various embodiments, the method for synthesizing the compound of Structure 1 or 2, the step of reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A" - R3 - B is carried out in a solution comprising water and a water miscible organic co-solvent. The water miscible organic co-solvent can comprise DMF (dimethylformamide), NMP (N-methyl-2-pyrrolidone), DMSO (dimethyl sulfoxide), or acetonitrile. The water miscible organic co-solvent can comprise about 10, 15, 20, 25, 30, 40, or 50 %V (v/v) of the solution.

[00211] In various embodiments, the compound is isolated or substantially pure. For example, the compound can be at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure. In one embodiment, the compound is about 85-95 % pure. Likewise, the methods for synthesizing the compounds and compositions according to the invention can result in a product that is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure. In one embodiment, the product is about 85-95 % pure. Preparations can be greater than or equal to 50% pure; preferably greater than or

equal to 75% pure; more preferably greater than or equal to 85% pure; and still more preferably, greater than or equal to 95% pure.

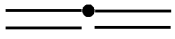
[00212] As used herein, the term about is used in accordance with its plain and ordinary meaning of approximately. For example, "about X" encompasses approximately the value X as stated, including similar amount that are within the measurement error for the value of X or amounts that are approximately the same as X and have essentially the same properties as X.

[00213] As used herein, isolated include compounds that are separated from other, unwanted substances. The isolated compound can be synthesized in a substantially pure state or separated from the other components of a crude reaction mixture, except that some amount of impurities, including residual amounts of other components of the crude reaction mixture, may remain. Similarly, pure or substantially pure means sufficiently free from impurities to permit its intended use (e.g., in a pharmaceutical formulation or as a material for a subsequent chemical reaction). X % pure means that the compound is X % of the overall composition by relevant measure, which can be for example by analytical methods such as HPLC.

[00214] ***Dimeric Compounds and Intermediates***

[00215] In various aspects, the invention provides dimeric defined multi-conjugate oligonucleotides. These compounds include homodimers (e.g., two oligonucleotides that are substantially the same, for example targeting the same gene *in vivo*) and heterodimers (e.g., two oligonucleotides that are substantially different, for example different sequences or targeting different genes *in vivo*)

[00216] In one aspect, the invention provides an isolated compound according to Structure 4:

 (Structure 4)

wherein:

each is a double stranded oligonucleotide designed to react with the same molecular target *in vivo*, and

• is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, and having the structure - R1 - R2 - A - R3 - A - R2 - R1 - wherein:

each R1 is independently a phosphodiester, thiophosphodiester, sulfate, amide, glycol, or is absent;

each R2 is independently a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

each A is independently the reaction product of a nucleophile and an electrophile, and

R3 is a C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

[00217] In one aspect, the invention provides an isolated compound according to Structure 5:

 (Structure 5)

wherein:

——— is a first single stranded oligonucleotide

 is a second single stranded oligonucleotide having a different sequence from the first, and

• is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, and having the structure - R1 - R2 - A - R3 - A - R2 - R1 - wherein:

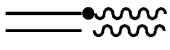
each R1 is independently a phosphate, sulfate, amide, glycol, or is absent;

each R2 is independently a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

each A is independently the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group, and

R3 is an C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

[00218] In one aspect, the invention provides an isolated compound according to Structure 6:

 (Structure 6)

wherein:

 is a first double stranded oligonucleotide

 is a second double stranded oligonucleotide having a different sequence from the first, and

• is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, and having the structure - R1 - R2 - A - R3 - A - R2 - R1 - wherein:

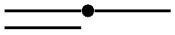
each R1 is independently a phosphate, sulfate, amide, glycol, or is absent;

each R2 is independently a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

each A is independently the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group, and

R3 is an C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

[00219] In one aspect, the invention provides an isolated compound according to Structure 11:

 (Structure 11)

wherein:

 is a double stranded oligonucleotide,

 is a single stranded oligonucleotide, and

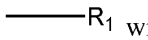
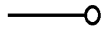
● is a covalent linker joining single strands of adjacent single stranded oligonucleotides.

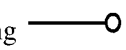
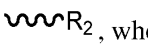
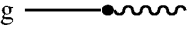
[00220] In various aspects, the invention provides methods for synthesizing dimeric defined multi-conjugate oligonucleotides.

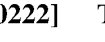
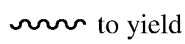
[00221] In one aspect, the invention provides a method for synthesizing a compound of Structure 5:

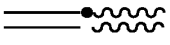
 (Structure 5)

wherein  is a first single stranded oligonucleotide, is a second single stranded oligonucleotide having a different sequence from the first, and ● is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

(i) reacting a first single stranded oligonucleotide  with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○ under conditions that produce the mono-substituted product ;

(ii) reacting  with a second single stranded oligonucleotide , wherein R2 is a chemical group capable of reacting with ○, thereby forming .

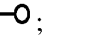
[00222] The method can further comprise the step of annealing complementary  and  to yield Structure 6:

 (Structure 6).

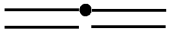
[00223] In one aspect, the invention provides a method for synthesizing an isolated compound of Structure 4:

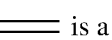
 (Structure 4)

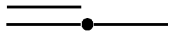
wherein each is a double stranded oligonucleotide and ● is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

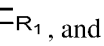
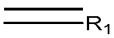
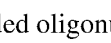
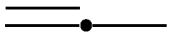
(i) reacting a first single stranded oligonucleotide  with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○, thereby forming a mono-substituted product ;

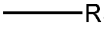
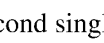
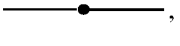
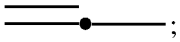
(ii) reacting  with a second single stranded oligonucleotide , wherein R2 is a chemical group capable of reacting with ○, thereby forming a single stranded dimer ;

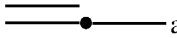
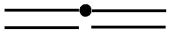
(iii) annealing single stranded oligonucleotides, at the same time or sequentially, thereby forming .

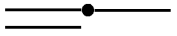
[00224] In one aspect, the invention provides a method for synthesizing an isolated compound of Structure 4:  (Structure 4) wherein each  is a double stranded oligonucleotide and • is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

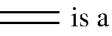
(i) forming  by:

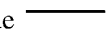
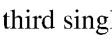
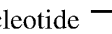
(a) annealing a first single stranded oligonucleotide  and a second single stranded oligonucleotide , thereby forming , and reacting with a third single stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ; or

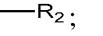
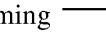
(b) reacting the second single stranded oligonucleotide  and the third single stranded oligonucleotide , thereby forming , and annealing the first single stranded oligonucleotide and , thereby forming ;

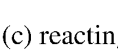
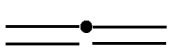
(ii) annealing  and a fourth single stranded oligonucleotide , thereby forming .

[00225] This methodology can be adapted for synthesizing an isolated compound according to  (Structure 11), for example by omitting step (ii).

[00226] In one aspect, the invention provides a method for synthesizing an isolated compound of Structure 4:  (Structure 4) wherein each  is a double stranded oligonucleotide and • is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

(a) annealing a first single stranded oligonucleotide  and a second single stranded oligonucleotide , thereby forming ;

(b) annealing a third single stranded oligonucleotide  and a fourth single stranded oligonucleotide , thereby forming ;

(c) reacting  and , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming .

[00227] As with the other compounds and compositions according to the invention, dimeric compounds and intermediates can include any one or more of the features described herein, including in the Examples. For example, the compounds can include any one or more of the nucleic acids (with or without modifications), targeting ligands, and/or linkers described above, or any of the specific structures or chemistries shown in the summary or Examples.

[00228] Example 4 provides an example methodology for preparing dimerized oligonucleotides and Example 5 provides an example methodology for annealing single stranded oligonucleotides to form double stranded oligonucleotides. Example 7 provides an example methodology for preparing various oligonucleotide precursors useful in the syntheses above. Example 8 provides an example methodology for preparing various oligonucleotide multimers, which are also useful in the syntheses above.

[00229] Examples of heterodimers are provided in Examples 9 and 10.

[00230] Examples of homodimers are provided in Examples 12-15.

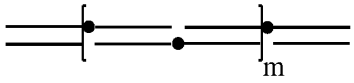
[00231] In various embodiments, R1, R2, and the bifunctional linking moiety ○ can form a covalent linker • as described and shown herein. For example, in various embodiments, R1 and R2 can each independently comprise a reactive moiety, for example an electrophile or nucleophile. In one embodiment, R1 and R2 can each independently be selected from the group consisting of a thiol, maleimide, vinylsulfone, pyridyldisulfide, iodoacetamide, acrylate, azide, alkyne, amine, and carboxyl group. In various embodiments, the bifunctional linking moiety ○ comprises two reactive moieties that can be sequentially reacted according to steps (i) and (ii) above, for example a second electrophile/nucleophile that can be reacted with an electrophile/nucleophile in R1 and R2. Examples of bifunctional linking moieties ○ include, but are not limited to, DTME, BM(PEG)2, BM(PEG)3, BMOE, BMH, or BMB.

[00232] These, as well as all other synthetic methods of the invention, can further comprise the step of adding a targeting ligand to the molecule. Example 6 provides an example methodology for adding a targeting ligand (e.g., GalNAc). Addition methods for adding targeting ligands are known in the art and can be adapted for the present invention by those skilled in the art.

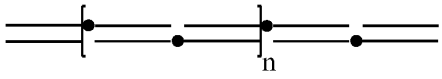
[00233] ***Multimeric (n>2) Compounds and Intermediates***

[00234] In various aspects, the invention provides multimeric (n>2) defined multi-conjugate oligonucleotides, including defined tri-conjugates and defined tetraconjugates.

[00235] In one aspect, the invention provides a compound according to Structure 7 or 8:



(Structure 7)



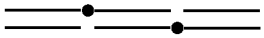
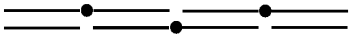
(Structure 8)

wherein:

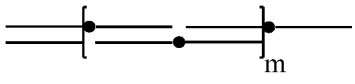
each ═══ is a double stranded oligonucleotide,

each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and

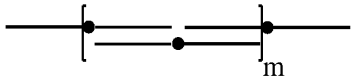
m is an integer ≥ 1 and n is an integer ≥ 0.

[00236] In one aspect, the invention provides a compound according to Structure 9 and wherein n = 0:  (Structure 9). In one aspect, the invention provides a compound according to Structure 10 and wherein m = 1:  (Structure 10).

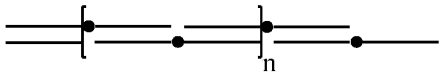
[00237] In one aspect, the invention provides a compound according to Structure 12, 13, 14, or 15:



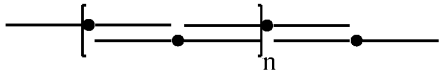
(Structure 12)



(Structure 13)



(Structure 14)



(Structure 15)

wherein:

each ═══ is a double stranded oligonucleotide,

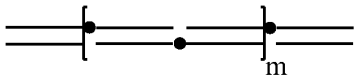
each ——— is a single stranded oligonucleotide,

each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and

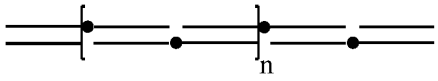
[00238] m is an integer ≥ 1 and n is an integer ≥ 0.

[00239] In various aspects, the invention provides methods for synthesizing multimeric (n>2) defined multi-conjugate oligonucleotides, including defined tri-conjugates and defined tetraconjugates.

[00240] In one aspect, the invention provides a method for synthesizing a compound according to Structure 7 or 8:

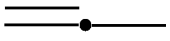


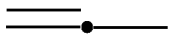
(Structure 7)

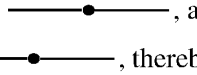
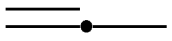


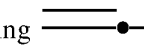
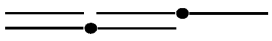
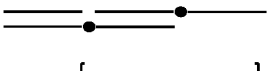
(Structure 8)

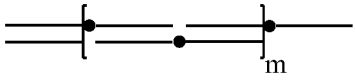
wherein: each ═══ is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and m is an integer ≥ 1 and n is an integer ≥ 0, the method comprising the steps of:

(i) forming  by:

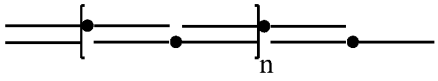
(a) annealing a first single stranded oligonucleotide ——— and a second single stranded oligonucleotide ———R_1, thereby forming ═══R_1, and reacting ═══R_1 with a third single stranded oligonucleotide ———R_2, wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ; or

(b) reacting the second single stranded oligonucleotide ———R_1 and the third single stranded oligonucleotide ———R_2, thereby forming , and annealing the first single stranded oligonucleotide ——— and , thereby forming ;

(ii) annealing  and a second single stranded dimer , thereby forming  and, optionally, annealing one or more additional single stranded dimers  to  thereby forming,



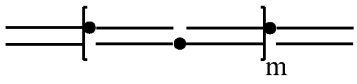
or



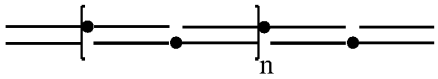
wherein m is an integer ≥ 1 and n is an integer ≥ 0; and

(iii) annealing a fourth single stranded oligonucleotide ——— to the product of step (ii), thereby forming structure 7 or 8.

[00241] In one aspect, the invention provides a method for synthesizing a compound according to Structure 7 or 8:



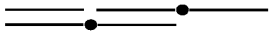
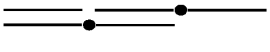
(Structure 7)

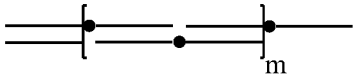


(Structure 8)

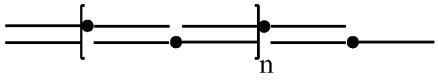
wherein: each ═══ is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and m is an integer ≥ 1 and n is an integer ≥ 0, the method comprising the steps of:

(i) annealing a first single stranded oligonucleotide ——— and a first single stranded dimer ———•———, thereby forming ═══•———;

(ii) annealing ═══•——— and a second single stranded dimer ———•———, thereby forming  and, optionally, annealing one or more additional single stranded dimers ———•——— to  thereby forming,

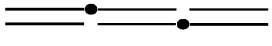


or



wherein m is an integer ≥ 1 and n is an integer ≥ 0; and

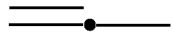
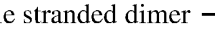
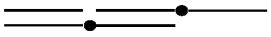
(iii) annealing a second single stranded oligonucleotide ——— to the product of step (ii), thereby forming structure 7 or 8.

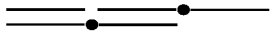
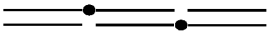
[00242] In one aspect, the invention provides a method for synthesizing a compound of Structure 9:  (Structure 9), wherein each ═══ is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, the method comprising the steps of:

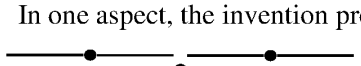
(i) forming ═══•——— by:

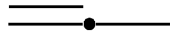
(a) annealing a first single stranded oligonucleotide ——— and a second single stranded oligonucleotide ———R_1, thereby forming ═══R_1, and reacting ═══R_1 with a third single stranded oligonucleotide ———R_2, wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ═══•———; or

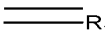
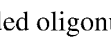
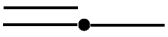
(b) reacting the second single stranded oligonucleotide ———R_1 and the third single stranded oligonucleotide ———R_2, thereby forming ———•———, and annealing the first single stranded oligonucleotide ——— and ———•———, thereby forming ═══•———;

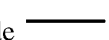
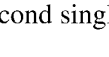
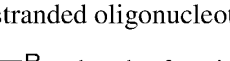
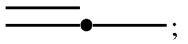
(ii) annealing  and a single stranded dimer , thereby forming ; and

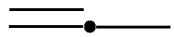
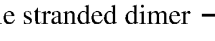
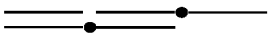
(iii) annealing  and a fourth single stranded oligonucleotide , thereby forming .

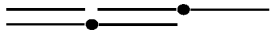
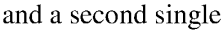
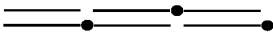
[00243] In one aspect, the invention provides a method for synthesizing a compound of Structure 10:  (Structure 10), wherein each is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, the method comprising the steps of:

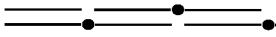
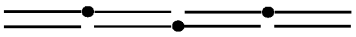
(i) forming  by:

(a) annealing a first single stranded oligonucleotide and a second single stranded oligonucleotide , thereby forming , and reacting with a third single stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ; or

(b) reacting the second single stranded oligonucleotide  and the third single stranded oligonucleotide , thereby forming , and annealing the first single stranded oligonucleotide and , thereby forming ;

(ii) annealing  and a single stranded dimer , thereby forming ;

(iii) annealing  and a second single stranded dimer , thereby forming ; and

(iv) annealing  and a fourth single stranded oligonucleotide , thereby forming .

[00244] As with the other compounds and compositions according to the invention, dimeric compounds and intermediates can include any one or more of the features described herein, including in the Examples. For example, the compounds can include any one or more of the nucleic acids (with or without modifications), targeting ligands, and/or linkers described above, or any of the specific structures or chemistries shown in the summary or Examples.

[00245] Example 7 provides an example methodology for preparing various oligonucleotide precursors useful in the syntheses above. Example 8 provides an example

methodology for preparing various oligonucleotide multimers, which are also useful in the syntheses above.

[00246] In various embodiments, R1, R2, and the bifunctional linking moiety ○ can form a covalent linker • as described and shown herein. For example, in various embodiments, R1 and R2 can each independently comprise a reactive moiety, for example an electrophile or nucleophile. In one embodiment, R1 and R2 can each independently be selected from the group consisting of a thiol, maleimide, vinylsulfone, pyridyldisulfide, iodoacetamide, acrylate, azide, alkyne, amine, and carboxyl group. In various embodiments, the bifunctional linking moiety ○ comprises two reactive moieties that can be sequentially reacted according to steps (i) and (ii) above, for example a second electrophile/nucleophile that can be reacted with an electrophile/nucleophile in R1 and R2. Examples of bifunctional linking moieties ○ include, but are not limited to, DTME, BM(PEG)2, BM(PEG)3, BMOE, BMH, or BMB.

[00247] In various embodiments comprising two or more covalent linkers • (e.g., in Structures 7-16), the linkers are all the same. Alternatively, the compound or composition can comprise two or more different covalent linkers •.

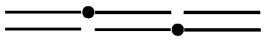
[00248] In various embodiments, each ——•—— may independently comprise two sense or two antisense oligonucleotides. For example, in the case of siRNA, a ——•—— may comprise two active strands or two passenger strands.

[00249] In various embodiments, each ——•—— may independently comprise one sense and one antisense oligonucleotide. For example, in the case of siRNA, a ——•—— may comprise one active strand and one passenger strand.

[00250] In various embodiments, the compound or composition comprises a homo-multimer of substantially identical double stranded oligonucleotides. The substantially identical double stranded oligonucleotides can each comprise an siRNA targeting the same molecular target *in vivo*.

[00251] In various embodiments, the compound or composition comprises a hetero-multimer of two or more substantially different double stranded oligonucleotides. The substantially different double stranded oligonucleotides can each comprise an siRNA targeting different genes.

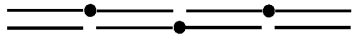
[00252] In various embodiments, the compound comprises Structure 9 and n = 0:



(Structure 9). The compound can further comprise a targeting ligand. The compound can further comprise 2 or 3 substantially different double stranded oligonucleotides ═══ each comprising an siRNA targeting a different molecular target *in vivo*. The compound can further comprise a targeting ligand, one ═══ comprising a first

siRNA guide strand targeting Factor VII and a first passenger strand hybridized to the guide strand, one  comprising a second siRNA guide strand targeting Apolipoprotein B and a second passenger strand hybridized to the second guide strand, and one  comprising a third siRNA guide strand targeting TTR and a third passenger strand hybridized to the third guide strand. The targeting ligand can comprise N-Acetylgalactosamine (GalNAc).

[00253] Examples of trimers are provided in Examples 17, 18, and 20.

[00254] In various embodiments, the compound comprises Structure 10 and m = 1:  (Structure 10). The compound can further comprise a targeting ligand. The compound can further comprise 2, 3, or 4 substantially different double stranded oligonucleotides  each comprising an siRNA targeting a different molecular target *in vivo*. The compound can further comprise a targeting ligand, one  comprising a first siRNA guide strand targeting Factor VII and a first passenger strand hybridized to the guide strand, one  comprising a second siRNA guide strand targeting Apolipoprotein B and a second passenger strand hybridized to the second guide strand, and one  comprising a third siRNA guide strand targeting TTR and a third passenger strand hybridized to the third guide strand. The targeting ligand can comprise N-Acetylgalactosamine (GalNAc).

[00255] Examples of tetramers are provided in Example 21.

[00256] In various embodiments, each double stranded oligonucleotide (e.g., , for example in Structure 4) comprises an siRNA guide strand targeting Factor VII and a passenger strand hybridized to the guide strand.

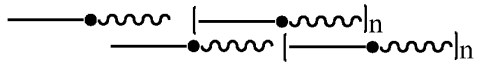
[00257] In various embodiments (e.g., in Structure 4), the compound further comprises a targeting ligand, each double stranded oligonucleotide (e.g., ) comprises an siRNA guide strand and a passenger strand hybridized to the guide strand, and the compound is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.

[00258] In various embodiments, at least one double stranded oligonucleotide (e.g., , for example in Structure 6) comprises a first siRNA guide strand targeting Factor VII and a first passenger strand hybridized to the guide strand, and at least one double stranded oligonucleotide (e.g., , for example in Structure 6) comprises a second siRNA guide strand targeting Apolipoprotein B and a second passenger strand hybridized the second guide strand.

[00259] ***Sense-antisense Multimeric Compounds***

[00260] In various aspects, the invention provides sense-antisense multi-conjugate oligonucleotides, as well as methods for their synthesis.

[00261] In one aspect, the invention provides a composition comprising a plurality of molecules, each molecule having Structure 16:


(Structure 16)

wherein n is an integer ≥ 1;

each ——— is a single stranded oligonucleotide;

each ∿∿∿ is a single stranded oligonucleotide that hybridizes with a ———;

∿∿∿
——— is a double stranded oligonucleotide; and

each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides.

[00262] Similarly, the invention provides a method for synthesizing composition comprising a plurality of molecules, each molecule having Structure 16, the methods comprising:

(i) reacting a first single stranded oligonucleotide ———R_1 with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○ under conditions that produce the mono-substituted product ———○;

(ii) reacting ———○ with a second single stranded oligonucleotide ∿∿∿R_2, wherein R2 is a chemical group capable of reacting with ○, thereby forming ———•∿∿∿; and

(iii) annealing a plurality of ———•∿∿∿, thereby forming a composition comprising a plurality of molecules, each molecule having Structure 16.

[00263] As with the other compounds and compositions according to the invention, molecules according to Structure 16 can include any one or more of the features described herein, including in the Examples. For example, the compounds can include any one or more of the nucleic acids (with or without modifications), targeting ligands, and/or linkers described above, or any of the specific structures or chemistries shown in the summary or Examples.

[00264] For example, in various embodiments, each ∿∿∿
——— has a length of 15-30 base pairs; each ∿∿∿
——— is an siRNA; and/or n is an integer from 1 to 100. Although specific examples of Structure 16 are shown as siRNA, the structure is not necessarily limited to siRNA.

[00265] In various embodiments, R1, R2, and the bifunctional linking moiety ○ can form a covalent linker • as described and shown herein. For example, in various embodiments, R1 and R2 can each independently comprise a reactive moiety, for example an electrophile or nucleophile. In one embodiment, R1 and R2 can each independently be selected from the group consisting of a thiol, maleimide, vinylsulfone, pyridyldisulfide, iodoacetamide, acrylate, azide,

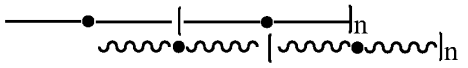
alkyne, amine, and carboxyl group. In various embodiments, the bifunctional linking moiety ○ comprises two reactive moieties that can be sequentially reacted according to steps (i) and (ii) above, for example a second electrophile/nucleophile that can be reacted with an electrophile/nucleophile in R1 and R2. Examples of bifunctional linking moieties ○ include, but are not limited to, DTME, BM(PEG)2, BM(PEG)3, BMOE, BMH, or BMB.

[00266] In one embodiment, each double stranded oligonucleotide has essentially the same sequence. In other embodiments, the double stranded oligonucleotides may vary. For example, each ——— can be an siRNA active strand with the same target, and each ∿∿∿ can be an siRNA passenger strand that is at least about 80, 85, 90, or 95 % complementary to ——— (e.g., the sequence of ∿∿∿ can vary as long as it hybridizes with ———).

[00267] ***Annealing Conditions for Multimeric Compounds***

[00268] In various aspects, the invention provides methods for synthesizing multi-conjugate oligonucleotides.

[00269] In one aspect, the invention provides method for synthesizing a composition comprising a plurality of molecule comprising Structure 17:

 (Structure 17), wherein n is an integer ≥ 1; each ——— is a single stranded oligonucleotide; each ∿∿∿ is a single stranded oligonucleotide that hybridizes with a ———; ——— is a double stranded oligonucleotide; and each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides to form ———•——— and ∿∿∿•∿∿∿, the method comprising the steps of:

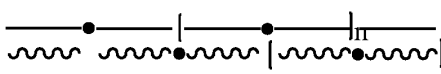
annealing a plurality of ———•——— and ∿∿∿•∿∿∿ at:

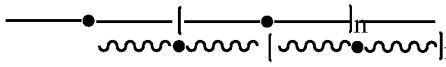
(i) a total oligonucleotide concentration of about 200-300 μM for ———•——— and ∿∿∿•∿∿∿,

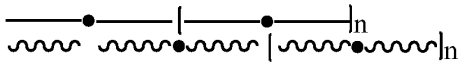
(ii) about 0.1-0.3x phosphate buffered saline (PBS), and

(iii) at a temperature of about 70-80 °C to about 20-30 °C for about 1.5-2.5 hours.

[00270] In various embodiments, the method further comprises the step of annealing a plurality of ——— and/or ∿∿∿, thereby forming a plurality of molecules comprising Structure 18:

 (Structure 18).

 (Structure 19), and/or

 (Structure 20).

[00271] In various embodiments, the method further comprises annealing ——— with the plurality of plurality of ———•——— and ~~~•~~~.

[00272] In various embodiments, the molar ratio of ——— to ~~~•~~~ is about 5:100, 10:100, 20:100, 30:100, 40:100, or 50:100.

[00273] In various embodiments, the method further comprises annealing ~~~ with the plurality of plurality of ———•——— and ~~~•~~~.

[00274] In various embodiments, the molar ratio of ~~~ to ———•——— is about 5:100, 10:100, 20:100, 30:100, 40:100, or 50:100.

[00275] In various embodiments, the molar ratio of ———•——— and ~~~•~~~ is about 1:1.

[00276] In various embodiments, the molar ratio of ———•——— to ~~~•~~~ or the molar ratio of ~~~•~~~ to ———•——— is about 100:90, 100:80, 100:75, 100:70, or 100:60.

[00277] In various embodiments, each ~~~ ——— has a length of 15-30 base pairs.

[00278] In various embodiments, each ~~~ ——— is an siRNA.

[00279] In various embodiments, each ~~~ ——— comprises siRNA guide strand targeting Factor VII and a passenger strand hybridized to the guide strand.

[00280] In various embodiments, n is an integer from 1 to 100.

[00281] In various embodiments, • is a cleavable or non-cleavable linker.

[00282] In various embodiments, the method further comprises formulating the plurality of molecules comprising Structure 17, 18, 19, and/or 20 in a nanoparticle.

[00283] As with the other compounds and compositions according to the invention, multimeric compounds and intermediates can include any one or more of the features described herein (including methods steps and including in the Examples). For example, the compounds can include any one or more of the nucleic acids (with or without modifications), targeting ligands, and/or linkers described above, or any of the specific structures or chemistries shown in the summary or Examples. Examples 22-24 illustrate exemplary embodiments of multimeric oligonucleotides.

[00284] ***Pharmaceutical Compositions***

[00285] In various aspects, the invention provides pharmaceutical compositions including any one or more of the compounds or compositions described above. As used herein, pharmaceutical compositions include compositions of matter, other than foods, that can be used to prevent, diagnose, alleviate, treat, or cure a disease. Similarly, the various compounds or compositions according to the invention should be understood as including embodiments for use as a medicament and/or for use in the manufacture of a medicament.

[00286] A pharmaceutical composition can include a compound or composition according to the invention and a pharmaceutically acceptable excipient. As used herein, an excipient can be a natural or synthetic substance formulated alongside the active ingredient. Excipients can be included for the purpose of long-term stabilization, increasing volume (e.g., bulking agents, fillers, or diluents), or to confer a therapeutic enhancement on the active ingredient in the final dosage form, such as facilitating drug absorption, reducing viscosity, or enhancing solubility. Excipients can also be useful manufacturing and distribution, for example, to aid in the handling of the active ingredient and/or to aid *in vitro* stability (e.g., by preventing denaturation or aggregation). As will be understood by those skilled in the art, appropriate excipient selection can depend upon various factors, including the route of administration, dosage form, and active ingredient(s).

[00287] Oligonucleotides can be delivered locally or systemically, and the pharmaceutical compositions of the invention can vary accordingly. For example, administration is not necessarily limited to any particular delivery system and may include, without limitation, parenteral (including subcutaneous, intravenous, intramedullary, intraarticular, intramuscular, or intraperitoneal injection), rectal, topical, transdermal, or oral. Administration to an individual may occur in a single dose or in repeat administrations, and in any of a variety of physiologically acceptable salt forms, and/or with an acceptable pharmaceutical carrier and/or additive as part of a pharmaceutical composition. Physiologically acceptable formulations and standard pharmaceutical formulation techniques, dosages, and excipients are well known to persons skilled in the art (see, e.g., Physicians' Desk Reference (PDR®) 2005, 59th ed., Medical Economics Company, 2004; and Remington: The Science and Practice of Pharmacy, eds. Gennado et al. 21th ed., Lippincott, Williams & Wilkins, 2005).

[00288] Pharmaceutical compositions can include an effective amount of the compound or composition according to the invention. As used herein, effective amount can be a concentration or amount that results in achieving a particular stated purpose, or more amount means an amount adequate to cause a change, for example in comparison to a placebo. Where

the effective amount is a therapeutically effective amount, it can be an amount adequate for therapeutic use, for example and amount sufficient to prevent, diagnose, alleviate, treat, or cure a disease. An effective amount can be determined by methods known in the art An effective amount can be determined empirically, for example by human clinical trials. Effective amounts can also be extrapolated from one animal (e.g., mouse, rat, monkey, pig, dog) for use in another animal (e.g., human), using conversion factors known in the art. See, e.g., Freireich et al., Cancer Chemother Reports 50(4):219-244 (1966).

[00289] ***Delivery Vehicles and Targeting Ligands***

[00290] In various aspects, the invention provides any one or more of the compounds or compositions described above formulated in a delivery vehicle. For example, the delivery vehicle can be a lipid nanoparticle (LNP), exosome, microvesicle, or viral vector. Similarly, in various aspects, the invention provides any one or more of the compounds or compositions described above and further comprising a targeting ligand. For example, the targeting ligand comprises *N*-Acetylgalactosamine (GalNAc), cholesterol, tocopherol, folate, 2-[3-(1,3-dicarboxypropyl)- ureido]pentanedioic acid (DUPA), or anisamide. The targeting ligand can be bound (e.g., directly) to the nucleic acid, for example through its 3' or 5' terminus. Additional examples that may be adapted for use with the invention are discussed below.

[00291] As will be understood by those skilled in the art, regardless of biological target or mechanism of action, therapeutic oligonucleotides must overcome a series of physiological hurdles to access the target cell in an organism (e.g., animal, such as a human, in need of therapy). For example, a therapeutic oligonucleotide generally must avoid clearance in the bloodstream, enter the target cell type, and then enter the cytoplasm, all without eliciting an undesirable immune response. This process is generally considered inefficient, for example, 95% or more of siRNA that enters the endosome *in vivo* may be degraded in lysosomes or pushed out of the cell without affecting any gene silencing.

[00292] To overcome these obstacles, scientists have designed numerous drug delivery vehicles. These vehicles have been used to deliver therapeutic RNAs in addition to small molecule drugs, protein drugs, and other therapeutic molecules. Drug delivery vehicles have been made from materials as diverse as sugars, lipids, lipid-like materials, proteins, polymers, peptides, metals, hydrogels, conjugates, and peptides. Many drug delivery vehicles incorporate aspects from combinations of these groups, for example, some drug delivery vehicle can combine sugars and lipids. In some other examples, drugs can be directly hidden in 'cell like' materials that are meant to mimic cells, while in other cases, drugs can be put into, or onto,

cells themselves. Drug delivery vehicles can be designed to release drugs in response to stimuli such as pH change, biomolecule concentration, magnetic fields, and heat.

[00293] Much work has focused on delivering oligonucleotides such as siRNA to the liver. The dose required for effective siRNA delivery to hepatocytes *in vivo* has decreased by more than 10,000 fold in the last ten years – whereas delivery vehicles reported in 2006 could require more than 10 mg/kg siRNA to target protein production, new delivery vehicles target protein production can now be reduced after a systemic injection of 0.001 mg/kg siRNA. The increase in oligonucleotide delivery efficiency can be attributed, at least in part, to developments in delivery vehicles.

[00294] Another important advance has been an increased understanding of the way helper components influence delivery. Helper components can include chemical structures added to the primary drug delivery system. Often, helper components can improve particle stability or delivery to a specific organ. For example, nanoparticles can be made of lipids, but the delivery mediated by these lipid nanoparticles can be affected by the presence of hydrophilic polymers and/or hydrophobic molecules. One important hydrophilic polymer that influences nanoparticle delivery is poly(ethylene glycol). Other hydrophilic polymers include non-ionic surfactants. Hydrophobic molecules that affect nanoparticle delivery include cholesterol, 1-2-Distearoyl-sn-glyerco-3-phosphocholine (DSPC), 1-2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and others.

[00295] Drug delivery systems have also been designed using targeting ligands or conjugate systems. For example, oligonucleotides can be conjugated to cholesterols, sugars, peptides, and other nucleic acids, to facilitate delivery into hepatocytes and/or other cell types.

[00296] One skilled in the art will appreciate that known delivery vehicles and targeting ligands can generally be adapted for use according to the present invention. Examples of delivery vehicles and targeting ligands, as well as their use, can be found in: Sahay, G., et al. Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling. Nat Biotechnol, 31: 653-658 (2013); Wittrup, A., et al. Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown. Nat Biotechnol (2015); Whitehead, K.A., Langer, R. & Anderson, D.G. Knocking down barriers: advances in siRNA delivery. Nature reviews. Drug Discovery, 8: 129-138 (2009); Kanasty, R., Dorkin, J.R., Vegas, A. & Anderson, D. Delivery materials for siRNA therapeutics. Nature Materials, 12: 967-977 (2013); Tibbitt, M.W., Dahlman, J.E. & Langer, R. Emerging Frontiers in Drug Delivery. J Am Chem Soc, 138: 704-717 (2016); Akinc, A., et al. Targeted delivery of RNAi therapeutics with

endogenous and exogenous ligand-based mechanisms. Molecular therapy: the journal of the American Society of Gene Therapy 18, 1357-1364 (2010); Nair, J.K., et al. Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. J Am Chem Soc, 136: 16958-16961 (2014); Ostergaard, M.E., et al. Efficient Synthesis and Biological Evaluation of 5'-GalNAc Conjugated Antisense Oligonucleotides. Bioconjugate chemistry (2015); Sehgal, A., et al. An RNAi therapeutic targeting antithrombin to rebalance the coagulation system and promote hemostasis in hemophilia. Nature Medicine, 21: 492-497 (2015); Semple, S.C., et al. Rational design of cationic lipids for siRNA delivery. Nat Biotechnol, 28: 172-176 (2010); Maier, M.A., et al. Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. Molecular therapy: the journal of the American Society of Gene Therapy, 21: 1570-1578 (2013); Love, K.T., et al. Lipid-like materials for low-dose, *in vivo* gene silencing. Proc Nat Acad USA, 107: 1864-1869 (2010); Akinc, A., et al. A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. Nat Biotechnol, 26: 561-569 (2008); Eguchi, A., et al. Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. Nat Biotechnol, 27: 567-571 (2009); Zuckerman, J.E., et al. Correlating animal and human phase Ia/Ib clinical data with CALAA-01, a targeted, polymer-based nanoparticle containing siRNA. Proc Nat Acad USA, 111: 11449-11454 (2014); Zuckerman, J.E. & Davis, M.E. Clinical experiences with systemically administered siRNA-based therapeutics in cancer. Nature Reviews. Drug Discovery, 14: 843-856 (2015); Hao, J., et al. Rapid Synthesis of a Lipocationic Polyester Library via Ring-Opening Polymerization of Functional Valerolactones for Efficacious siRNA Delivery. J Am Chem Soc, 29: 9206-9209 (2015); Siegwart, D.J., et al. Combinatorial synthesis of chemically diverse core-shell nanoparticles for intracellular delivery. Proc Nat Acad USA, 108: 12996-13001 (2011); Dahlman, J.E., et al. *In vivo* endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. Nat Nano 9, 648-655 (2014); Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R. & Rudzinski, W.E. Biodegradable polymeric nanoparticles as drug delivery devices. Journal of controlled release: official journal of the Controlled Release Society 70, 1-20 (2001); Kim, H.J., et al. Precise engineering of siRNA delivery vehicles to tumors using polyion complexes and gold nanoparticles. ACS Nano, 8: 8979-8991 (2014); Krebs, M.D., Jeon, O. & Alsberg, E. Localized and sustained delivery of silencing RNA from macroscopic biopolymer hydrogels. J Am Chem Soc 131, 9204-9206 (2009); Zimmermann, T.S., et al. RNAi-mediated gene silencing in non-human primates. Nature, 441: 111-114 (2006); Dong, Y., et al. Lipopeptide nanoparticles for potent and selective siRNA

delivery in rodents and nonhuman primates. Proc Nat Acad USA, 111: 3955-3960 (2014); Zhang, Y., et al. Lipid-modified aminoglycoside derivatives for *in vivo* siRNA delivery. Advanced Materials, 25: 4641-4645 (2013); Molinaro, R., et al. Biomimetic proteolipid vesicles for targeting inflamed tissues. Nat Mater (2016); Hu, C.M., et al. Nanoparticle biointerfacing by platelet membrane cloaking. Nature, 526: 118-121 (2015); Cheng, R., Meng, F., Deng, C., Klok, H.-A. & Zhong, Z. Dual and multi-stimuli responsive polymeric nanoparticles for programmed site-specific drug delivery. Biomaterials, 34: 3647-3657 (2013); Qiu, Y. & Park, K. Environment-sensitive hydrogels for drug delivery. Advanced Drug Delivery Reviews, 64, Supplement, 49-60 (2012); Mui, B.L., et al. Influence of Polyethylene Glycol Lipid Desorption Rates on Pharmacokinetics and Pharmacodynamics of siRNA Lipid Nanoparticles. Mol Ther Nucleic Acids 2, e139 (2013); Draz, M.S., et al. Nanoparticle-Mediated Systemic Delivery of siRNA for Treatment of Cancers and Viral Infections. Theranostics, 4: 872-892 (2014); Otsuka, H., Nagasaki, Y. & Kataoka, K. PEGylated nanoparticles for biological and pharmaceutical applications. Advanced Drug Delivery Reviews, 55: 403-419 (2003); Kauffman, K.J., et al. Optimization of Lipid Nanoparticle Formulations for mRNA Delivery *in vivo* with Fractional Factorial and Definitive Screening Designs. Nano Letters, 15: 7300-7306 (2015); Zhang, S., Zhao, B., Jiang, H., Wang, B. & Ma, B. Cationic lipids and polymers mediated vectors for delivery of siRNA. Journal of Controlled Release 123, 1-10 (2007); Illum, L. & Davis, S.S. The organ uptake of intravenously administered colloidal particles can be altered using a non-ionic surfactant (Poloxamer 338). FEBS Letters, 167: 79-82 (1984); Felgner, P.L., et al. Improved Cationic Lipid Formulations for *In vivo* Gene Therapy. Annals of the New York Academy of Sciences, 772: 126-139 (1995); Meade, B.R. & Dowdy, S.F. Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. Advanced Drug Delivery Reviews, 59: 134-140 (2007); Endoh, T. & Ohtsuki, T. Cellular siRNA delivery using cell-penetrating peptides modified for endosomal escape. Advanced Drug Delivery Reviews, 61: 704-709 (2009); and Lee, H., et al. Molecularly self-assembled nucleic acid nanoparticles for targeted *in vivo* siRNA delivery. Nat Nano, 7: 389-393 (2012).

[00297] In various embodiments, the compounds and compositions of the invention can be conjugated to or delivered with other chemical or biological moieties, including, e.g., biologically active moieties. A biologically active moiety is any molecule or agent that has a biological effect, preferably a measurable biological effect. Chemical or biological moieties include, e.g., proteins, peptides, amino acids, nucleic acids (including, e.g., DNA, RNA of all types, RNA and DNA aptamers, antisense oligonucleotides, and antisense miRNA inhibitors),

targeting ligands, carbohydrates, polysaccharides, lipids, organic compounds, and inorganic chemical compounds.

[00298] As used herein, the term targeting ligand can include a moiety that can be made accessible on the surface of a nanoparticle or as part of a delivery conjugate for the purpose of delivering the payload of the nanoparticle or delivery conjugate to a specific target, such as a specific bodily tissue or cell type, for example, by enabling cell receptor attachment of the nanoparticle or delivery conjugate. Examples of suitable targeting ligands include, but are not limited to, cell specific peptides or proteins (e.g., transferrin and monoclonal antibodies), aptamers, cell growth factors, vitamins (e.g., folic acid), monosaccharides (e.g., galactose and mannose), polysaccharides, arginine-glycine-aspartic acid (RGD), and asialoglycoprotein receptor ligands derived from *N*-acetylgalactosamine (GalNac). The ligand may be incorporated into the foregoing compounds of the invention using a variety of techniques known in the art, such as via a covalent bond such as a disulfide bond, an amide bond, or an ester bond, or via a non-covalent bond such as biotin-streptavidin, or a metal-ligand complex. Additional biologically active moieties within the scope of the invention are any of the known gene editing materials, including for example, materials such as oligonucleotides, polypeptides and proteins involved in CRISPR/Cas systems, Tales, Talens, and zinc fingers.

[00299] In various embodiments, the compounds and compositions of the invention can be encapsulated in a carrier material to form nanoparticles for intracellular delivery. Known carrier materials include cationic polymers, lipids or peptides, or chemical analogs thereof. Jeong et al., BIOCONJUGATE CHEM., Vol. 20, No. 1, pp. 5-14 (2009). Examples of a cationic lipid include dioleyl phosphatidylethanolamine, cholesterol dioleyl phosphatidylcholine, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 1,2-dioleoyl-3-(4'-trimethyl-ammonio)butanoyl-sn-glycerol(DOTB), 1,2-diacyl-3-dimethylammonium-propane (DAP), 1,2-diacyl-3-trimethylammonium-propane (TAP), 1,2-diacyl-sn-glycerol-3-ethylphosphocholin, 3 beta-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Cholesterol), dimethyldioctadecylammonium bromide (DDAB), and copolymers thereof. Examples of a cationic polymer include polyethyleneimine, polyamine, polyvinylamine, poly(alkylamine hydrochloride), polyamidoamine dendrimer, diethylaminoethyl-dextran, polyvinylpyrrolidone, chitin, chitosan, and poly(2-dimethylamino)ethyl methacrylate. In one embodiment, the carrier contains one or more acylated amines, the properties of which may be better suited for use *in vivo* as compared to other known carrier materials.

[00300] In one embodiment, the carrier is a cationic peptide, for example KALA (a cationic fusogenic peptide), polylysine, polyglutamic acid or protamine. In one embodiment, the carrier is a cationic lipid, for example dioleyl phosphatidylethanolamine or cholesterol dioleyl phosphatidylcholine. In one embodiment, the carrier is a cationic polymer, for example polyethyleneimine, polyamine, or polyvinylamine.

[00301] In various embodiments, the compounds and compositions of the invention can be encapsulated in exosomes. Exosomes are cell-derived vesicles having diameters between 30 and 100 nm that are present in biological fluids, including blood, urine, and cultured medium of cell cultures. Exosomes, including synthetic exsosomes and exosome mimetics can be adapted for use in drug delivery according to the skill in the art. See, e.g., "A comprehensive overview of exosomes as drug delivery vehicles - endogenous nanocarriers for targeted cancer therapy" Biochim Biophys Acta. 1846(1):75-87 (2014); "Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges" Acta Pharmaceutica Sinica B, Available online 8 March 2016 (In Press); and "Exosome mimetics: a novel class of drug delivery systems" International Journal of Nanomedicine, 7: 1525-1541 (2012).

[00302] In various embodiments, the compounds and compositions of the invention can be encapsulated in microvesicles. Microvesicles (sometimes called, circulating microvesicles, or microparticles.) are fragments of plasma membrane ranging from 100 nm to 1000 nm shed from almost all cell types and are distinct from smaller intracellularly generated extracellular vesicles known as exosomes. Microvesicles play a role in intercellular communication and can transport mRNA, miRNA, and proteins between cells. Microvesicles, including synthetic microvesicles and microvesicle mimetics can be adapted for use in drug delivery according to the skill in the art. See, e.g., "Microvesicle- and exosome-mediated drug delivery enhances the cytotoxicity of Paclitaxel in autologous prostate cancer cells" Journal of Controlled Release, 220: 727-737 (2015); "Therapeutic Uses of Exosomes" J Circ Biomark, 1:0 (2013).

[00303] In various embodiments, the compounds and compositions of the invention can be delivered using a viral vector. Viral vectors are tools commonly used by molecular biologists to deliver genetic material into cells. This process can be performed inside a living organism (*in vivo*) or in cell culture (*in vitro*). Viral vectors can be adapted for use in drug delivery according to the skill in the art. See, e.g., "Viruses as nanomaterials for drug delivery" Methods Mol Biol, 26: 207-21 (2011); "Viral and nonviral delivery systems for gene delivery" Adv Biomed Res, 1:27 (2012); and "Biological Gene Delivery Vehicles: Beyond Viral Vectors" Molecular Therapy, 17(5): 767-777 (2009).

[00304] General procedures for LNP formulation and characterization are provided in the Examples below, as are working examples of LNP formulations and other *in vitro* and *in vivo* tests. Other methods are known in the art and can be adapted for use with the present invention by those of ordinary skill.

[00305] ***Methods of Treatment, Reducing Gene Expression***

[00306] In various aspects, the invention provides methods for using multi-conjugate oligonucleotides, for example for medical treatments, research, or for producing new or altered phenotypes in animals and plants.

[00307] In one aspect, the invention provides a method for treating a subject comprising administering an effective amount of a compound or composition according to the invention to a subject in need thereof. In such therapeutic embodiments, the oligonucleotide will be a therapeutic oligonucleotide, for example an siRNA or miRNA.

[00308] In this, and other embodiments, the compositions and compounds of the invention can be administered in the form of a pharmaceutical composition, in a delivery vehicle, or coupled to a targeting ligand.

[00309] In one aspect, the invention provides a method for silencing or reducing gene expression comprising administering an effective amount of a compound or composition according to the invention to a subject in need thereof. In such therapeutic embodiments, the oligonucleotide will be a oligonucleotide that silences or reduces gene expression, for example an siRNA or antisense oligonucleotide.

[00310] Similarly, the invention provides a method for silencing or reducing expression of two or more genes comprising administering an effective amount of a compound or composition according to the invention to a subject in need thereof, wherein the compound or composition comprises oligonucleotides targeting two or more genes. The compound or composition can comprise oligonucleotides targeting two, three, four, or more genes.

[00311] In one aspect, the invention provides a method for delivering two or more oligonucleotides to a cell per targeting ligand binding event comprising administering an effective amount of a compound or composition according to the invention to a subject in need thereof, wherein the compound or composition comprises a targeting ligand.

[00312] In one aspect, the invention provides a method for delivering a predetermined stoichiometric ratio of two or more oligonucleotides to a cell comprising administering an effective amount of a compound or composition according to the invention to a subject in need thereof, wherein the compound or composition comprises the predetermined stoichiometric ratio of two or more oligonucleotides.

[00313] As used herein, subject includes a cell or organism subject to the treatment or administration. The subject can be an animal, for example a mammal such a laboratory animal (mouse, monkey) or veterinary patient, or a primate such as a human. Without limitation, a subject in need of the treatment or administration can include a subject having a disease (e.g., that may be treated using the compounds and compositions of the invention) or a subject having a condition (e.g., that may be addressed using the compounds and compositions of the invention, for example one or more genes to be silenced or have expression reduced).

[00314] General procedures for measurement of gene knockdown and animal experiments are provided in the Examples below, as are working example of other *in vitro* and *in vivo* tests. Other methods are known in the art and can be adapted for use with the present invention by those of ordinary skill.

[00315] The following examples are illustrative and not restrictive. Many variations of the technology will become apparent to those of skill in the art upon review of this disclosure. The scope of the technology should, therefore, be determined not with reference to the examples, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

EXAMPLES

[00316] **General Procedure: Single Chain Oligonucleotide Synthesis**

[00317] Oligoribonucleotides were assembled on ABI 394 and 3900 synthesizers (Applied Biosystems) at the 10 µmol scale, or on an Oligopilot 10 synthesizer at 28 µmol scale, using phosphoramidite chemistry. Solid supports were polystyrene loaded with 2'-deoxythymidine (Glen Research, Sterling, Virginia, USA), or controlled pore glass (CPG, 520Å, with a loading of 75 µmol/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^{Ac}), 6-*N*-benzoyl-2'-*O*-methyl-adenosine (2'-OMe-A^{bz}) and 2-*N*-isobutyrlguanosine (2'-OMe-G^{iBu}) 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-1*H*-tetrazole (ETT, 0.5 M in Acetonitrile) as activator. Phosphorothioate linkages were introduced using 50 mM 3-((Dimethylamino-

methylidene)amino)-3*H*-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),

[00318] 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.

[00319] 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.

[00320] **General Procedure: Lipid Nanoparticle Formulation**

[00321] 1,2-distearoyl-3-phosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). α-[3'-(1,2-dimyristoyl-3-propanoxy)-carboxamide-propyl]-ω-methoxy-polyoxyethylene (PEG-c-DOMG) was obtained from NOF (Bouwelven, Belgium). Cholesterol was purchased from Sigma-Aldrich (Taufkirchen, Germany).

[00322] The proprietary aminolipids KL22 and KL52 are disclosed in the patent literature (*Constien et al.* "Novel Lipids and Compositions for Intracellular Delivery of Biologically Active Compounds" US 2012/0295832 A1). Stock solutions of KL52 and KL22 lipids, DSPC, cholesterol, and PEG-c-DOMG were prepared at concentrations of 50 mM in ethanol and stored at -20°C. The lipids were combined to yield various molar ratios (see individual examples below) and diluted with ethanol to a final lipid concentration of 25 mM. siRNA stock solutions at a concentration of 10 mg/mL in H_2O were diluted in 50 mM sodium citrate buffer, pH 3. KL22 and KL52 are sometimes referred to as XL 7 and XL 10, respectively, in the examples that follow.

[00323] The lipid nanoparticle (LNP) formulations were prepared by combining the lipid solution with the siRNA solution at total lipid to siRNA weight ratio of 7:1. The lipid ethanolic solution was rapidly injected into the aqueous siRNA solution to afford a suspension containing 33% ethanol. The solutions were injected by the aid of a syringe pump (Harvard Pump 33 Dual Syringe Pump Harvard Apparatus Holliston, MA).

[00324] Subsequently, the formulations were dialyzed 2 times against phosphate buffered saline (PBS), pH 7.4 at volumes 200-times that of the primary product using a Slide-A-Lyzer cassettes (Thermo Fisher Scientific Inc. Rockford, IL) with a MWCO of 10 kD (RC membrane) to remove ethanol and achieve buffer exchange. The first dialysis was carried out at room temperature for 3 hours and then the formulations were dialyzed overnight at 4°C. The resulting nanoparticle suspension was filtered through 0.2 μm sterile filter (Sarstedt, Nümbrecht, Germany) into glass vials and sealed with a crimp closure.

[00325] **General Procedure: LNP Characterization**

[00326] Particle size and zeta potential of formulations were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK) in 1X PBS and 15 mM PBS, respectively.

[00327] The siRNA concentration in the liposomal formulation was measured by UV-vis. Briefly, 100 μL of the diluted formulation in 1X PBS was added to 900 μL of a 4:1 (v/v) mixture of methanol and chloroform. After mixing, the absorbance spectrum of the solution was recorded between 230 nm and 330 nm on a DU 800 spectrophotometer (Beckman Coulter, Beckman Coulter, Inc., Brea, CA). The siRNA concentration in the liposomal formulation was calculated based on the extinction coefficient of the siRNA used in the formulation and on the difference between the absorbance at a wavelength of 260 nm and the baseline value at a wavelength of 330 nm.

[00328] Encapsulation of siRNA by the nanoparticles was evaluated by the Quant-iT™ RiboGreen® RNA assay (Invitrogen Corporation Carlsbad, CA). Briefly, the samples were diluted to a concentration of approximately 5 μg/mL in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). 50 μL of the diluted samples were transferred to a polystyrene 96 well plate, then either 50 μL of TE buffer or 50 μL of a 2% Triton X-100 solution was added. The plate was incubated at a temperature of 37°C for 15 minutes. The RiboGreen reagent was diluted 1:100 in TE buffer, 100 μL of this solution was added to each well. The fluorescence intensity was measured using a fluorescence plate reader (Wallac Victor 1420 Multilabel Counter; Perkin Elmer, Waltham, MA) at an excitation wavelength of ~480 nm and an emission wavelength of ~520 nm. The fluorescence values of the reagent blank were subtracted from

that of each of the samples and the percentage of free siRNA was determined by dividing the fluorescence intensity of the intact sample (without addition of Triton X-100) by the fluorescence value of the disrupted sample (caused by the addition of Triton X-100).

[00329] General Procedure: Animal Experiments

[00330] Mouse strain C57BL/6N was used for all *in vivo* experiments. Animals were obtained from Charles River (Sulzfeld, Germany) and were between 6 and 8 weeks old at the time of experiments. Intravenously administered LNP formulations were injected by infusion of 200 µL into the tail vein. Subcutaneously administered compounds were injected in a volume of 100-200 µL. Blood was collected by submandibular vein bleed the day before injection ("prebleed") and during the experiment post injection at times indicated. Serum was isolated with serum separation tubes (Greiner Bio-One, Frickenhausen, Germany) and kept frozen until analysis. 7 days after compound administration, mice were anaesthetized by CO_2 inhalation and killed by cervical dislocation. Blood was collected by cardiac puncture and serum isolated as described above. Tissue for mRNA quantification was harvested and immediately snap frozen in liquid nitrogen.

[00331] General Procedures: Measurement of Gene Knockdown

[00332] Determination of serum protein levels was achieved using the following: Factor VII was analyzed using the chromogenic enzyme activity assay BIOPHEN FVII (#221304, Hyphen BioMed, MariaEnzersdorf, Austria) following the manufacturer's recommendations. Mouse serum was diluted 1:3000 before analysis. Absorbance of colorimetric development at 405 nm was measured using a Victor 3 multilabel counter (Perkin Elmer, Wiesbaden, Germany).

[00333] ApoB protein in serum was measured by ELISA (CloudClone Corp. / Hoelzel Diagnostics, Cologne, Germany, #SEC003Mu). A 1:5000 dilution of mouse serum was processed according to the manufacturer's instructions and absorbance at 450 nm measured using a Victor 3 multilabel counter (Perkin Elmer, Wiesbaden, Germany).

[00334] Transthyretin (TTR, also known as prealbumin) protein in serum was measured by ELISA (#KA2070, Novus Biologicals, / Biotechne, Wiesbaden, Germany). A 1:4000 dilution of mouse serum was processed according to the manufacturer's instructions and absorbance at 450 nm measured using a Victor 3 multilabel counter (Perkin Elmer, Wiesbaden, Germany).

[00335] For quantification of mRNA levels, frozen tissue pieces (30-50 mg) were transferred to a chilled 1.5 mL reaction tube. 1 mL Lysis Mixture (Epicenter Biotechnologies, Madison, USA) containing 3,3 µL/ml Proteinase K (50µg/µL) (Epicenter Biotechnologies,

Madison, USA) was added and tissues were lysed by sonication for several seconds using a sonicator (HD2070, Bandelin, Berlin, Germany) and digested with Proteinase K for 30 min at 65°C in a thermomixer (Thermomixer comfort, Eppendorf, Hamburg, Germany). Lysates were stored at -80°C until analysis. For mRNA analysis, lysates were thawed and mRNA levels were quantified using either QuantiGene 1.0 (FVII, ApoB and GAPDH) or Quantigene 2.0 (TTR) branched DNA (bDNA) Assay Kit (Panomics, Fremont, Calif., USA, Cat-No: QG0004) according to the manufacturer's recommendations. As assay readout, the chemiluminescence signal was measured in a Victor 2 Light luminescence counter (Perkin Elmer, Wiesbaden, Germany) as relative light units (RLU). The signal for the corresponding mRNA was divided by the signal for GAPDH mRNA from the same lysate. Values are reported as mRNA expression normalized to GAPDH.

[00336] Example 1: Generation of Thiol-terminated siRNA

[00337] Where necessary 3'- or 5'-terminal thiol groups were introduced via 1-*O*-Dimethoxytrityl-hexyl-disulfide,1'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite linker (NucleoSyn, Olivet Cedex, France). Upon completion of the solid phase synthesis and final 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 6 hours at 10°C. Subsequently, the crude oligonucleotides were purified by anion-exchange high-performance liquid chromatography (HPLC) on an AKTA Explorer System (GE Healthcare, Freiburg, Germany). Purified (C_6SSC_6)-oligonucleotides were precipitated by addition of ethanol and overnight storage in the freezer. Pellets were collected by centrifugation. 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 and RP HPLC.

[00338] Each disulfide containing oligomer was then reduced using a 100 mM DL-Dithiothreitol (DTT) solution. 1.0 M DTT stock solution (Sigma-Aldrich Chemie GmbH, Munich, Germany, #646563,) was diluted with Triethylammonium bicarbonate buffer (TEABc, 1M, pH 8.5, Sigma, #90360) and water to give a solution 100 mM each in DTT and TEABc. The oligonucleotide was dissolved in TEABc buffer (100mM, pH 8.5) to yield a 1 mM solution. To accomplish the disulfide reduction a 50-100 fold molar DTT excess is 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.

[00339] Example 2: General Procedure for Preparation of Mono-DTME Oligomer

[00340] 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.

[00341] Example 3: General Procedure for Preparation of Dimer via DTME Functionality

[00342] The DTME modified oligonucleotide prepared according to the procedure in Example 2 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).

[00343] Example 4: General Procedure for Annealing of Single Stranded RNAs (ssRNAs) to Form Double Stranded RNA (dsRNA)

[00344] dsRNAs were generated from RNA single strands by mixing equimolar amounts of complementary sense and antisense strands 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.

[00345] Example 5: General Procedure for Preparation of 3'- or 5'- NH_2 Derivatized Oligonucleotides

[00346] RNA equipped with a C-6-aminolinker at the 5´-end of the sense strand was produced by standard phosphoramidite chemistry on solid phase at a scale of 140 µmol using an ÄKTA Oligopilot 100 (GE Healthcare, Freiburg, Germany) and controlled pore glass (CPG) as solid support (Prime Synthesis, Aston, PA, USA). Oligomers containing 2´-O-methyl and 2'-F nucleotides were generated employing the corresponding 2'-OMe-phosphoramidites, 2´-F-methyl phosphoramidites. The 5'-aminohexyl linker at the 5'-end of the sense strand was introduced employing the TFA-protected hexylaminolinker phosphoramidite (Sigma-Aldrich, SAFC, Hamburg, Germany). In case the hexylamino-linker was needed at the 3'-position, a phtalimido protected hexylamino-linker immobilized on CPG (Prime Synthesis, Aston, PA, USA) was used. Cleavage and deprotection was accomplished using a mixture of 41% methylamine in water and concentrated aqueous ammonia (1:1 v/v). Crude oligonucleotides were purified using anion exchange HPLC and a column (2.5 x 18 cm) packed with Source 15Q resin obtained from GE Healthcare.

[00347] Example 6: General method for GalNAc ligand conjugation

[00348] The trivalent GalNAc ligand was prepared as outlined in *Hadwiger et al.*, patent application US2012/0157509 A1. The corresponding carboxylic acid derivative was activated using NHS chemistry according to the following procedure:

[00349] 3GalNAc-COOH (90 µmol, 206 mg) was dissolved in 2.06 mL DMF. To this solution *N*-Hydroxysuccinimide (NHS, 14.3 mg (99 µmol, 1.1 eq.) and Diisopropylcarbodiimide (DIC, 18.29 µL, 1.05 eq., 94 µmol) were added at 0°C. This solution was stirred overnight at ambient temperature. Completion of the reaction was monitored by TLC (DCM:MeOH=9:1).

[00350] The precursor oligonucleotide equipped with an aminohexyl linker was dissolved in sodium carbonate buffer (pH 9.6):DMSO 2:3 v/v to give a 4.4 mM solution. To this solution an aliquot of the NHS activated GalNAc solution (1.25 eq, 116 µL) was added. After shaking for 1 hour at 25°C, another aliquot (116 µL) of the NHS activated GalNAc was added. Once RP HPLC analysis showed at least more than 85% conjugated material, the crude conjugate was precipitated by addition of ethanol and storage in the freezer overnight. The pellet was collected by centrifugation. The pellet was dissolved in 1 mL concentrated aqueous ammonia and agitated for 4 hours at room temperature in order to remove the O-acetates from the GalNAc sugar residues. After confirmation of quantitative removal of the *O*-acetates by RP HPLC ESI MS, the material was diluted with 100 mM Triethyl ammonium acetate (TEAA) and the crude reaction mixture was purified by RP HPLC using an XBridge Prep C18 (5 µm, 10x 50 mm, Waters) column at 60°C on an ÄKTA explorer HPLC system. Solvent A was 100

mM aqueous TEAA and solvent B was 100 mM TEAA in 95% CAN, both heated to 60°C by means of a buffer pre-heater. A gradient from 5% to 25% B in 60 min with a flow rate of 3.5 mL/min was employed. Elution of compounds was observed at 260 and 280 nm. Fractions with a volume of 1.0 mL were collected and analyzed by analytical RP HPLC/ESI-MS. Fractions containing the target conjugate with a purity of more than 85% were combined. The correct molecular weight was confirmed by ESI/MS.

[00351] Example 7: Oligonucleotide Precursors

[00352] Using the methodologies described in the above examples the following single-stranded monomers, dimers and GalNAc tagged monomers and dimers were prepared:

Table 1: Oligonucleotide Precursors – Single Strands ("X")

SEQ ID	ID	FVII sense strands (5'-3')
1	X18791	(C_6SSC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C_6NH_2)
2	X18792	(C_6SSC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C_6NH)($GalNAc_3$)
3	X18793	(SHC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C_6NH)($GalNAc_3$)
4	X18794	(C_6SSC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)
5	X19569	(SHC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)
6	X19574	(DTME)(SHC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)
	ID	FVII antisense strands (5'-3')
7	X18796	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C_6SSC_6)dT
8	X18797	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C_6SH)
9	X18798	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C_6SH)(DTME)
	ID	ApoB sense strands (5'-3')
10	X19577	(C_6SSC_6)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)
11	X19578	(SHC_6)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)
12	X19579	(DTME)(SHC_6)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)

Table 2: Oligonucleotide Single Stranded Sense and Antisense Pairs; and Resulting Duplexes ("XD") After Annealing.

SEQ ID	Duplex ID	SEQ ID	Single Strand ID	Sequence (5'-3')	Target/strand
15	XD-00376	13	X01162	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT	FVIIs
		14	X00549	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT	FVIIas
18	XD-00030	16	X00116	GcAAAGGcGuGccAAcucAdTsdT	FVIIs
		17	X00117	UGAGUUGGcACGCCUUUGCdTsdT	FVIIas

21	XD-01078	19	X02943	GGAAUCuuAuAuuuGAUCcAsA	ApoBs
		20	X02944	uuGGAUcAAAuAuAAGAuUCcscsU	Apoas
24	XD-00194	22	X00539	cuuAcGcuGAGuAcuucGAdTsdT	LUCs
		23	X00540	UCGAAGuACUcAGCGuAAGdTsdT	LUCas

Table 3: Derivatized Oligonucleotide Single Stranded Sense and Antisense Pairs; and Resulting Duplexes After Annealing.

SEQ ID	Duplex ID	SEQ ID	Single Strand ID	Sequence (5'-3')	Target
27	XD-06328	25	X18790	(GalNAc3)(NHC$_6$)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	FVII
		26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	
30	XD-06728	28	X20124	(GalNAc3)(NHC$_6$)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)	ApoB
		29	X19583	UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu	
33	XD-06386	31	X20216	(GalNAc3)(NHC$_6$)sAfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)	TTR
		32	X19584	usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu	
		34	X19571	gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C$_6$NH)(GalNAc3)	FVII
36	XD-05961	35	X18788	gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	FVII
		26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	

Table 4: Single Stranded Oligonucleotide Dimers Linked by DTME

SEQ ID	ID	Sequence (5'-3')	Target/strand
37	X15049	GGAAUCuuAuAuuuGAUCcAsA(SHC$_6$)(DTME)GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC$_6$)	ApoBs/F7as
38	X12714	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC$_6$)(DTME)GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC$_6$)	F7s/F7as
39	X19575	(SHC$_6$)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C$_6$NH)(GalNAc$_3$)(DTME)(SHC$_6$)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	F7s/F7s

40	X19819	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C_6SH)(DTME)UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C_6SH)	F7as/F7as
41	X20336	(SHC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C_6NH)(GalNAc$_3$)(DTME)(SHC_6)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)	F7s/ApoBs

Table 5: Single Strand DTME Dimers and Corresponding Monomers; and Resulting Duplexes After Annealing

SEQ ID	Duplex ID	SEQ ID	Single Strand ID	Sequence (5'-3')	Target/Strand
42	XD-05311	37	X15049	GGAAUCuuAuAuuuGAUCcAsA(SHC6)(DTME)GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)	ApoBs--FVIIs
		14	X00549	5'-GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT-3' +	FVIIas
		20	X02944	5'-uuGGAUcAAAuAuAAGAuUCcscsU-3'	ApoBas
43	XD-05312	38	X12714	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)(DTME)GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)	FVIIs--FVIIas
		13	X01162	5'-GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT-3'	FVIIs
		14	X00549	5'-GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT-3'	FVIIas

Table 6: Chemically Synthesized Disulfide-Linked Dimers and Trimers

SEQ ID	Single Strand ID	Sequence (5'-3')	Target/Strand
44	X20366	usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu(C6SSC6)UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu	TTRas/ApoBas
45	X22413	AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)(C6SSC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	FVIIs/TTRs
46	X20256	(SHC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C6NH)(GalNAc3)(SPDP)(NHC6)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)(C6SSC6)AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)	FVII/ApoB/TTr
47	X20366	usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu(C_6SSC_6)UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu	TTRas/ApoBas

48	X22413	AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)(C_6SSC_6)gcA faAfgGfcGfuGfcCfaAfcUfcAf(invdT)	FVIIs/ TTRs

[00353] Key: In the Sequence portion of Tables 1-6 above (and those that follow): 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; and "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. "(BMPEG2)" represents the non-cleavable homobifunctional crosslinker 1,8-bismaleimido-diethyleneglycol. "C6NH2" and "C6NH" are used interchangeably to represent the aminohexyl linker. "C6SSC6" represents The dihexyldisulfide linker. "GalNAc3" and "GalNAc" are used interchangeably to represent the tri-antennary N-acetylgalactosamine ligand, whose chemical structure is shown in Fig. 1. "SPDP" represents the reaction product of the reaction of succinimidyl 3-(2-pyridyldithio)propionate with the aminolinker equipped RNA. "InvdT" means inverted thymidine. In general, sequences are written left to right from the 5'- to the 3'-terminus; however, sequences comprising the DTME or BMPEG2 crosslinker are linked via the 3'-ends, and in these sequences the second half needs to be read from right to left (to maintain 5'-to 3' orientation); or if the second half is read from left to right, then the orientation is 3'-to 5'.

[00354] In the Target/Strand portion of the chart: "F7" or "FVII" designates an siRNA sequence targeting the Factor VII transcript (or mRNA) (also known as Factor VII). "ApoB" designates an siRNA sequence targeting the apolipoprotein B transcript. "TTR" designates an siRNA sequence targeting the transthyretin transcript. Sense strand is designated "s"; antisense strand is designated "as".

[00355] Example 8: General Procedure to Generate Dimeric, Trimeric and Tetrameric siRNAs by Sequential Annealing

[00356] For the preparation of dimeric, trimeric and tetrameric siRNAs, a stepwise annealing procedure was performed. The 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").

[00357] 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.

[00358] Example 9: Preparation of FVII-DTME-ApoB Heterodimer (XD-05311)

[00359] The FVII-ApoB heterodimer (XD-05311) was prepared in high purity by sequential combination of the following single strands using the methodology described in Example 8 and depicted in Fig. 2.

[00360] The single stranded heterodimer X15049 was purified on an ÄKTA explorer 100 (GE Healthcare) equipped with a ResourceQ column obtained from 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 but contained 500 mM sodium perchlorate. The column was maintained at 60°C using a column oven. The flow rate was 4 mL/min. The crude material was loaded on to the column using the instrument's sample pump. Elutuin was recorded at 280 nm and a gradient from 15%B to 45%B in 45 minutes was employed. Appropriate fractions were pooled and precipitated by the addition of 3M sodium acetate (NaOAc, pH5.2)/ethanol 1/32 (v/v) and storage at minus 20°C over night. The pellet was collected by centrifugation and the material reconstituted in water. The material was analyzed for purity using analytical AEX HPLC. Using a Dionex DNA Pac 200 column (4x 250 mm) the material had a purity of 92.8% area. In addition, the material was analyzed by RP HPLC on a XBridge C18 Oligo BEH column from waters (2.5 µm; 2.1x50 mm). Using this technique the material had 96.5% area.

[00361] Example 10: *In vivo* Analysis of LNP-Formulated FVII-ApoB Heterodimer (XD-05311) (Animal Experiment MausRNAi-TV30)

[00362] A heterodimeric siRNA targeting Factor VII and ApoB (XD-05311) was formulated into LNP according to the General Procedure: Lipid Nanoparticle Formulation (above) and using the standard KL52 (XL10) formulation consisting of KL52/DSPC/Cholesterol/PEG-c-DMOG 50:10:38.5/1.5 mole%. A canonical siRNA for ApoB (XD-01078) and a canonical siRNA for FVII (XD-00030) were each formulated in the same LNP (XL10), and an additional canonical siRNA for FVII (XD-00030) was formulated in the standard KL22 (XL7) formulation consisting of KL22/DSPC/Cholesterol/PEG-c-DMOG 50:10:38.5/1.5 mole%. The LNP formulations, summarized in Table 7 below, were characterized according to General Procedure: LNP Characterization (above) and tested for *in vivo* efficacy in an animal experiment as described in General Procedure: Animal Experiments (above). Group size was n=3 mice for treatment groups and n=6 for saline control. All compounds were injected intravenously at a dose of 0.35 mg/kg. Blood was collected prior to injection, and at 24 hours, 69 hours, and 7 days post-injection at the time points described above and analyzed for FVII enzyme activity according to General Procedures: Measurement of Gene Knockdown (above). Results are shown in Fig. 3. mRNA levels of FVII and ApoB in liver lysates were measured at day 7 post injection, and are shown in Fig. 4.

Table 7: LNP-formulations used in animal experiment MausRNAi-TV30

Formulation ID	siRNA	Lipid
NPA-640-1	Heterodimer FVII-ApoB (XD-05311)	XL10 Std
NPA-641-1	ApoB (XD-01078)	XL10 Std
NPA-194-3	FVII (XD-00030)	XL10 Std
NPA-624-1	FVII (XD-00030)	XL7 Std

[00363] Example 11: Preparation of 5'-GalNAc-FVII Canonical Control (XD-06328)

[00364] 5'-GalNAc-FVII Canonical Control (XD-06328) (see Fig. 5) was prepared by annealing ssRNA strands X18790 and X18795 by the methods described in Example 4. The product was obtained in 91.6% purity as determined by HPLC analysis.

[00365] Example 12: Preparation of 3'-GalNAc-FVII-DTME-FVII Homodimer with Cleavable Linker Joining 3' Antisense Strands and GalNAc Conjugated to External 3' End of Sense Strand (XD-06330)

[00366] GalNAc-conjugated homodimeric siRNA XD-06330 targeting FVII (Fig. 6) was prepared (10mg, 323 nmol) by combining the single stranded dimer X19819 stepwise with

X18788 and X19571 according to the duplex titration method described in Example 8. The isolated material was essentially pure by HPLC analysis.

Table 8: Stoichiometries of Oligomers Used in Synthesis of GalNAc-FVII-DTME- FVII Homodimer (XD-06330)

SEQ ID	ID	Target	E (L/mol*cm)	Nmol/ OD	MW (free Acid)	MW Na salt	Req OD
40	X19819	FVIIas-FVIIas	389000	2.57	14405.6	15372.9	174
36	X18788	FVIIs	193000	5.18	6545.3	6962.9	62.3
34	X19571	FVIIs	193000	5.18	8161.0	8600.6	62.3
49	XD-06330				29111.9	30936.4	

[00367] Example 13: Preparation of 3'-GalNAc-FVII-DTME-FVII Homodimer with Cleavable Linker Joining 5' Sense Strands and GalNAc Conjugated to External 3' End of Sense Strand (XD-06360)

[00368] GalNAc-conjugated homodimeric siRNA XD-06360 targeting FVII was prepared (11 mg, 323 nmol) by combining single strands stepwise using the synthesis strategy depicted in Fig. 7 and the methodology described in Example 8.

[00369] All reactive steps produced high quality material, with oligomer X19575 being determined to be 91.7 and 93.4 % pure by ion exchange and reverse phase chromatography respectively, and oligomer XD-06360 being isolated in 86.8% purity as determined by non-denaturing reverse phase HPLC. The stoichiometries of the various oligomers used in the synthesis are shown in Table 9.

Table 9: Stoichiometries of Oligomers Used in Synthesis of GalNAc-FVII-FVII Homodimer (XD-06360)

SEQ ID	ID	Target	E (L/mol*cm)	Nmol/OD	MW (free Acid)	MW Na salt	Req OD

39	X19575	FVIIs-FVIIs	384800	2.60	15413.1	16314.4	137
26	X18795	FVIIas	194800	5.13	6849.4 x2	7289.1 x2	139
50	XD06360				29111.9	30892.6	

[00370] Example 14: Preparation of 5'-GalNAc-FVII-DTME-FVII Homodimer with Cleavable Linker Joining 3' Antisense Strands and GalNAc Conjugated to Internal 5' end of Sense Strand (XD-06329)

[00371] GalNAc-conjugated homodimeric siRNA XD-06329 targeting FVII [SEQ ID 51] was prepared as depicted in Fig. 8 by annealing 1150 nmol of X18788 and 1150 nmol X18798. The sum of the ODs of the individual strands was 450 ODs and the combined solution, i.e. the duplex, had 394 ODs due to the hyperchromicity (394 ODs = 1150 nmol duplex). This DTME modified duplex was reacted with 1150 nmol X18797 (3'-SH modified FVII antisense) (224 ODs). After HPLC purification, 364 ODs "half-dimer" siRNA was isolated. "Half-dimer" FVII siRNA (10 mg,323 nmol, 174 ODs) was then annealed with 5'GalNAc-FVII sense (X18790) (323 nmol,62.3 OD) to yield final product XD-06329.

[00372] Example 15: Determination of *In vivo* FVII Gene Knockdown by FVII Homodimeric GalNAc Conjugates (XD-06329, XD-06330 and XD-06360).

[00373] Three different variants of homodimeric, GalNAc-conjugated siRNAs targeted against Factor VII (XD-06329, XD-06330 and XD-06360) and a monomeric GalNAc-conjugated FVII-siRNA (XD-06328) were tested for *in vivo* efficacy in an animal experiment as described above (General Procedure: Animal Experiments). Group size was n=4 mice for treatment groups and n=5 for saline control. All compounds were injected subcutaneously at different doses (25mg/kg or 50 mg/kg) in a volume of 0.2 mL. Blood was collected 1 day prior to treatment, and at 1, 3 and 7 days post-treatment, and analyzed for FVII enzyme activity. Results are shown in Fig. 9.

[00374] Silencing activity, onset of action, and potency of the homodimeric GalNAc-conjugates (XD-06329, XD-06330 and XD-06360) was comparable to the monomeric, canonical control (XD-06328) on a knockdown per unit weight basis. No signs of toxicity were observed (e.g., weight loss, abnormal behavior).

However, upon normalizing for GalNAc content, the homodimeric GalNAc conjugates were all more effective at FVII knockdown than GalNAc monomer, thereby demonstrating more efficient siRNA uptake per ligand/receptor binding event. These results are shown in Figs. 10A and 10B and 10C.

[00375] Figure 10A. Factor VII serum activity after subcutaneous administration of GalNAc conjugates or PBS. Factor VII serum values at each time point are normalized to control mice, which were injected with 1X PBS. In this case, the amount of GalNAc injected in the animals was kept nearly constant. Factor VII serum activity was measured three days before injection (-3), or 1, 3, or 7 days following injection. Data are plotted as average +/- S.E.M., and N=3 mice / group. The bars at each datapoint (days -3, 1, 3, and 7) correspond, left to right, to X06328, X06329, X06330, and X06360, respectively.

[00376] Figure 10B. Factor VII serum activity after subcutaneous administration of GalNAc conjugates or PBS. Factor VII serum values at each time point are normalized to control Factor VII values taken 3 days before injection. In this case, the amount of GalNAc injected in the animals was kept nearly constant. Factor VII serum activity was measured three days before injection (-3), or 1, 3, or 7 days following injection. Data are plotted as average +/- S.E.M. and N=3 mice / group. The bars at each data point (days -3, 1, 3, and 7) correspond, left to right, to X06328, X06329, X06330, and X06360, respectively.

[00377] **Example 16: Preparation of Canonical GalNAc-siRNAs independently targeting FVII (XD-06328), ApoB (XD-06728) and TTR (XD-06386).**

[00378] Three canonical siRNAs independently targeting FVII (XD-06328), ApoB (XD-06728) and TTR (XD-06386) (see Fig. 11) were independently prepared by solid phase synthesis. Three sense strands (X18790, X20124, X20216, respectively) were separately prepared with a 5'-hexylamine linker. Following cleavage and deprotection of the oligonucleotides and HPLC purification of the crude material conjugation of a per-acetylated GalNAc cluster to each oligo was achieved using NHS chemistry. Removal of the *O*-acetates by saponification was mediated by aqueous ammonia. The complementary antisense strands (X18795, X19583, and X19584, respectively) were synthesized by standard procedures provided above, followed by annealing to the GalNAc conjugated single strands to yield siRNAs targeting FVII (XD-06328), ApoB (XD-06728) and TTR (XD-06386) in 99.7, 93.1 and 93.8% purity respectively.

Table 10: GalNAc-siRNA Conjugates

SEQ ID	Duplex ID	ssRNA	Sequence 5'--3'	
27	XD-	X18790	(GalNAc3)(NHC$_6$)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invd	FVII

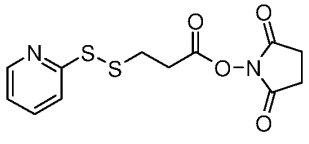
	06328		T)	
		X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	
30	XD-06728	X20124	(GalNAc3)(NHC$_6$)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)	ApoB
		X19583	UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu	
33	XD-06386	X20216	(GalNAc3)(NHC$_6$)sAfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)	TTR
		X19584	usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu	

[00379] Example 17: Preparation of GalNAc-FVII-ApoB-TTR Trimer with Cleavable Linkages on Sense Strands (XD06726)

[00380] A heterotrimer targeting FVII, ApoB and TTR conjugated to GalNAc (see Fig. 12) was synthesized using a hybrid strategy of solid phase and solution phase, as depicted in Fig. 13.

[00381] Fig. 13: Synthesis Strategy for GalNAc-Conjugated Heterotrimer (XD06726)

[00382] The dimer X19581 was made using solid phase chemistry with an aminohexyl linker on the 5'-end using the corresponding commercially available TFA protected phosphoramidite (SAFC Proligo, Hamburg, Germany). The sequence was cleaved from the solid support, deprotected and purified according to the conditions outlined above. In order to install an additional disulfide linker, the oligonucleotide's 5'-aminohexyllinker was reacted with SPDP (succinimidyl 3-(2-



pyridyldithio)propionate) available from Sigma (#P3415). 928 nmol (400 OD) oligonucleotide was dissolved in 4.7 mL 100 mM TEAB, pH8.5, containing 20% Dimethyl formamide (DMF). To this solution was added a solution of 1.4 mg (4.6 µmol, 5 eq) SPDP in 100 µL DMF. Once analytical RP HPLC indicated consumption of the starting material, the crude reaction mixture was purified on a C18 XBridge column (10x 50 mm) purchased from Waters. RP purification was performed on an ÄKTA explorer HPLC system. Solvent A was 100 mM aqueous TEAA and solvent B was 100 mM TEAA in 95% ACN. Solvents were heated to 60°C by means of a buffer

pre-heater and the column was kept in an oven at the same temperature. A gradient from 0% to 35% B in 45 min with a flow rate of 4 mL/min was employed. Elution of compounds was observed at 260 and 280 nm. Fractions with a volume of 1.5 mL were collected and analyzed by analytical RP HPLC/ESI-MS. Suitable fractions were combined and the oligonucleotide X19582 precipitated at minus 20°C after addition of ethanol and 3M NaOAc (pH5.2). Identity was confirmed by RP-HPLC ESI-MS.

[00383] In order to prepare the single stranded trimer, the above oligonucleotide X19582 (255 nmol) was dissolved in 1.3 mL water. To this solution 306 nmol (1.2 eq) of the thiol modified oligonucleotide X18793 was added. The reaction mixture contained 200 mM TEAA and 20% acetonitrile. Progress of the reaction was followed by RP HPLC. Once the starting material was consumed the reaction mixture was purified using the same conditions as described in the previous paragraph, with the exception that the gradient was run from 0%B to 30%B in 45 min.

[00384] The single-stranded heterotrimer X20256 (containing linked sense strands of siFVII, siApoB and siTTR) was obtained in high purity. The sequence of X20256 is shown in Table 11.

Table 11:

SEQ ID	ID	Sequence	Target/ Strand
52	X20256	(SHC$_6$)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C$_6$NH)(GalNAc$_3$)(SPDP)(NHC$_6$)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)(C$_6$SSC$_6$)AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)	FVIIs/ApoBs/TTRs

[00385] Note: In principle the above sequence is accessible through a single solid phase synthesis. In this case, SPDP and C_6NH_2 would be replaced by the C_6SSC_6 phosphoramidite. However, due to the sequence length of the entire construct such a synthesis would be challenging.

[00386] Thereafter, the heterotrimeric duplex construct (XD-06726), simultaneously targeting FVII, ApoB and TTR, 7 mg (150 nmol), was prepared by sequentially adding the antisense single strands stepwise to the sense-strand heterotrimeric intermediate (X20256) according to the duplex titration method described Example 8. 7 mg of material was obtained which was essentially pure by HPLC.

Table 12: Stoichiometries of Oligomers Used in Synthesis of GalNAc-FVII-ApoB-TTR Trimer (XD-06726).

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

[00387] Example 18: Preparation of GalNAc-FVII-ApoB-TTR Trimer with Cleavable Linkages on Alternating Sense and Antisense Strands (XD06727).

[00388] 9 mg (192 nmol) of Trimeric siRNA XD-06727 (see Fig. 14), simultaneously targeting FVII, ApoB and TTR, was prepared in high purity by combining single strands stepwise as depicted in Fig. 15, using the methodology described in Example 8.

Table 13: Stoichiometries of Oligomers used in synthesis of GalNAc-siFVII-siApoB-siTTR Trimer (XD-06727)

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

[00389] The synthesis that produced the heterotrimer (XD-06727) is highly efficient. In this example, nearly 100% conversion of the reactants was achieved at each step. See Figs. 16, 17, and 18.

[00390] **Example 19: Preparation of LNP Formulation of Pooled siRNAs Individually Targeting FVII, ApoB and TTR**

[00391] Monomeric siRNAs targeting FVII (XD-00030), ApoB (XD-01078) and TTR (XD-06729 were formulated in Lipid Nanoparticles and characterized using the methodologies described in General Procedure: Lipid Nanoparticle Formulation and General Procedure: LNP Characterization. The lipid composition was XL10:DSPC:Cholesterol:PEG-DOMG/50:10:38.5:1.5 molar percent. 88% encapsulation was achieved and the resulting particles were 83nm in size with a zeta potential of 2.2mV and a PDI of 0.04.

[00392] **Example 20: Assessment of mRNA Knockdown by GalNAc-Conjugated Heterotrimeric SiRNAs**

[00393] To determine the *in vivo* efficacy of heterotrimeric GalNAc-conjugated siRNAs (targeted to FVII, ApoB and TTR), an animal experiment was performed as described above (General Procedure: Animal Experiments) using a group size of n=4 mice for treatment groups and n=5 for saline controls. The heterotrimers XD-06726 and XD-06727 as well as a pool of 3 monomeric GalNAc-conjugated siRNAs (XD-06328 targeting FVII; XD-06386 targeting TTR and XD-06728 targeting ApoB) were injected subcutaneously (0.1 mL volume) at a concentration of 50 mg/kg total RNA for the trimers and 17 mg/kg for each of the monomeric conjugates. For comparison, a pool of LNP-formulated siRNAs (NPA-741-1) directed against the same targets (FVII (XD-00030), ApoB (XD-01078) and TTR (XD-06729)) was injected intravenously at 0.5 mg/kg per siRNA. Blood was collected as described above (General Procedure: Animal Experiments) 1 day prior to treatment and at 1, 3 and 7 days post-treatment, and serum levels of FVII, ApoB and TTR measured according to the General Procedures: Measurement of Gene Knockdown. Results are shown in Figs. 19A and 19B, 20A and 20B, and 21A and 21B. mRNA levels in liver lysates were measured at day 7 post injection (Figs. 22A and 22B).

[00394] One animal in group A (XD-06726) did not show any effect on TTR serum levels. The second of the two TTR protein graphs shows data with values omitted for the non-responding animal.

[00395] For comparison, the values from the animal showing poor TTR response have been omitted from the second FVII graph.

[00396] ApoB serum levels show a high variation, both within the animals of one group and between the different time-points of the saline control.

[00397] Knockdown of all three genes was also measured using a bDNA assay for mRNA from liver tissue according to the General Procedures: Measurement of Gene Knockdown, above. Target gene levels were normalized to the housekeeper GAPDH.

[00398] **Example 21: Preparation GalNAc-FVII-ApoB-TTR-FVII Tetramer (XD-07140)**

[00399] 12.4 nmol of the tetrameric siRNA XD-07140 (see Fig. 23), simultaneously targeting FVII, ApoB and TTR, was prepared by combining single strands stepwise as depicted in Fig. 24, and according to the duplex titration method described in Example 8. HPLC analysis showed the product was obtained in high purity.

Table 14: Stoichiometries of Oligomers used in Synthesis of GalNAc-FVII-ApoB-TTR-FVII Tetramer (XD--07140)

SEQ ID	ID	Target	E (L/mol*cm)	1 OD	MW (free Acid)	MW Na salt	Req OD
42	X20336	FVIIs-ApoBs	404300	2.47 nmol	15440.1	16341.4	5
49	X20366	ApoBas-TTRas	446700	2.24 nmol	14748.9	15716.1	5.5
45	X22413	TTRs-FVIIs	412100	2.52 nmol	14041.3	14964.5	4.9
26	X18795	FVIIas	194800	5.13 nmol	6849.4 x2	7289.1 x2	4.8
55	XD-07140				57929.1	61600.2	

[00400] **Example 22: Generation of Mixtures of Multimeric siRNAs**

[00401] Mixtures of multimeric siRNAs in dynamic equilibria, and methods of manufacturing them, are described in Mok et al., "Multimeric small interfering ribonucleic acid for highly efficient sequence-specific gene silencing," NATURE MATERIALS, Vol. 9, March 2010. As described therein, the mixtures comprise linear chains of chemically linked siRNAs, wherein the chains are of varying lengths. In this

Example, there follows a series of experiments conducted to investigate the effect of different annealing conditions on the efficiency of the Mok et al. synthesis reaction and the characteristics of the final product.

Table 15: Starting Materials for Multimeric siRNA Mixtures

SEQ ID	Description	Axo ID	Sequence (5'--3')
13	F7 sense (s)	X01162	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT
56	3'-thiol-F7s	X12006	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)
14	F7 antisense (as)	X00549	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT
57	3'-thiol-F7as	X12007	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)
58	F7 homodimer (s-c-s)	X12710	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)(DTME)GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)
59	F7 homodimer (as-c-as)	X12711	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)(DTME)GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)
60	homodimer (s-nc-s)	X12712	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)(BMPEG2)GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)
61	homodimer (as-nc-as)	X12713	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)(BMPEG2)GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)

[00402] Key: In the Description portion of the chart: “F7” means siRNA sequence targeting the Factor VII gene (also known as Factor VII). Sense strand is designated “s”; antisense strand is designated “as”. The chemical linkers joining the strands are designated as “c” for cleavable and “nc’ for noncleavable.

[00403] In the Sequence portion of this chart (and those that follow): upper case letters “A”, “C”, “G” and “U” represent RNA nucleotides. Lower case letter “s” represents phosphorothioate and “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. “(BMPEG2)” represents the non-cleavable homobifunctional crosslinker 1,8-bismaleimido-diethyleneglycol. In general, sequences are written from the 5’- to the 3’-terminus; however, sequences comprising the DTME or BMPEG2

crosslinker are linked via the 3'-ends, and in these sequences the second half needs to be read from right to left, i.e. 3'-to 5'.

[00404] The sequences shown in Table 15 were made following the synthetic methodology described above in General Procedure: Single Chain Oligonucleotide Synthesis.

[00405] Subsequent to the solid phase assembly of the sequences, deprotection and preparative AEX HPLC purification of the sense and antisense oligonucleotides, the single stranded homodimers were formed by combining the 3'-thiol modified single stranded oligonucleotide and the respective homobifunctional crosslinker (DTME or BMPEG2, both purchased from Pierce). To this end, the oligonucleotide was dissolved in 100 mM triethylammonium acetate, pH 7, to give a 1.4 mM solution. A freshly prepared solution of the homobifunctional crosslinker (5 mg/mL) in acetonitrile was added to the solution containing the oligonucleotide. The reaction mixture was agitated at 25°C on a thermoshaker. The reaction was monitored by analytical AEX HPLC using a Dionex DNA Pac 200 column (4x 250 mm). Once the starting material was consumed the reaction was quenched by the addition of a mixture of sodium acetate (3M, pH 5.2) and ethanol 1:32 (v/v). The crude material was precipitated overnight in the freezer. The pellet was dissolved in water and purified by AEX HPLC using a column filled with source 15 Q resin (GE Healthcare). Fractions of appropriate purity were combined and precipitated again. The pellet was dissolved in water and quantified by measuring the absorption at 260 nm. A generic depiction of the synthesis of a homodimer using BMPEG2 as linker is shown in Fig. 26.

[00406] Analytical data for the sequences shown in the Table 15 above are listed in Table 16, as follows:

Table 16: Analytical Data for Starting Materials

Description	ID	Purity (IEX HPLC, %)	Mol weight (calculated)	Mol weight (measured)
F7 sense (s)	X01162	93.5	6629.1	6629.4
3'-thiol-s	X12006	93.4	6826.4	6825.3
F7 antisense (as)	X00549	94.2	6726.2	6726.0
3'-thiol-as	X12007	94.2	6923.4	6922.1
homodimer (s-c-s)	X12710	84.9	13967.2	13969.3
homodimer (as-c-	X12711	87.2	14159.2	14157.7

as)				
homodimer (s-nc-s)	X12712	89.9	13961.1	13959.7
homodimer (as-nc-as)	X12713	87.2	14155.1	14153.1

[00407] Table 17 shows the single stranded homodimers that were annealed to produce duplexed multimeric siRNA mixtures, XD-05305 (having noncleavable linkages) and XD-05306 (having cleavable linkages). Initially, generic annealing conditions were used: complementary single strands were combined in 1x PBS and placed into a water bath kept at 70°C for 10 minutes. Then, the water bath was cooled down to 25°C over a period of 3 hours.

Table 17: Sequences Used in Production of Duplexed Multimeric siRNA Mixtures

Duplex-ID	Description	Axo ssRNA ID	Sequence (5'--3')
XD-05305	(s-nc-s)	X12712	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)(BMPEG2)GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)
	(as-nc-as)	X12713	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)(BMPEG2)GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)
XD-05306	(s-c-s)	X12710	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)(DTME)GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)
	(as-c-as)	X12711	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)(DTME)GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)

[00408] In order to establish a baseline for potentially improved annealing conditions over those shown in the prior art (Mok et al, NATURE MATERIALS, Vol. 9, March 2010), the non-cleavable homodimers X12712 and X12713 were used. A comparison of the annealing conditions published by Mok et al. (hereinafter "Park") and a proprietary set of standard annealing conditions (hereinafter "Axolabs" conditions) was conducted. The Park conditions were: 1x PBS, 1 hour, 37°C. The Axolabs conditions were: 1x PBS, 10 minutes at 70°C, cooling down to 25°C over a period of 3 hours. Fig. 27 demonstrates that SEC HPLC separates single stranded dimers from multimeric

siRNA. The NC-sense-dimer corresponds to the middle peak, the NC-antisense-dimer corresponds to the right peak, and the multimeric siRNA corresponds to the left peak.

[00409] Further, Fig. 28 demonstrates that the integral of the multimeric siRNA appears to be independent of the annealing conditions; whereas the fraction of short dsRNA is not. The higher peak in the circled region around t = 15.0 min corresponds to the "Park's conditions" and the lower peak in the circled region corresponds to the "Axolabs' conditions."

[00410] The Axolabs annealing conditions shift more material into the multimeric siRNA fraction.

[00411] Scouting of a series of different native HPLC conditions (buffer, temperature, content of acetonitrile) resulted in the ability to partially separate the multimeric siRNAs, as demonstrated in Fig. 29. The highest peak corresponds to the NC-sense-dimer, the leftmost peak corresponds to the NC-antisense dimer, the highest peak in the "multimeric siRNA" region around t = 9-11 min corresponds to the "Axolabs' annealing conditions," and the second highest peak in the "multimeric siRNA" region corresponds to "Park's annealing conditions."

[00412] Further, this analysis confirmed that annealing at higher temperature minimizes shorter multimers and favors longer ones. Further, we found that using SEC and IEX HPLC analysis, it was not possible to assign the distribution of X-mer siRNAs in the mixtures analyzed.

[00413] Baseline separation of various siRNA constituents of the multimeric siRNA mixtures was achieved by SEC HPLC Analysis, as shown in Fig. 30. Multimeric siRNA is the left-hand peak; dimeric siRNA is the middle peak; and canonical siRNA is the right-hand peak.

[00414] Using HPLC-based methods and a Superdex 200 10/300 GL column, additional annealing conditions were investigated with the aim to minimize the fraction of material that rarely oligomerizes. Specifically, Table 18 shows the parameters that were analyzed and the results that were obtained using the noncleavable F7 homodimers.

Table 18: Annealing conditions and Results

Annealing Conditions	Results
Temperature versus Cooling Rate: 1x PBS, 250 μM, at 70°C , 80°C and 90°C	No significant changes observed. Quick snap cooling does not offer any advantage.

(for 10 min each time point) with subsequent slow cooling to room temperature (2h) versus quick cooling by placing samples in ice water bath	No significant benefit from increased temperature.
Temperature versus High Salt Concentration: 10x PBS, 250 µM, at 70°C , 80°C and 90°C (for 10 min each time point) with subsequent slow cooling to room temperature (2h)	Higher salt concentration increases the portion of smaller multimeric siRNA species.
Temperature versus Low Salt Concentration: 0.2x PBS, 250 µM, at 70°C , 80°C and 90°C (for 10 min each time point) with subsequent slow cooling to room temperature (2h)	Lower salt concentration appears to be optimal for the formation of multimeric siRNAs. Again, temperature has less of an impact.
Temperature, Diluted Annealing, Baseline Salt: 1x PBS, 25 µM (1:10), at 70°C , 80°C and 90°C (for 10 min each time point) with subsequent slow cooling to room temperature (2h)	Higher temperatures destroyed the formation of multimeric siRNA
Temperature, Diluted Annealing, Higher Salt: 10x PBS, 25 µM (1:10), at 70°C , 80°C and 90°C (for 10 min each time point) with subsequent slow cooling to room temperature (2h)	Increasing the salt concentration at least partially restored the formation of multimeric siRNAs at 80°C , but not at 90°C.
Temperature, Diluted Annealing, Lower Salt: 0.2x PBS, 25 µM (1:10), at 70°C , 80°C and 90°C (for 10 min each time point) with subsequent slow cooling to room temperature (2h)	Consistent with what was observed with annealing at 250 µM

[00415] Based on these results, it was determined that optimized annealing conditions comprise high RNA concentration (≥250 µM), low salt concentration (~0.2x

PBS), reaction temperature around 70 -80 (for about 10 min), and slow cooling to room temperature (2h).

[00416] Fig. 31 demonstrates the effect of salt concentration and reaction temperature on the multimeric siRNA mixture.

[00417] Next the optimized annealing conditions were selected and two additional experiments were run to determine if it is possible to control the reaction so as to minimize the amount of extremely high molecular weight multimeric species in the final mixture (on the theory that the lower molecular weight species will be more active *in vivo* and potentially more easily formulated into LNP, if LNP is used as a delivery vehicle).

[00418] In the first of these, the optimized annealing conditions were repeated in the presence of 0.1, 0.3 and 0.9 equivalents of single stranded monomer (lacking any linker) acting as a termination strand. The results, shown in Fig. 32 (below), show that the higher the concentration of termination strand (in this case, the antisense strand was used as the terminator), the smaller the multimerized siRNA fraction.

[00419] In the second experiment, the optimized annealing conditions were performed with sub-stoichiometric amounts of the non-cleavable sense homodimer X12712; specifically, 90 mol%, 75 mol% and 60 mol% of the sense homodimer versus 100 mol% of the antisense homodimer X12711 were used in the annealing reaction. The results, shown in Fig. 33, show that the smaller the concentration of sense homodimer, the smaller the multimerized siRNA fraction.

[00420] After annealing, the various "terminator" samples and "sub-stoichometric" samples were analyzed on a 2% agarose gel in TAE buffer employing 140 mA for 2 hours. Bands were made visible using GelRed Staining. Fig. 34A represents the gel for sample nos. 1-15. Fig. 34B represents the gel for sample Nos. 1'-10'.

Table 19 lists the samples present in the gels and their characteristics:

Molar Ratios	Reaction Conditions	Sample #
+0.5x_unlinkered_as	70C_250µM_0.2xPBS	1
+1.5x_unlinkered_as	70C_250µM_0.2xPBS	2
+4.5x_unlinkered_as	70C_250µM_0.2xPBS	3
4.5_s + 5_as	70C_250µM_0.2xPBS	4
3.75_s + 5_as	70C_250µM_0.2xPBS	5
3.0_s + 5_as	70C_250µM_0.2xPBS	6
5_s + 5_as	70C_250µM_1xPBS	7

5_s + 5_as	80C_250µM_1xPBS	8
5_s + 5_as	90C_250µM_1xPBS	9
5_s + 5_as	70C_250µM_10xPBS	10
5_s + 5_as	80C_250µM_10xPBS	11
5_s + 5_as	90C_250µM_10xPBS	12
5_s + 5_as	70C_250µM_0.2xPBS	13
5_s + 5_as	80C_250µM_0.2xPBS	14
5_s + 5_as	90C_250µM_0.2xPBS	15
5_s + 5_as	70C_25µM_1xPBS	1'
5_s + 5_as	80C_25µM_1xPBS	2'
5_s + 5_as	90C_25µM_1xPBS	3'
5_s + 5_as	70C_25µM_10xPBS	4'
5_s + 5_as	80C_25µM_10xPBS	5'
5_s + 5_as	90C_25µM_10xPBS	6'
5_s + 5_as	70C_25µM_0.2xPBS	7'
5_s + 5_as	80C_25µM_0.2xPBS	8'
5_s + 5_as	90C_25µM_0.2xPBS	9'
5_s + 5_as	37C_250µM_1xPBS	10'

[00421] Key: Left-hand column: "+0.5x_unlink_as" means that the sample (#1) was prepared with 10% excess unlinked antisense strand X00549 (as terminator); "+1.5x_unlinkered_as" means that the sample (#2) was prepared with 30% excess unlinked antisense strand X00549 (as terminator); "+4.5x_unlinkered_as" means that the sample (#3) was prepared with 90% excess unlinked antisense strand X00549 (as terminator); for the remaining samples #4-10', X_s + Y_as means the sample was prepared with X nmol sense linked homodimer and Y nmol linked antisense homodimer (e.g., Sample #4, was prepared with 4.5 nmol of linked sense homodimer and 5 nmol of linked antisense homodimer). Sequence X00549: 5'-GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT-3'. Middle column: reaction conditions are provided in terms of temperature (°C), RNA concentration (µM), and salt concentration (as PBS).

[00422] In summary, these experiments demonstrate that analysis of the multimeric siRNA mixtures is challenging due to the large size of the multimerized siRNA units within the mixture. SEC HPLC analysis is well suited to establish a ration of multimerized (up to the 5- or 6-mer) versus dimerized siRNA units, but failed to provide

insights with respect to the extent of multimerization in a given sample. Native agarose gel helps to visualize the extent of multimerization. Further, annealing conditions have a profound influence on the extent of multimerization in the final mixture. For example, when equimolar annealings are performed, very high molecular weight multimeric siRNA formations can be observed (e.g., more than 6000 bp equivalents). Generally, annealing should be performed at high RNA concentrations (≥250 μM), low salt concentrations (e.g., ~0.2x PBS) and reaction temperatures around 70-80°C. The extent of multimerization can be reduced by performing non-equimolar annealings. Multimerizations concentrated in the range of 200-500 DNA bp equivalent (e.g., gel lanes 3 and 6 in Fig. 34A) can be made either by the addition of a terminator single strand or by reducing the amount of one strand significantly.

[00423] The samples from gel lane 6 in Fig. 34A and gel lane 10' in Fig. 34B were selected for testing in mice after formulation into LNPs. Sample #10' had an RNA concentration of 250 μM consisting of equal parts sense homodimer and antisense homodimer, and was annealed for 1 hour at 37°C in 1x PBS. Sample #6, at an RNA concentration of 250 μM consisting of a molar ratio of 3 (sense homodimer) to 5 (antisense homodimer) and 0.2x PBS, was placed into a water bath at 70°C and cooled down over a period of 3 hours. The resulting multimeric siRNA mixtures were formulated into LNPs according to General Procedure: Lipid Nanoparticle Formulation and analyzed according to General Procedure: LNP Characterization. The compositions and analytical data for the LNP experiment are presented in Tables 20, 21 and 22.

Table 20:

Formulation ID	siRNA	Formulation composition mole %	Size (nm)	PDI	Zeta (mV)	Conc (mg/ml)	Entrap %
NPA-624-1	FVII (XD-00030)	KL22/DSPC/Cholesterol/PEG-c-DOMG 50:10:38.5:1.5	69,73	0,05	-0,4	0,71	63%
NPA-194-3	FVII (XD-00030)	KL52/DSPC/Cholesterol/PEG-c-DOMG 50:10:38.5:1.5	91,02	0,07	-2,6	0,51	83%
NPA-625-1	Multimer lane 6 (XD-05305) (X12712K1 +X12713K1)	KL52/DOPE/Cholesterol/PEG-c-DOMG 40:10:48.5:1.5	113,2	0,10	-4,4	0,11	76%
NPA-626-1	Multimer lane 6 (XD-05305) (X12712K1 +X12713K1)	KL52/DOPE/Cholesterol/PEG-c-DOMG 40:30:28.5:1.5	106,2	0,05	-4,6	0,14	75%
NPA-627-1	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)	KL52/DOPE/Cholesterol/PEG-c-DOMG 40:10:48.5:1.5	129,6	0,10	0,0	0,13	92%
NPA-628-1	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)	KL52/DOPE/Cholesterol/PEG-c-DOMG 40:15:43.5:1.5	116,4	0,07	-5,3	0,14	89%
NPA-629-2	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)	KL52/DOPE/Cholesterol/PEG-c-DOMG 40:20:38.5:1.5	142,2	0,09	-6,7	0,15	99%
NPA-630-1	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)	KL52/DOPE/Cholesterol/PEG-c-DOMG 40:25:33.5:1.5	118,9	0,04	-5,6	0,15	86%
NPA-631-1	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)	KL52/DOPE/Cholesterol/PEG-c-DOMG 40:30:28.5:1.5	102,8	0,03	-3,7	0,16	90%
NPA-632-1	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)	KL52/DOPE/Cholesterol/PEG-c-DOMG 40:40:18.5:1.5	90,88	0,06	-1,8	0,16	83%
NPA-623-2	Multimer lane 6 (XD-05305) (X12712K1 +X12713K1)	KL52/DOPE/Cholesterol/PEG-c-DOMG 40:20:38.5:1.5	129,1	0,06	-4,9	0,15	95%

Table 21:

Formulation-ID	siRNA	Formulation composition mole %	Size (nm)	PDI	Zeta (mV)	Conc (mg/ml)	Entrap %
NPA-642-1	Multimer cleavable lane 6 (XD-05306) (X12710K1 +X12711K1)	KL22/DOPE/Cholesterol/PEG-c-DOMG 40:20:38.5:1.5	62,45	0,07	-2,4	0,19	93%
NPA-643-1	Multimer cleavable lane 10' (XD-05306) (X12710K1 +X12711K1)	KL22/DOPE/Cholesterol/PEG-c-DOMG 40:20:38.5:1.5	61,67	0,08	-2,5	0,19	92%
NPA-644-1	Multimer cleavable lane 6 (XD-05306) (X12710K1 +X12711K1)	Invivofectamine 2.0	69,52	0,02	1,2	0,42	97%
NPA-645-1	Multimer cleavable lane 10' (XD-05306) (X12710K1 +X12711K1)	Invivofectamine 2.0	87,71	0,11	1,2	0,44	100%
NPA-646-1	FVII (XD-00376)	Invivofectamine 2.0	67,04	0,07	2,2	0,46	100%

Table 22: Defined-length Dimeric (2-mer) siRNA Duplexes. Defined dimeric siRNA:

Duplex-ID	Description	ssRNA ID	Sequence (5'--3')
XD-04600	F (s-c-s)	X12710	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)(DTME)GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)
		X00549	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT
XD-04601	H (s-nc-s)	X12712	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)(BMPEG2)GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)
	B	X00549	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT

[00424] Example 23: Analysis of LNP-formulated Mixtures of FVII Multimeric siRNA (Animal Experiment MausRNAi-TV29/30)

[00425] To determine the *in vivo* efficacy of mixtures of multimeric siRNAs (targeted to FVII) formulated in LNPs of different lipid composition (listed in Table 23), an animal experiment was performed as described above (General Procedure: Animal Experiments). Compounds were injected intravenously at a dose of 0.35 mg/kg siRNA. Multimeric siRNAs formulated with Invivofectamine 2.0 were injected at a dose of 1 mg/kg and 3 mg/kg. LNP-formulated canonical FVII siRNA (XD-00030) was included as positive control. Group size was n=3 mice for treatment groups and n=6 for saline controls. Blood was collected at the time points noted in the graphs below and analyzed for FVII enzyme activity. Results are shown in Fig. 35.

Table 23: LNP-formulations used for animal experiment MausRNAi-TV29

Formulation-ID	lipids	siRNA
NPA-625-1	XL10 DOPE 10	Multimer lane 6 (XD-05305) (X12712K1 +X12713K1)
NPA-626-1	XL10 DOPE 30	Multimer lane 6 (XD-05305) (X12712K1 +X12713K1)
NPA-627-1	XL10 DOPE 10	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)
NPA-628-1	XL10 DOPE 15	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)
NPA-630-1	XL10 DOPE 25	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)
NPA-631-1	XL10 DOPE 30	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)
NPA-632-1	XL10 DOPE 40	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)
NPA-623-2	XL10 DOPE 20	Multimer lane 6 (XD-05305) (X12712K1 +X12713K1)
NPA-629-2	XL10 DOPE 20	Multimer cleavable (XD-05306) (X12710K1 +X12711K1)
NPA-194-3	XL10 std	XD-00030 (FVII monomer)
NPA-624-1	XL7 std	XD-00030 (FVII monomer)

[00426] When comparing different payloads (payloads having cleavable vs. noncleavable linkers) in the same formulations, we observe that the cleavable payloads performed better than the non-cleavable payloads (e.g., NPA-625-1 vs. NPA-62701; NPA-626-1 vs. NPA-631-1; and NPA-629-1 vs. NPA-623).

Table 24: LNP-formulations used in animal experiment MausRNAi-TV30

Formulation-ID	siRNA	lipids
NPA-642-1	Multimer cleavable lane 6 (XD-05306) (X12710K1 +X12711K1)	XL7 DOPE 20
NPA-643-1	Multimer cleavable lane 10' (XD-05306) (X12710K1 +X12711K1)	XL7 DOPE 20
NPA-644-1	Multimer cleavable lane 6 (XD-05306) (X12710K1 +X12711K1)	Invivofectamine 2.0
NPA-645-1	Multimer cleavable lane 10' (XD-05306) (X12710K1 +X12711K1)	Invivofectamine 2.0
NPA-646-1	FVII (XD-00376)	Invivofectamine 2.0

[00427] Example 24: Manufacture of FVII HeteroDimer of FVIIsense:FVIIantisense (X12714) and Resulting Mixture of Multimers (XD-05312)

[00428] A variation on the F7-ApoB heterodimer of Example 9 was made from chemically linking siF7 sense strand to siF7 antisense strand to form a single-stranded heterodimer targeting F7, as depicted in Fig. 37.

[00429] Heterodimer X12714 appears in lane 12 in the gel depicted in Fig. 38. Gel analysis conditions were: 1,5 µg/lane; 2% agarose gel in 1xTAE; 140mA; 130 min; Gel red staining (1:10000).

[00430] Example 25: Sequence Selection for ApoB Screen

[00431] No suitable siRNA against Murine ApoB was known before the present invention. Accordingly, dsRNA design was carried out to identify specific dsRNAs targeting mouse ApoB. First, known mRNA sequences of mouse (Mus musculus) ApoB (NM_009693.2 listed as SEQ ID NO:62 and XM_006515078.1 listed as SEQ ID NO:63 were downloaded from NCBI Reference Sequence database, release 73).

[00432] From this initial set of sequences those harbouring a SNP (single nucleotide polymorphism) in their corresponding target site sequence (positions 2 - 18 of 19mer) in mouse ApoB mRNA (SEQ ID NO. 64) as indicated by the NCBI dbSNP (build 146) were excluded.

[00433] In identifying RNAi agents, the selection was limited to 19mer sense and antisense sequences, having at least 1 or 2 mismatches, respectively, to any other sequence in the mouse NCBI Ref Seq database (release 73), which we assumed to represent the comprehensive mouse transcriptome.

[00434] Selection of candidates was further limited by elimination of 19mer sense and antisense strands harbouring seed sequences (nucleotides 2-7 of the 5' terminus) identical to known mouse miRNA seed sequences (nucleotides 2-7 of the 5' terminus) as listed in miRBase (University of Manchester, release 21).

[00435] In addition, all sense and antisense sequences containing five or more consecutive G's (poly-G sequences) were excluded from the selection. The sequences identified are presented in Table 25.

Table 25: Core sequences of double stranded RNAs (dsRNAs) targeting mouse ApoB mRNA.

SEQ ID NO	Sense strand core sequence (5´-3´)	SEQ ID NO	Antisense strand core sequence (5´-3´)
65	CAACCAGUGUACCCUUAAA	77	UUUAAGGGUACACUGGUUG
66	CUGUGUACGAAGUACAAAA	78	UUUUGUACUUCGUACACAG
67	CAACCUAUGAACUCCUAAA	79	UUUAGGAGUUCAUAGGUUG
68	GCUUACGGCUCAACAAUUU	80	AAAUUGUUGAGCCGUAAGC
69	GCACGUGAUGGACUAUCAA	81	UUGAUAGUCCAUCACGUGC
70	CUAUUUGGAGAGAAAUCGA	82	UCGAUUUCUCUCCAAAUAG
71	GAGAUUAUUGAUCGAAUCA	83	UGAUUCGAUCAAUAAUCUC
72	CCGUGUAAAUCUAGCAAAA	84	UUUUGCUAGAUUUACACGG
73	GCAUUUAGAUCAAUUGAGA	85	UCUCAAUUGAUCUAAAUGC
74	GGUUUUAAUGGAUAAAUCA	86	UGAUUUAUCCAUUAAAACC
75	GACUUUGCAGAGCAAUAUU	87	AAUAUUGCUCUGCAAAGUC
76	CUUACGGGUCAUCCAAAAA	88	UUUUUGGAUGACCCGUAAG

[00436] The selected sequences from Table 25 were synthesized with chemical modifications as presented in Table 26A and 26B.

[00437] Table 26A:

SEQ ID	Duplex-ID	SEQ ID	ss-ID	Sequence (5'--3')
91	XD-05962	89	X18815	caAfcCfaGfuGfuAfcCfcUfuAfaAfdTsdT
94	XD-05963	92	X18817	cuGfuGfuAfcGfaAfgUfaCfaAfaAfdTsdT
97	XD-05964	95	X18819	caAfcCfuAfuGfaAfcUfcCfuAfaAfdTsdT
100	XD-05965	98	X18821	gcUfuAfcGfgCfuCfaAfcAfaUfuUfdTsdT
103	XD-05966	101	X18823	gcAfcGfuGfaUfgGfaCfuAfuCfaAfdTsdT
106	XD-05967	104	X18825	cuAfuUfuGfgAfgAfgAfaAfuCfgAfdTsdT
109	XD-05968	107	X18827	gaGfaUfuAfuUfgAfuCfgAfaUfcAfdTsdT
112	XD-05969	110	X18829	ccGfuGfuAfaAfuCfuAfgCfaAfaAfdTsdT
115	XD-05970	113	X18831	gcAfuUfuAfgAfuCfaAfuUfgAfgAfdTsdT
118	XD-05971	116	X18833	ggUfuUfuAfaUfgGfaUfaAfaUfcAfdTsdT
121	XD-05972	119	X18835	gaCfuUfuGfcAfgAfgCfaAfuAfuUfdTsdT
124	XD-05973	122	X18837	cuUfaCfgGfgUfcAfuCfcAfaAfaAfdTsdT

Table 26B:

SEQ ID	Duplex-ID	SEQ ID	as-ID	Sequence (5'--3')
91	XD-05962	90	X18816	UfUfuAfaGfgGfuAfcAfcUfgGfuUfgdTsdT
94	XD-05963	93	X18818	UfUfuUfgUfaCfuUfcGfuAfcAfcAfgdTsdT
97	XD-05964	96	X18820	UfUfuAfgGfaGfuUfcAfuAfgGfuUfgdTsdT
100	XD-05965	99	X18822	AfAfaUfuGfuUfgAfgCfcGfuAfaGfcdTsdT
103	XD-05966	102	X18824	UfUfgAfuAfgUfcCfaUfcAfcGfuGfcdTsdT
106	XD-05967	105	X18826	UfCfgAfuUfuCfuCfuCfcAfaAfuAfgdTsdT
109	XD-05968	108	X18828	UfGfaUfuCfgAfuCfaAfuAfaUfcUfcdTsdT
112	XD-05969	111	X18830	UfUfuUfgCfuAfgAfuUfuAfcAfcGfgdTsdT
115	XD-05970	114	X18832	UfCfuCfaAfuUfgAfuCfuAfaAfuGfcdTsdT
118	XD-05971	117	X18834	UfGfaUfuUfaUfcCfaUfuAfaAfaCfcdTsdT
121	XD-05972	120	X18836	AfAfuAfuUfgCfuCfuGfcAfaAfgUfcdTsdT
124	XD-05973	123	X18838	UfUfuUfuGfgAfuGfaCfcCfgUfaAfgdTsdT

[00438] wherein lower case letters “c”, “g”, “a” and “u” represent 2’-O-methyl-modified nucleotides, “s” represents phosphorothioate and “dT” represents

deoxythymidine residues. Upper case letters A, C, G, U followed by "f" indicate 2'-fluoro nucleotides. The modified dsRNAs presented in Tables 26A and 26B correspond to the unmodified dsRNAs presented in Table 25, as follows: SEQ ID NO:89-124 are the modified sequences corresponding to the unmodified sequences presented as SEQ ID NO:65-88.

[00439] Example 26: *In vitro* evaluation of siRNAs targeting ApoB

[00440] The activity of the siRNAs in Table X directed against mouse ApoB mRNA was tested in the murine liver cell line NMuLi.

[00441] ApoB mRNA content was quantified by branched DNA in total mRNA isolated from cells incubated with ApoB specific siRNAs. Cells were obtained from American Type Culture Collection (Rockville, Md., Cat. No. CCL-1638). NMuLi cells were cultured in Dulbeccos modified Eagle's medium (DMEM, Biochrom #F0435) supplemented with 10% fetal calf serum (FCS, Biochrom AG, Berlin, Germany, cat. No. S0115) and Penicillin 100 U/ml, Streptomycin 100 mg/ml (Biochrom AG, Berlin, Germany, Cat. No. A2213).

[00442] Transfection of siRNAs was performed directly after seeding 15,000 NMuLi cells/well on a 96-well plate, and was carried out with the transfection reagent RNAiMax (Invitrogen GmbH, Karlsruhe, Germany, Cat. No. 13778-150) as described by the manufacturer. In a dose response experiment performed in quadruplicates, siRNA concentrations started at 50 nM and decreased in 5-fold dilution steps down to 16 pM. After transfection cells were incubated for 24 h at 37°C and 5% CO_2 in a humidified incubator (Heraeus GmbH, Hanau, Germany). Transfection reagent only ("mock")-treated cells served as negative control. ApoB mRNA levels were quantified using a Quantigene Explore Kit QG1.0 (Panomics, Fremont, Calif., USA, cat. No. QG0004). Cells were harvested and lysed at 53°C following procedures recommended by the manufacturer. After incubation and lysis, cell lysates were incubated with probe-sets specific to mouse ApoB and mouse GAPDH (as housekeeper for normalization). Assays were processed according to the manufacturer's protocol. Chemoluminescence was measured in a Victor2-Light (Perkin Elmer, Wiesbaden, Germany) as RLUs (relative light units) and values obtained with the ApoB probe-set were normalized to the respective GAPDH values for each well. For graphical representation, ApoB mRNA levels at 10 nM and 0.4 nM are shown relative to the levels of mock-treated cells set as 1 (Fig. 39). IC_{50} (target mRNA reduced by 50 %) and IC_{80} (target mRNA reduced by 80 %)

values were determined using the XLfit software (IDBS, Guildford, UK) and are shown in Table 27. The siRNA XD-05967 was chosen as best candidate for the multimer experiments as it had the lowest IC_{80} value, and XD-05970 as backup candidate due to the best IC_{50} value.

Table 27: IC50 and IC80 values of ApoB targeted siRNAs

siRNA	IC50 (nM)	IC80 (nM)
XD-05962	1.77	n.a.
XD-05963	n.a.	n.a.
XD-05964	n.a.	n.a.
XD-05965	1.34	n.a.
XD-05966	1.84	n.a.
XD-05967	0.29	9.12
XD-05968	n.a.	n.a.
XD-05969	0.54	n.a.
XD-05970	0.17	44.63
XD-05971	n.a.	n.a.
XD-05972	n.a.	n.a.
XD-05973	n.a.	n.a.

CLAIMS

1. A compound according to Structure 1:

X - R1 - R2 - A - R3 - B (Structure 1)

wherein:

X is a nucleic acid bonded to R1 through its 3' or 5' terminus;

R1 a phosphodiester, thiophosphodiester, sulfate, amide, glycol, or is absent;

R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

A is the reaction product of a nucleophile and an electrophile;

R3 is a C2-C10 alkyl, alkoxy, aryl, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and

B is a nucleophile or electrophile.

2. The compound of claim 1, wherein the nucleophile and electrophile of A comprise a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group.

3. The compound of any of claims 1 to 2, wherein the nucleophile or electrophile of B comprise a thiol, maleimide, vinylsulfone, pyridyldisulfide, iodoacetamide, acrylate, azide, alkyne, amine, or carboxyl group.

4. The compound of claim 1, wherein:

R1 is a phosphodiester or thiophosphodiester;

R2 is a C2-C10 alkyl;

A is the reaction product of a thiol and maleimide;

R3 is a disulfide; and

B is a thiol or maleimide.

5. The compound of any of claims 1 to 4, wherein X is an siRNA.

6. The compound of any of claims 1 to 5, wherein the nucleic acid is RNA, DNA, or comprises an artificial or non-natural nucleic acid analog.

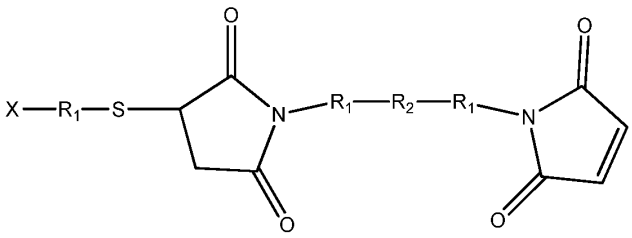
7. The compound of claim 6, wherein the nucleic acid is DNA.

8. The compound of claim 7, wherein the DNA is an antisense DNA (aDNA) or antisense gapmer.

9. The compound of claim 6, wherein the nucleic acid is RNA.

10. The compound of claim 9, wherein the RNA is an antisense RNA (aRNA), CRISPR RNA (crRNA), long noncoding RNA (lncRNA), microRNA (miRNA), piwi-interacting RNA (piRNA), small interfering RNA (siRNA), messenger RNA (mRNA), short hairpin RNA (shRNA), small activating (saRNA), antagomir, or ribozyme.
11. The compound of any of claims 1 to 10, wherein the nucleic acid is an aptamer.
12. The compound of any of claims 1 to 11, wherein the nucleic acid further comprises a chemical modification.
13. The compound of claim 12, wherein the chemical modification comprises a modified nucleoside, modified backbone, modified sugar, or modified terminus.
14. The compound of any of claims 1 to 13, further comprising a targeting ligand.
15. The compound of claim 14, wherein the targeting ligand is bound to the nucleic acid.
16. The compound if claim 15, wherein the targeting ligand is bonded to the nucleic acid through its 3' or 5' terminus.
17. The compound of any of claims 14 to 16, wherein the targeting ligand comprises *N*-Acetylgalactosamine (GalNAc), cholesterol, tocopherol, folate, 2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid (DUPA), or anisamide.
18. The compound of any of claims 1 to 17, wherein the nucleic acid is single stranded.
19. The compound of any of claims 1 to 17, wherein the nucleic acid is double stranded.
20. The compound of any of claims 1 to 19, wherein the nucleic acid is 15-30, 17-27, 19-26, 20-25, 40-50, 40-150, 100-300, 1000-2000, or up to 10000 nucleotides in length.
21. The compound of any one of claims 1 to 20, wherein R1 is a phosphodiester or thiophosphodiester.
22. The compound of any one of claims 1 to 21, wherein R2 is C2-C10, C3-C6, or C6 alkyl.
23. The compound of any one of claims 1 to 22, wherein A is the reaction product of a thiol and maleimide group.
24. The compound of any one of claims 1 to 23, wherein R3 is a thiopropionate or disulfide.

25. The compound of any one of claims 1 to 24, wherein R3 comprises a linker that is cleavable under intracellular conditions.
26. The compound of any one of claims 1 to 25, wherein B comprises one of the same groups as A.
27. The compound of any one of claims 1 to 26, wherein B comprises a different group than A.
28. The compound of any one of claims 1 to 27, wherein the compound is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.
29. The compound of any one of claims 1 to 27, wherein the compound is about 85-95 % pure.
30. A compound according to Structure 2:



(Structure 2)

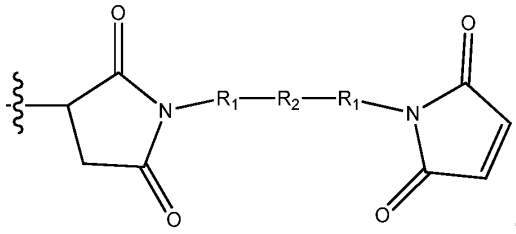
wherein:

X is a nucleic acid bonded to R1 via a phosphate or thiophosphate at its 3' or 5' terminus;

each R1 is independently a C2-C10 alkyl, alkoxy, or aryl group; and

R2 is a thiopropionate or disulfide group.

31. The compound of claim 30, wherein R1-R2-R1 is cleavable under intracellular conditions.
32. The compound of claim 30, wherein X comprises an siRNA.
33. The compound of claim 30, wherein the compound is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.
34. The compound of claim 30, wherein the compound is about 85-95 % pure.
35. The compound of any of claims 30 to 34, wherein the moiety



comprises the reaction product of a DTME (dithiobismaleimidoethane), BM(PEG)2 (1,8-bis(maleimido)diethylene glycol), BM(PEG)3 (1,11-bismaleimido-triethyleneglycol), BMOE (bismaleimidoethane), BMH (bismaleimidohexane), or BMB (1,4-bismaleimidobutane).

36. A compound according to Structure 3:

X - R1 - R2 - A - R3 - B (Structure 3)

wherein:

X is a nucleic acid bonded to R1 through its 3' or 5' terminus;

R1 a phosphate, thiophosphate, sulfate, amide, glycol, or is absent;

R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

A is the reaction product of a first and a second reactive moiety;

R3 is an C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and

B is a third reactive moiety.

37. The compound of claim 36, wherein A-R3-B is cleavable under intracellular conditions.

38. The compound of claim 36, wherein X comprises an siRNA.

39. The compound of claim 36, wherein the compound is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.

40. The compound of claim 36, wherein the compound is about 85-95 % pure.

41. A method for synthesizing a compound according to any one of claims 1 to 40, the method comprising:

reacting a functionalized nucleic acid X - R1 - R2 - A' and a covalent linker A'' - R3 - B, wherein A' and A'' comprise a nucleophile and an electrophile, in a dilute solution of X - R1 - R2 - A' and with a stoichiometric excess of A'' - R3 – B, thereby forming the compound X - R1 - R2 - A - R3 - B (Structure 1)

wherein:

X is a nucleic acid bonded to R1 through its 3' or 5' terminus;

R1 a phosphodiester, thiophosphodiester, sulfate, amide, glycol, or is absent;

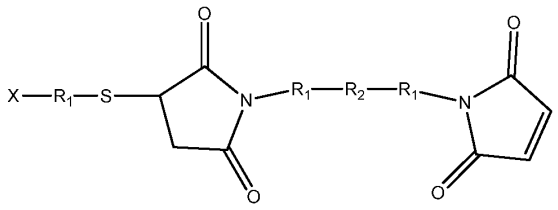
R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

A is the reaction product of a nucleophile and an electrophile;

R3 is a C2-C10 alkyl, alkoxy, aryl, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and

B is a nucleophile or electrophile.

42. The method of claim 41, further comprising the step of synthesizing the functionalized nucleic acid X - R1 - R2 - A', wherein A' comprises a thiol (-SH) by (i) introducing a the thiol during solid phase synthesis of the nucleic acid using phosphoramidite oligomerization chemistry or (ii) reduction of a disulfide introduced during the solid phase synthesis.

43. The method of any one of claims 41 to 42, comprising synthesizing the compound of claim 30  (Structure 2).

44. The method of any one of claims 41 to 43, wherein the reaction is carried out under conditions that substantially favor the formation of Structure 1 or 2 and substantially prevent dimerization of X.

45. The method of any one of claims 41 to 44, wherein reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out at a X - R1 - R2 - A' concentration of below about 1 mM, 500 μM, 250 μM, 100 μM, or 50 μM.

46. The method of any one of claims 41 to 44, wherein reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out at a X - R1 - R2 - A' concentration of about 1 mM, 500 μM, 250 μM, 100 μM, or 50 μM.

47. The method of any one of claims 41 to 46, wherein reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out with a molar excess of A'' - R3 – B of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100.

48. The method of any one of claims 41 to 46, wherein reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out with a molar excess of A'' - R3 - B of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100.

49. The method of any one of claims 41 to 48, wherein reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out at a pH of below about 7, 6, 5, or 4.

50. The method of any one of claims 41 to 48, wherein reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out at a pH of about 7, 6, 5, or 4.

51. The method of any one of claims 41 to 50, wherein reacting the functionalized nucleic acid X - R1 - R2 - A' and the covalent linker A'' - R3 - B is carried out in a solution comprising water and a water miscible organic co-solvent.

52. The method of claim 51, wherein the water miscible organic co-solvent comprises DMF, NMP, DMSO, or acetonitrile.

53. The method of any one of claim 51 or 52, wherein the water miscible organic co-solvent comprises about 10, 15, 20, 25, 30, 40, or 50 % (v/v) of the solution.

54. An isolated compound according to Structure 4:

 (Structure 4)

wherein:

each is a double stranded oligonucleotide designed to react with the same molecular target *in vivo*, and

• is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, and having the structure - R1 - R2 - A - R3 - A - R2 - R1 - wherein:

each R1 is independently a phosphodiester, thiophosphodiester, sulfate, amide, glycol, or is absent;

each R2 is independently a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

each A is independently the reaction product of a nucleophile and an electrophile, and

R3 is a C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

55. The isolated compound of claim 54, wherein each ═══ comprises an siRNA guide strand targeting Factor VII and a passenger strand hybridized to the guide strand.

56. The isolated compound of any one of claims 54 to 55, further comprising a targeting ligand, wherein each ═══ comprises an siRNA guide strand and a passenger strand hybridized to the guide strand, and wherein the isolated compound is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.

57. An isolated compound according to Structure 5:

 (Structure 5)

wherein:

——— is a first single stranded oligonucleotide

 is a second single stranded oligonucleotide having a different sequence from the first, and

● is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, and having the structure - R1 - R2 - A - R3 - A - R2 - R1 - wherein:

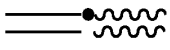
each R1 is independently a phosphate, sulfate, amide, glycol, or is absent;

each R2 is independently a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

each A is independently the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group, and

R3 is an C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

58. An isolated compound according to Structure 6:

 (Structure 6)

wherein:

═══ is a first double stranded oligonucleotide

 is a second double stranded oligonucleotide having a different sequence from the first, and

● is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, and having the structure - R1 - R2 - A - R3 - A - R2 - R1 - wherein:

each R1 is independently a phosphate, sulfate, amide, glycol, or is absent;

each R2 is independently a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

each A is independently the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group, and

R3 is an C2-C10 alkyl, alkoxy, aryl, alkoxy, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

59. The isolated compound of claim 58, wherein:

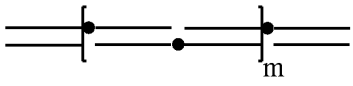
 comprises a first siRNA guide strand targeting Factor VII and a first passenger strand hybridized to the guide strand, and

 comprises a second siRNA guide strand targeting Apolipoprotein B and a second passenger strand hybridized the second guide strand.

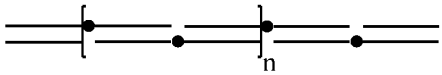
60. The isolated compound of any one of claims 54 to 59, wherein the nucleophile and electrophile of A comprise a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group.

61. The isolated compound of any one of claims 54 to 60, wherein the covalent linker comprises the reaction product of a DTME (dithiobismaleimidoethane), BM(PEG)2 (1,8-bis(maleimido)diethylene glycol), BM(PEG)3 (1,11-bismaleimido-triethyleneglycol), BMOE (bismaleimidoethane), BMH (bismaleimidohexane), or BMB (1,4-bismaleimidobutane).

62. A compound according to Structure 7 or 8:



(Structure 7)



(Structure 8)

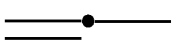
wherein:

each is a double stranded oligonucleotide,

each ● is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and

m is an integer ≥ 1 and n is an integer ≥ 0.

63. A compound according to Structure 11:



(Structure 11)

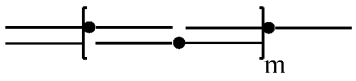
wherein:

═══ is a double stranded oligonucleotide,

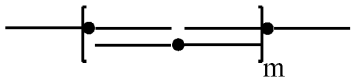
——— is a single stranded oligonucleotide, and

● is a covalent linker joining single strands of adjacent single stranded oligonucleotides.

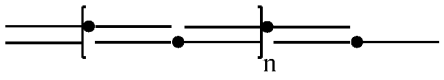
64. A compound according to Structure 12, 13, 14, or 15:



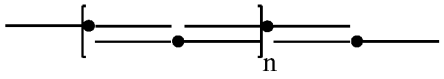
(Structure 12)



(Structure 13)



(Structure 14)



(Structure 15)

wherein:

each ═══ is a double stranded oligonucleotide,

each ——— is a single stranded oligonucleotide,

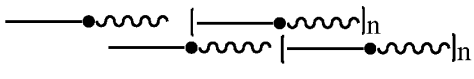
each ● is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and

m is an integer ≥ 1 and n is an integer ≥ 0.

65. The compound of any of claims 62 to 64, wherein each ———●——— may independently comprise two sense or two antisense oligonucleotides.

66. The compound of any of claims 62 to 64, wherein each ———●——— may independently comprise one sense and one antisense oligonucleotide.

67. A composition comprising a plurality of molecules, each molecule having Structure 16:



(Structure 16), wherein n is an integer ≥ 1;

each ——— is a single stranded oligonucleotide;

each ∿∿∿ is a single stranded oligonucleotide that hybridizes with a ———;

∿∿∿
——— is a double stranded oligonucleotide; and

each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides.

68. The composition of claim 67, wherein each ~~~~ has a length of 15-30 base pairs.

69. The composition of any one of claims 67 to 68, where each ~~~~ is an siRNA.

70. The composition of any one of claims 67 to 69, wherein n is an integer from 1 to 100.

71. The compound of any of claims 62 to 70, wherein each covalent linker • is the same.

72. The compound of any of claims 62 to 70, comprising two or more different covalent linkers •.

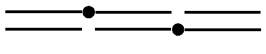
73. The compound of any of claims 62 to 72, comprising a homo-multimer of substantially identical double stranded oligonucleotides.

74. The compound of claim 73, wherein the substantially identical double stranded oligonucleotides each comprise an siRNA targeting the same molecular target *in vivo*.

75. The compound of any of claims 62 to 66, comprising a hetero-multimer of two or more substantially different double stranded oligonucleotides ——.

76. The compound of claim 75, wherein the substantially different double stranded oligonucleotides —— each comprise an siRNA targeting different genes.

77. The compound of claim 62, comprising Structure 9 and wherein n = 0:

 (Structure 9).

78. The compound of claim 77, further comprising a targeting ligand.

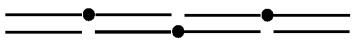
79. The compound of any one of claims 77 to 78, further comprising 2 or 3 substantially different double stranded oligonucleotides —— each comprising an siRNA targeting a different molecular target *in vivo*.

80. The compound of any one of claims 77 to 79, further comprising a targeting ligand, and wherein one —— comprises a first siRNA guide strand targeting Factor VII and a first passenger strand hybridized to the guide strand, one —— comprises a second siRNA guide strand targeting Apolipoprotein B and a second passenger strand hybridized to the second guide strand, and one ——

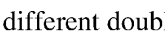
comprises a third siRNA guide strand targeting TTR and a third passenger strand hybridized to the third guide strand.

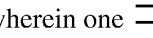
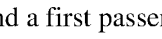
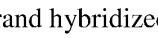
81. The compound of any one of claims 77 to 80, wherein the targeting ligand comprises N-Acetylgalactosamine (GalNAc).

82. The compound of claim 62, comprising Structure 10 and wherein m = 1:

 (Structure 10).

83. The compound of claim 82, further comprising a targeting ligand.

84. The compound of any one of claims 82 to 83, further comprising 2, 3, or 4 substantially different double stranded oligonucleotides  each comprising an siRNA targeting a different molecular target *in vivo*.

85. The compound of any one of claims 82 to 84, further comprising a targeting ligand, and wherein one  comprises a first siRNA guide strand targeting Factor VII and a first passenger strand hybridized to the guide strand, one  comprises a second siRNA guide strand targeting Apolipoprotein B and a second passenger strand hybridized to the second guide strand, and one  comprises a third siRNA guide strand targeting TTR and a third passenger strand hybridized to the third guide strand.

86. The compound of any one of claims 82 to 85, wherein the targeting ligand comprises N-Acetylgalactosamine (GalNAc).

87. The compound of any one of claims 62 to 86, wherein the covalent linkers • comprise the reaction product of a nucleophile and electrophile.

88. The compound of claim 87, wherein the covalent linkers • comprise the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group.

89. The compound of claim 87, wherein the covalent linkers • comprise the reaction product of a thiol and DTME (dithiobismaleimidoethane), BM(PEG)2 (1,8-bis(maleimido)diethylene glycol), BM(PEG)3 (1,11-bismaleimido-triethyleneglycol), BMOE (bismaleimidoethane), BMH (bismaleimidohexane), or BMB (1,4-bismaleimidobutane).

90. The compound of any one of claims to 54 to 89, wherein one or more double stranded oligonucleotides comprises blunt ends.

91. The compound of any one of claims to 54 to 90, wherein one or more double stranded oligonucleotides comprises overhang.

92. The compound of any one of claims to 54 to 91, wherein each covalent linker • independently joins adjacent single stranded oligonucleotides 3' to 3', 5' to 5', or 3' to 5'.

93. The compound of any one of claims to 54 to 92, wherein at least one oligonucleotide further comprises a chemical modification.

94. The compound of claim 93, wherein the chemical modification comprises a modified nucleoside, modified backbone, modified sugar, or modified terminus.

95. The compound of any one of claims to 54 to 94, wherein the compound is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.

96. The compound of any one of claims to 54 to 95, wherein the compound is about 85-95 % pure.

97. The compound of any one of claims to 54 to 78, 81 to 83, or 86 to 96, wherein at least one of the oligonucleotides is an siRNA.

98. The compound of any one of claims to 54 to 77, 82, or 87 to 96, wherein the nucleic acid is RNA, DNA, or comprises an artificial or non-natural nucleic acid analog.

99. The compound of claim 98, wherein the nucleic acid is DNA.

100. The compound of claim 99, wherein the DNA is an antisense DNA (aDNA) or antisense gapmer.

101. The compound of claim 98, wherein the nucleic acid is RNA.

102. The compound of claim 101, wherein the RNA is an antisense RNA (aRNA), CRISPR RNA (crRNA), long noncoding RNA (lncRNA), microRNA (miRNA), piwi-interacting RNA (piRNA), small interfering RNA (siRNA), messenger RNA (mRNA), short hairpin RNA (shRNA), small activating (saRNA), antagomir, or ribozyme.

103. The compound of any one of claims to 54 to 77, 82, or 87 to 96, wherein the nucleic acid is an aptamer.

104. The compound of any one of claims to 54 to 77, 82, or 87 to 96, wherein the nucleic acid is 15-30, 17-27, 19-26, 20-25, 40-50, 40-150, 100-300, 1000-2000, or up to 10000 nucleotides in length.

105. The compound of any one of claims to 54 to 77, 82, or 87 to 96, further comprising a targeting ligand.

106. The compound of claim 105, wherein the targeting ligand is bound to the nucleic acid.

107. The compound of claim 106, wherein the targeting ligand is bonded to the nucleic acid through its 3' or 5' terminus.

108. The compound of any one of claims to 105 to 107, wherein the targeting ligand comprises *N*-Acetylgalactosamine (GalNAc), cholesterol, tocopherol, folate, 2-[3-(1,3-dicarboxypropyl)- ureido]pentanedioic acid (DUPA), or anisamide.

109. The compound of any one of claims to 54 to 108, wherein one or more of • comprises a cleavable covalent linker.

110. The composition of claim 109, wherein the cleavable covalent linker comprises an acid cleavable ester bond, hydrazine bond, or acetal bond.

111. The composition of claim 109, wherein the cleavable covalent linker comprises a reductant cleavable bond.

112. The composition of claim 111, wherein the reductant cleavable bond is a disulfide bond.

113. The composition of claim 109, wherein the cleavable covalent linker is cleavable under intracellular conditions.

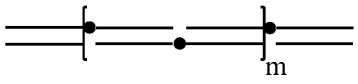
114. The composition of claim 109, wherein the cleavable covalent linker comprises a biocleavable bond.

115. The composition of claim 109, wherein the cleavable covalent linker comprises an enzyme cleavable bond.

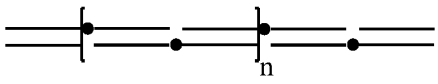
116. The compound of any one of claims to 54 to 108, wherein one or more of • comprises a noncleavable covalent linker.

117. The composition of claim 116, wherein the noncleavable covalent linker comprises an amide bond or urethane bond.

118. A method for synthesizing a compound according to Structure 7 or 8:



(Structure 7)



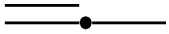
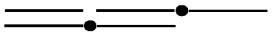
(Structure 8)

wherein: each  is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and m is an integer ≥ 1 and n is an integer ≥ 0, the method comprising the steps of:

(i) reacting a first single stranded oligonucleotide  with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○ under conditions that produce the mono-substituted product ;

(ii) reacting  with a second single stranded oligonucleotide , wherein R2 is a chemical group capable of reacting with ○, thereby forming a single stranded dimer ;

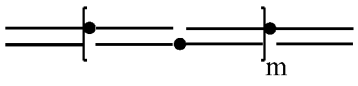
(iii) annealing with a third single stranded oligonucleotide , thereby forming ;

(iv) annealing  and a single stranded dimer , thereby forming ;

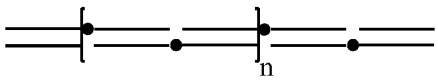
(v) optionally annealing one or more additional single stranded dimers ,

(vi) annealing the product of step (iv) or step (v) and a fourth single stranded oligonucleotide , thereby forming Structure 7 or 8.

119. A method for synthesizing a compound according to Structure 7 or 8:

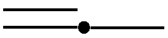


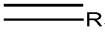
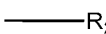
(Structure 7)

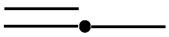


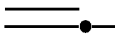
(Structure 8)

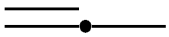
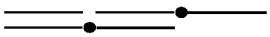
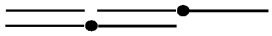
wherein: each is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and m is an integer ≥ 1 and n is an integer ≥ 0, the method comprising the steps of:

(i) forming  by:

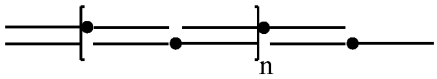
(a) annealing a first single stranded oligonucleotide  and a second single stranded oligonucleotide , thereby forming , and reacting  with a third single stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting

directly or indirectly to form a covalent linker •, thereby forming ; or

(b) reacting the second single stranded oligonucleotide and the third single stranded oligonucleotide , thereby forming , and annealing the first single stranded oligonucleotide and , thereby forming ;

(ii) annealing  and a second single stranded dimer , thereby forming  and, optionally, annealing one or more additional single stranded dimers to  thereby forming,



or


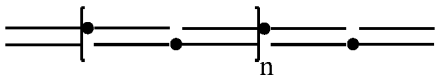
wherein m is an integer ≥ 1 and n is an integer ≥ 0; and

(iii) annealing a fourth single stranded oligonucleotide to the product of step (ii), thereby forming structure 7 or 8.

120. A method for synthesizing a compound according to Structure 7 or 8:

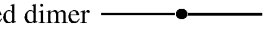
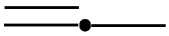


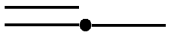
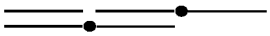
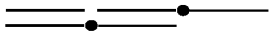
(Structure 7)

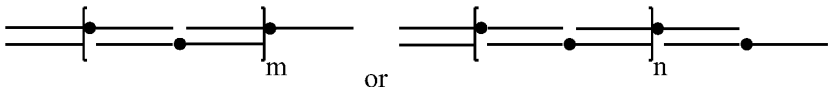


(Structure 8)

wherein: each is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, and m is an integer ≥ 1 and n is an integer ≥ 0, the method comprising the steps of:

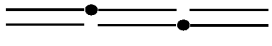
(i) annealing a first single stranded oligonucleotide and a first single stranded dimer , thereby forming ;

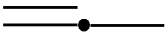
(ii) annealing  and a second single stranded dimer , thereby forming  and, optionally, annealing one or more additional single stranded dimers to  thereby forming,

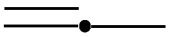


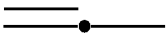
wherein m is an integer ≥ 1 and n is an integer ≥ 0; and

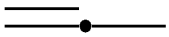
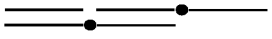
(iii) annealing a second single stranded oligonucleotide to the product of step (ii), thereby forming structure 7 or 8.

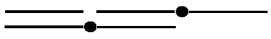
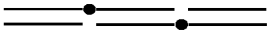
121. A method for synthesizing a compound of Structure 9:  (Structure 9), wherein each is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, the method comprising the steps of:

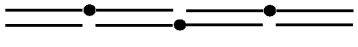
(i) forming  by:

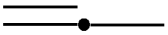
(a) annealing a first single stranded oligonucleotide and a second single stranded oligonucleotide , thereby forming , and reacting with a third single stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ; or

(b) reacting the second single stranded oligonucleotide and the third single stranded oligonucleotide , thereby forming , and annealing the first single stranded oligonucleotide and , thereby forming ;

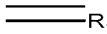
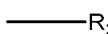
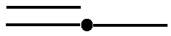
(ii) annealing  and a single stranded dimer , thereby forming ; and

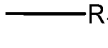
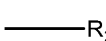
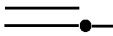
(iii) annealing  and a fourth single stranded oligonucleotide , thereby forming .

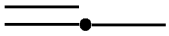
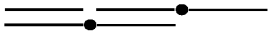
122. A method for synthesizing a compound of Structure 10:  (Structure 10), wherein each is a double stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides, the method comprising the steps of:

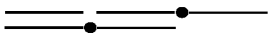
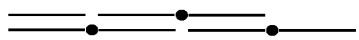
(i) forming  by:

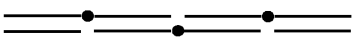
(a) annealing a first single stranded oligonucleotide and a second single stranded oligonucleotide , thereby forming ,

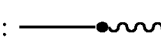
and reacting  with a third single stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ; or

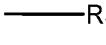
(b) reacting the second single stranded oligonucleotide  and the third single stranded oligonucleotide , thereby forming , and annealing the first single stranded oligonucleotide and , thereby forming ;

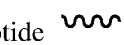
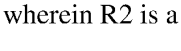
(ii) annealing  and a single stranded dimer , thereby forming ;

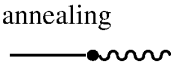
(iii) annealing  and a second single stranded dimer , thereby forming ; and

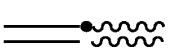
(iv) annealing  and a fourth single stranded oligonucleotide , thereby forming .

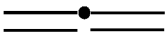
123. A method for synthesizing a compound of Structure 5:  (Structure 5) wherein  is a first single stranded oligonucleotide, is a second single stranded oligonucleotide having a different sequence from the first, and • is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

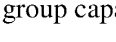
(i) reacting a first single stranded oligonucleotide  with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○ under conditions that produce the mono-substituted product ;

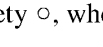
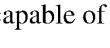
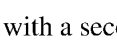
(ii) reacting  with a second single stranded oligonucleotide , wherein R2 is a chemical group capable of reacting with ○, thereby forming .

124. The method of claim 123, further comprising the step of annealing complementary and to yield Structure 6:  (Structure 6).

125. The method of claim 124, wherein the yield of  is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.

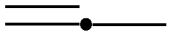
126. A method for synthesizing an isolated compound of Structure 4:  (Structure 4) wherein each is a double stranded oligonucleotide and • is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

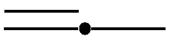
(i) reacting a first single stranded oligonucleotide  with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○, thereby forming a mono-substituted product ;

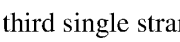
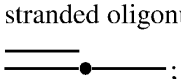
(ii) reacting  with a second single stranded oligonucleotide , wherein R2 is a chemical group capable of reacting with ○, thereby forming a single stranded dimer ;

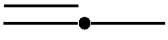
(iii) annealing single stranded oligonucleotides, at the same time or sequentially, thereby forming .

127. A method for synthesizing an isolated compound of Structure 4:  (Structure 4) wherein each is a double stranded oligonucleotide and • is a covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

(i) forming  by:

(a) annealing a first single stranded oligonucleotide and a second single stranded oligonucleotide , thereby forming , and reacting  with a third single stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ; or

(b) reacting the second single stranded oligonucleotide and the third single stranded oligonucleotide , thereby forming , and annealing the first single stranded oligonucleotide and , thereby forming ;

(ii) annealing  and a fourth single stranded oligonucleotide , thereby forming .

128. A method for synthesizing an isolated compound of Structure 4:  (Structure 4) wherein each is a double stranded oligonucleotide and • is a

covalent linker joining single strands of adjacent single stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

(a) annealing a first single stranded oligonucleotide —— and a second single stranded oligonucleotide ——R_1, thereby forming ═══R_1;

(b) annealing a third single stranded oligonucleotide ——R_2 and a fourth single stranded oligonucleotide ——, thereby forming ═══R_2;

(b) reacting ═══R_1 and ═══R_2 with wherein R1 and R2 ——R_2, wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming ——•——.

129. A method for synthesizing the composition of claim 67, comprising:

(i) reacting a first single stranded oligonucleotide ——R_1 with a bifunctional linking moiety ○, wherein R1 is a chemical group capable of reacting with ○ under conditions that produce the mono-substituted product ——○;

(ii) reacting ——○ with a second single stranded oligonucleotide ∿∿R_2, wherein R2 is a chemical group capable of reacting with ○, thereby forming ——•∿∿;

(iii) annealing a plurality of ——•∿∿, thereby forming the composition of claim 67.

130. A method for synthesizing a composition comprising a plurality of molecule comprising Structure 17:

——•——|——•——|$_n$
∿∿•∿∿|∿∿•∿∿|$_n$ (Structure 17), wherein n is an integer ≥ 1; each —— is a single stranded oligonucleotide; each ∿∿ is a single stranded oligonucleotide that hybridizes with a ——; ∿∿ —— is a double stranded oligonucleotide; and each • is a covalent linker joining single strands of adjacent single stranded oligonucleotides to form ——•—— and ∿∿•∿∿, the method comprising the steps of:

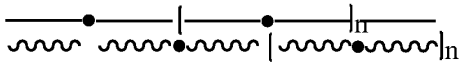
annealing a plurality of ——•—— and ∿∿•∿∿ at:

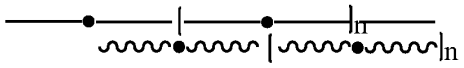
(i) a total oligonucleotide concentration of about 200-300 μM for ——•—— and ∿∿•∿∿,

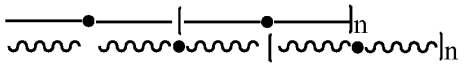
(ii) about 0.1-0.3x phosphate buffered saline (PBS), and

(iii) at a temperature of about 70-80 °C to about 20-30 °C for about 1.5-2.5 hours.

131. The method of claim 130, further comprising the step of annealing a plurality of ——— and/or ∿∿∿, thereby forming a plurality of molecules comprising Structure 18:

 (Structure 18).

 (Structure 19), and/or

 (Structure 20).

132. The method of claim 130 or 131, further comprising annealing ——— with the plurality of plurality of ———●——— and ∿∿∿●∿∿∿.

133. The method of claim 131 or 132, wherein the molar ratio of ——— to ∿∿∿●∿∿∿ is about 5:100, 10:100, 20:100, 30:100, 40:100, or 50:100.

134. The method of claim 130 or 131, further comprising annealing ∿∿∿ with the plurality of plurality of ———●——— and ∿∿∿●∿∿∿.

135. The method of claim 131 or 132, wherein the molar ratio of ∿∿∿ to ———●——— is about 5:100, 10:100, 20:100, 30:100, 40:100, or 50:100.

136. The method of any one of claims 130-135, wherein the molar ratio of ———●——— and ∿∿∿●∿∿∿ is about 1:1.

137. The method of any one of claims 130-135, wherein the molar ratio of ———●——— to ∿∿∿●∿∿∿ or the molar ratio of ∿∿∿●∿∿∿ to ———●——— is about 100:90, 100:80, 100:75, 100:70, or 100:60.

138. The method of any one of claims 130-137, wherein each ∿∿∿ ——— has a length of 15-30 base pairs.

139. The method of any one of claims 130-138, where each ∿∿∿ ——— is an siRNA.

140. The method of any one of claims 130-139, where each ∿∿∿ ——— comprises siRNA guide strand targeting Factor VII and a passenger strand hybridized to the guide strand.

141. The method of any one of claims 130-140, wherein n is an integer from 1 to 100.

142. The method of any one of claims 130-141, wherein • is a cleavable or non-cleavable linker.

143. The method of any one of claims 130-142, further comprising formulating the plurality of molecules comprising Structure 17, 18, 19, and/or 20 in a nanoparticle.

144. The methods of any one of claims 118 to 122 or 129 to 143, wherein each covalent linker • is the same.

145. The methods of any one of claims 118 to 122 or 129 to 143, comprising two or more different covalent linkers •.

146. The methods of any one of claims 118 to 122 or 129 to 143, comprising a homo-multimer of substantially identical double stranded oligonucleotides.

147. The method of claim 146, wherein the substantially identical double stranded oligonucleotides each comprise an siRNA targeting the same molecular target *in vivo*.

148. The method of any one of claims 118 to 122, comprising a hetero-multimer of two or more substantially different double stranded oligonucleotides $\overline{\text{———}}$.

149. The method of claim 148, wherein the substantially different double stranded oligonucleotides $\overline{\text{———}}$ each comprise an siRNA targeting different genes.

150. The method of any one of claims 118 to 149, wherein the covalent linkers • comprise the reaction product of a nucleophile and electrophile.

151. The method of claim 150, wherein the covalent linkers • comprise the reaction product of a thiol and maleimide, a thiol and vinylsulfone, a thiol and pyridyldisulfide, a thiol and iodoacetamide, a thiol and acrylate, an azide and alkyne, or an amine and carboxyl group.

152. The compound of claim 150, wherein the covalent linkers • comprise the reaction product of a thiol and DTME (dithiobismaleimidoethane), BM(PEG)2 (1,8-bis(maleimido)diethylene glycol), BM(PEG)3 (1,11-bismaleimido-triethyleneglycol), BMOE (bismaleimidoethane), BMH (bismaleimidohexane), or BMB (1,4-bismaleimidobutane).

153. The method of any one of claims 118 to 152, wherein one or more double stranded oligonucleotides comprises blunt ends.

154. The method of any one of claims 118 to 153, wherein one or more double stranded oligonucleotides comprises overhang.

155. The method of any one of claims 118 to 154, wherein each covalent linker • independently joins adjacent single stranded oligonucleotides 3' to 3', 5' to 5', or 3' to 5'.
156. The method of any one of claims 118 to 155, wherein at least one oligonucleotide further comprises a chemical modification.
157. The method of claim 156, wherein the chemical modification comprises a modified nucleoside, modified backbone, modified sugar, or modified terminus.
158. The method of any one of claims 118 to 157, wherein the product is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.
159. The method of any one of claims 118 to 157, wherein the product is about 85-95 % pure.
160. The method of any one of claims 118 to 159, wherein at least one of the oligonucleotides is an siRNA.
161. The method of any one of claims 118 to 159, wherein the nucleic acid is RNA, DNA, or comprises an artificial or non-natural nucleic acid analog.
162. The method of claim 161, wherein the nucleic acid is DNA.
163. The method of claim 162, wherein the DNA is an antisense DNA (aDNA) or antisense gapmer.
164. The method of claim 161, wherein the nucleic acid is RNA.
165. The method of claim 164, wherein the RNA is an antisense RNA (aRNA), CRISPR RNA (crRNA), long noncoding RNA (lncRNA), microRNA (miRNA), piwi-interacting RNA (piRNA), small interfering RNA (siRNA), messenger RNA (mRNA), short hairpin RNA (shRNA), small activating (saRNA), antagomir, or ribozyme.
166. The method of any one of claims 118 to 159, wherein the nucleic acid is an aptamer.
167. The method of any one of claims 118 to 166, wherein the nucleic acid is 15-30, 17-27, 19-26, 20-25, 40-50, 40-150, 100-300, 1000-2000, or up to 10000 nucleotides in length.
168. The method of any one of claims 118 to 167, further comprising a targeting ligand.
169. The method of claim 168, wherein the targeting ligand is bound to the nucleic acid.

170. The method of claim 168, wherein the targeting ligand is bonded to the nucleic acid through its 3' or 5' terminus.

171. The method of claim 168 to 170, wherein the targeting ligand comprises *N*-Acetylgalactosamine (GalNAc), cholesterol, tocopherol, folate, 2-[3-(1,3-dicarboxypropyl)- ureido]pentanedioic acid (DUPA), or anisamide.

172. The method of any one of claims 118 to 171, wherein one or more of • comprises a cleavable covalent linker.

173. The method of claim 172, wherein the cleavable covalent linker comprises an acid cleavable ester bond, hydrazine bond, or acetal bond.

174. The method of claim 172, wherein the cleavable covalent linker comprises a reductant cleavable bond.

175. The method of claim 174, wherein the reductant cleavable bond is a disulfide bond.

176. The method of claim 172, wherein the cleavable covalent linker is cleavable under intracellular conditions.

177. The method of claim 172, wherein the cleavable covalent linker comprises a biocleavable bond.

178. The method of claim 172, wherein the cleavable covalent linker comprises an enzyme cleavable bond.

179. The method of any one of claims 118 to 171, wherein one or more of • comprises a noncleavable covalent linker.

180. The method of claim 179, wherein the noncleavable covalent linker comprises an amide bond or urethane bond.

181. A composition comprising the compound of any one of claims 54 to 117 and a pharmaceutically acceptable excipient.

182. A composition comprising the compound of any one of claims 54 to 117 for use a medicament.

183. A composition comprising the compound of any one of claims 54 to 117 for use in the manufacture of a medicament.

184. The composition of claims 182 or 183 wherein the medicament is for silencing or reducing the expression of at least one overexpressed gene.

185. The composition of claim 184 for silencing or reducing the expression of two, three, four, or more overexpressed genes.

186. A composition comprising the compound of any one of claims 54 to 117, formulated in lipid nanoparticles (LNP), exosomes, microvesicles, or viral vectors.

187. A method for reducing gene expression comprising administering an effective amount of the compound or composition according to any one of claims 54 to 117 or 181 to 186 to a subject in need thereof.

188. A method for treating a subject comprising administering an effective amount of the compound or composition according to any one of claims 54 to 117 or 181 to 186 to a subject in need thereof.

189. A method for silencing two or more genes comprising administering an effective amount of a compound or composition according to any one of the preceding claims to a subject in need thereof, wherein the compound or composition comprises oligonucleotides targeting two or more genes.

190. The methods of claim 189, wherein the compound or composition comprises oligonucleotides targeting two, three, four, or more genes.

191. A method for delivering two or more oligonucleotides to a cell per targeting ligand binding event comprising administering an effective amount of a compound or composition according to any one of the preceding claims to a subject in need thereof, wherein the compound or composition comprises a targeting ligand.

192. A method for delivering a predetermined stoichiometric ratio of two or more oligonucleotides to a cell comprising administering an effective amount of a compound or composition according to any one of the preceding claims to a subject in need thereof, wherein the compound or composition comprises the predetermined stoichiometric ratio of two or more oligonucleotides.

193. The method of any one of claims claim 187 to 192, wherein the subject is a cell, mammal, or human.

194. An siRNA having SEQ ID NO:106.

195. An siRNA having SEQ ID NO:115.