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(54) Title: MULTIMERIC OLIGONUCLEOTIDES WITH ENHANCED BIOACTIVITY

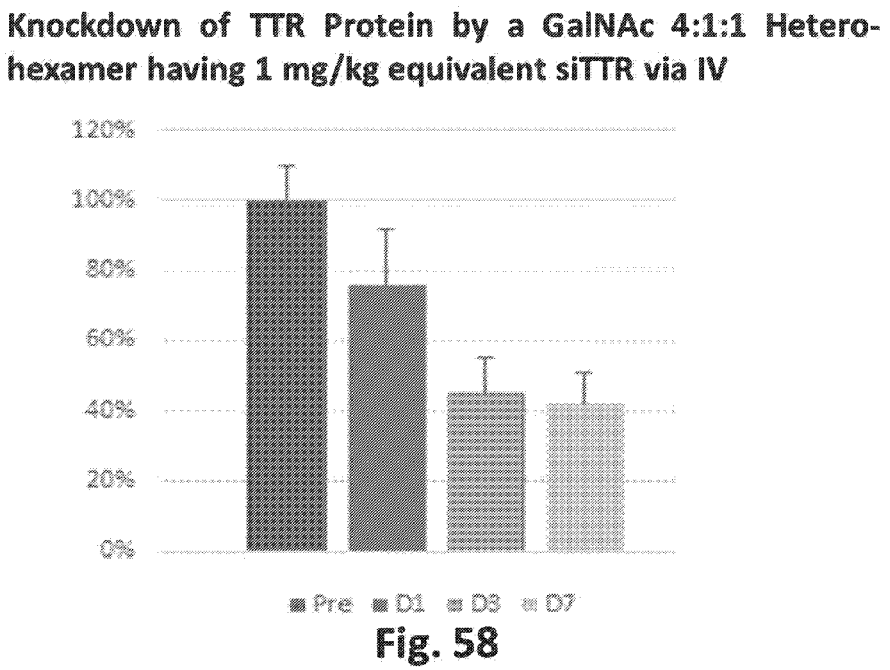


Fig. 58

(57) Abstract: The present disclosure relates to multimeric oligonucleotides having monomeric subunits joined by linkers and methods of administering multimeric oligonucleotides to a subject. The multimeric oligonucleotides have a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form. The present disclosure also relates to such multimeric oligonucleotides and methods of synthesizing such multimeric oligonucleotides.

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[Continued on next page]

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MULTIMERIC OLIGONUCLEOTIDES WITH ENHANCED BIOACTIVITY

RELATED APPLICATION INFORMATION

[0001] This application claims priority to U.S. Serial No. 62/813,599, filed March 4, 2019, and U.S. Serial No. 62/824,771, filed March 27, 2019, both of which are hereby incorporated herein by reference in their entireties.

FIELD

[0002] The present disclosure relates to oligonucleotide-based therapeutics. More specifically, the present disclosure relates to multimeric therapeutic oligonucleotides containing multiple sub-units each with increased bioactivity in a subject relative to the corresponding monomers.

BACKGROUND

[0003] Oligonucleotides are now a well-established class of therapeutics with multiple applications (e.g., RNA interference, or RNAi) and ongoing clinical trials. However, many factors still limit oligonucleotide therapeutics, for example, the delivery of the oligonucleotide to a target cell and the subsequent internalization of the oligonucleotide into the target cell in sufficient quantities to achieve a desired therapeutic effect.

[0004] In an attempt to address these delivery and internalization limitations, many parties have investigated lipid nanoparticles (LNPs, e.g., lipid spheroids including positively charged lipids to neutralize the negative charge of the oligonucleotide and to facilitate target cell binding and internalization). While LNPs can in some cases facilitate delivery and internalization, they suffer from major drawbacks, for example poor targeting and toxicity, resulting in a narrowed therapeutic window.

[0005] Oligonucleotides conjugated to ligands targeting specific cell surface receptors have been also investigated. The use of one such ligand, *N*-acetylgalactosamine (GalNAc), has become a method of choice for oligonucleotide delivery to hepatocytes. However, while the toxicological profiles of GalNAc-conjugates can be better than LNPs, delivery is not as efficient. This limitation necessitates increased dosages, often by an order of magnitude or more. Increased dosages can be undesirable due to toxicity, side effects, and/or cost.

[0006] Although individual oligonucleotide therapeutics are quite large molecules compared to traditional drugs, they are nonetheless small enough to be easily absorbed and secreted via the kidney. This is a major problem as the amount of therapeutic material reaching the target cells is consequently reduced.

[0007] In order to minimalize secretion of the oligonucleotide via the kidney one approach has been to maximize the number of phosphorothioate internucleotide linkages in the molecule. Phosphorothioate groups were originally introduced to reduce cleavage by nucleases but were found to promote binding to proteins. Because the affinity of phosphorothioate oligonucleotides for proteins is length-dependent but largely sequence-independent (Stein CA, et al. Biochemistry. 1993; 32:4855–4861), oligonucleotides containing a large proportion of such groups bind to proteins circulating in the blood, thereby increasing the effective molecular size of the oligonucleotide and decreasing the rate of secretion via the kidney. However, the use of a high number of phosphorothioate groups has many drawbacks. For example, phosphorothioate oligonucleotides of the appropriate length can block the binding of biologically relevant proteins to their natural receptors resulting in toxic side effects (Stein, CA. J Clin Invest. 2001 Sep 1; 108(5): 641–644). Hence, the facilitation of protein binding that is an advantage of high levels of thiophosphorylation is simultaneously a major disadvantage. Increased toxicity and reduction of gene silencing was also observed when phosphorothioates have been applied to siRNAs (Lam et al., Mol Ther Nucleic Acids, 2015, 4(9): e252; Chiu et al., RNA, 2003, 9: 1034–1048; Amarzguioui et al., Nucleic Acids Res, 2003, 31: 589–595; Choung et al., Biochem Biophys Res Commun, 2006, 342: 919–927).

[0008] Thus, the use of high levels of phosphorothioate groups to minimize losses of oligonucleotides from the blood stream via kidney filtration is inapplicable to siRNAs and similar double-stranded molecules, such as miRNAs, and is limited to a subset of antisense oligonucleotides. There is therefore a need for a method to increase the bioactivity of all classes of oligonucleotide therapeutics.

SUMMARY

[0009] The present disclosure relates to compositions and related methods to increase the biological activity in a subject of an oligonucleotide therapeutic agent. The disclosure is applicable to all types of oligonucleotide therapeutics, including siRNAs and

miRNAs as well as antisense oligonucleotides, independent of phosphorothioate content and resulting protein binding characteristics.

[0010] The present disclosure provides a multimeric oligonucleotide wherein an oligonucleotide therapeutic agent (a "subunit") is linked via a covalent linker to a number of copies of the same or differing subunits and wherein the biological activity of each of the agents is increased relative to the activity of the agent alone. In one embodiment, the increase in bioactivity is independent of the phosphorothioate content. In another embodiment, the increase in bioactivity is independent of the total phosphorothioate content of the multimeric oligonucleotide. In yet another embodiment, the increase in bioactivity is independent of the ratio of phosphorothioates to nucleotide residues in the multimeric oligonucleotide. In other embodiments, the multimeric oligonucleotide may contain one or more double-stranded subunits, or may contain four or more subunits overall, or may have a molecular weight of at least about 45 kilodaltons (kD). The improved and advantageous properties of the multimers according to the disclosure may be described in terms of increased *in vivo* activity. The relative increase in *in vivo* bioactivity of each of the subunits in the multimer as compared to the corresponding monomer may be in the range of 2-10 and higher; for example, the relative increase may be 2, 5, 10, or more times that of the corresponding monomer.

[0011] The present disclosure also relates to new synthetic intermediates and methods of synthesizing the multi-conjugate oligonucleotides. The present disclosure also relates to methods of using the multi-conjugate oligonucleotides, for example in reducing gene expression, biological research, treating or preventing medical conditions, and/or to produce new or altered phenotypes.

[0012] In one aspect, the disclosure provides a multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and the increase in activity of one or more subunits within the multimeric oligonucleotide is independent of phosphorothioate content in the multimeric oligonucleotide.

[0013] In another aspect, the disclosure provides a multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and at least one subunit within the multimeric oligonucleotide is a double-stranded oligonucleotide.

[0014] In yet another aspect, the disclosure provides a multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and the multimeric oligonucleotide comprises 4 or more subunits.

[0015] In still another aspect, the disclosure provides a multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

[0016] In an embodiment, at least one subunit within the multimeric oligonucleotide is a double-stranded oligonucleotide.

[0017] In an embodiment, the multimeric oligonucleotide comprises 4 or more subunits.

[0018] In an embodiment, the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

[0019] In another aspect, the disclosure provides a multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to

another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to decrease its clearance due to glomerular filtration; and the molecular weight of the multimeric oligonucleotide is at least about 45 kD, wherein the multimeric oligonucleotide comprises a hetero-multimer of six or more subunits -------, wherein at least two subunits ------- are substantially different.

[0020] In yet another aspect, the disclosure provides a multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide comprises five or more subunits -------; and wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

[0021] In still another aspect, the disclosure provides a multimeric oligonucleotide comprising subunits ------ forming Structure 119: -----•-----•-----•-----, wherein: each subunit is independently a single or double-stranded oligonucleotide; each of the subunits is joined to another subunit by a covalent linker •; and wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

[0022] In another aspect, the disclosure provides a multimeric oligonucleotide comprising two subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to the other subunit by a covalent linker •; the molecular weight of the compound is at least about 45 kD; and at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

[0023] In an embodiment, at least two subunits ------- are substantially different.

[0024] In an embodiment, all of the subunits are substantially different.

[0025] In an embodiment, at least two subunits ------- are substantially the same or are identical.

[0026] In an embodiment, all of the subunits ------- are substantially the same or are identical.

[0027] In an embodiment, the multimeric oligonucleotide comprises five, six, seven, eight, nine, or ten subunits -------.

[0028] In an embodiment, the multimeric oligonucleotide comprises six subunits -------.

[0029] In an embodiment, the multimeric oligonucleotide comprises seven, eight, nine, or ten subunits -------.

[0030] In an embodiment, each subunit ------- is independently 10-30, 17-27, 19-26, or 20-25 nucleotides in length.

[0031] In an embodiment, one or more subunits are double-stranded. In an embodiment, one or more subunits are single-stranded. In an embodiment, the subunits comprise a combination of single-stranded and double-stranded oligonucleotides.

[0032] In an embodiment, one or more nucleotides within an oligonucleotide is an RNA, a DNA, or an artificial or non-natural nucleic acid analog.

[0033] In an embodiment, at least one of the subunits is RNA.

[0034] In an embodiment, at least one of the subunits is a siRNA, a saRNA, or a miRNA.

[0035] In an embodiment, at least one of the subunits is a siRNA. In an embodiment, at least one of the subunits is a miRNA. In an embodiment, at least one of the subunits is an antisense oligonucleotide.

[0036] In an embodiment, at least one of the subunits is a double-stranded siRNA.

[0037] In an embodiment, two or more siRNA subunits are joined by covalent linkers attached to the sense strands of the siRNA.

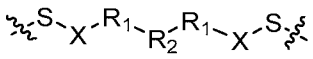
[0038] In an embodiment, two or more siRNA subunits are joined by covalent linkers attached to the antisense strands of the siRNA.

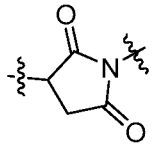
[0039] In an embodiment, two or more siRNA subunits are joined by covalent linkers attached to the sense strand of a first siRNA and the antisense strand of a second siRNA.

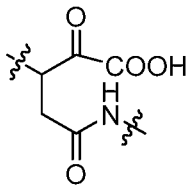
[0040] In an embodiment, one or more of the covalent linkers • comprise a cleavable covalent linker.

[0041] In an embodiment, the cleavable covalent linker contains an acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond.

[0042] In an embodiment, the cleavable covalent linker is cleavable under intracellular conditions.

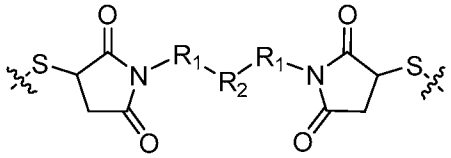
[0043] In an embodiment, at least one covalent linker comprises a disulfide bond or a compound of Formula (I):  wherein: S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; R_2 is a thiopropionate or disulfide

group; and each X is independently selected from:  or



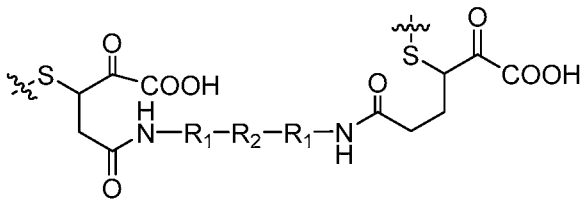
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[0044] In an embodiment, the compound of Formula (I) is



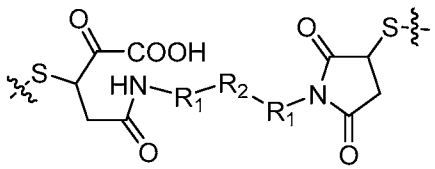
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[0045] In an embodiment, the compound of Formula (I) is



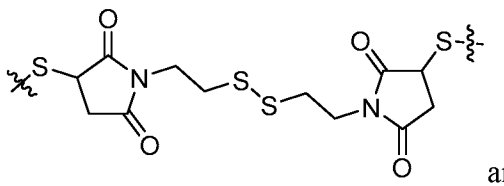
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[0046] In an embodiment, the compound of Formula (I) is



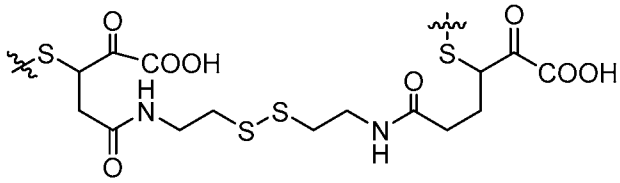
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[0047] In an embodiment, the compound of Formula (I) is



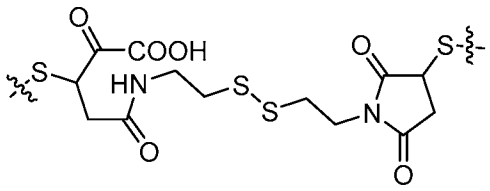
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

[0048] In an embodiment, the compound of Formula (I) is

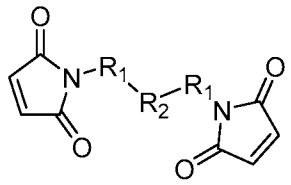


and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

[0049] In an embodiment, the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

[0050] In an embodiment, the covalent linker of Formula (I) is formed from a covalent linking precursor of Formula (II):  wherein: each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[0051] In an embodiment, one or more of the covalent linkers • comprise a nucleotide linker.

[0052] In an embodiment, the nucleotide linker is between 2-6 nucleotides in length. In an embodiment, the nucleotide linker is 3, 4, or 5 nucleotides in length.

[0053] In an embodiment, the nucleotide linker is a dinucleotide linker.

[0054] In an embodiment, each covalent linker • is the same. In an embodiment, the covalent linkers • comprise two or more different covalent linkers.

[0055] In an embodiment, at least two subunits are joined by covalent linkers • between the 3' end of a first subunit and the 3' end of a second subunit. In an embodiment, at least two subunits are joined by covalent linkers • between the 3' end of a first subunit and the 5' end of a second subunit. In an embodiment, at least two subunits are joined by covalent linkers • between the 5' end of a first subunit and the 3' end of a second subunit. In an embodiment, at least two subunits are joined by covalent linkers • between the 5' end of a first subunit and the 5' end of a second subunit.

[0056] In an embodiment, the multimeric oligonucleotide further comprises one or more targeting ligands. In an embodiment, at least one of the subunits is a targeting ligand. In an embodiment, the targeting ligand is an aptamer. In an embodiment, the targeting ligand comprises N-Acetylgalactosamine (GalNAc). In an embodiment, the targeting ligand comprises an immunostimulant. In an embodiment, the targeting ligand comprises a CpG oligonucleotide. In an embodiment, the CpG oligonucleotide comprises the sequence TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 162). In an embodiment, the CpG oligonucleotide comprises the sequence GGTGCATCGATGCAGGGGG (SEQ ID NO: 163).

[0057] In an embodiment, the multimeric oligonucleotide is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% pure.

[0058] In an embodiment, at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

[0059] In an embodiment, the subunit with complementarity to TTR mRNA comprises increased activity *in vivo* relative to a monomeric oligonucleotide with complementarity to TTR mRNA.

[0060] In an embodiment, the oligonucleotide with complementarity to TTR mRNA comprises UUAUAGAGCAAGAACACUGUUUU (SEQ ID NO: 164).

[0061] In an embodiment, the multimeric oligonucleotide is administered *in vivo* by intravenous injection.

[0062] In an embodiment, the multimeric oligonucleotide is administered *in vivo* by intravenous injection and has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered subcutaneously in monomeric form.

[0063] In an embodiment, the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide is at least a 2-fold increase relative to in vivo activity of the same subunit when administered in monomeric form.

[0064] In an embodiment, the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide is at least a 5-fold increase relative to in vivo activity of the same subunit when administered in monomeric form. In an embodiment, the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide is at least a 10-fold increase relative to in vivo activity of the same subunit when administered in monomeric form.

[0065] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and the increase in activity of one or more subunits within the multimeric oligonucleotide is independent of phosphorothioate content in the multimeric oligonucleotide.

[0066] In another aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and at least one subunit within the multimeric oligonucleotide is a double-stranded oligonucleotide.

[0067] In yet another aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and the multimeric oligonucleotide comprises 4 or more subunits.

[0068] In still another aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising

administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

[0069] In an embodiment, at least one subunit within the multimeric oligonucleotide is a double-stranded oligonucleotide

[0070] In an embodiment, the multimeric oligonucleotide comprises 4 or more subunits.

[0071] In an embodiment, the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

[0072] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD; and wherein the multimeric oligonucleotide comprises a hetero-multimer of six or more subunits -------, wherein at least two subunits ------- are substantially different.

[0073] In another aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity

of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; the multimeric oligonucleotide comprises five or more subunits -------; and wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

[0074] In yet another aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; wherein the multimeric oligonucleotide comprises Structure 117: ------•------•------•------; and wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

[0075] In still another aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein: each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •; the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; wherein the multimeric oligonucleotide comprises two subunits -------; wherein the molecular weight of the compound is at least about 45 kD; and wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

[0076] In an embodiment, the administering comprises intravenous injection.

[0077] In an embodiment, the number of subunits contained in the multimeric oligonucleotide is m, m being an integer selected to enable the multimeric oligonucleotide to have the molecular weight and/or size configured to increase *in vivo*

activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form.

[0078] In an embodiment, at least two subunits ------- are substantially different.

[0079] In an embodiment, all of the subunits are substantially different.

[0080] In an embodiment, at least two subunits ------- are substantially the same or are identical.

[0081] In an embodiment, all of the subunits ------- are substantially the same or are identical.

[0082] In an embodiment, the multimeric oligonucleotide comprises five, six, seven, eight, nine, or ten subunits -------.

[0083] In an embodiment, the multimeric oligonucleotide comprises six subunits -------.

[0084] In an embodiment, the multimeric oligonucleotide comprises seven, eight, nine, or ten subunits -------.

[0085] In an embodiment, each subunit ------- is independently 10-30, 17-27, 19-26, or 20-25 nucleotides in length.

[0086] In an embodiment, one or more subunits are double-stranded. In an embodiment, one or more subunits are single-stranded. In an embodiment, the subunits comprise a combination of single-stranded and double-stranded oligonucleotides.

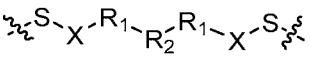
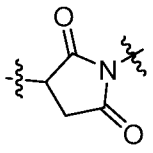
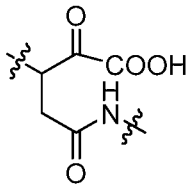
[0087] In an embodiment, one or more nucleotides within an oligonucleotide is an RNA, a DNA, or an artificial or non-natural nucleic acid analog.

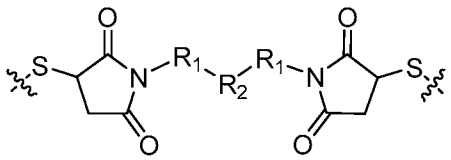
[0088] In an embodiment, at least one of the subunits is RNA. In an embodiment, at least one of the subunits is a siRNA, a saRNA, or a miRNA. In an embodiment, at least one of the subunits is a siRNA. In an embodiment, at least one of the subunits is a miRNA. In an embodiment, at least one of the subunits is an antisense oligonucleotide. In an embodiment, at least one of the subunits is a double-stranded siRNA.

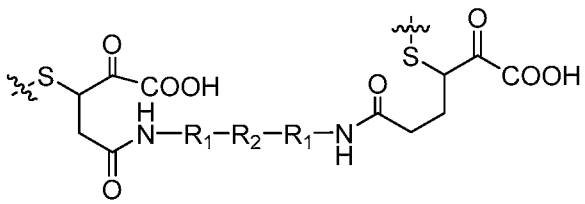
[0089] In an embodiment, two or more siRNA subunits are joined by covalent linkers attached to the sense strands of the siRNA. In an embodiment, two or more siRNA subunits are joined by covalent linkers attached to the antisense strands of the siRNA. In an embodiment, two or more siRNA subunits are joined by covalent linkers attached to the sense strand of a first siRNA and the antisense strand of a second siRNA.

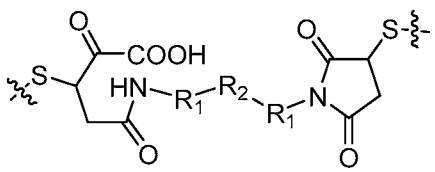
[0090] In an embodiment, one or more of the covalent linkers • comprise a cleavable covalent linker. In an embodiment, the cleavable covalent linker contains an

acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond. In an embodiment, the cleavable covalent linker is cleavable under intracellular conditions.

[0091] In an embodiment, at least one covalent linker comprises a disulfide bond or a compound of Formula (I):  wherein: S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; R_2 is a thiopropionate or disulfide group; and each X is independently selected from:  or .

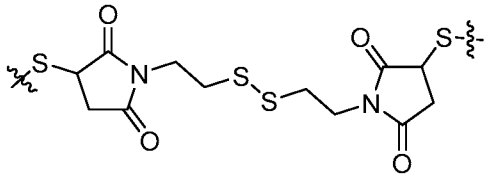
[0092] In an embodiment, the compound of Formula (I) is  wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[0093] In an embodiment, the compound of Formula (I) is  wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[0094] In an embodiment, the compound of Formula (I) is  wherein S is attached by a covalent bond or by a linker

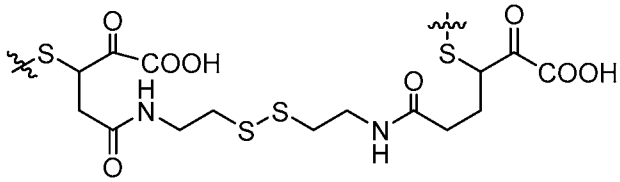
to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[0095] In an embodiment, the compound of Formula (I) is



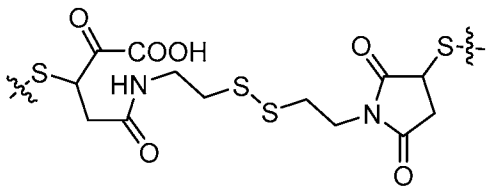
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

[0096] In an embodiment, the compound of Formula (I) is

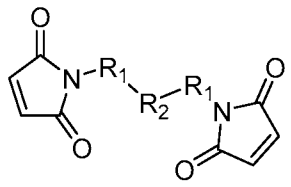


and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

[0097] In an embodiment, the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

[0098] In an embodiment, the covalent linker of Formula (I) is formed from a covalent linking precursor of Formula (II):  wherein: each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[0099] In an embodiment, one or more of the covalent linkers • comprise a nucleotide linker.

[00100] In an embodiment, the nucleotide linker is between 2-6 nucleotides in length. In an embodiment, the nucleotide linker is 3, 4, or 5 nucleotides in length. In an embodiment, the nucleotide linker is a dinucleotide linker.

[00101] In an embodiment, each covalent linker • is the same. In an embodiment, the covalent linkers • comprise two or more different covalent linkers.

[00102] In an embodiment, at least two subunits are joined by covalent linkers • between the 3' end of a first subunit and the 3' end of a second subunit. In an embodiment, at least two subunits are joined by covalent linkers • between the 3' end of a first subunit and the 5' end of a second subunit. In an embodiment, at least two subunits are joined by covalent linkers • between the 5' end of a first subunit and the 3' end of a second subunit. In an embodiment, at least two subunits are joined by covalent linkers • between the 5' end of a first subunit and the 5' end of a second subunit.

[00103] In an embodiment, the multimeric oligonucleotide further comprises one or more targeting ligands. In an embodiment, at least one of the subunits is a targeting ligand. In an embodiment, the targeting ligand is an aptamer. In an embodiment, the targeting ligand comprises N-Acetylgalactosamine (GalNAc). In an embodiment, the targeting ligand comprises an immunostimulant. In an embodiment, the targeting ligand comprises a CpG oligonucleotide. In an embodiment, the CpG oligonucleotide comprises the sequence TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 162). In an embodiment, the CpG oligonucleotide comprises the sequence GGTGCATCGATGCAGGGGG (SEQ ID NO: 163).

[00104] In an embodiment, the multimeric oligonucleotide is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% pure.

[00105] In an embodiment, at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

[00106] In an embodiment, the subunit with complementarity to TTR mRNA comprises increased activity *in vivo* relative to a monomeric oligonucleotide with complementarity to TTR mRNA.

[00107] In an embodiment, the oligonucleotide with complementarity to TTR mRNA comprises UUAUAGAGCAAGAACACUGUUUU (SEQ ID NO: 164).

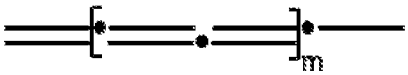
[00108] In an embodiment, the multimeric oligonucleotide is administered *in vivo* by intravenous injection.

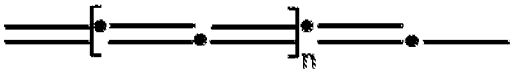
[00109] In an embodiment, the multimeric oligonucleotide is administered *in vivo* by intravenous injection and has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered subcutaneously in monomeric form.

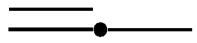
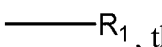
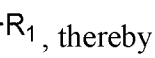
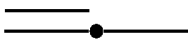
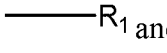
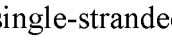
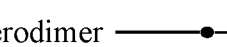
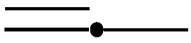
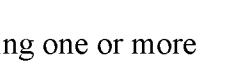
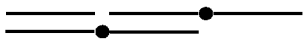
[00110] In an embodiment, the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide is at least a 2-fold increase relative to in vivo

activity of the same subunit when administered in monomeric form. In an embodiment, the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide is at least a 5-fold increase relative to in vivo activity of the same subunit when administered in monomeric form. In an embodiment, the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide is at least a 10-fold increase relative to in vivo activity of the same subunit when administered in monomeric form.

[00111] In one aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 92 or Structure 93:

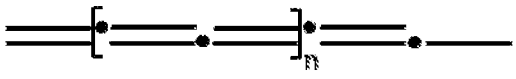

(Structure 92); or

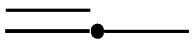
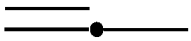
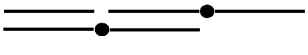
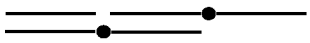
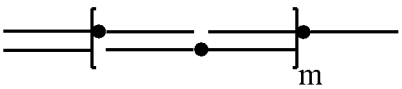
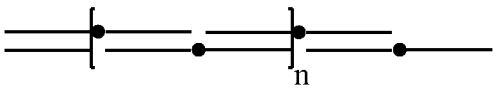

(Structure 93),

wherein each —— is independently a single-stranded oligonucleotide, each ═══ is independently a double stranded oligonucleotide, each • is a covalent linker joining adjacent oligonucleotides, and m is an integer ≥ 0 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 a heterodimer , and annealing the first single-stranded oligonucleotide —— and the heterodimer , thereby forming ; (ii) optionally annealing  and a single-stranded dimer , thereby forming ; (iii) optionally annealing one or more additional single-stranded dimers , thereby forming Structure 92 or Structure 93.

[00112] In one aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 92 or Structure 93:


(Structure 92); or


(Structure 93),

wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each • is a covalent linker joining adjacent oligonucleotides, and m is an integer ≥ 0 and n is an integer ≥ 0, the method comprising (i) annealing a first single-stranded oligonucleotide ——— and a first single-stranded heterodimer ———•———, thereby forming ; (ii) optionally annealing  and a second single-stranded dimer ———•———, thereby forming , and (iii) optionally annealing one or more additional single-stranded dimers ———•——— to  thereby forming,  or 

, wherein m is an integer ≥ 0 and n is an integer ≥ 0.

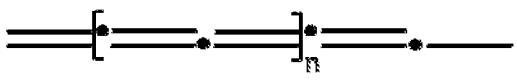
[00113] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising:


(Structure 94) or

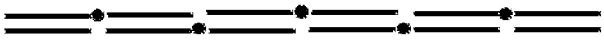
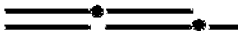
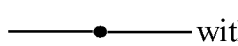
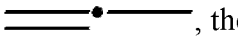
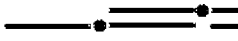
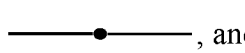
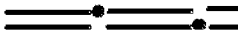

(Structure 95) or


(Structure 96)

wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each • is a covalent linker joining adjacent oligonucleotides, and p is an integer ≥ 0, q is an integer ≥ 0, and r is an integer ≥ 0, the method comprising: (i) annealing Structure 92 and Structure 93:


(Structure 92) 

(Structure 93), or (ii) annealing a first Structure 92 with a second Structure 92, or (iii) annealing a first Structure 93 and a second Structure 93, thereby forming Structure 94, Structure 95, or Structure 96, wherein m is independently an integer ≥ 0 and n is independently an integer ≥ 0.

[00114] In still another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 97:  (Structure 97) wherein each is independently a single-stranded oligonucleotide, each is independently a double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the steps of: (i) forming a first  by: (a) annealing a first single-stranded oligonucleotide and a second single-stranded oligonucleotide , thereby forming , 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 , and annealing with , thereby forming ; or (b) reacting the second single-stranded oligonucleotide and the third single-stranded oligonucleotide , thereby forming a heterodimer , annealing the first single-stranded oligonucleotide and the heterodimer , thereby forming , and annealing with , thereby forming ; (ii) forming a second  by the steps of (i) (a) or (i) (b); and (iii) forming  by annealing , , and , thereby forming .

[00115] In an embodiment, a terminus of the multimeric oligonucleotide is conjugated to a targeting ligand.

[00116] In an embodiment, each and is independently 10-30, 17-27, 19-26, or 20-25 nucleotides in length.

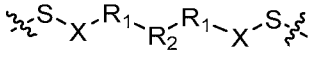
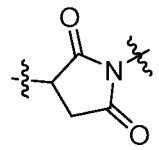
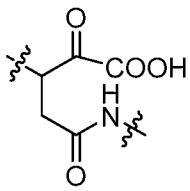
[00117] In an embodiment, one or more nucleotides within and is an RNA, a DNA, or an artificial or non-natural nucleic acid analog.

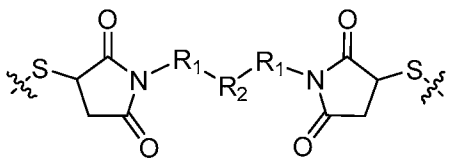
[00118] In an embodiment, at least one of and is a RNA.

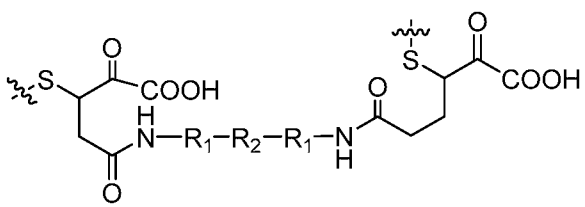
[00119] In an embodiment, at least one of and is a siRNA, a saRNA, or a miRNA. In an embodiment, at least one of and is a siRNA. In an embodiment, at least one of and is a miRNA. In an embodiment, at least one of is an antisense oligonucleotide.

[00120] In an embodiment, two or more siRNA are joined by covalent linkers attached to the sense strands of the siRNA. In an embodiment, two or more siRNA are joined by covalent linkers attached to the antisense strands of the siRNA. In an embodiment, two or more siRNA are joined by covalent linkers attached to the sense strand of a first siRNA and the antisense strand of a second siRNA.

[00121] In an embodiment, one or more of the covalent linkers • comprise a cleavable covalent linker. In an embodiment, the cleavable covalent linker contains an acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond. In an embodiment, the cleavable covalent linker is cleavable under intracellular conditions.

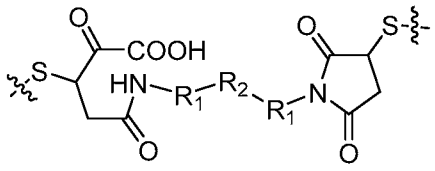
[00122] In an embodiment, at least one covalent linker comprises a disulfide bond or a compound of Formula (I):  wherein: S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; R_2 is a thiopropionate or disulfide group; and each X is independently selected from:  or .

[00123] In an embodiment, the compound of Formula (I) is  wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00124] In an embodiment, the compound of Formula (I) is  wherein S is attached by a covalent bond or

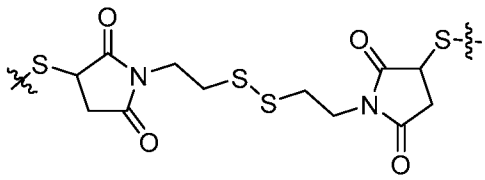
by a linker to the 3' or 5' terminus of ——— or ═══; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00125] In an embodiment, the compound of Formula (I) is



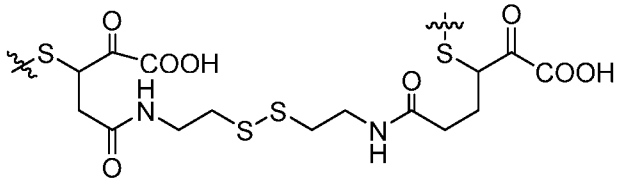
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00126] In an embodiment, the compound of Formula (I) is



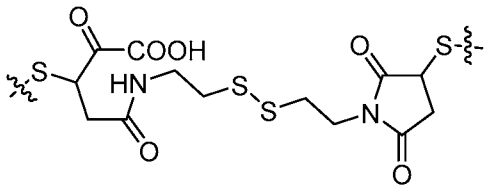
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══.

[00127] In an embodiment, the compound of Formula (I) is



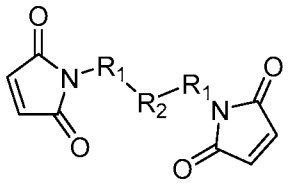
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══.

[00128] In an embodiment, the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══.

[00129] In an embodiment, the covalent linker of Formula (I) is formed from a covalent linking precursor of Formula (II):



wherein: each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00130] In an embodiment, one or more of the covalent linkers • comprise a nucleotide linker. In an embodiment, the nucleotide linker is between 2-6 nucleotides in length. In an embodiment, the nucleotide linker is 3, 4, or 5 nucleotides in length. In an embodiment, the nucleotide linker is a dinucleotide linker.

[00131] In an embodiment, each covalent linker • is the same. In an embodiment, the covalent linkers • comprise two or more different covalent linkers.

[00132] In an embodiment, two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 3' end of a first subunit and the 3' end of a second subunit. In an embodiment, two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 3' end of a first subunit and the 5' end of a second subunit. In an embodiment, two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 5' end of a first subunit and the 3' end of a second subunit. In an embodiment, two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 5' end of a first subunit and the 5' end of a second subunit.

[00133] In an embodiment, the multimeric oligonucleotide further comprises one or more targeting ligands. In an embodiment, at least one of the oligonucleotide subunits is a targeting ligand. In an embodiment, the targeting ligand is an aptamer. In an embodiment, a terminus of the multimeric oligonucleotide is conjugated to a targeting ligand. In an embodiment, the targeting ligand comprises N-Acetylgalactosamine (GalNAc).

[00134] In an embodiment, the multimeric oligonucleotide is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% pure.

[00135] In an embodiment, at least one of the oligonucleotide subunits comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

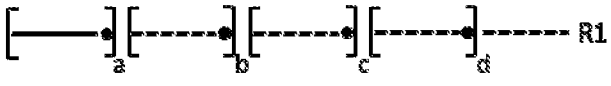
[00136] In an embodiment, the oligonucleotide with complementarity to TTR mRNA comprises UUAUAGAGCAAGAACACUGUUUU (SEQ ID NO: 164).

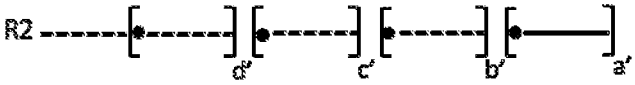
[00137] In an embodiment, one or more subunits comprise one or more phosphorothioate modifications. In an embodiment, one or more subunits comprise 1-3 phosphorothioate modifications at the 5' and/or 3' end. In an embodiment, each subunit comprises 0-15 phosphorothioate modifications, or 1-12 phosphorothioate modifications, or 2-8 phosphorothioate modifications.

[00138] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 100

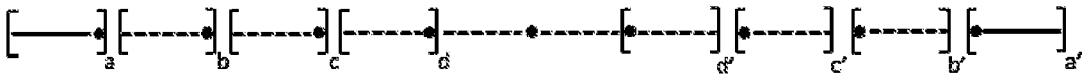


(Structure 100), wherein each ——— is independently a single-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the steps of:

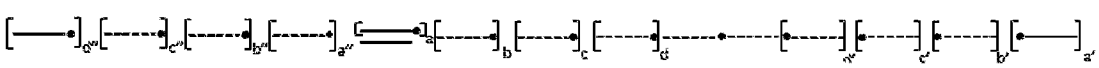
a) reacting Structure 98  (Structure 98)

with Structure 99  (Structure 99),

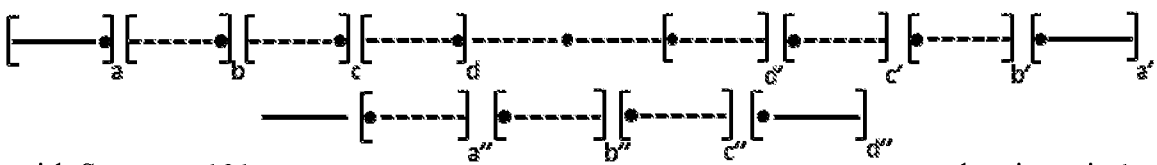
wherein: a, a', b, b', c, c', d and d' are each independently 0 or 1, and R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming Structure 100

. In other aspects, the sum of a+a'+b+b'+c+c'+d+d' is greater than or equal to 1, greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+b+b'+c+c'+d+d' is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

[00139] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 102

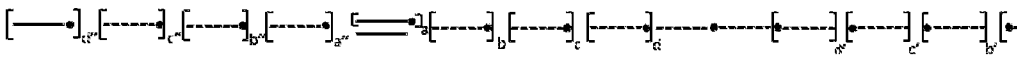


(Structure 102), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 100



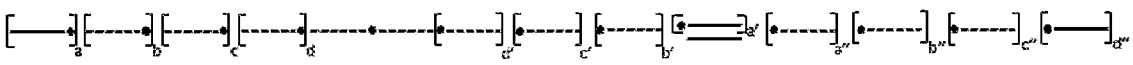
with Structure 101 , wherein: a is 1, and a', a'', b, b', b'', c, c', c'', d, d', and d'' are each independently 0 or 1, thereby

forming Structure 102

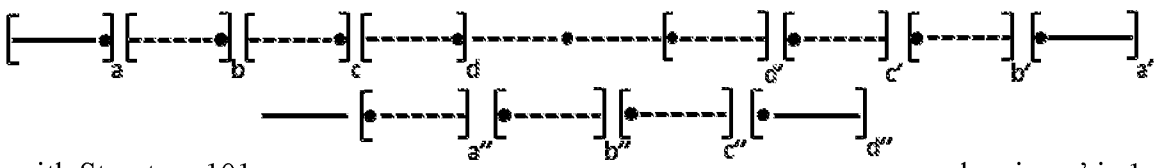


. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is 2, 3, 4, 5, 6, 7, 8, 9, or 10.

[00140] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 103

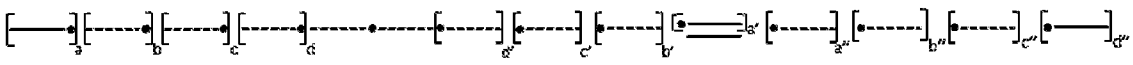


(Structure 103), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 100



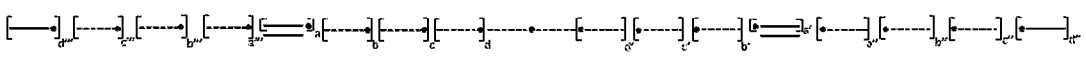
with Structure 101

, wherein: a' is 1, and a, a'', b, b', b'', c, c', c'', d, d', and d'' are each independently 0 or 1, thereby forming Structure 103



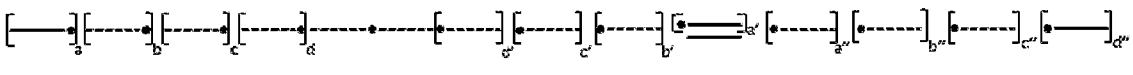
. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is 2, 3, 4, 5, 6, 7, 8, 9, or 10.

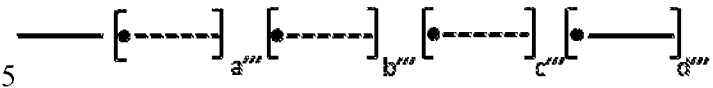
[00141] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 104

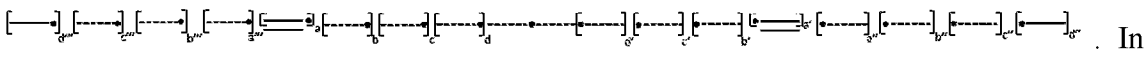


(Structure 104), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is

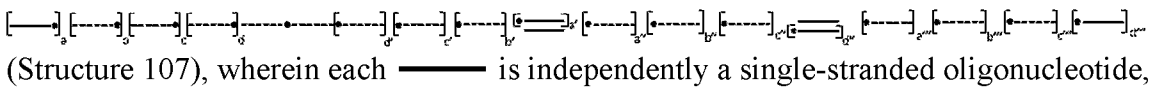
independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 103



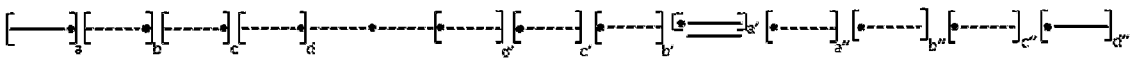
with Structure 105 , wherein: a and a' are each 1, and a'', a''', b, b', b'', b''', c, c', c'', c''', d, d', d'', and d''' are each independently 0 or 1, thereby forming Structure 104

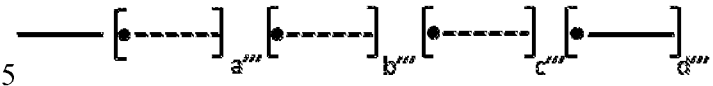
. In other aspects, the sum of a+a'+a"+a'"+b+b'+b"+b'"+c+c'+c"+c'"+d+d'+d"+d'" is greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+a"+a'"+b+b'+b"+b'"+c+c'+c"+c'"+d+d'+d"+d'" is 3, 4, 5, 6, 7, 8, 9, or 10.

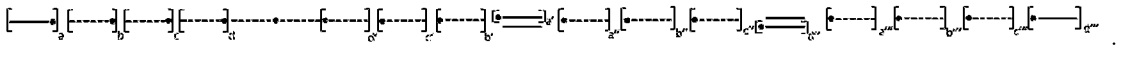
[00142] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 107



(Structure 107), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 103



with Structure 105 , wherein: a' and d'' are each 1, and a, a'', a''', b, b', b'', b''', c, c', c'', c''', d, d', and d''' are each independently 0 or 1, thereby forming Structure 107

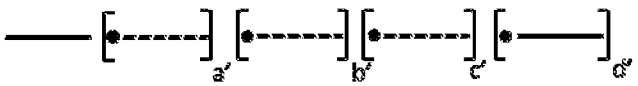
.

In other aspects, the sum of a+a'+a"+a'"+b+b'+b"+b'"+c+c'+c"+c'"+d+d'+d"+d'" is greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+a"+a'"+b+b'+b"+b'"+c+c'+c"+c'"+d+d'+d"+d'" is 3, 4, 5, 6, 7, 8, 9, or 10.

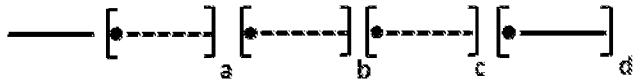
[00143] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 108



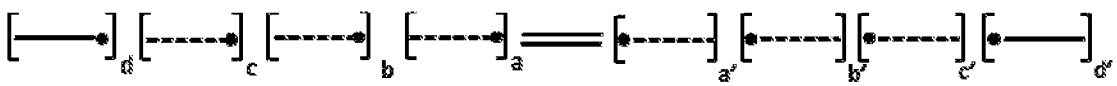
(Structure 108), wherein each —— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure



109 with Structure 110

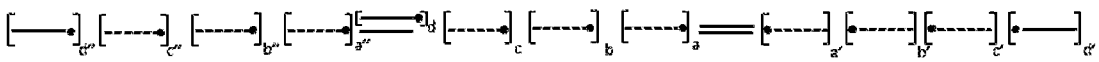


, wherein: a, a', b, b', c, c', d, and d' are each independently 0 or 1, thereby forming Structure 108



In other aspects, the sum of a+a'+b+b'+c+c'+d+d' is greater than or equal to 1, greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, greater than or equal to 10, or greater than or equal to 11. In other aspects, the sum of a+a'+b+b'+c+c'+d+d' is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.

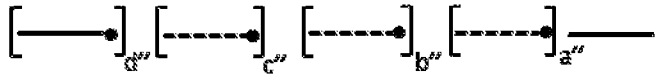
[00144] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 111



(Structure 111), wherein each —— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 108

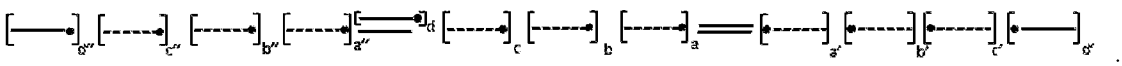


with Structure 112



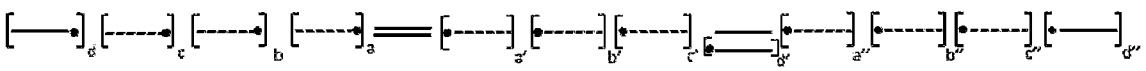
, wherein: d is 1, and a, a', a'', b, b', b'', c, c', c'', d' and d'' are each independently 0 or 1, thereby

forming Structure 111

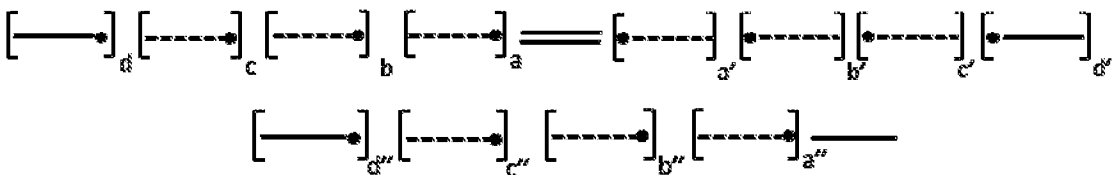


In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, greater than or equal to 10, or greater than or equal to 11. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.

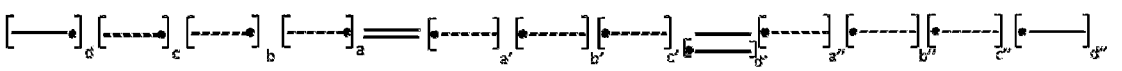
[00145] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 113



(Structure 113), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 108

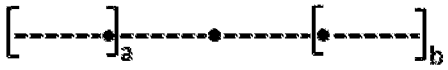


with Structure 112 , wherein: d' is 1, and a, a', a'', b, b', b'', c, c', c'', d and d'' are each independently 0 or 1, thereby forming Structure 113

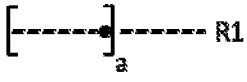


In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, greater than or equal to 10, or greater than or equal to 11. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.

[00146] In another aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 114



(Structure 114), wherein each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker

joining adjacent oligonucleotides, the method comprising reacting Structure 115  (Structure 115) with Structure 116  (Structure 116), wherein: R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker ●, a and b are each independently an integer ≥ 0, with the proviso that the sum of a and b is ≥ 4, thereby forming Structure 114 . In other aspects, the sum of a and b is greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a and b is 5, 6, 7, 8, 9, or 10.

[00147] In an embodiment, the method further comprises annealing one or more single-stranded oligonucleotides ——— with a complementary single-stranded oligonucleotide ——— in Structure 98 to Structure 113, thereby forming a double-stranded oligonucleotide ═══.

[00148] In an embodiment, each single-stranded oligonucleotide ——— and each single or double strand oligonucleotide ------ comprises 0-15 phosphorothioate modifications, or 1-12 phosphorothioate modifications, or 2-8 phosphorothioate modifications.

[00149] In an embodiment, at least one ------ is a double-stranded oligonucleotide.

[00150] In an embodiment, the total number of ——— and ------ in the multimeric oligonucleotide is at least 4.

[00151] In an embodiment, the multimeric oligonucleotide is at least about 45 kD.

[00152] In an embodiment, the multimeric oligonucleotide is ------●------, ------●------●------, or ------●------●------●------, and each ------ is substantially the same or different.

[00153] In an embodiment, the multimeric oligonucleotide is

------●------●------●------● ------,

------●------●------●------● ------●------,

------●------●------●------● ------●------●------, or

------●------●------●------● ------●------●------●------, and each ------ is substantially the same.

[00154] In an embodiment, a terminus of the multimeric oligonucleotide is conjugated to a targeting ligand.

[00155] In an embodiment, each ———, ═══, and ------ is independently 10-30, 17-27, 19-26, or 20-25 nucleotides in length.

[00156] In an embodiment, one or more nucleotides within ———, ═══, and ------ is an RNA, a DNA, or an artificial or non-natural nucleic acid analog.

[00157] In an embodiment, at least one of ———, ═══, and ------ is a RNA.

[00158] In an embodiment, at least one of ———, ═══, and ------ is a siRNA, a saRNA, or a miRNA.

[00159] In an embodiment, at least one of ———, ═══, and ------ is a siRNA.

[00160] In an embodiment, at least one of ———, ═══, and ------ is a miRNA.

[00161] In an embodiment, at least one of ——— and ------ is an antisense oligonucleotide.

[00162] In an embodiment, two or more siRNA are joined by covalent linkers attached to the sense strands of the siRNA. In an embodiment, two or more siRNA are joined by covalent linkers attached to the antisense strands of the siRNA.

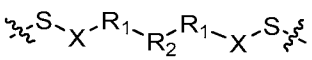
[00163] In an embodiment, two or more siRNA are joined by covalent linkers attached to the sense strand of a first siRNA and the antisense strand of a second siRNA.

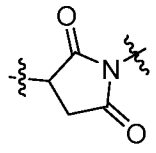
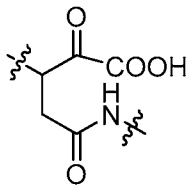
[00164] In an embodiment, one or more of the covalent linkers • comprise a cleavable covalent linker.

[00165] In an embodiment, the cleavable covalent linker contains an acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond.

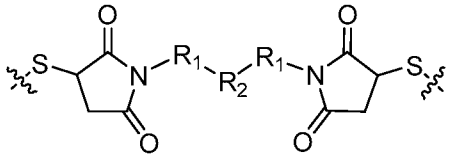
[00166] In an embodiment, the cleavable covalent linker is cleavable under intracellular conditions.

[00167] In an embodiment, at least one covalent linker comprises a disulfide bond or a compound of Formula (I):

 wherein: S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ———, ═══, or ------; each R_1 is independently a C_2-C_{10}

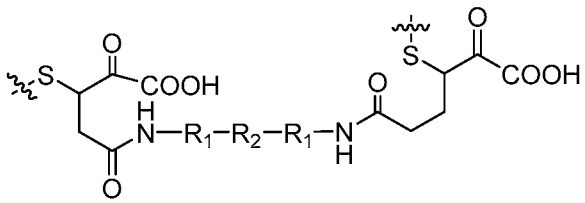
alkyl, alkoxy, or aryl group; R_2 is a thiopropionate or disulfide group; and each X is independently selected from:  or .

[00168] In an embodiment, the compound of Formula (I) is



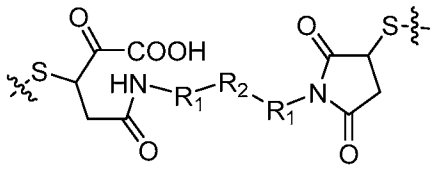
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——, ═══, or ------; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00169] In an embodiment, the compound of Formula (I) is



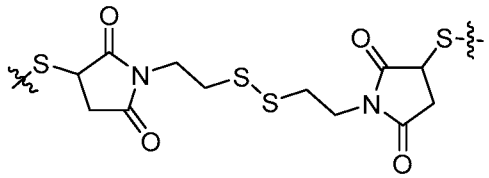
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——, ═══, or ------; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00170] In an embodiment, the compound of Formula (I) is



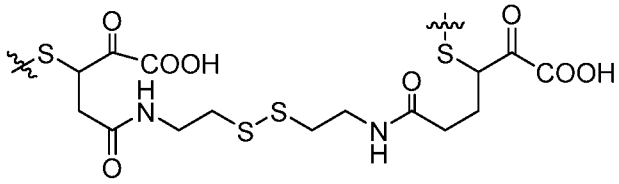
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——, ═══, or ------; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00171] In an embodiment, the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——, ═══, or ------.

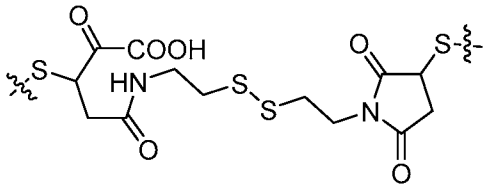
[00172] In an embodiment, the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of

——, ═══, or - - - - - -.

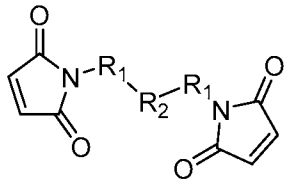
[00173] In an embodiment, the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of

——, ═══, or - - - - - -.

[00174] In an embodiment, the covalent linker of Formula (I) is formed from a covalent linking precursor of Formula (II):



wherein: each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00175] In an embodiment, one or more of the covalent linkers • comprise a nucleotide linker.

[00176] In an embodiment, the nucleotide linker is between 2-6 nucleotides in length. In an embodiment, the nucleotide linker is 3, 4, or 5 nucleotides in length. In an embodiment, the nucleotide linker is a dinucleotide linker.

[00177] In an embodiment, each covalent linker • is the same. In an embodiment, the covalent linkers • comprise two or more different covalent linkers.

[00178] In an embodiment, two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 3' end of a first subunit and the 3' end of a second subunit. In an embodiment, two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 3' end of a first subunit and the 5' end of a second subunit. In an embodiment, two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 5' end of a first subunit and the 3' end of a second subunit. In an embodiment, two or more adjacent oligonucleotide subunits are

joined by covalent linkers • between the 5' end of a first subunit and the 5' end of a second subunit.

[00179] In an embodiment, the multimeric oligonucleotide further comprises one or more targeting ligands. In an embodiment, at least one of the oligonucleotide subunits is a targeting ligand.

[00180] In an embodiment, the targeting ligand is an aptamer. In an embodiment, the targeting ligand comprises N-Acetylgalactosamine (GalNAc).

[00181] In an embodiment, a terminus of the multimeric oligonucleotide is conjugated to a targeting ligand.

[00182] In an embodiment, the multimeric oligonucleotide is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% pure.

[00183] In an embodiment, at least one of the oligonucleotide subunits comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

[00184] In an embodiment, the oligonucleotide with complementarity to TTR mRNA comprises UUAUAGAGCAAGAACACUGUUUU (SEQ ID NO: 164).

[00185] 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

[00186] FIG. 1A presents the chemical structure of a tri-antennary *N*-acetylgalactosamine ligand.

[00187] FIG. 1B presents the chemical structure of a dithio-bis-maleimidoethane.

[00188] FIG. 2 presents a 5'-GalNAc-FVII canonical control, which is discussed in connection with Example 9.

[00189] FIG. 3 presents a GalNAc-homodimer conjugate (XD-06330), which is discussed in connection with Example 10.

[00190] FIG. 4 presents a schematic diagram of a synthesis of a GalNAc-homodimer conjugate (XD-06360), which is discussed in connection with Example 11.

[00191] FIG. 5 presents a schematic diagram of a synthesis of a GalNAc-homodimer conjugate (XD-06329), which is discussed in connection with Example 12.

[00192] FIG. 6 presents data showing FVII activity in mouse serum (knockdown by FVII homodimeric GalNAc conjugates), which are discussed in connection with Example 13.

[00193] FIGS. 7A and 7B and 7C present data showing FVII activity in mouse serum (knockdown by FVII homodimeric GalNAc conjugates normalized for GalNAc content), which are discussed in connection with Example 13.

[00194] FIG. 8 presents canonical GalNAc-siRNAs independently targeting FVII, ApoB and TTR, which are discussed in connection with Example 14.

[00195] FIG. 9 presents a GalNAc-heterotrimer conjugate (XD-06726), which is discussed in connection with Example 15. Key: In this Example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00196] FIG. 10 presents a schematic diagram for a synthesis strategy for a GalNAc-conjugated heterotrimer (XD-06726), which is discussed in connection with Example 15. Key: In this Example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00197] FIG. 11 presents a GalNAc-heterotrimer conjugate (XD-06727), which is discussed in connection with Example 16. Key: In this Example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00198] FIG. 12 presents a schematic diagram for a synthesis strategy for GalNAc-conjugated heterotrimer (XD-06727), which is discussed in connection with Example 16. Key: In this Example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00199] FIG. 13 presents data for an HPLC analysis of the addition of X20336 to X20366, which are discussed in connection with Example 16.

[00200] FIG. 14 presents data for an HPLC analysis of the further addition of X19580 to the reaction product of X20336 and X20366, which are discussed in connection with Example 16.

[00201] FIG. 15 presents data for an HPLC analysis of the further addition of X18795 (5'-siFVIIantisense-3') to the reaction product of X20336, X20366, and X19580 to yield XD-06727, which are discussed in connection with Example 16.

[00202] FIGS. 16A and 16B present data for TTR protein levels in serum samples (measured by ELISA), which are discussed in connection with Example 18.

[00203] FIGS. 17A and 17B present data for FVII enzymatic activity in serum samples, which are discussed in connection with Example 18.

[00204] FIGS. 18A and 18B present data for ApoB protein levels in serum samples (measured by ELISA), which are discussed in connection with Example 18.

[00205] FIGS. 19A and 19B present target knockdown in liver data, which are discussed in connection with Example 18.

[00206] FIG. 20 presents a GalNAc-heterotetramer conjugate (XD-07140), which are discussed in connection with Example 19. Key: In this Example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00207] FIG. 21 presents a schematic diagram for synthesis of a GalNAc-heterotetramer conjugate (XD-07140), which is discussed in connection with Example 19. Key: In this Example, "GeneA" is siFVII; "GeneB" is siApoB; and "GeneC" is siTTR.

[00208] FIG. 22 presents HPLC results of the GalNAc-siFVII-siApoB-siTTR-siFVII Tetramer (XD-07140), which are discussed in connection with Example 19.

[00209] FIG. 23 presents a schematic diagram illustrating the steps for synthesizing a homo-hexamer, which is discussed in connection with Example 23.

[00210] FIGS. 24A and 24B present RP-HPLC results showing yield and purity of the ssRNA X30835, which are discussed in connection with Example 24.

[00211] FIGS. 24C and 24D present RP-HPLC results showing yield and purity of the ssRNA X30837, which are discussed in connection with Example 24.

[00212] FIG. 24E presents RP-HPLC results for X30838, which are discussed in connection with Example 24.

[00213] FIG. 24F presents RP-HPLC results for X30838, X18795 and XD-09795, which are discussed in connection with Example 24.

[00214] FIG. 25 presents data showing serum concentrations of FVII antisense RNA in mice at various times after injection of XD-09795 or XD-09794, which are discussed in connection with Example 25.

[00215] FIGS. 26A-J present data showing serum levels of various cytokines in mice at various times after injection of XD-09795 or XD-09794, which are discussed in connection with Example 26.

[00216] FIG. 27A presents a schematic diagram for a synthesis strategy for monomer of FVII siRNA, which is discussed in connection with Example 28.

[00217] FIG. 27B presents RP-HPLC results for XD-09794, which are discussed in connection with Example 28.

[00218] FIG. 28A presents a schematic diagram for a synthesis strategy for homo-dimer of FVII siRNA, which are discussed in connection with Example 29.

[00219] FIG. 28B presents RP-HPLC results for XD-10635, which are discussed in connection with Example 29.

[00220] FIG. 29A presents a schematic diagram for a synthesis strategy for homo-trimer of FVII siRNA, which is discussed in connection with Example 30.

[00221] FIG. 29B presents RP-HPLC results for XD-10636, which are discussed in connection with Example 30.

[00222] FIG. 30A presents a schematic diagram for a synthesis strategy for homo-tetramer of FVII siRNA, which is discussed in connection with Example 31.

[00223] FIG. 30B presents RP-HPLC results for XD-10637, which are discussed in connection with Example 31.

[00224] FIG. 31A presents a schematic diagram for a synthesis strategy for homo-pentamer of FVII siRNA, which is discussed in connection with Example 32.

[00225] FIG. 31B presents RP-HPLC results for XD-10638, which is discussed in connection with Example 32.

[00226] FIG. 32A presents a schematic diagram for a synthesis strategy for homo-hexamer of FVII siRNA, which is discussed in connection with Example 33.

[00227] FIG. 32B presents RP-HPLC results for XD-10639, which are discussed in connection with Example 33.

[00228] FIG. 33A presents a schematic diagram for a synthesis strategy for homo-hexamer of FVII siRNA via mono-DTME conjugate, which is discussed in connection with Example 34.

[00229] FIG. 33B presents RP-HPLC results for XD-09795, which are discussed in connection with Example 34.

[00230] FIG. 34A presents a schematic diagram for a synthesis strategy for homo-heptamer of FVII siRNA via mono-DTME conjugate, which is discussed in connection with Example 35.

[00231] FIG. 34B presents RP-HPLC results for XD-10640, which are discussed in connection with Example 35.

[00232] FIG. 35A presents a schematic diagram for a synthesis strategy for homo-octamer of FVII siRNA via mono-DTME conjugate, which is discussed in connection with Example 36.

[00233] FIG. 35B presents RP-HPLC results for XD-10641, which are discussed in connection with Example 36.

[00234] FIG. 36A presents a smooth line scatter plot of FVII siRNA levels in serum for various FVII siRNA multimers over time which is discussed in connection with Example 37.

[00235] FIG. 36B presents a straight marked scatter plot of FVII siRNA levels in serum for various FVII siRNA multimers over time, which are discussed in connection with Example 37.

[00236] FIGS. 37A-D present bar charts of FVII siRNA levels in serum for FVII siRNA multimers at various times after administration of the respective oligonucleotides, which are discussed in connection with Example 37.

[00237] FIG. 38A presents a bar chart of FVII siRNA exposure levels in serum (area under the curve) for FVII multimers, which are discussed in connection with Example 37.

[00238] FIG. 38B presents a bar chart of total FVII siRNA levels in serum (area under the curve) for FVII multimers normalized to monomer, which are discussed in connection with Example 37.

[00239] FIG. 39 presents a bar chart of time taken for multimers to reach the same FVII siRNA serum concentrations as the monomer at 5 minutes, which is discussed in connection with Example 38.

[00240] FIG. 40 represents a schematic diagram for a synthesis strategy for homo-tetrameric siRNA, which is discussed in connection with Example 20.

[00241] FIG. 41 represents a schematic diagram for a synthesis strategy for homo-tetrameric siRNA having linkages on alternating strands, which is discussed in connection with Example 20.

[00242] FIG. 42 represents a schematic diagram showing a synthesis strategy for hetero-hexameric siRNA in the format of 4:1:1 FVII:ApoB:TTR targeting siRNA.

[00243] FIG. 43 represents a schematic diagram for the preparation of FVII targeting sense strands.

[00244] FIG. 44 depicts RP-HPLC and MS data for the FVII targeting sense strand X39850.

[00245] FIG. 45 depicts RP-HPLC and MS data for the FVII targeting sense strand X39851.

[00246] FIG. 46 depicts RP-HPLC and MS data for the FVII targeting antisense strand X18795.

[00247] FIG. 47 depicts RP-HPLC and MS data for the FVII targeting antisense strand linked to the ApoB targeting antisense strand via a disulfide linkage and designated X39855.

[00248] FIG. 48 depicts RP-HPLC data for the annealed duplex of X39850 and X18795 (X39850-X18795).

[00249] FIG. 49 depicts RP-HPLC data for the product of the conjugation between the FVII duplex X39850-X18795 and the FVII targeting sense strand X39851 (X39850-X18795-X39851).

[00250] FIG. 50 depicts RP-HPLC data for the product of annealing X39850-X18795-X39851 to the dimeric FVII / ApoB targeting antisense strand X39855 (X39850-X18795-X39851-X39855).

[00251] FIG. 51 depicts RP-HPLC and MS data for the FVII targeting sense strand linked to the TTR targeting sense strand via a disulfide linkage and designated X39852.

[00252] FIG. 52 depicts RP-HPLC and MS data for the FVII targeting antisense strand linked to the TTR targeting antisense strand via a disulfide linkage and designated X39854.

[00253] FIG. 53 depicts RP-HPLC and MS data for the FVII targeting sense strand linked to the ApoB targeting sense strand via a disulfide linkage and designated X39853.

[00254] FIG. 54 depicts RP-HPLC data for the product of annealing the dimeric sense strand X39852 to the FVII targeting antisense strand X18795 (X39852-X18795).

[00255] FIG. 55 depicts RP-HPLC data for the product of annealing the dimeric antisense strand X39854 to X39852-X18795 (X39852-X18795-X39854).

[00256] FIG. 56 depicts RP-HPLC data for the product of annealing the dimeric sense strand X39853 to X39852-X18795-X39854 (X39852-X18795-X39854-X39853).

[00257] FIG. 57A depicts RP-HPLC (FIG. 57A) and MS (FIG. 57B) data for the product of annealing X39852-X18795-X39854-X39853 of FIG. 56 to X39850-X18795-X39851-X39855 of FIG. 50 to form the final hetero-hexameric siRNA (X39850-X18795-X39851-X39855-X39852-X18795-X39854-X39853).

[00258] FIG. 58 depicts knockdown of TTR protein by a 4:1:1 FVII:ApoB:TTR hetero-hexamer at 6 mg/kg, equivalent to 1 mg/kg siTTR monomer.

[00259] FIG. 59 illustrates an evaluation of the stability of disulfide linkers.

[00260] While the disclosure 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 disclosure to the embodiments illustrated.

DETAILED DESCRIPTION

[00261] The disclosures of any patents, patent applications, and publications referred to herein are hereby incorporated by reference in their entireties into this application in order to more fully describe the state of the art known to those skilled therein as of the date of the disclosure described and claimed herein.

[00262] The present disclosure relates to methods of administering to a subject multimeric oligonucleotides having monomeric subunits joined by covalent linkers. The multimeric oligonucleotide can have a molecular weight of at least about 45 kD, such that clearance due to glomerular filtration of the multimeric oligonucleotide is reduced. The present disclosure also relates to the multimeric oligonucleotide and methods of synthesizing the multimeric oligonucleotide. For example, whereas a typical siRNA (e.g., double-stranded monomer) may have a molecular weight of about 15 kD and relatively low circulation half-life (e.g., have a glomerular filtration rate similar to urea or glucose), an oligonucleotide multimer according to the disclosure may have a molecular weight of at least about 45 kD and have a relatively higher circulation half-life (e.g., have a lower rate of clearance due to glomerular filtration rate). The improved and advantageous properties of the multimers according to the disclosure can be described in terms of increased *in vivo* circulation half-life. They may also be described in terms of increased *in vivo* activity, or increased bioactivity. Increased bioactivity may be represented by decreased levels of a target protein or mRNA after administration of the multimeric oligonucleotide. The increased bioactivity may be observed depending on the administration route for the multimeric oligonucleotide. For example, increased bioactivity may be observed when the multimeric oligonucleotide is administered via the intravenous (i.v.) route compared to the subcutaneous (s.c.) route. This increased bioactivity may be observed relative to a monomeric oligonucleotide. For example, a multimeric oligonucleotide administered via the i.v. route may achieve better bioactivity (i.e., increased reduction of the target protein or mRNA) compared to a monomeric oligonucleotide administered via the i.v. route.

[00263] When combined with a targeting ligand, the multi-conjugate can also deliver a higher payload per ligand/receptor binding event than the monomeric equivalent. The present disclosure also relates to new synthetic intermediates and methods of synthesizing the multi-conjugate oligonucleotides. The present disclosure also relates to methods of using the multi-conjugate oligonucleotides, for example in reducing gene expression, biological research, treating or preventing medical conditions, and/or to produce new or altered phenotypes.

[00264] ***Methods of Administering Multimeric Oligonucleotide to a Subject***

[00265] In various aspects, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein:

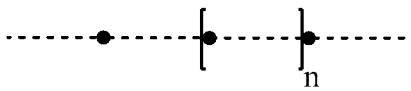
each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to decrease its clearance due to glomerular filtration; and

the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

[00266] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, wherein the number of subunits contained in the multimeric oligonucleotide is m, m being an integer selected to enable the multimeric oligonucleotide to have the molecular weight and/or size configured to decrease its clearance due to glomerular filtration. In various aspects, m is (i) ≥ 2; (ii) ≥ 3; (iii) ≥ 4; (iv) ≥ 4 and ≤ 17; (v) ≥ 4 and ≤ 8; or (vi) 4, 5, 6, 7, or 8.

[00267] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the multimeric oligonucleotide comprises Structure 21:



(Structure 21) and n is an integer ≥ 0.

[00268] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the subunits are single-stranded oligonucleotides.

[00269] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, wherein n is ≥ 1.

[00270] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the subunits are double-stranded oligonucleotides.

[00271] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, wherein:

when n = 0, the clearance of the multimeric oligonucleotide due to glomerular filtration is decreased relative to that of a monomeric subunit ••••••• and/or a dimeric subunit -------●------- of the multimeric oligonucleotide; and

when n ≥ 1, the clearance of the multimeric oligonucleotide due to glomerular filtration is decreased relative to that of a monomeric subunit •••••••, a dimeric subunit -------●-------, and/or a trimeric subunit -------●-------●----- of the multimeric oligonucleotide.

[00272] ***Methods of Measuring Decreased Clearance of Multimeric Oligonucleotide***

[00273] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the decreased clearance due to glomerular filtration results in increased *in vivo* circulation half-life of the multimeric oligonucleotide.

[00274] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the decreased clearance due to glomerular filtration is determined by measuring the *in vivo* circulation half-life of the multimeric oligonucleotide after administering the multimeric oligonucleotide to the subject.

[00275] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the decreased clearance due to glomerular filtration is determined by measuring the time required for the serum concentration of the multimeric oligonucleotide to decrease to a predetermined value. The predetermined value can be 90%, 80%, 70%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, or 1% of the administered dose.

[00276] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the decreased clearance due to glomerular filtration is determined by measuring the serum concentration of the multimeric oligonucleotide at a predetermined time after administering the multimeric oligonucleotide to the subject.

[00277] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the decreased clearance due to glomerular filtration is determined by measuring the area under a curve of a graph representing serum concentration of the multimeric oligonucleotide over time after administering the multimeric oligonucleotide to the subject.

[00278] ***Effects of Decreased Clearance of Multimeric Oligonucleotide Administered to Subjects***

[00279] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the decreased clearance due to glomerular filtration increases *in vivo* bioavailability of the multimeric oligonucleotide.

[00280] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the decreased clearance due to glomerular filtration increases *in vivo* cellular uptake of the multimeric oligonucleotide.

[00281] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, in which the decreased clearance due to glomerular filtration increases *in vivo* therapeutic index/ratio of the multimeric oligonucleotide.

[00282] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, wherein the measured parameter has a sigmoidal relationship with respect to the number of subunits in a monomeric, dimeric, trimeric and higher number multimeric oligonucleotides, for example, as shown in FIGS. 37A-37D.

[00283] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, wherein the measured parameter for the multimeric oligonucleotide and each of its subunits starting with a monomeric

subunit, when plotted, define a sigmoidal curve, for example, as shown in FIGS. 38A-38B.

[00284] ***Multimeric Oligonucleotide***

[00285] In various aspects, the disclosure provides a multimeric oligonucleotide comprising subunits -------, wherein:

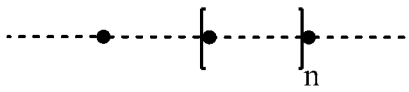
each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to decrease its clearance due to glomerular filtration; and

the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

[00286] In one aspect, the disclosure provides a multimeric oligonucleotide wherein the number of subunits contained in the multimeric oligonucleotide is m, m being an integer selected to enable the multimeric oligonucleotide to have the molecular weight and/or size configured to decrease its clearance due to glomerular filtration. In various aspects, m is (i) ≥ 2; (ii) ≥ 3; (iii) ≥ 4; (iv) ≥ 4 and ≤ 17; (v) ≥ 4 and ≤ 8; or (vi) 4, 5, 6, 7, or 8.

[00287] A multimeric oligonucleotide as in any one of claims 16 and 17 comprising Structure 21:



(Structure 21)

wherein at least one of the subunits ------- comprises a single strand having one of the covalent linkers • joined to its 3' terminus and another of the covalent linkers joined to its 5' terminus, and n is an integer ≥ 0.

[00288] In one aspect, the disclosure provides a multimeric oligonucleotide in which each subunit ------- is 15-30, 17-27, 19-26, or 20-25 nucleotides in length.

[00289] In one aspect, the disclosure provides a multimeric oligonucleotide wherein $n \geq 1$ and $n \leq 17$.

[00290] In one aspect, the disclosure provides a multimeric oligonucleotide in which $n \geq 1$ and $n \leq 5$.

[00291] In one aspect, the disclosure provides a multimeric oligonucleotide in which n is 1, 2, 3, 4, or 5.

[00292] In one aspect, the disclosure provides a multimeric oligonucleotide wherein each subunit is a double-stranded RNA and $n \geq 1$.

[00293] In one aspect, the disclosure provides a multimeric oligonucleotide in which each subunit is a single-stranded oligonucleotide.

[00294] In one aspect, the disclosure provides a multimeric oligonucleotide in which each subunit is a double-stranded oligonucleotide.

[00295] In one aspect, the disclosure provides a multimeric oligonucleotide in which the subunits comprise a combination of single-stranded and double-stranded oligonucleotides.

[00296] In one aspect, the disclosure provides a multimeric oligonucleotide in which each subunit is an RNA, a DNA, or an artificial or non-natural nucleic acid analog.

[00297] In one aspect, the disclosure provides a multimeric oligonucleotide in which each subunit is a RNA.

[00298] In one aspect, the disclosure provides a multimeric oligonucleotide in which each subunit is a siRNA, a saRNA, or a miRNA.

[00299] In one aspect, the disclosure provides a multimeric oligonucleotide in which each subunit is a double-stranded siRNA and each of the covalent linkers joins sense strands of the siRNA.

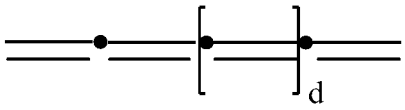
[00300] In one aspect, the disclosure provides a multimeric oligonucleotide in which the multimeric oligonucleotide comprises a homo-multimer of substantially identical subunits -------.

[00301] In one aspect, the disclosure provides a multimeric oligonucleotide in which the multimeric oligonucleotide comprises a hetero-multimer of two or more substantially different subunits-------.

[00302] In one aspect, the disclosure provides a multimeric oligonucleotide in which the multimeric oligonucleotide is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% pure.

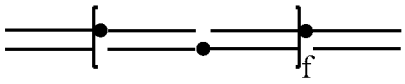
[00303] In one aspect, the disclosure provides a multimeric oligonucleotide wherein each subunit ------- is independently a double-stranded oligonucleotide ——, and wherein n is an integer ≥ 1.

[00304] In one aspect, the disclosure provides a multimeric oligonucleotide wherein each subunit ------- is independently a double-stranded oligonucleotide ——, wherein n is an integer ≥ 1, and wherein each covalent linker • is on the same strand:

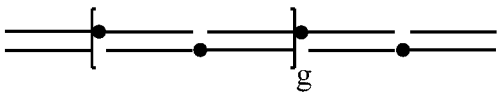


(Structure 54), wherein d is an integer ≥ 1.

[00305] In one aspect, the disclosure provides a multimeric oligonucleotide comprising Structure 22 or 23:



(Structure 22)



(Structure 23)

where each —— is a double-stranded oligonucleotide, each • is a covalent linker joining adjacent double-stranded oligonucleotides, f is an integer ≥ 1, and g is an integer ≥ 0.

[00306] In one aspect, the disclosure provides a plurality of a multimeric oligonucleotide wherein substantially all of the multimeric oligonucleotides have a predetermined value of n and/or predetermined molecular weight.

[00307] ***Target Ligands and Aptamers***

[00308] In one aspect, the disclosure provides a multimeric oligonucleotide in which the multimeric oligonucleotide further comprises one or more targeting ligands.

[00309] In one aspect, the disclosure provides a multimeric oligonucleotide in which at least one of the subunits is a targeting ligand.

[00310] In one aspect, the disclosure provides a multimeric oligonucleotide in which the targeting ligand is an aptamer.

[00311] ***Linkers***

[00312] In one aspect, the disclosure provides a multimeric oligonucleotide in which one or more of the covalent linkers • comprise a cleavable covalent linker and include nucleotide linkers, for example, as discussed in Examples 20, 22B, 27, and 41. Nucleotide linker is a linker that contains one or more nucleotides and it can be chosen such that it does not carry out any other designated function.

[00313] In one aspect, the disclosure provides a multimeric oligonucleotide in which the cleavable covalent linker contains an acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond.

[00314] In one aspect, the disclosure provides a multimeric oligonucleotide in which the cleavable covalent linker is cleavable under intracellular conditions.

[00315] In one aspect, the disclosure provides a multimeric oligonucleotide in which each covalent linker • is the same.

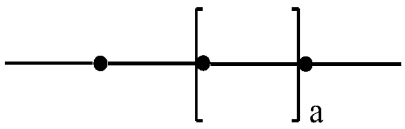
[00316] In one aspect, the disclosure provides a multimeric oligonucleotide in which the covalent linkers • comprise two or more different covalent linkers.

[00317] In one aspect, the disclosure provides a multimeric oligonucleotide in which each covalent linker • joins two monomeric subunits -------.

[00318] In one aspect, the disclosure provides a multimeric oligonucleotide in which at least one covalent linker • joins three or more monomeric subunits -------.

[00319] ***Method of Synthesis of Multimeric Oligonucleotide***

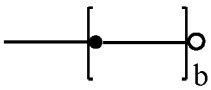
[00320] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 51:



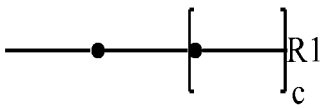
(Structure 51)

wherein each —— is a single-stranded oligonucleotide, each • is a covalent linker joining adjacent single-stranded oligonucleotides, and a is an integer ≥ 1, the method comprising the steps of:

(i) reacting

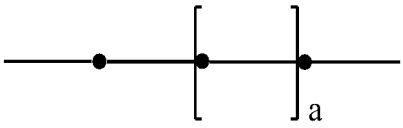


(Structure 52) and



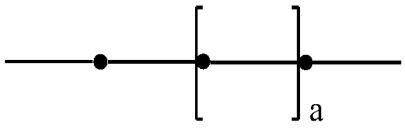
(Structure 53),

wherein O is a linking moiety, R1 is a chemical group capable of reacting with the linking moiety O, b and c are each independently an integer ≥ 0, b and c cannot both simultaneously be zero, and b + c = a, thereby forming Structure 51:

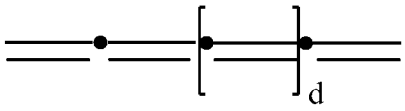


(Structure 51), and

(ii) optionally annealing Structure 51:

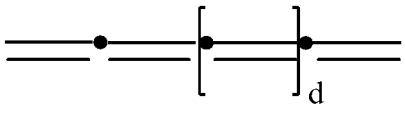


(Structure 51) with complementary single-stranded oligonucleotides ——, thereby forming Structure 54:



(Structure 54). In various aspects, a is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, or greater than or equal to 9. In various aspects, a is 2, 3, 4, 5, 6, 7, 8, or 9.

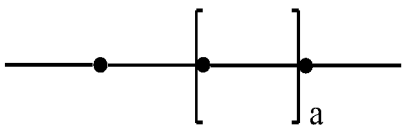
[00321] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 54:



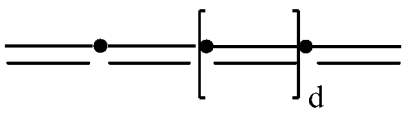
(Structure 54)

wherein each —— is a single-stranded oligonucleotide, each • is a covalent linker joining adjacent single-stranded oligonucleotides, and $a \geq 1$, the method comprising the steps of:

(i) annealing Structure 51:



(Structure 51) with complementary single-stranded oligonucleotides ——, thereby forming Structure 54:



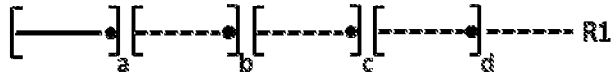
(Structure 54). In various aspects, a is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, or greater than or equal to 9. In various aspects, a is 2, 3, 4, 5, 6, 7, 8, or 9.

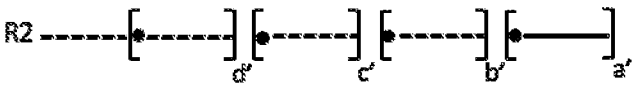
[00322] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 100



(Structure 100), wherein each —— is independently a single-stranded oligonucleotide,

each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the steps of:

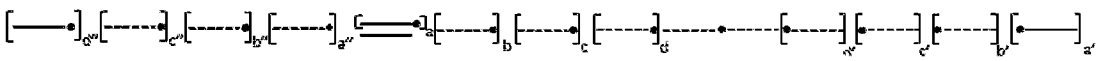
a) reacting Structure 98  (Structure 98)

with Structure 99  (Structure 99),

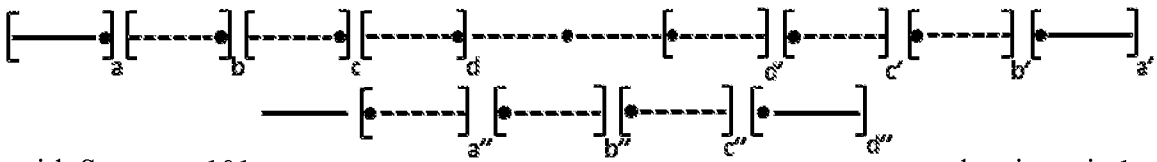
wherein: a, a', b, b', c, c', d and d' are each independently 0 or 1, and R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming Structure 100

. In other aspects, the sum of a+a'+b+b'+c+c'+d+d' is greater than or equal to 1, greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+b+b'+c+c'+d+d' is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

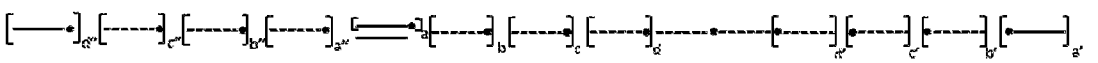
[00323] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 102



(Structure 102), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 100



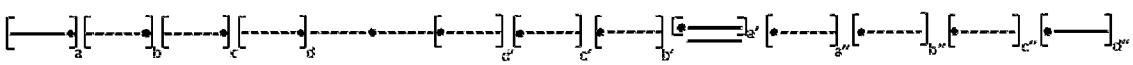
with Structure 101 , wherein: a is 1, and a', a'', b, b', b'', c, c', c'', d, d', and d'' are each independently 0 or 1, thereby forming Structure 102

.

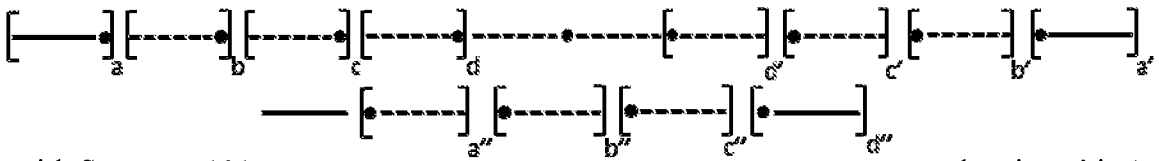
In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or

equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is 2, 3, 4, 5, 6, 7, 8, 9, or 10.

[00324] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 103

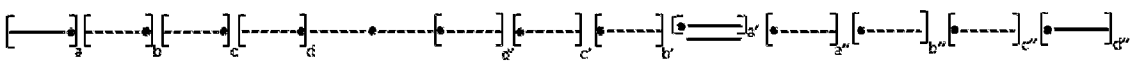


(Structure 103), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 100



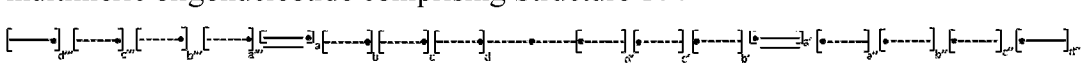
with Structure 101

, wherein: a' is 1, and a, a'', b, b', b'', c, c', c'', d, d', and d'' are each independently 0 or 1, thereby forming Structure 103

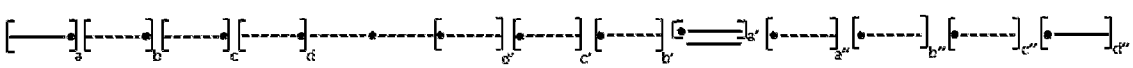


In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is 2, 3, 4, 5, 6, 7, 8, 9, or 10.

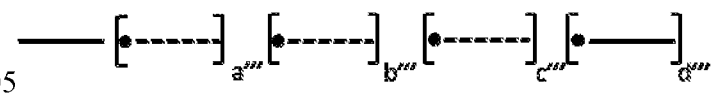
[00325] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 104



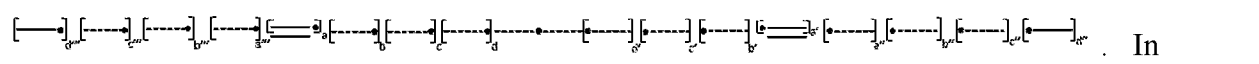
(Structure 104), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 103

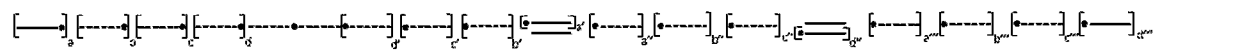
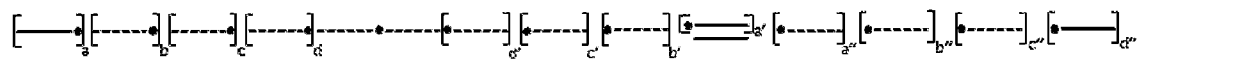
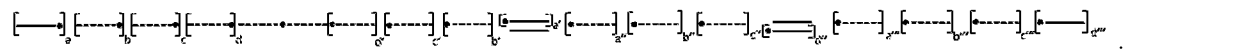


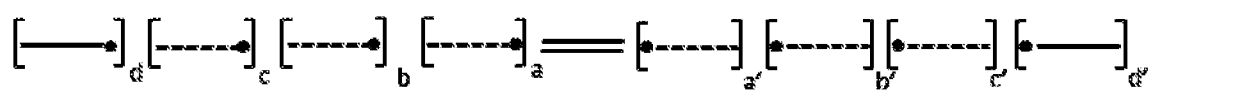
with Structure 105



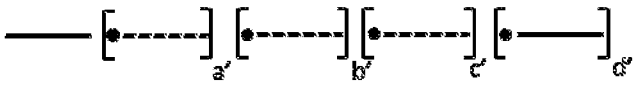
, wherein: a

and a' are each 1, and a'', a''', b, b', b'', b''', c, c', c'', c''', d, d', d'', and d''' are each independently 0 or 1, thereby forming Structure 104 . In other aspects, the sum of a+a'+a"+a'"+b+b'+b"+b'"+c+c'+c"+c'"+d+d'+d"+d'" is greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+a"+a'"+b+b'+b"+b'"+c+c'+c"+c'"+d+d'+d"+d'" is 3, 4, 5, 6, 7, 8, 9, or 10.

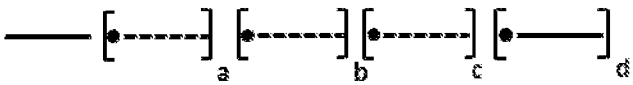
[00326] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 107  (Structure 107), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 103  with Structure 105 , wherein: a' and d'' are 1, and a, a'', a''', b, b', b'', b''', c, c', c'', c''', d, d', and d''' are each independently 0 or 1, thereby forming Structure 107 . In other aspects, the sum of a+a'+a"+a'"+b+b'+b"+b'"+c+c'+c"+c'"+d+d'+d"+d'" is greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a+a'+a"+a'"+b+b'+b"+b'"+c+c'+c"+c'"+d+d'+d"+d'" is 3, 4, 5, 6, 7, 8, 9, or 10.

[00327] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 108  (Structure 108), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is

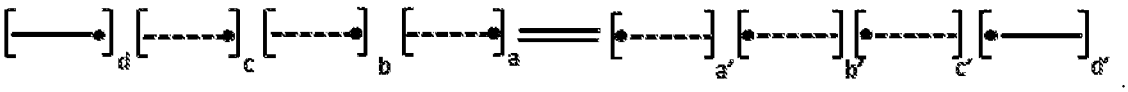
independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure



109 with Structure 110

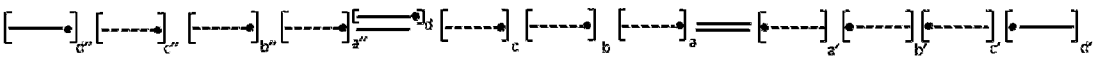


, wherein: a, a', b, b', c, c', d, and d' are each independently 0 or 1, thereby forming Structure 108

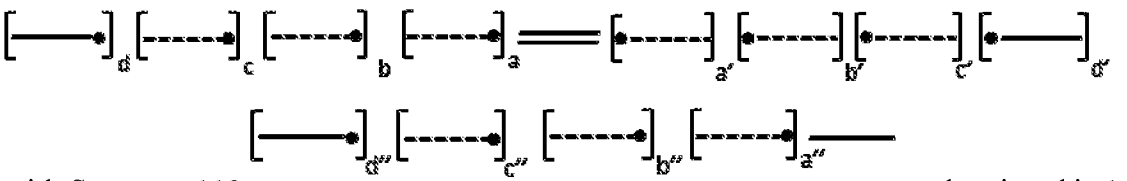


In other aspects, the sum of a+a'+b+b'+c+c'+d+d' is greater than or equal to 1, greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, greater than or equal to 10, or greater than or equal to 11. In other aspects, the sum of a+a'+b+b'+c+c'+d+d' is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.

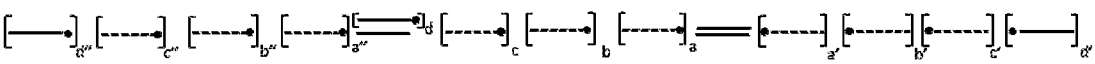
[00328] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 111



(Structure 111), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 108



with Structure 112 , wherein: d is 1, and a, a', a'', b, b', b'', c, c', c'', d' and d'' are each independently 0 or 1, thereby forming Structure 111



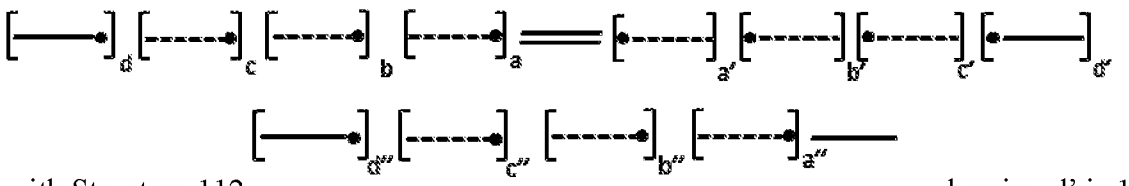
. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or

equal to 9, greater than or equal to 10, or greater than or equal to 11. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.

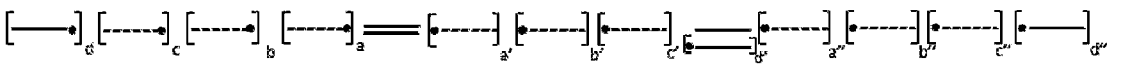
[00329] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 113



(Structure 113), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 108



with Structure 112 , wherein: d' is 1, and a, a', a'', b, b', b'', c, c', c'', d and d'' are each independently 0 or 1, thereby forming Structure 113



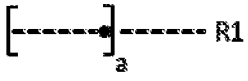
In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is greater than or equal to 2, greater than or equal to 3, greater than or equal to 4, greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, greater than or equal to 10, or greater than or equal to 11. In other aspects, the sum of a+a'+a"+b+b'+b"+c+c'+c"+d+d'+d" is 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.

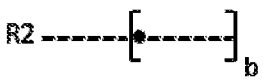
[00330] In one aspect, the method further comprises annealing one or more single-stranded oligonucleotides ——— with a complementary single-stranded oligonucleotide ——— in Structure 98 to Structure 113, thereby forming a double-stranded oligonucleotide ═══.

[00331] In various aspects, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising Structure 114



(Structure 114), wherein each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising reacting Structure 115



(Structure 115) with Structure 116 

(Structure 116), wherein: R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, a and b are each independently an integer ≥ 0, with the proviso that the sum of a and b is ≥ 4, thereby forming Structure 114 

. In other aspects, the sum of a and b is greater than or equal to 5, greater than or equal to 6, greater than or equal to 7, greater than or equal to 8, greater than or equal to 9, or greater than or equal to 10. In other aspects, the sum of a and b is 5, 6, 7, 8, 9, or 10.

[00332] In one aspect, Structure 115 and/or Structure 116 further comprise one or more targeting ligands. In an embodiment, the targeting ligand is a terminal targeting ligand.

[00333] In one aspect, a is an integer of 4, 5, 6, 7, 8, 9, or 10. In another aspect, b is an integer of 4, 5, 6, 7, 8, 9, or 10.

[00334] ***Subjects***

[00335] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof. Examples of subjects include, but are not limited to, mammals, such as primates, rodents, and agricultural animals. Examples of a primate subject includes, but is not limited to, a human, a chimpanzee, and a rhesus monkey. Examples of a rodent subject includes, but is not limited to, a mouse and a rat. Examples of an agricultural animal subject includes, but is not limited to, a cow, a sheep, a lamb, a chicken, and a pig.

[00336] Mouse glomerular filtration rate (GFR) can be about 0.15-0.25 ml/min. Human GFR can be about 1.8 ml/min/kg (Mahmood I: (1998) Interspecies scaling of renally secreted drugs. Life Sci 63:2365–2371).

[00337] Mice can have about 1.46 ml of blood. Therefore, the time for glomerular filtration of total blood volume in mice can be about 7.3 minutes (1.46/0.2). Humans can have about 5 liters of blood and weigh about 70 kg. Therefore, the time for glomerular filtration of total blood volume in humans can be 39.7 mins [5000/126(1.8*70)].

[00338] A person of ordinary skill in the art would recognize that different species can have different rates of clearance by glomerular filtration, at least for the above reasons. A person of ordinary skill in the art can infer that a ratio of rate of clearance by

glomerular filtration between human and mouse times can be about 1:5 or 1:6. In other words, the rate of clearance of a certain substance (e.g., a particular oligonucleotide) by humans can be 5-6 times slower than that of a mouse.

[00339] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, wherein the *in vivo* circulation half-life is measured between 30 and 120 minutes after administering the multimeric oligonucleotide to the subject.

[00340] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, wherein the predetermined time is between 30 and 120 minutes after administering the multimeric oligonucleotide to the subject.

[00341] In one aspect, the disclosure provides a method of administering a multimeric oligonucleotide to a subject in need thereof, wherein the area under the curve is calculated based on serum concentration of the multimeric oligonucleotide between x and y minutes after administering the multimeric oligonucleotide to the subject. In some embodiments, x can be 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 75, 90, 120, 180, 240, or 300 minutes and y can be 90, 120, 180, 240, 300, 360, 420, 480, 540, 600, 720, 840, 960, 1080, 1200, 1320, 1440, or 1600 minutes. For example, the time range can be 30-120 minutes, 1-1600 minutes, or 300-600 minutes.

[00342] In one aspect, the disclosure provides a multimeric oligonucleotide or a method for increasing *in vivo* circulation half-life of the multimeric oligonucleotide, wherein the multimeric oligonucleotide is not formulated in a nanoparticle (NP) or a lipid nanoparticle (LNP).

[00343] The present disclosure also relates to multi-conjugate oligonucleotides having improved pharmacodynamics and/or pharmacokinetics. For example, the multi-conjugate oligonucleotides (e.g., multimeric oligonucleotides including 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more siRNA) can have increased *in vivo* circulation half-life and/or increased *in vivo* activity, relative to that of the individual monomeric subunits. When conjugated to a targeting ligand, the multi-conjugate can also deliver a higher oligonucleotide payload per ligand/receptor binding event than the monomeric equivalent. The present disclosure also relates to new synthetic intermediates and methods of synthesizing the multi-conjugate oligonucleotides. The present disclosure also

relates to methods of using the multi-conjugate oligonucleotides, for example in reducing gene expression, biological research, treating or preventing medical conditions, and/or to produce new or altered phenotypes.

[00344] Various features of the disclosure are discussed, in turn, below.

[00345] ***Nucleic Acids***

[00346] 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).

[00347] 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.

[00348] 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.

[00349] In various embodiments, the nucleic acid or oligonucleotide is an aptamer.

[00350] siRNA (small interfering RNA) is a short double-stranded RNA normally composed of 19-22 nucleic acids, the sense strand of which has a nucleic acid sequence identical to that of a region of a target messenger RNA (mRNA) of a gene in order to suppress expression of that gene by decomposing the mRNA (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).

[00351] Another class of nucleic acid, useful in the methods of the disclosure, 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.

[00352] 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.

[00353] In some embodiments, the RNA can be 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.

[00354] 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.

[00355] In various embodiments, the oligonucleotide is double-stranded and complementary. Complementarity 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.

[00356] 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. 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)).

[00357] 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.

[00358] 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)).

[00359] 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 antagomirs are described for example, in US Patent No. 7,232,806.

[00360] In various embodiments, the oligonucleotide has a specific sequence, for example any one of the sequences disclosed herein.

[00361] A general procedure for oligonucleotide synthesis is provided in the examples below. Other methods that can be adapted for use with the disclosure are known in the art.

[00362] ***Modifications to Nucleic Acids***

[00363] 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.

[00364] Modifications include phosphorus-containing linkages, which 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 adjacent nucleoside units that are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'.

[00365] In various embodiments, the oligonucleotides contained in the multi-conjugate may comprise one or more phosphorothioate groups. The oligonucleotides may

comprise 1-3 phosphorothioate groups at the 5' end. The oligonucleotides may comprise 1-3 phosphorothioate groups at the 3' end. The oligonucleotides may comprise 1-3 phosphorothioate groups at the 5' end and the 3' end. In various embodiments, each oligonucleotide contained in the multi-conjugate may comprise 0-15 total phosphorothioate groups. In certain embodiments, each oligonucleotide may comprise fewer than 10, fewer than 9, fewer than 8, fewer than 7, fewer than 6, fewer than 5, fewer than 4, or fewer than 3 total phosphorothioate groups. In certain embodiments, the oligonucleotides contained in the multi-conjugate may possess increased *in vivo* activity with fewer phosphorothioate groups relative to the same oligonucleotides in monomeric form with more phosphorothioate groups.

[00366] The oligonucleotides contained in the multi-conjugates of this disclosure 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.

[00367] 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), an alkyne (-C≡C-), or an alkene (-CH=CH-).

[00368] The oligonucleotides contained in the multi-conjugates of this disclosure may be modified to 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 °C. (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 the 3' end or the 5' end.

[00369] ***Linkers***

[00370] In various aspects and embodiments of the disclosure, one or more oligonucleotides may be conjugated to one or more additional oligonucleotides or targeting ligands. The oligonucleotides and targeting ligands may be conjugated via any means known in the art, including, but is not limited to, covalent bonds, ionic bonds, hydrogen bonds, and magnetic linkage.

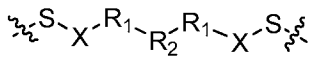
[00371] In various aspects and embodiments of the disclosure, oligonucleotides are linked covalently. Linkers may be cleavable (e.g., under intracellular conditions, to facilitate oligonucleotide delivery and/or action) or non-cleavable. Although generally described below and in the Examples in the context 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 disclosure.

[00372] In various embodiments, a covalent linker can comprise the reaction product of nucleophilic and electrophilic groups. 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 group is encompassed by a second molecule (e.g., linking agent) that ultimately links two oligonucleotides (e.g., maleimide in DTME).

[00373] In various embodiments, a covalent linker can comprise an unmodified di-nucleotide linkage or a reaction product of thiol and maleimide.

[00374] In various embodiments, a covalent linker can comprise a nucleotide linker of 2-6 nucleotides in length. In various embodiments, the nucleotide linker is 3, 4, or 5 nucleotides in length.

[00375] In various embodiments, a covalent linker can comprise a disulfide bond or a compound of Formula (I):



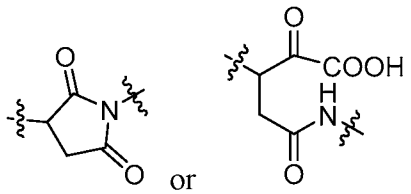
wherein:

S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit;

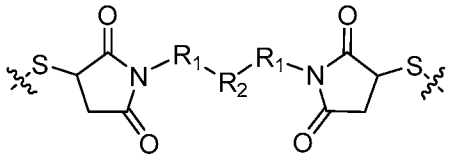
each R1 is independently a C2-C10 alkyl, alkoxy, or aryl group;

R2 is a thiopropionate or disulfide group; and

each X is independently selected from:

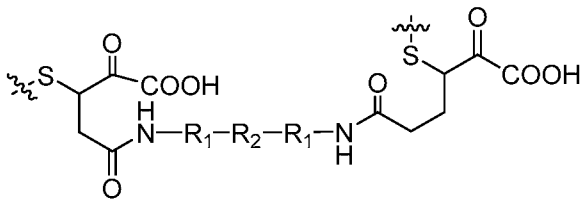


[00376] In an embodiment, the compound of Formula (I) is



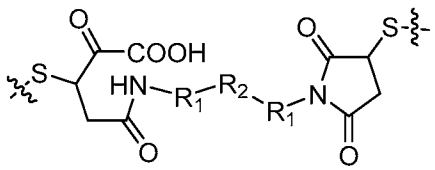
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00377] In an embodiment, the compound of Formula (I) is



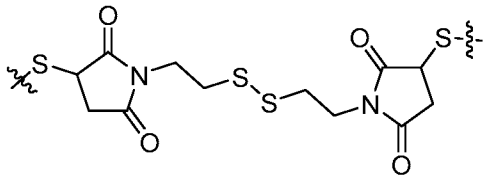
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00378] In an embodiment, the compound of Formula (I) is



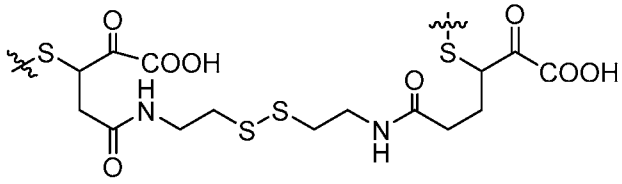
wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit; each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and R_2 is a thiopropionate or disulfide group.

[00379] In certain embodiments, the compound of Formula (I) is



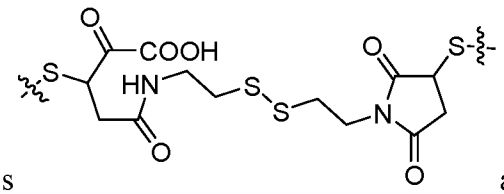
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

[00380] In certain embodiments, the compound of Formula (I) is

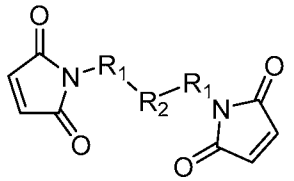


and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

[00381] In certain embodiments, the compound of Formula (I)

is  and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

[00382] In various embodiments, the covalent linker of Formula (I) is formed from a covalent linking precursor of Formula (II):



wherein:

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

R2 is a thiopropionate or disulfide group.

[00383] In various embodiments, two or more linkers of a multimeric oligonucleotide can comprise two orthogonal types of bio-cleavable linkages. In one such embodiment, the two orthogonal bio-cleavable linkages can comprise an unmodified di-nucleotide and a reaction product of thiol and maleimide.

[00384] 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 C1-8 alkyl, C2-8 alkenyl, C2-8 alkynyl, heterocyclyl, aryl, and heteroaryl, branched alkyl, aryl, halo-aryl, and/or other carbon-based connectors. 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). In a preferred embodiment, the nucleic acid or oligonucleotide

is connected to the linker via a C6 alkyl. Alternatively, these moieties (e.g., R1 and/or R2 in Structure 1) are optional and a direct linkage is possible.

[00385] 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).

[00386] 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.

[00387] 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.

[00388] The linking agent may have a molecular weight of 100-10,000 Daltons. Examples of such linking agent include dithio-bis-maleimidoethane (DTME), 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.

[00389] 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 disclosure 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 disclosure 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 (e.g., a peptide 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 disclosure without limitation.

[00390] 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-maleimidoethane (DTME); 1,8-Bis-maleimidodiethyleneglycol (BM(PEG)2); or dithiobis(sulfosuccinimidyl propionate) (DTSSP).

[00391] 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.

[00392] ***Monomeric Intermediate Compounds***

[00393] In various aspects, the disclosure 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.

[00394] In one aspect, the disclosure 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 is a derivative of phosphoric acid, a derivative of thiophosphoric acid, a sulfate, amide, glycol, or is absent;

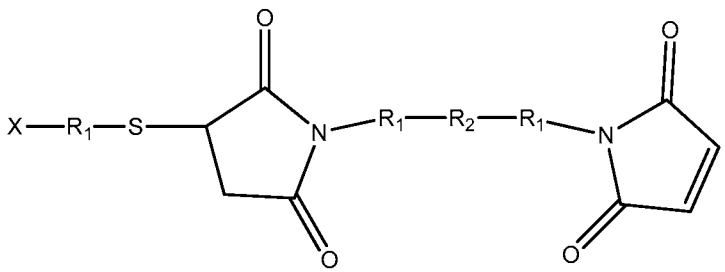
R2 is a C2-C10 alkyl, alkoxy, or aryl group, or is absent;

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).

[00395] In one aspect, the disclosure provides a compound according to Structure 2:



(Structure 2)

wherein:

X is a nucleic acid bonded to R1 via a phosphate or derivative thereof, or thiophosphate or derivative thereof 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.

[00396] In one aspect, the disclosure 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 is a derivative of phosphoric acid such as phosphate, phosphodiester, phosphotriester, phosphonate, phosphoramidate and the like, a derivative of thiophosphoric acid such as

thiophosphate, thiophosphodiester, thiophosphotriester, thiophosphoramidate and the like, a 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, alkyldithio group, ether, thioether, thiopropionate, or disulfide; and
B is a third reactive moiety.

[00397] In various aspects, the disclosure also provides methods for synthesizing an oligonucleotide coupled to a covalent linker.

[00398] In one aspect, the disclosure 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).

[00399] 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 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.

[00400] In various embodiments, the method for synthesizing the compound of Structure 1 further comprises synthesizing the compound of Structure 2.

[00401] 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 herein, or any of the specific structures or chemistries shown in the summary, description, 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.

[00402] In various embodiments, the method for synthesizing the compound of Structure 1, 2 or 3 is carried out under conditions that substantially favor the formation of Structure 1, 2 or 3 and substantially prevent dimerization of X. The conditions can improve the yield of the reaction (e.g., improve the purity of the product).

[00403] In various embodiments, the method for synthesizing the compound of Structure 1, 2 or 3, 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.

[00404] In various embodiments, the method for synthesizing the compound of Structure 1, 2 or 3, 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.

[00405] In various embodiments, the method for synthesizing the compound of Structure 1, 2 or 3, 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.

[00406] In various embodiments, the method for synthesizing the compound of Structure 1, 2 or 3, 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.

[00407] 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 disclosure 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.

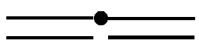
[00408] As used herein, the term about is used in accordance with its plain and ordinary meaning of approximately. For example, "about X" encompasses approximately the value X as stated, including similar amounts that are within the measurement error for the value of X or amounts that are approximately the same as X and have essentially the same properties as X.

[00409] As used herein, isolated includes 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.

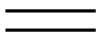
[00410] ***Dimeric Compounds and Intermediates***

[00411] In various aspects, the disclosure 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*)

[00412] In one aspect, the disclosure 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 a derivative of phosphoric acid such as phosphate, phosphodiester, phosphotriester, phosphonate, phosphoramidate and the like, a derivative of thiophosphoric acid such as thiophosphate, thiophosphodiester, thiophosphotriester, thiophosphoramidate and the like,

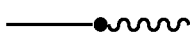
a sulfate, amide, glycol, or is absent;

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, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

[00413] In one aspect, the disclosure 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 a derivative of phosphoric acid such as phosphate, phosphodiester, phosphotriester, phosphonate, phosphoramidate and the like, a derivative of thiophosphoric acid such as thiophosphate, thiophosphodiester, thiophosphotriester, thiophosphoramidate and the like,

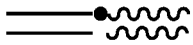
a sulfate, amide, glycol, or is absent;

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, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

[00414] In one aspect, the disclosure 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:

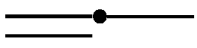
each R1 is a derivative of phosphoric acid such as phosphate, phosphodiester, phosphotriester, phosphonate, phosphoramidate and the like, a derivative of thiophosphoric acid such as thiophosphate, thiophosphodiester, thiophosphotriester, thiophosphoramidate and the like, a sulfate, amide, glycol, or 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, alkyldithio group, ether, thioether, thiopropionate, or disulfide.

[00415] In one aspect, the disclosure 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.

[00416] In various aspects, the disclosure provides methods for synthesizing dimeric defined multi-conjugate oligonucleotides.

[00417] In one aspect, the disclosure 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 .

[00418] The method can further comprise the step of annealing complementary and to yield Structure 6:

 (Structure 6).

[00419] In one aspect, the disclosure provides a method for synthesizing an isolated compound of Structure 4:

 (Structure 4)

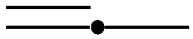
wherein each is a double-stranded oligonucleotide and • is a covalent linker joining single strands of adjacent single-stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

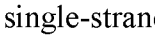
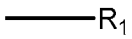
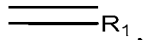
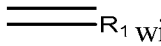
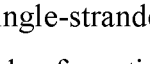
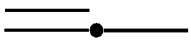
(i) reacting a first single-stranded oligonucleotide  with a bifunctional linking moiety , wherein R1 is a chemical group capable of reacting with , thereby forming a mono-substituted product ;

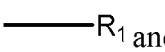
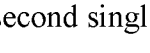
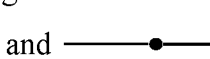
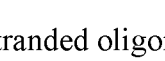
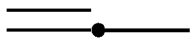
(ii) reacting with a second single-stranded oligonucleotide , wherein R2 is a chemical group capable of reacting with , thereby forming a single-stranded dimer ;

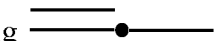
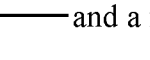
(iii) annealing single-stranded oligonucleotides, at the same time or sequentially, thereby forming .

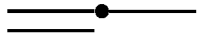
[00420] In one aspect, the disclosure provides a method for synthesizing an isolated compound of Structure 4:  (Structure 4) wherein each is a double-stranded oligonucleotide and • is a covalent linker joining single strands of adjacent single-stranded oligonucleotides at their 3' or 5' termini, the method comprising the steps of:

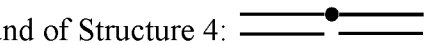
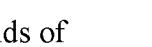
(i) forming  by:

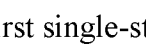
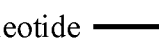
(a) annealing a first single-stranded oligonucleotide  and a second single-stranded oligonucleotide , thereby forming , and reacting  with a third single-stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby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

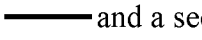
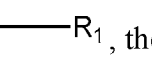
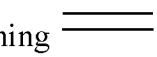
(b) reacting the second single-stranded oligonucleotide  and the third single-stranded oligonucleotide , thereby forming , and annealing the first single-stranded oligonucleotide  and , thereby forming ;

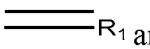
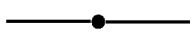
(ii) annealing  and a fourth single-stranded oligonucleotide , thereby forming .

[00421] This methodology can be adapted for synthesizing an isolated compound according to  (Structure 11), for example by omitting step (ii).

[00422] In one aspect, the disclosure 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 .

[00423] As with the other compounds and compositions according to the disclosure, 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 herein, or any of the specific structures or chemistries shown in the summary, description, or Examples.

[00424] Example 3 provides an example methodology for preparing dimerized oligonucleotides and Example 4 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.

[00425] Examples of heterodimers are provided in Examples 9 and 10.

[00426] Examples of homodimers are provided in Examples 12-15.

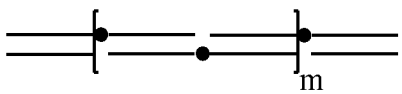
[00427] In various embodiments, R1, R2, and the bifunctional linking moiety ○ can form a covalent linker • as described and shown herein. For example, in various embodiments, R1 and R2 can each independently comprise a reactive moiety, for example an electrophile or nucleophile. In one embodiment, R1 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.

[00428] These, as well as all other synthetic methods of the disclosure, 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). Additional methods for adding targeting ligands are known in the art and can be adapted for the present disclosure by those skilled in the art.

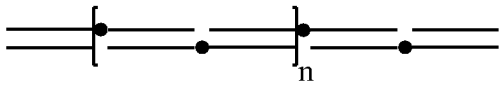
[00429] ***Multimeric (n>2) Compounds and Intermediates***

[00430] In various aspects, the disclosure provides multimeric (n>2) defined multi-conjugate oligonucleotides, including defined tri-conjugates and defined tetraconjugates.

[00431] In one aspect, the disclosure provides a compound according to Structure 7 or 8:



(Structure 7)



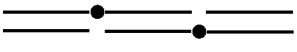
(Structure 8)

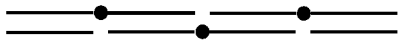
wherein:

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.

[00432] In one aspect, the disclosure provides a compound according to Structure 9 and wherein n = 0:  (Structure 9). In one aspect, the disclosure provides a compound according to Structure 10 and wherein m = 1:

 (Structure 10).

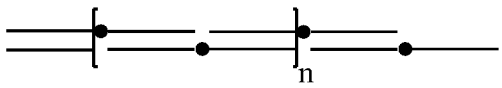
[00433] In one aspect, the disclosure provides a compound according to Structure 12, 13, 14, or 15:



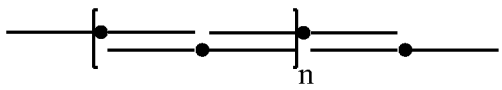
(Structure 12)



(Structure 13)



(Structure 14)



(Structure 15)

wherein:

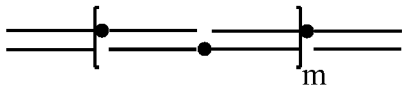
each ═══ is a double-stranded oligonucleotide,

each ——— is a single-stranded oligonucleotide,

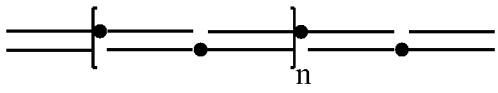
each ● is a covalent linker joining single strands of adjacent single-stranded oligonucleotides, and m is an integer ≥ 1 and n is an integer ≥ 0.

[00434] In various aspects, the disclosure provides methods for synthesizing multimeric (n>2) defined multi-conjugate oligonucleotides, including defined tri-conjugates and defined tetra-conjugates.

[00435] In one aspect, the disclosure 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:

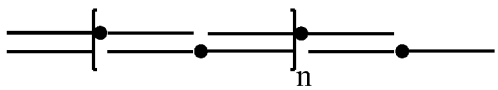
(a) annealing a first single-stranded oligonucleotide ——— and a second single-stranded oligonucleotide ———R_1, thereby forming ═══R_1, and reacting ═══R_1 with a third single-stranded oligonucleotide ———R_2, wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a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



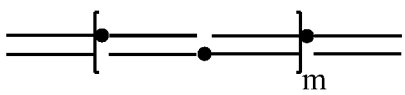
or



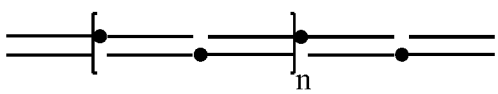
wherein m is an integer ≥ 1 and n is an integer ≥ 0; and

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

[00436] In one aspect, the disclosure provides a method for synthesizing a compound according to Structure 7 or 8:

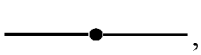
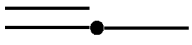


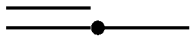
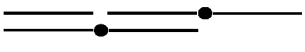
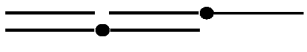
(Structure 7)

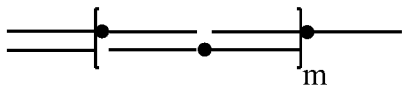


(Structure 8)

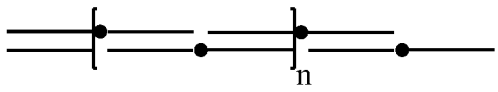
wherein: each  is a double-stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single-stranded oligonucleotides, and m is an integer ≥ 1 and n is an integer ≥ 0, the method comprising the steps of:

(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,

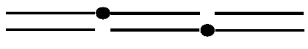


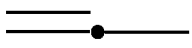
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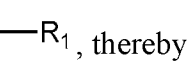
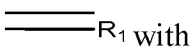
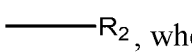
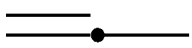


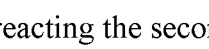
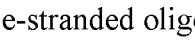
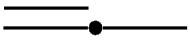
wherein m is an integer ≥ 1 and n is an integer ≥ 0; and

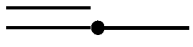
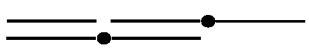
(iii) annealing a second single-stranded oligonucleotide to the product of step (ii), thereby forming structure 7 or 8.

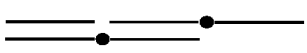
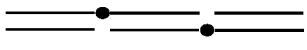
[00437] In one aspect, the disclosure provides a method for synthesizing a compound of Structure 9:  (Structure 9), wherein each is a double-stranded oligonucleotide, each • is a covalent linker joining single strands of adjacent single-stranded oligonucleotides, the method comprising the steps of:

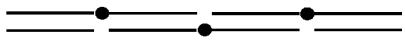
(i) forming  by:

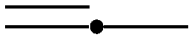
(a) annealing a first single-stranded oligonucleotide and a second single-stranded oligonucleotide , thereby forming , and reacting  with a third single-stranded oligonucleotide , wherein R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming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

[00438] In one aspect, the disclosure 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 .

[00439] As with the other compounds and compositions according to the disclosure, 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 herein, or any of the specific structures or chemistries shown in the summary, description, or Examples.

[00440] 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.

[00441] 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.

[00442] 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 ●.

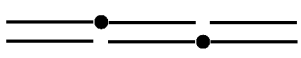
[00443] In various embodiments, each ——●—— may independently comprise two sense or two antisense oligonucleotides. For example, in the case of siRNA, a ——●—— may comprise two active strands or two passenger strands.

[00444] 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.

[00445] 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*.

[00446] 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.

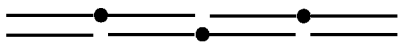
[00447] In various embodiments, the compound comprises Structure 9 and n = 0:



(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).

[00448] Examples of trimers are provided in Examples 17, 18, and 20.

[00449] 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).

[00450] Examples of tetramers are provided in Example 21.

[00451] 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.

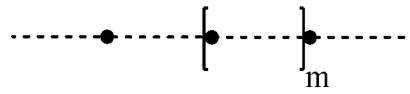
[00452] In various embodiments (e.g., in Structure 4), the compound further comprises a targeting ligand, each double-stranded oligonucleotide (e.g., ———) comprises an siRNA guide strand and a passenger strand hybridized to the guide strand, and the compound is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100 % pure.

[00453] 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.

[00454] ***Oligonucleotides Having Increased Circulation Half-Life and/or Activity in vivo***

[00455] The disclosure provides multimeric oligonucleotides having increased circulation half-life and/or activity *in vivo*, as well as compositions including the multimeric oligonucleotides and methods for their synthesis and use.

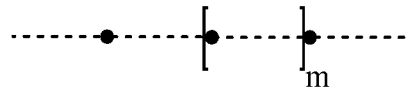
[00456] In various aspects, the disclosure provides a multimeric oligonucleotide comprising Structure 21:



(Structure 21)

wherein each monomeric subunit ------- is independently a single or double-stranded oligonucleotide, m is an integer ≥ 1, each ● is a covalent linker joining adjacent monomeric subunits -------, and at least one of the monomeric subunits ------- comprises a single strand having one of the covalent linkers ● joined to its 3' terminus and another of the covalent linkers joined to its 5' terminus.

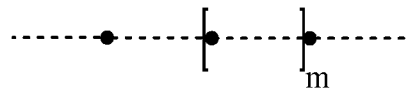
[00457] In various aspects, the disclosure provides a multimeric oligonucleotide comprising Structure 21:



(Structure 21)

wherein each monomeric subunit ------- is independently a single or double-stranded oligonucleotide, each ● is a covalent linker joining adjacent monomeric subunits -------, and m is an integer ≥ 0 selected to (a) increase *in vivo* circulation half-life of the multimeric oligonucleotide relative to that of the individual monomeric subunits ------- and/or (b) increase *in vivo* activity of the multimeric oligonucleotide relative to that of the individual monomeric subunits -------.

[00458] In various aspects, the disclosure provides a multimeric oligonucleotide comprising Structure 21:

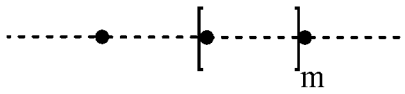


(Structure 21)

wherein each monomeric subunit ------- is independently a single or double-stranded oligonucleotide, each ● is a covalent linker joining adjacent monomeric subunits -------, m is an integer ≥ 0, and

wherein the multimeric oligonucleotide has molecular size and/or weight configured to (a) increase *in vivo* circulation half-life of the multimeric oligonucleotide relative to that of the individual monomeric subunits ------- and/or (b) increase *in vivo* activity of the multimeric oligonucleotide relative to that of the individual monomeric subunits -------.

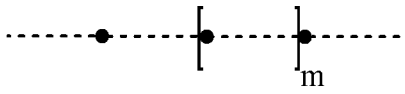
[00459] In various aspects, the disclosure provides a method for increasing *in vivo* circulation half-life and/or *in vivo* activity of one or more oligonucleotides, the method comprising administering to a subject the one or more oligonucleotides in the form of a multimeric oligonucleotide comprising Structure 21:



(Structure 21)

wherein each monomeric subunit ------- is independently a single or double-stranded oligonucleotide, each • is a covalent linker joining adjacent monomeric subunits -------, and m is an integer ≥ 0 selected to (a) increase *in vivo* circulation half-life of the multimeric oligonucleotide relative to that of the individual monomeric subunits ------- and/or (b) increase *in vivo* activity of the multimeric oligonucleotide relative to that of the individual monomeric subunits -------.

[00460] In various aspects, the disclosure provides a method for increasing *in vivo* circulation half-life and/or *in vivo* activity of one or more oligonucleotides, the method comprising administering to a subject the one or more oligonucleotides in the form of a multimeric oligonucleotide comprising Structure 21:



(Structure 21)

wherein each monomeric subunit ------- is independently a single or double-stranded oligonucleotide, each • is a covalent linker joining adjacent monomeric subunits -------, m is an integer ≥ 0, and

wherein the multimeric oligonucleotide has molecular size and/or weight configured to (a) increase *in vivo* circulation half-life of the multimeric oligonucleotide relative to that of the individual monomeric subunits ------- and/or (b) increase *in vivo* activity of the multimeric oligonucleotide relative to that of the individual monomeric subunits -------.

[00461] In various aspects, the disclosure provides a multimeric oligonucleotide comprising m monomeric subunits -------, wherein each of the monomeric subunits

------- is independently a single or double-stranded oligonucleotide, each of the monomeric subunits ------- is joined to another monomeric subunit by a covalent linker ●, and m is an integer ≥ 3 selected to (a) increase *in vivo* circulation half-life of the multimeric oligonucleotide relative to that of the individual monomeric subunits ------- and/or (b) increase *in vivo* activity of the multimeric oligonucleotide relative to that of the individual monomeric subunits -------.

[00462] In various aspects, the disclosure provides a multimeric oligonucleotide comprising m monomeric subunits -------, wherein each of the monomeric subunits ------- is independently a single or double-stranded oligonucleotide, each of the monomeric subunits ------- is joined to another monomeric subunit by a covalent linker ●, m is an integer ≥ 3, and the multimeric oligonucleotide has molecular size and/or weight configured to (a) increase *in vivo* circulation half-life of the multimeric oligonucleotide relative to that of the individual monomeric subunits ------- and/or (b) increase *in vivo* activity of the multimeric oligonucleotide relative to that of the individual monomeric subunits -------.

[00463] In various embodiments, the increase is relative to the circulation half-life and/or activity of a monomeric subunit of the multimeric oligonucleotide. Circulation half-life (and its relationship to other properties such as glomerular filtration) is discussed in further detail in the *Oligonucleotide Uptake and Clearance* section and in Examples 25 and 37 below. In various embodiments, the *in vivo* circulation half-life increases by a factor of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 500, or 1,000. The *in vivo* circulation half-life can increase by a factor of at least 2. The *in vivo* circulation half-life can increase by a factor of at least 10. In various embodiments, the increase in *in vivo* activity is measured as the ratio of *in vivo* activity at t_{max}. In various embodiments, the *in vivo* activity increases by a factor of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 500, or 1,000. The *in vivo* activity can increase by a factor of at least 2. The *in vivo* activity can increase by a factor of at least 10. In one embodiment, the increase is in a mouse. In one embodiment, the increase is in a human.

[00464] In various embodiments, m is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12.

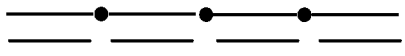
[00465] In various embodiments, m is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12.

[00466] In various embodiments, each of the monomeric subunits ------- comprises an siRNA and each of the covalent linkers joins sense strands of the siRNA.

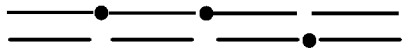
[00467] In various embodiments, each of the covalent linkers • joins two monomeric subunits -------.

[00468] In various embodiments, at least one of the covalent linkers • joins three or more monomeric subunits -------.

[00469] In various embodiments, each monomeric subunit ------- is independently a double-stranded oligonucleotide ═══, and m is 1:

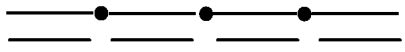


(Structure 28) or



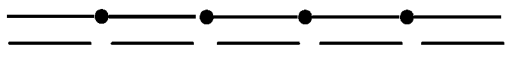
(Structure 29).

[00470] In various embodiments, each monomeric subunit ------- is independently a double-stranded oligonucleotide ═══, m is 1, and each covalent linker • is on the same strand:

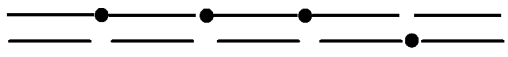


(Structure 28).

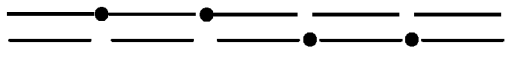
[00471] In various embodiments, each monomeric subunit ------- is independently a double-stranded oligonucleotide ═══, and m is 2:



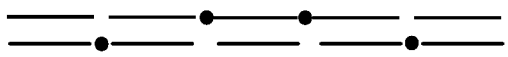
(Structure 30),



(Structure 31),

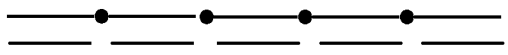


(Structure 32), or



(Structure 33).

[00472] In various embodiments, each monomeric subunit ------- is independently a double-stranded oligonucleotide ═══, and m is 2, and each covalent linker • is on the same strand:



(Structure 33).

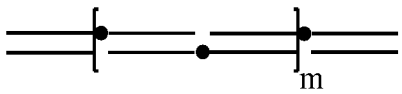
[00473] In various embodiments, each monomeric subunit ------- is independently a double-stranded oligonucleotide ═══, and m is 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12.

[00474] In various embodiments, each monomeric subunit ------- is independently a double-stranded oligonucleotide ═══, m is 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12, and each covalent linker • is on the same strand.

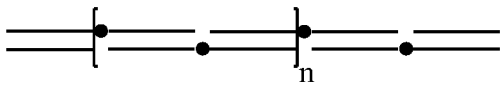
[00475] In various embodiments, each monomeric subunit ------- is independently a double-stranded oligonucleotide ═══, and m is $\geq$ 13.

[00476] In various embodiments, each monomeric subunit ------- is independently a double-stranded oligonucleotide ═══, m is ≥ 13, and each covalent linker ● is on the same strand.

[00477] In various embodiments, Structure 21 is Structure 22 or 23:



(Structure 22)



(Structure 23)

where each ═══ is a double-stranded oligonucleotide, each ● is a covalent linker joining adjacent double-stranded oligonucleotides, m is an integer ≥ 1, and n is an integer ≥ 0.

[00478] In various embodiments, Structure 21 is not a structure disclosed in PCT/US2016/037685.

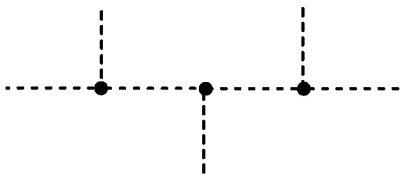
[00479] In various embodiments, each oligonucleotide ------- is a single-stranded oligonucleotide.

[00480] In various embodiments, each oligonucleotide ------- is a double-stranded oligonucleotide.

[00481] In various embodiments, the oligonucleotides ------- comprise a combination of single and double-stranded oligonucleotides.

[00482] In various embodiments, the multimeric oligonucleotide comprises a linear structure wherein each of the covalent linkers ● joins two monomeric subunits -------.

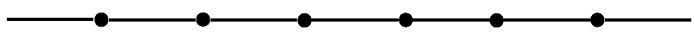
[00483] In various embodiments, the multimeric oligonucleotide comprises a branched structure wherein at least one of the covalent linkers ● joins three or more monomeric subunits --------. For example, Structure 21 could be

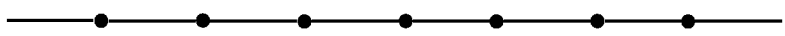


Structure 41.

[00484] In various embodiments, each monomeric subunit ------- is independently a single-stranded oligonucleotide ———. In some such embodiments, m is 1 ———●———●———●——— (Structure 34); m is 2———●———●———●———●——— (Structure 39); m is 3

 (Structure 35); m is 4

 (Structure 40); or m is 5

 (Structure 37). In some such embodiments, m is 6, 7, 8, 9, 10, 11, or 12. In some such embodiments, m is an integer $\geq$ 13. In one such embodiment, at least one single-stranded oligonucleotide ——— is an antisense oligonucleotide. In one such embodiment, each single-stranded oligonucleotide ——— is independently an antisense oligonucleotide.

[00485] In various embodiments, the multimeric oligonucleotide comprises a homo-multimer of substantially identical oligonucleotides. The substantially identical oligonucleotides can be siRNAs targeting the same molecular target *in vivo*. The substantially identical oligonucleotides can be miRNAs targeting the same molecular target *in vivo*. The substantially identical oligonucleotides can be antisense oligonucleotides targeting the same molecular target *in vivo*. The substantially identical oligonucleotides can be a combination of siRNA, miRNA, and/or antisense RNA targeting the same molecular target *in vivo*.

[00486] In various embodiments, the multimeric oligonucleotide comprises a hetero-multimer of two or more substantially different oligonucleotides. The substantially different oligonucleotides can be siRNAs targeting different molecular targets *in vivo*. The substantially different oligonucleotides can be miRNAs targeting different molecular targets *in vivo*. The substantially different oligonucleotides can be antisense oligonucleotides targeting different molecular targets *in vivo*. The substantially different oligonucleotides can be a combination of siRNA, miRNA, and/or antisense RNA targeting different molecular targets *in vivo*.

[00487] Polymer linkers such as polyethylene glycol (PEG) have been used in attempts to increase the circulation half-life of certain drugs. Such approaches can have drawbacks, including "diluting" the therapeutic agent (e.g., less active agent per unit mass). The present disclosure can be distinguished from such approaches. For example, in various embodiments, the multimeric oligonucleotide does not comprise PEG. In various embodiments, the multimeric oligonucleotide does not comprise a polyether compound. In various embodiments, the multimeric oligonucleotide does not comprise a polymer other than the oligonucleotides.

[00488] Nanoparticles (NP), such as lipid nanoparticles (LNP) have been used in attempts to increase the circulation half-life of certain drugs. Such approaches can have

drawbacks, including increased toxicity (e.g., from cationic lipids). The present disclosure can be distinguished from such approaches. For example, in various embodiments, the multimeric oligonucleotide is not formulated in an NP or LNP.

[00489] Phosphorothioate groups have been used in attempts to increase the circulation half-life of certain drugs. Such approaches can have the drawbacks, including lower activity (e.g., due to oligonucleotide/plasma protein aggregation). The present disclosure can be distinguished from such approaches. For example, in various embodiments, the multimeric oligonucleotide does not comprise a phosphorothioate.

[00490] In various embodiments, the multimeric oligonucleotide further comprises one or more targeting ligands. In various embodiments, the multimeric oligonucleotide consists essentially of Structure 21 and an optional targeting ligand. The multimeric oligonucleotide can use any of the targeting ligands discussed herein (see, e.g., the *Targeting Ligands* section below). In various embodiments, a targeting ligand is conjugated to an oligonucleotide subunit, and/or to a linker between adjacent oligonucleotide subunits. In various embodiments, a targeting ligand can be conjugated to an oligonucleotide through its 3' or 5' terminus.

[00491] The multimeric oligonucleotide can use any of the linkers discussed herein (see, e.g., the *Linkers* section above). In various embodiments, each covalent linker • is the same. In various embodiments, the multimeric oligonucleotide comprises two or more different covalent linkers •. In various embodiments, one or more of • comprises a cleavable covalent linker. Cleavable linkers can be particularly advantageous in some situations. For example, intracellular cleavage can convert a single multimeric oligonucleotide into multiple biologically active oligonucleotides after cellular targeting and entry (e.g., a single siRNA construct can deliver four or more active siRNA), increasing potency and decreasing undesired side effects.

[00492] In various embodiments, one or more of • comprises nucleotide linker (e.g., a cleavable nucleotide linker such as UUU). Alternatively, in some embodiments, the multimeric oligonucleotide expressly excludes nucleotide linkers.

[00493] 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 disclosure 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.

[00494] In various embodiments, each oligonucleotide is RNA, DNA, or comprises an artificial or non-natural nucleic acid analog. In various embodiments, at least one oligonucleotide is an siRNA, miRNA, or antisense oligonucleotide. Various other possible oligonucleotides and substitutions are discussed, for example, in the *Nucleic Acids* section above.

[00495] In various embodiments, each oligonucleotide is 15-30, 17-27, 19-26, or 20-25 nucleotides in length. 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.

[00496] In various embodiments, the multimeric oligonucleotides comprising structure 21 have a molecular weight of at least about 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, or 65 kD. In various embodiments, the multimeric oligonucleotides comprising structure 21 have a molecular weight of at least about 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, or 70-75 kD. Molecular weight can include everything conjugated to the multimeric oligonucleotide, such a targeting ligands and linkers.

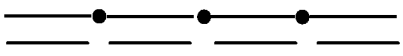
[00497] Although the multimeric oligonucleotides comprising Structure 21 can be synthesized by various methods (e.g., those described herein for making tetrameric or greater multimers), certain results may call for specific methodologies. For example, the following method (as well as those shown in Example 22) is designed to efficiently produce multimers having each covalent linker ● on the same strand.

[00498] For example, in one aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising structure 34:

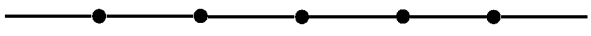
——●——●——●—— (Structure 34)

wherein each —— is a single-stranded oligonucleotide and each ● is a covalent linker joining adjacent single-stranded oligonucleotides, the method comprising the steps of:

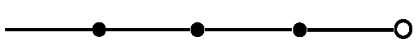
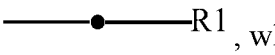
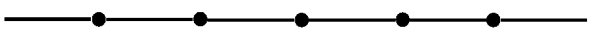
(i) reacting ——●——○ and ——●——R1, wherein ○ is a linking moiety and R_1 is a chemical group capable of reacting with the linking moiety ○, thereby forming ——●——●——●—— (Structure 34), and

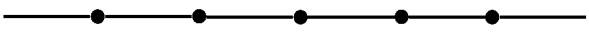
[00499] (ii) optionally annealing  (Structure 34) with complementary single-stranded oligonucleotides, thereby forming  (Structure 28).

[00500] For example, in one aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising structure 35:

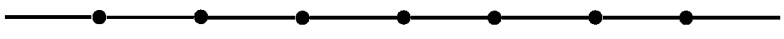
 (Structure 35)

wherein each ——— is a single-stranded oligonucleotide and each ● is a covalent linker joining adjacent single-stranded oligonucleotides, the method comprising the steps of:

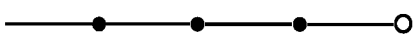
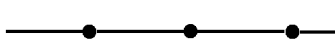
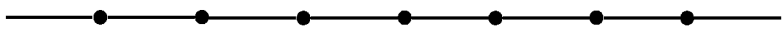
(i) reacting  and , wherein ○ is a linking moiety and R_1 is a chemical group capable of reacting with the linking moiety ○, thereby forming  (Structure 35), and

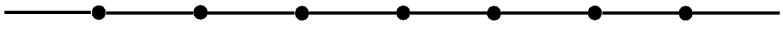
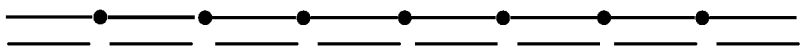
(ii) optionally annealing  (Structure 35) with complementary single-stranded oligonucleotides, thereby forming  (Structure 36).

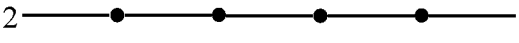
[00501] For example, in one aspect, the disclosure provides a method of synthesizing a multimeric oligonucleotide comprising structure 37:

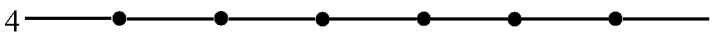
 (Structure 37)

wherein each ——— is a single-stranded oligonucleotide and each ● is a covalent linker joining adjacent single-stranded oligonucleotides, the method comprising the steps of:

(i) reacting  and , wherein ○ is a linking moiety and R_1 is a chemical group capable of reacting with the linking moiety ○, thereby forming  (Structure 37), and

(ii) optionally annealing  (Structure 37) with complementary single-stranded oligonucleotides, thereby forming  (Structure 38).

[00502] The disclosure also provides methods for synthesizing single-stranded multimeric oligonucleotides, for example wherein m is 2  (Structure 39); m is

 (Structure 40); m is 6, 7, 8, 9, 10, 11, or 12; or m is ≥ 13 (see Example 22 below).

[00503] The multimeric compounds can include any one or more of the features disclosed herein. For example, the compounds can include any one or more of the nucleic acids (with or without modifications), targeting ligands, and/or linkers described herein, or any of the specific structures or chemistries shown in the summary, description, or Examples. Likewise, the compounds can be prepared in an of the compositions (e.g., for experimental or medical use) shown in the summary, description, or Examples. Illustrative examples are provided in the *Pharmaceutical Compositions* section below.

[00504] ***Oligonucleotide Uptake and Clearance***

[00505] The bioavailability of a drug in the blood stream can be characterized as the balance between target cell uptake versus kidney clearance. From a practical perspective, *in vivo* circulation half-life and/or *in vivo* activity are good proxies for kidney clearance/glomerular filtration because they can be readily quantified and measured and because their improvement (e.g., increase) can correlate with improved pharmacodynamics and/or pharmacokinetics.

[00506] The uptake rate of a therapeutic agent such as an oligonucleotide (ONT) in the blood is a function of a number of factors, which can be represented as: Rate of Uptake = f {(ONT Concentration) x (Rate Blood Flow) x (Receptor Copy Number/cell) x (Number of Cells) x (equilibrium dissociation constant K_D) x (Internalization Rate)}. For a given ligand/receptor pair, the Copy Number, K_D, Number of cells and Internalization Rate will be constant. This can explain why the GalNAc ligand system is so effective for hepatocytes – it targets the ASGP receptor, which is present at high copy number. The K_D of some ASGP/GalNAc variants is in the nanomolar range and the internalization rate is very high.

[00507] However, effective targeting is also dependent on the ONT concentration, which rapidly decreases over time due to clearance from the blood stream. The rate of clearance of a therapeutic can be represented as: Rate of Clearance = f {(Blood Flow Rate) x (Kidney Filtration Rate) x (Other clearance mechanisms)}. The resulting concentration of ONT at time t can be represented as: (ONT Concentration)t = f {(Initial Concentration) – (Rate of Clearance x t)}.

[00508] In humans, clearance is mainly due to glomerular filtration in the kidney. In general, molecules less than about 45 kD have a half-life of about 30 minutes. In mice, the rate of clearance is even faster, the circulation half-life being about 5 minutes. Without wishing to be bound by any particular theory, it is believed that the disclosure can reduce glomerular filtration using specifically configured multimeric oligonucleotides (e.g., specific composition, size, weight, etc.), leading to a lower rate of clearance, resulting in a higher concentration of ONT in circulation at a given time t (e.g., increased serum half-life, higher overall uptake, and higher activity).

[00509] Again, without wishing to bound by any particular theory, actual glomerular filtration rates can be difficult to measure directly. For example, compounds that pass through the glomerular capillaries are readily absorbed by cells such as tubular epithelial cells, which can retain compounds like siRNA for significant periods of time (see, e.g., Henry, S. P. et al; Toxicology, 301, 13-20 (2012) and van de Water, F.M et al; Drug metabolism and Disposition, 34, No 8, 1393-1397 (2006)). In addition, absorbed compounds can be metabolized to breakdown products, which are then secreted in urine. Thus, the concentration (e.g., in urine) of a therapeutic agent such as an siRNA at a specific time point may not necessarily be representative of the glomerular filtration rate. However, serum half-life, which is related to glomerular filtration and which is directly measurable, may be considered to be a suitable proxy for glomerular filtration.

[00510] Table 1 below shows the dramatic effect increasing the circulation half-life ($t_{1/2}$) of a component can have on the resulting concentration of the component at time t:

Table 1 – Effect of increasing circulation half-life ($t_{1/2}$) on concentration at time t.

t (min):	0	30	60	90	120	150	180	210	240
30 min $t_{1/2}$	100	50	25	12.5	6.25	3.13	1.56	0.78	0.4
60 min $t_{1/2}$	100		50		25		12.5		6.25
90 min $t_{1/2}$	100			50			25		
120 min $t_{1/2}$	100				50				25

Values are presented as % initial dose at time t.

[00511] Thus, increasing the half-life of a component by a factor of 2 increases its residual concentration at 2 hours by a factor of 4. Increasing the half-life by a factor of four leads to even more dramatic improvements in residual concentration - by factors of eight and greater than sixty at 2 and 4 hours, respectively.

[00512] A typical siRNA (e.g., double-stranded monomer) has a molecular weight of about 15kD. A siRNA tetramer according to the disclosure can have a molecular weight of about 60 kD. Without wishing to be bound by any particular theory, it is believed that such multimers (tetramers, pentamers, etc.) can be configured to have a molecular size and/or weight resulting in decreased glomerular filtration *in vivo*. Such multimers would have an increased circulation half-life. Thus, multimers according to the disclosure can be configured to have increased *in vivo* circulation half-life and/or increased *in vivo* activity, relative to that of the individual monomeric subunits. Further, if directed by a suitable targeting ligand the multimer (e.g., tetramer) would deliver many (e.g., four) times the payload per ligand/receptor binding event than the monomeric equivalent. In combination, these effects can lead to a dramatic increase in the bio-availability and uptake of the therapeutic agent. This can be especially advantageous in cases where some combination of the copy number, KD, number of target cells and internalization rate of a given ligand/receptor pair are sub-optimal.

[00513] Accordingly, the multimeric oligonucleotide has a structure selected to (a) increase *in vivo* circulation half-life of the multimeric oligonucleotide relative to that of the individual monomeric subunits and/or (b) increase *in vivo* activity of the multimeric oligonucleotide relative to that of the individual monomeric subunits. For example, the multimeric oligonucleotide can have a molecular size and/or weight configured for this purpose.

[00514] ***Pharmaceutical Compositions***

[00515] In various aspects, the disclosure 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 disclosure should be understood as including embodiments for use as a medicament and/or for use in the manufacture of a medicament.

[00516] A pharmaceutical composition can include a compound or composition according to the disclosure 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).

[00517] Oligonucleotides can be delivered locally or systemically, and the pharmaceutical compositions of the disclosure 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).

[00518] Pharmaceutical compositions can include an effective amount of the compound or composition according to the disclosure. 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).

[00519] ***Delivery Vehicles and Targeting Ligands***

[00520] In various aspects, the disclosure 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 disclosure 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), anisamide, phospholipid, phosphatidyl choline, lecithin, or an immunostimulant. The immunostimulant may be a CpG oligonucleotide, for example, the CpG oligonucleotides of TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 162) or GGTGCATCGATGCAGGGGG (SEQ ID NO: 163). The targeting ligand can be bound to the multimeric oligonucleotide construct directly or indirectly to the nucleic acid, for example through its 3' or 5' terminus, to an internal nucleic acid residue, or to a linker within the construct. In some embodiments, two targeting ligands are conjugated to the multimeric oligonucleotide, where one ligand is conjugated through the 3' terminus and the other ligand is conjugated through the 5' terminus of the oligonucleotide. One or more targeting ligands can be conjugated to the sense strand or the antisense strand of the oligonucleotide, or both the sense strand and the antisense strand. Additional examples that may be adapted for use with the disclosure are discussed below.

[00521] 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.

[00522] 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 vehicles 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.

[00523] 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, with 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.

[00524] 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.

[00525] 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. Such conjugate systems may facilitate delivery into specific cell types by binding to specific receptors.

[00526] One skilled in the art will appreciate that known delivery vehicles and targeting ligands can generally be adapted for use according to the present disclosure.

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).

[00527] In various embodiments, the compounds and compositions of the disclosure 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.

[00528] 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 (e.g., multi-conjugate oligonucleotide, or multimeric oligonucleotide) 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), phospholipid, phosphatidyl choline, lecithin, 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 disclosure 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.

[00529] Additional biologically active moieties within the scope of the disclosure 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 finger nucleases (ZFNs).

[00530] In various embodiments, the compounds and compositions of the disclosure 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.

[00531] 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.

[00532] In various embodiments, the compounds and compositions of the disclosure 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).

[00533] In various embodiments, the compounds and compositions of the disclosure 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).

[00534] In various embodiments, the compounds and compositions of the disclosure 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).

[00535] 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 disclosure by those of ordinary skill.

[00536] ***Methods of Treatment or Reducing Gene Expression***

[00537] In various aspects, the disclosure provides methods for using multi-conjugate oligonucleotides, for example for medical treatments, research, or for producing new or altered phenotypes in animals and plants.

[00538] In one aspect, the disclosure provides a method for treating a subject comprising administering an effective amount of a compound or composition according to the disclosure to a subject in need thereof. In such therapeutic embodiments, the oligonucleotide will be a therapeutic oligonucleotide, for example an siRNA or miRNA. For example, in an embodiment, a multimeric oligonucleotide comprises one or more therapeutic oligonucleotides that are useful for the treatment of cancer.

[00539] In this, and other embodiments, the compositions and compounds of the disclosure can be administered in the form of a pharmaceutical composition, in a delivery vehicle, or coupled to a targeting ligand.

[00540] In one aspect, the disclosure provides a method for silencing or reducing gene expression comprising administering an effective amount of a compound or composition according to the disclosure to a subject in need thereof. In such therapeutic embodiments, the oligonucleotide will be an oligonucleotide that silences or reduces gene expression, for example an siRNA or antisense oligonucleotide.

[00541] Similarly, the disclosure 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 disclosure 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.

[00542] In one aspect, the disclosure 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 disclosure to a subject in need thereof, wherein the compound or composition comprises a targeting ligand.

[00543] In one aspect, the disclosure 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 disclosure to a subject in need thereof, wherein the compound or composition comprises the predetermined stoichiometric ratio of two or more oligonucleotides.

[00544] 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 disclosure) or a subject having a condition (e.g., that may be addressed using the compounds and compositions of the disclosure, for example one or more genes to be silenced or have expression reduced).

[00545] General procedures for measurement of gene knockdown and animal experiments are provided in the Examples below, as are working examples of other *in vitro* and *in vivo* tests. Other methods are known in the art and can be adapted for use with the present disclosure by those of ordinary skill.

[00546] 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

[00547] **General Procedure 1: Single Chain Oligonucleotide Synthesis**

[00548] 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-1H-tetrazole (ETT, 0.5 M in Acetonitrile) as activator. Phosphorothioate linkages were introduced using 50 mM 3-((Dimethylamino-methylidene)amino)-3H-1,2,4-dithiazole-3-

thione (DDTT, AM Chemicals, Oceanside, California, USA) in a 1:1 (v/v) mixture of pyridine and Acetonitrile.

[00549] 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).

[00550] 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.

[00551] 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.

[00552] **General Procedure 2: Lipid Nanoparticle Formulation**

[00553] 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).

[00554] 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.

[00555] 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).

[00556] 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.

[00557] General Procedure 3: LNP Characterization

[00558] 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.

[00559] 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.

[00560] 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).

[00561] General Procedure 4: Animal Experiments

[00562] 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 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.

[00563] General Procedure 5: Measurement of Gene Knockdown

[00564] 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).

[00565] 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).

[00566] 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).

[00567] 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.

[00568] Additional General Procedure 1: Single Chain Oligonucleotide Synthesis

[00569] 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-CAc), 6-N-benzoyl-2'-O-methyl-adenosine (2'-OMe-Abz) and 2-N-isobutyrlguanosine (2'-OMe-GiBu) were used to build the oligomer sequences. 2'-Fluoro modifications were introduced employing the corresponding phosphoramidites carrying the same nucleobase protecting groups as the 2'-OMe RNA building blocks. Coupling time for all phosphoramidites (70 mM in Acetonitrile) was 3 min employing 5-Ethylthio-1H-tetrazole (ETT, 0.5 M in Acetonitrile) as activator. Phosphorothioate linkages were introduced using 50 mM 3-((Dimethylamino-methylidene)amino)-3H-1,2,4-dithiazole-3-thione (DDTT, AM Chemicals, Oceanside, California, USA) in a 1:1 (v/v) mixture of pyridine and Acetonitrile.

[00570] 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).

[00571] 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.

[00572] 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.

[00573] 5'-aminohexyl linkers were introduced employing the TFA-protected hexylamino-linker phosphoramidite (Sigma-Aldrich, SAFC, Hamburg, Germany). 3'-

hexylamino-linkers were introduced using a phtalimido protected hexylamino-linker immobilized on CPG (Prime Synthesis, Aston, PA, USA). Deprotection and purification was performed as above.

[00574] Additional General Procedure 2: Generation of Thiol-terminated siRNA

[00575] 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). After deprotection and purification as above each disulfide containing oligomer was reduced using Dithiothreitol (DTT) (0.1 M DTT stock solution (Sigma-Aldrich Chemie GmbH, Munich, Germany, #646563) in Triethylammonium bicarbonate buffer (TEABc, 0.1M, pH 8.5, Sigma, #90360). The oligonucleotide was dissolved in TEABc buffer (100mM, pH 8.5) to yield a 1 mM solution. To accomplish the disulfide reduction a 50-100 fold molar DTT excess was added to the oligonucleotide solution. The progress of the reduction was monitored by analytical AEX HPLC on a Dionex DNA Pac 200 column (4x 250 mm) obtained from Thermo Fisher. The reduced material, i.e. the corresponding thiol (C6SH), elutes prior to the starting material. After completion of the reaction, excess reagent is removed by size exclusion chromatography using a HiPrep column from GE Healthcare and water as eluent. Subsequently, the oligonucleotide is precipitated using 3 M NaOAc (pH 5.2) and ethanol and stored at minus 20 °C.

[00576] Additional General Procedure 3: General Procedure for Annealing of Single-stranded RNAs (ssRNAs) to Form Double-stranded RNA (dsRNA)

[00577] dsRNAs were generated from RNA single strands by mixing a slight excess of the required complementary antisense strand(s) relative to sense strand and annealing in 20 mM NaCl/4 mM sodium phosphate pH 6.8 buffer. Successful duplex formation was confirmed by native size exclusion HPLC using a Superdex 75 column (10 x 300 mm) from GE Healthcare. Samples were stored frozen until use.

[00578] In the sequences described herein, upper case letters "A", "C", "G" and "U" represent RNA nucleotides. Lower case letters "c", "g", "a", and "u" represent 2'-O-methyl-modified nucleotides; "s" represents phosphorothioate; 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, "C6NH2" and "C6NH" are used interchangeably to represent the aminohexyl linker. "C6SSC6" represents the dihexyldisulfide linker. "InvdT" means inverted thymidine.

[00579] Additional General Procedure 4: General Procedure to Generate Multimeric siRNAs by Sequential Annealing

[00580] Preparation of multimeric siRNAs via stepwise annealing was performed in water and utilized stepwise addition of complementary strands. No heating/cooling of the solution was required. After each addition, an aliquot of the annealing solution was removed and monitored for duplex formation using analytical RP HPLC under native conditions (20°C). The required amounts to combine equimolar amounts of complementary single strands were calculated based on the extinction coefficients for the individual single strands computed by the nearest neighbor method. If the analytical RP HPLC trace showed excess single strand, additional amounts of the corresponding complementary strand were added to force duplex formation ("duplex titration").

[00581] 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.

[00582] Example 1: Generation of Thiol-terminated siRNA

[00583] 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.

[00584] 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.

[00585] Example 2: General Procedure for Preparation of Mono-DTME Oligomer

[00586] 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.

[00587] Example 3: General Procedure for Preparation of Dimer via DTME Functionality

[00588] 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).

[00589] Example 4: General Procedure for Annealing of Single-stranded RNAs (ssRNAs) to Form Double-stranded RNA (dsRNA)

[00590] 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.

[00591] Example 5: General Procedure for Preparation of 3'- or 5'- NH_2 Derivatized Oligonucleotides

[00592] 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.

[00593] Example 6: General Method for GalNAc Ligand Conjugation

[00594] 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:

[00595] 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).

[00596] 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.

[00597] Example 7: Oligonucleotide Precursors

[00598] Using the methodologies described in the above Examples, Tables 2-7 below describes the single-stranded monomers, dimers and GalNAc tagged monomers and dimers that were prepared:

Table 2: Oligonucleotide Precursors – Single Strands ("X")

SEQ ID NO:	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 3: Oligonucleotide Single-stranded Sense and Antisense Pairs; and Resulting Duplexes ("XD-") After Annealing.

Duplex ID	SEQ ID NO:	Single Strand ID	Sequence (5'-3')	Target/strand
XD-00376	13	X01162	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT	FVIIs
	14	X00549	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT	FVIIas
XD-	16	X00116	GcAAAGGcGuGccAAcucAdTsdT	FVIIs

00030	17	X00117	UGAGUUGGcACGCCUUUGCdTsdT	FVIIas
XD-01078	19	X02943	GGAAUCuuAuAuuuGAUCcAsA	ApoBs
	20	X02944	uuGGAUcAAAuAuAAGAuUCcscsU	ApoBas
XD-00194	22	X00539	cuuAcGcuGAGuAcuucGAdTsdT	LUCs
	23	X00540	UCGAAGuACUcAGCGuAAGdTsdT	LUCas

Table 4: Derivatized Oligonucleotide Single-stranded Sense and Antisense Pairs; and Resulting Duplexes After Annealing.

Duplex ID	SEQ ID NO:	Single Strand ID	Sequence (5'-3')	Target
XD-06328	25	X18790	(GalNAc3)(NHC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	FVII
	26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	
XD-06728	28	X20124	(GalNAc3)(NHC_6)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)	ApoB
	29	X19583	UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu	
XD-06386	31	X20216	(GalNAc3)(NHC_6)sAfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)	TTR
	32	X19584	usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu	
	34	X19571	gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C_6NH)(GalNAc3)	FVII
XD-05961	35	X18788	gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	FVII
	26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	

Table 5: Single-stranded Oligonucleotide Dimers Linked by DTME

SEQ ID NO:	ID	Sequence (5'-3')	Target/strand
37 & 125	X15049	GGAAUCuuAuAuuuGAUCcAsA(SHC_6)(DTME)GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC_6)	ApoBs/F7s
38 & 126	X12714	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC_6)(DTME)GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC_6)	F7s/F7as
39 & 127	X19575	(SHC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C_6NH)(GalNAc$_3$)(DTME)(SHC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	F7s/F7s
40 & 128	X19819	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C_6SH)(DTME)UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C_6SH)	F7as/F7as
41 & 129	X20336	(SHC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C_6NH)(GalNAc$_3$)(DTME)(SHC_6)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)	F7s/ApoBs

Table 6: Single Strand DTME Dimers and Corresponding Monomers; and Resulting Duplexes After Annealing

Duplex ID	SEQ ID	Single Strand ID	Sequence (5'-3')	Target/Strand

XD-05311	37 & 130	X15049	GGAAUCuuAuAuuuGAUCcAsA(SHC6)(DTME)GGAUfCfAUfCfUfCfAAGUfCfUfUfUfACfdTsdT(SHC6)	ApoBs-FVIIs
	14	X00549	5'-GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT-3' +	FVIIas
	20	X02944	5'-uuGGAUcAAAuAuAAGAuUCcscsU-3'	ApoBas
XD-05312	38 & 131	X12714	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT(SHC6)(DTME)GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT(SHC6)	FVIIs-FVIIas
	13	X01162	5'-GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT-3'	FVIIs
	14	X00549	5'-GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT-3'	FVIIas

Table 7: Chemically Synthesized Disulfide-Linked Dimers and Trimers

SEQ ID	Single Strand ID	Sequence (5'-3')	Target/Strand
44 & 132	X20366	usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu(C6SSC6)UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu	TTRas/ApoBas
45 & 133	X22413	AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)(C6SSC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	FVIIs/TTRs
46 & 134 & 135	X20256	(SHC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C6NH)(GalNAc3)(SPDP)(NHC6)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)(C6SSC6)AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)	FVIIs/ApoBs/TTRs
47 & 136	X20366	usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu(C_6SSC_6)UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu	TTRas/ApoBas
48 & 137	X22413	AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)(C_6SSC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	FVIIs/TTRs

[00599] 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, whose structure is shown in FIG. 1B. "(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. 1A. "SPDP" represents the reaction product of the reaction of succinimidyl 3-(2-pyridyldithio)propionate with the aminolinker equipped RNA. "InvdT" means inverted thymidine.

[00600] In the Target/Strand portion of the chart: "F7" or "FVII" designates an siRNA sequence targeting the Factor VII transcript (mRNA). "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".

[00601] **Example 8: General Procedure to Generate Dimeric, Trimeric and Tetrameric siRNAs by Sequential Annealing**

[00602] 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").

[00603] 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.

[00604] Example 9: Preparation of 5'-GalNAc-FVII Canonical Control (XD-06328)

[00605] 5'-GalNAc-FVII Canonical Control (XD-06328) (see FIG. 2) 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.

[00606] Example 10: 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)

[00607] GalNAc-conjugated homodimeric siRNA XD-06330 targeting FVII (FIG. 3) 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 9: Stoichiometry of Oligomers Used in Synthesis of GalNAc-FVII-DTME- FVII Homodimer (XD-06330)

SEQ ID NO:	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	

[00608] Example 11: 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)

[00609] 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. 4 and the methodology described in Example 8.

[00610] 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 stoichiometry of the various oligomers used in the synthesis are shown in Table 10.

Table 10: Stoichiometry of Oligomers Used in Synthesis of GalNAc-FVII-FVII Homodimer (XD-06360)

SEQ ID NO:	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	XD-06360				29111.9	30892.6	

[00611] Example 12: 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)

[00612] GalNAc-conjugated homodimeric siRNA XD-06329 targeting FVII was prepared as depicted in FIG. 5 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.

[00613] Example 13: Determination of *In vivo* FVII Gene Knockdown by FVII Homodimeric GalNAc Conjugates (XD-06329, XD-06330 and XD-06360).

[00614] 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 (25 mg/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. 6.

[00615] 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 the data 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. 7A and 7B.

[00616] Figure 7A. Factor VII serum values at each time point are normalized to control mice injected with 1X PBS. The bars at each datapoint correspond, left to right, to saline, XD-06328, XD-06329, XD-06330, and XD-06360, respectively.

[00617] Figure 7B. Factor VII serum values at each time point are normalized to the prebleed value for each individual group. The bars at each data point correspond, left to right, to saline, XD-06328, XD-06329, XD-06330, and XD-06360, respectively.

[00618] Example 14: Preparation of Canonical GalNAc-siRNAs independently targeting FVII (XD-06328), ApoB (XD-06728) and TTR (XD-06386).

[00619] Three canonical siRNAs independently targeting FVII (XD-06328), ApoB (XD-06728) and TTR (XD-06386) (see FIG. 8) 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.

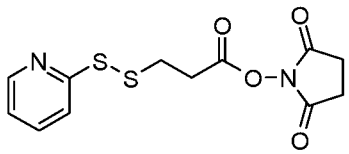
Table 11: GalNAc-siRNA Conjugates

Duplex ID	SEQ ID NO:	ssRNA	Sequence 5'-3'	
XD-06328	138	X18790	(GalNAc3)(NHC_6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)	FVII
	139	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	
XD-06728	140	X20124	(GalNAc3)(NHC_6)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)	ApoB
	141	X19583	UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu	
XD-06386	142	X20216	(GalNAc3)(NHC_6)sAfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)	TTR
	143	X19584	usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu	

[00620] Example 15: Preparation of GalNAc-FVII-ApoB-TTR Trimer with Cleavable Linkages on Sense Strands (XD-06726)

[00621] A heterotrimer targeting FVII, ApoB and TTR conjugated to GalNAc (see FIG. 9) was synthesized using a hybrid strategy of solid phase and solution phase, as depicted in FIG. 10.

[00622] 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, pH 8.5, 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 (pH 5.2). Identity was confirmed by RP-HPLC ESI-MS.

[00623] 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.

[00624] 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 12.

Table 12: Single-Stranded Heterotrimer

SEQ ID NO:	ID	Sequence	Target/Strand
52 & 144 & 145	X20256	(SHC$_6$)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C$_6$NH)(GalNAc$_3$)(SPDP)(NHC$_6$)cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)(C$_6$SSC$_6$)AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)	FVIIs/ApoBs/TTRs

[00625] Note: In principle the above sequence is accessible through a single solid phase synthesis. In this case, SPDP and C$_6$NH$_2$ would be replaced by the C$_6$SSC$_6$ phosphoramidite. However, due to the sequence length of the entire construct such a synthesis would be challenging.

[00626] 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 in Example 8. 7 mg of material was obtained which was essentially pure by HPLC.

Table 13: Stoichiometry of Oligomers Used in Synthesis of GalNAc-FVII-ApoB-TTR Trimer (XD-06726).

SEQ ID NO:	ID	Target	E (L/mol*cm)	Nmol/OD	MW (free Acid)	MW Na salt	Req OD
52 & 144 & 145	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	

[00627] Example 16: Preparation of GalNAc-FVII-ApoB-TTR Trimer with Cleavable Linkages on Alternating Sense and Antisense Strands (XD-06727).

[00628] 9 mg (192 nmol) of Trimeric siRNA XD-06727 (see FIG. 11), simultaneously targeting FVII, ApoB and TTR, was prepared in high purity by combining single strands stepwise as depicted in FIG. 12, using the methodology described in Example 8.

Table 14: Stoichiometry of Oligomers used in synthesis of GalNAc-siFVII-siApoB-siTTR Trimer (XD-06727)

SEQ ID NO:	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	

[00629] 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. 13, 14, and 15.

[00630] Example 17: Preparation of LNP Formulation of Pooled siRNAs Individually Targeting FVII, ApoB and TTR

[00631] 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 83 nm in size with a zeta potential of 2.2 mV and a PDI of 0.04.

Table 15: Monomeric siRNA targeting TTR (XD-06729)

dsRNA ID	ssRNA ID	SEQ ID NO:	Sequence	Target/Strand
XD-06729	X21072	154	cAGuGuucuuGcucuAuAAdTsdT	TTRs
	X21073	155	UuAuAGAGcAAGAAcACUGdTsdT	TTRas

[00632] Example 18: Assessment of mRNA Knockdown by GalNAc-Conjugated Heterotrimeric SiRNAs

[00633] 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. 16A and 16B, 17A and 17B, and 18A and 18B. mRNA levels in liver lysates were measured at day 7 post injection (FIGS. 19A and 19B).

[00634] One animal in group A (XD-06726) did not show any effect on TTR serum levels. The first of the two TTR protein graphs shows data with values omitted for the non-responding animal.

[00635] For comparison, the values from the animal showing poor TTR response have been omitted from the second FVII graph.

[00636] ApoB serum levels show a high variation, both within the animals of one group and between the different time-points of the saline control.

[00637] 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.

[00638] Example 19: Preparation GalNAc-FVII-ApoB-TTR-FVII Tetramer (XD-07140)

[00639] 12.4 nmol of the tetrameric siRNA XD-07140 (see FIG. 20), simultaneously targeting FVII, ApoB and TTR, was prepared by combining single

strands stepwise as depicted in FIG. 21, and according to the duplex titration method described in Example 8. HPLC analysis showed the product was obtained in high purity.

Table 16: Stoichiometry of Oligomers used in Synthesis of GalNAc-FVII-ApoB-TTR-FVII Tetramer (XD-07140)

SEQ ID NO:	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	

[00640] Example 20: Synthesis of Homo-tetramer

[00641] Multimeric oligonucleotide according to the disclosure can be synthesized by any of the methods disclosed herein. Two example methods are provided below for homo-tetramers. These Examples can be readily adapted to synthesize longer multimers (e.g., pentamers, hexamers, etc.)

[00642] A homo-tetrameric siRNA with linkages on a single strand can be synthesized by preparing a tetramer of the sense strand, each sense strand linked via a cleavable linker, on a synthesizer and then subsequently adding a targeting ligand and annealing the anti-sense strands, as shown in FIG. 40. The cleavable linkers of the sense strand may be disulfides (as shown) or other labile linkages (e.g., chemically unmodified nucleic acid sequences such as UUU/Uridine-Uridine-Uridine).

[00643] Variations on the scheme shown in FIG. 40 can include using alternative linkers, linking anti-sense strands and annealing sense strands, synthesizing longer multimers, or where the technical limits of machine-based synthesis are reached, synthesizing one or more multimers and then joining said multimers together using one or more solution phase chemical reactions (e.g., synthesizing two tetramers per scheme 1, one with ligand, the other without, one or both strands modified, as appropriate, with a functional group to facilitate linking, and then linking the two tetramers together via the formation of a covalent bond, with or without the addition of a linking moiety such as, e.g., DTME).

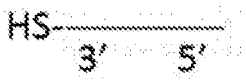
[00644] Alternatively, the homo-tetramer could be assembled as shown in FIG. 41 with linkages on alternating strands.

[00645] In FIG. 41, "-SH" represents a sulfhydryl group, "Mal" represents DTME, "-CL-" represents a cleavable linker. Variations on the scheme shown in FIG 41 can include using alternative linkers and synthesizing longer multimers.

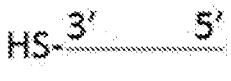
[00646] **Example 21: Synthesis of Ligand Conjugates**

[00647] The ligand conjugate shown in FIG. 41 can be synthesized as follows:

[00648] 3'-Sulfydryl derivatives of both sense and antisense strands of the monomer are synthesized:

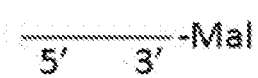


(Structure 61)

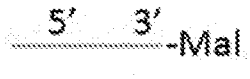


(Structure 62)

[00649] Portions of each are converted to the corresponding mono-maleimide derivative:



(Structure 63)



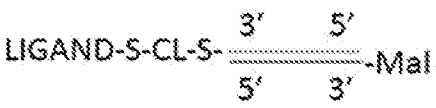
(Structure 64)

[00650] A portion of the sense-strand maleimide derivative thus obtained is then treated with a sulfhydryl derivative of the targeting ligand of choice:



(Structure 65)

[00651] A slight molar excess of anti-sense-maleimide derivative is then added and the desired ligand-ds-siRNA-maleimide product isolated by preparative chromatography:



(Structure 66)

[00652] A slight molar excess of each of the sense and anti-sense components of the homo-tetramer are then added in the sequence as outlined in FIG. 41, the products at each step being purified by preparative chromatography when required.

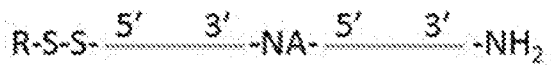
[00653] Example 22: Synthesis of Multimeric Oligonucleotides

[00654] Multimeric oligonucleotide according to the disclosure can be synthesized by any of the methods disclosed herein or adapted from the art. Example methods are provided below for homo-multimers, but the present synthesis can also be readily adapted to synthesize hetero-multimers.

[00655] These Examples can also be adapted to synthesize multimers of different lengths. For example, one can use essentially the same synthesis and linking chemistry to combine a tetramer and monomer (or trimer and dimer) to produce a pentamer. Likewise, one can combine a tetramer and a trimer to produce a septamer, etc. Complementary linking chemistries (e.g., click chemistry) can be used to assemble larger multimers.

[00656] Example 22A: Synthesis of Homo-Tetramer of siRNA Via Pre-Synthesized Homodimers

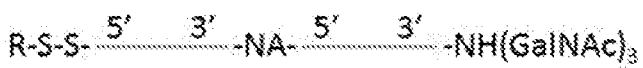
[00657] Step 1: A sense strand homodimer is synthesized wherein the two sense strands are linked by a nuclease cleavable oligonucleotide (NA) and terminated with an amino function and a disulfide moiety.



(Structure 67)

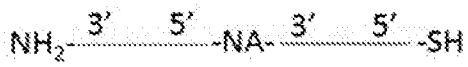
Individual strands (for this and other steps) are synthesized as outlined above in the *General Procedure: Single Chain Oligonucleotide Synthesis* section. Other methods for oligonucltide strand synthesis, linking, and chemical modification can be adapted from the art.

[00658] Step 2: A tri-antennary GalNAc ligand is then added to the terminal amino function of one part of the sense strand homo-dimer via reaction with an acyl activated triantennary GalNAc ligand.



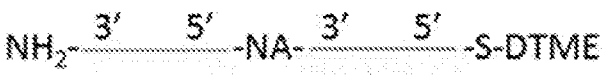
(Structure 68)

[00659] Step 3: The remainder of the sense strand homodimer is treated with a molar excess of dithiothreitol to cleave the disulfide group to generate a thiol terminated sense strand homodimer.



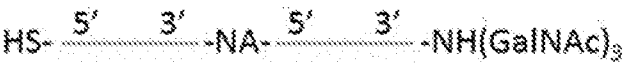
(Structure 69)

[00660] Step 4: This material is mono-derivatized with dithiobismaleimidoethane (DTME) according to the procedure used to prepare hetero-multimers (see above).



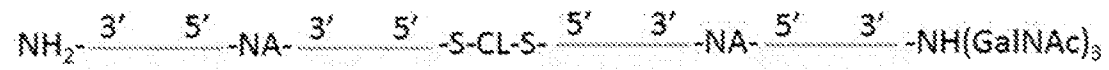
(Structure 70)

[00661] Step 5: The disulfide group of the GalNAc derivitized homodimer is also cleaved by treatment with a molar excess of dithiothreitol.



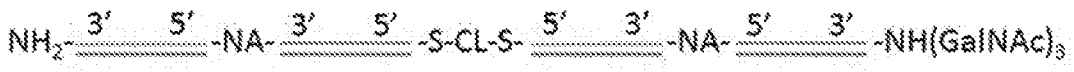
(Structure 71)

[00662] Step 6: The GalNAc terminated homodimer is then linked to the mono-DTME derivatized homodimer via reaction of the terminal thiol-group to yield single-stranded homo-tetramer. "-S-CL-S-" represents the cleavable disulfide group in DTME, e.g., a Cleavable linker (CL).



(Structure 72)

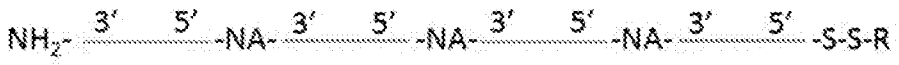
[00663] Step 7: This material is then annealed with 4 molecular equivalents of antisense monomer to yield the desired double-stranded homo-tetramer (this annealing step is optional and can be omitted, for example to prepare single-stranded multimers such as antisense oligonucleotides).



(Structure 73)

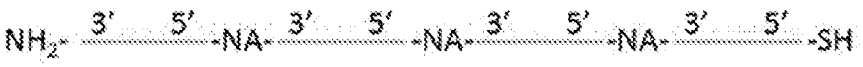
[00664] Example 22B: Synthesis of Homo-Hexamer of siRNA Via Presynthesized Homodimer and Homo-tetramer

[00665] Step 1: A sense strand homo-tetramer is synthesized wherein the four sense strands are linked by a nuclease cleavable oligonucleotide and terminated with an amino function and a disulfide moiety.



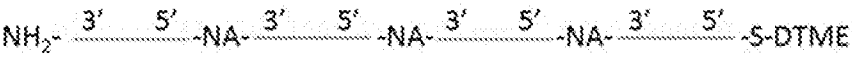
(Structure 74)

[00666] Step 2: This material is treated with a molar excess of dithiothreitol to cleave the disulfide group



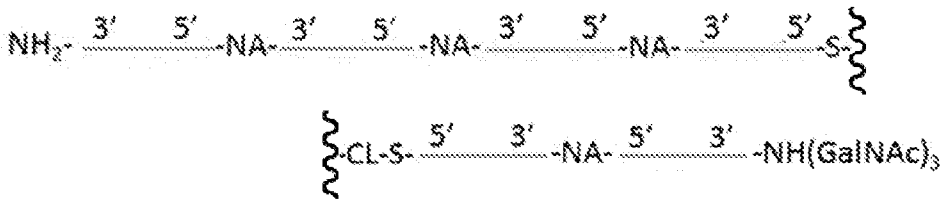
(Structure 75)

[00667] Step 3: This material is monoderivatized with dithiobismaleimidoethane (DTME) according to the procedure used to prepare hetero-multimers (see above).



(Structure 76)

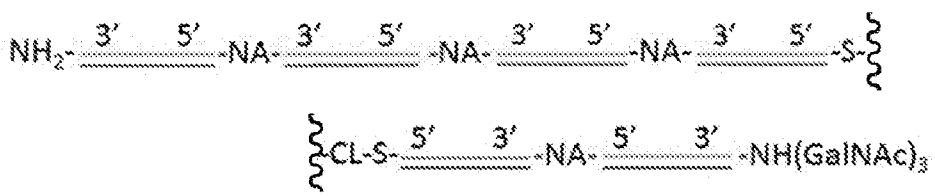
[00668] Step 4: This material is reacted with the thiol terminated GalNAc homodimer to yield the single-stranded homo-hexamer.



(Structure 77)

[00669] Note: In Structures 77, 78, 81, 82, 89, and 91, a single contiguous structure is broken into two parts by the symbol .

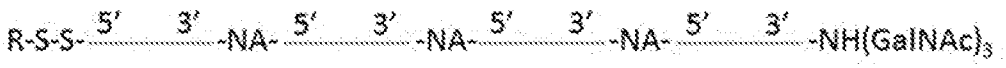
[00670] Step 5: This material is then annealed with 6 molecular equivalents of antisense monomer to yield the desired double-stranded homo-hexamer (this annealing step is optional and can be omitted, for example to prepare single-stranded multimers such as antisense oligonucleotides).



(Structure 78)

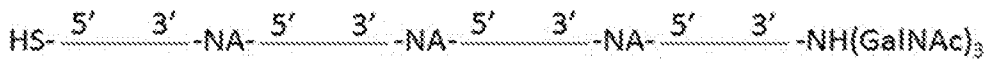
[00671] Example 22C: Synthesis of Homo-Octamer of siRNA Via Presynthesized Homo-tetramer

[00672] Step 1: One part of the amino-terminal homo-tetramer synthesized above is converted to the corresponding GalNAc derivative by reaction with an acyl activated triantennary GalNAc ligand.



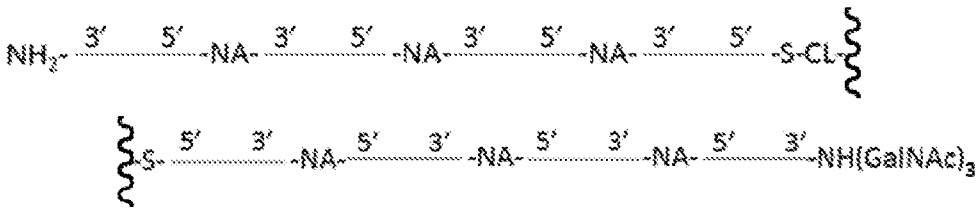
(Structure 79)

[00673] Step 2: This material is treated with a molar excess of dithiothreitol to cleave the disulfide group



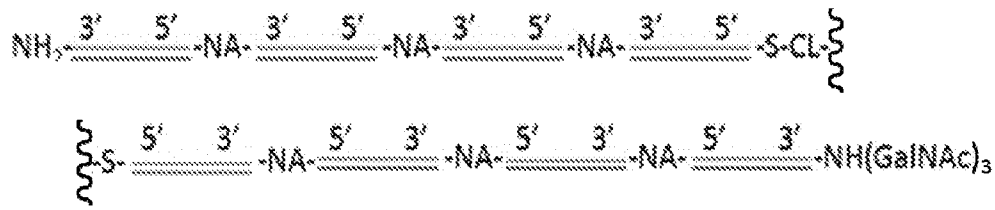
(Structure 80)

[00674] Step 3: This material is reacted with the mono-DTME derivatized tetramer to yield the terminal GalNAc derivatized single-stranded octamer.



(Structure 81)

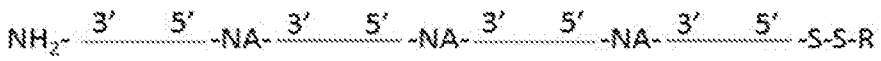
[00675] Step 4: This material is then annealed with 8 molecular equivalents of antisense monomer to yield the desired double-stranded homo-octamer (this annealing step is optional and can be omitted, for example to prepare single-stranded multimers such as antisense oligonucleotides).



(Structure 82)

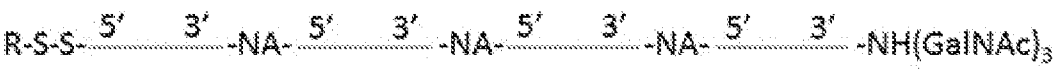
[00676] Example 22D: Synthesis of Homo-Dodecamer of Anti-Sense Oligonucleotide via Pre-synthesized Homo-tetramers Using Combination of Thiol/maleimide and Azide/acetylene ("Click") Linkers

[00677] Step 1: A homo-tetramer of anti-sense oligonucleotides is synthesized containing 3 nuclease cleavable oligonucleotide linkers and terminal disulfide and amino groups.



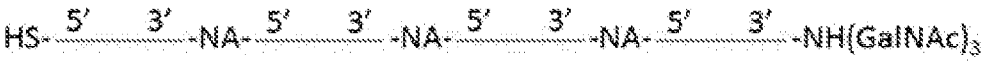
(Structure 83)

[00678] Step 2: This material is converted to the corresponding GalNAc derivative by reaction with an acyl activated triantennary GalNAc ligand.



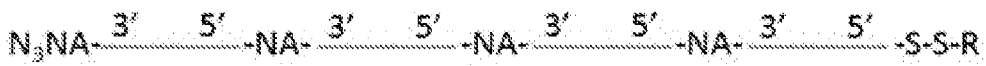
(Structure 84)

[00679] Step 3: This material is treated with a molar excess of dithiothreitol to cleave the disulfide group



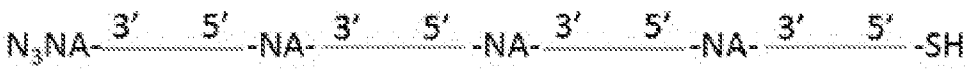
(Structure 85)

[00680] Step 4: Separately, a homo-tetramer of anti-sense oligonucleotides is synthesized containing 3 nuclease cleavable oligonucleotide linkers and terminal disulfide and azide groups.



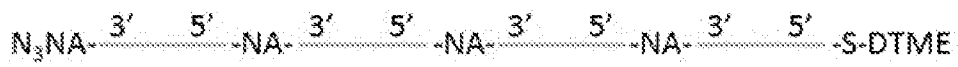
(Structure 86)

[00681] Step 5: This material is treated with a molar excess of dithiothreitol to cleave the disulfide group



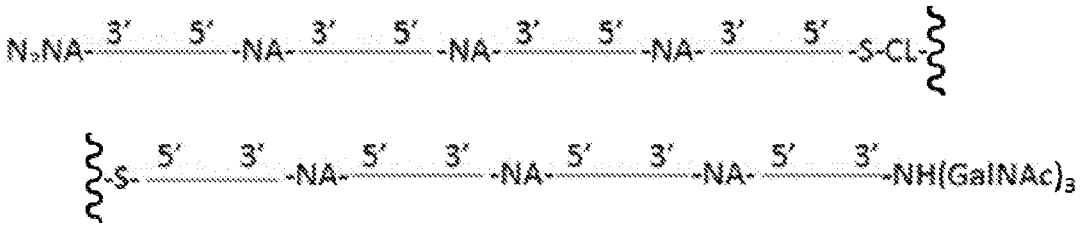
(Structure 87)

[00682] Step 6: This material is mono-derivatized with dithiobismaleimidoethane (DTME) according to the procedure used to prepare siRNA hetero-multimers (see above).



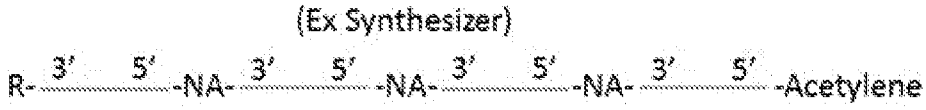
(Structure 88)

[00683] Step 7: This material is reacted with the thiol-terminated GalNAc derivatized tetramer to yield the terminal GalNAc derivatized single-stranded anti-sense octamer.



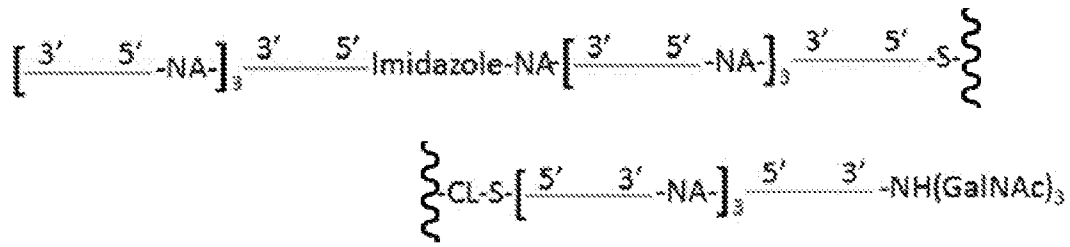
(Structure 89)

[00684] Step 8: Separately, a third homo-tetramer of anti-sense oligonucleotides is synthesized containing 3 nuclease cleavable oligonucleotide linkers and a terminal acetylene group. The latter can be underivatized or a sterically strained derivative such as dibenzocyclooctyne (DBCO, Glen Research, VA, USA)



(Structure 90)

[00685] Step 9: This material is then reacted with the azide-terminated octamer prepared in Step 7 to yield the desired Anti-Sense Homo-Dodecamer. If the terminal acetylene on the tetramer is underivatized, a metal salt catalyst such as copper 1 chloride will be required to effect the linking. By contrast if the terminal acetylene is DBCO then the coupling reaction will be spontaneous.



(Structure 91)

[00686] This methodology, or methods using alternative linking chemistry, can also be used to make multimers of other lengths (e.g., 9, 10, 11, 13, 14, 15, … oligonucleotides). Such multimers can be made double-stranded by annealing the single-stranded multimer with complementary oligonucleotides.

[00687] Example 23: Synthesis of Homo-hexamer siRNA

[00688] A homo-hexamer of FVII siRNA was constructed containing two orthogonal types of bio-cleavable linkages, i) an unmodified di-nucleotide linkage easily introduced on the synthesizer, and ii) the thiol/maleimide derivative that was introduced

post-synthesis. The FVII homo-hexamer (XD-09795) was assembled by combining a homodimer (X30835) and a homo-tetramer (X30837) as illustrated in FIG. 23. Both the homodimer and homo-tetramer synthesized on solid support via standard techniques with an amino- and disulfide group at each terminus. After unblocking and purification the homodimer and homo-tetramer were then linked together via the thiol/maleimide reaction and annealed with antisense strand X18795 to give the FVII homo-hexamer (XD-09795).

[00689] The sequences of the single-stranded homodimer X30835, the single-stranded homo-tetramer X30837, the resultant single-stranded homo-hexamer X30838, as well as the double-stranded hexamer XD-09795 and the double-stranded monomer XD-09794 are shown in Table 17.

Table 17: Sequences of oligonucleotides in Example 23

Duplex-ID	SEQ ID NO:	ss-ID	Sequence (5'-3')
	146	X30835	(DTME)(SHC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(NH2C6)
	147	X30837	(SHC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(NH2C6)
XD09794	148	X18789	(NH2C6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)
	26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu
XD09795	146 & 147	X30838	[(DTME)(SHC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(NH2C6)](SHC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(NH2C6)
	26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu

[00690] Example 24: Purity and Yield in Synthesis of Homo-hexamer siRNA

[00691] The synthesis steps described in Example 23 resulted in high yield and purity of the intermediate products, homodimer (X30835), homo-tetramer (X30837), and homo-hexamer (X30878), as well as the resultant dsRNA homo-hexamer (XD-09795), as presented by HPLC trace data in FIGS. 24A-24B, 24C-24D, 24E, and 24F, respectively.

[00692] Example 25: Comparison of *in vivo* Circulation Half-life Between Homo-hexamer siRNA and Corresponding Monomer

[00693] The serum half-lives of the FVII homo-hexamer XD-09795 and the corresponding FVII monomer XD-09794 were determined in mice. Briefly, the homo-

hexamer or the corresponding monomer were administered via intravenous (IV) bolus injection into 3 cohorts of 4 C57/BL6N female mice aged approximately 11 weeks per cohort. Dosage was 20mg/kg for both FVII monomer and FVII hexamer and blood samples were drawn 5, 30, 60 and 120 minutes after the IV bolus injection. The concentration of FVII antisense was determined at various time-points via a fluorescent PNA probe complementary to the antisense strand and the results are shown in FIG. 25.

[00694] As shown in FIG. 25, only approximately 10% of administered FVII monomer remained in circulation after 5 minutes, and all had essentially disappeared after 30 minutes. By contrast, nearly all of the administered FVII hexamer remained in circulation after 5 minutes with one third of the initial dose remaining after 30 minutes. The data shows that the in-vivo circulation half-life of the hexamer was approximately 30-fold greater than the monomer.

[00695] Example 26: Determination of Levels of Cytokines in Blood Samples Taken at t= 5, 30, 60, and 120 Minutes Using MSD U-Plex Platform

[00696] To assess any adverse toxicological response to the hexamer, analysis of cytokine levels in the blood samples was performed using a MSD U-Plex platform. Blood samples from the monomer XD-09794 and homo-hexamer XD-09795 treated cohorts were analyzed for cytokine levels at the various time points. Serum levels of ten cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, KC-GRO, TNF-α, and GM-CSF) were assayed and shown in FIGS. 26 A-J. Of the ten cytokines assayed, the serum levels of 4 cytokines were unchanged between monomer and hexamer, and the serum levels were virtually identical in the remaining 6.

[00697] Example 27: Synthesis Homo-multimers

[00698] Homo-multimers of an siRNA directed against FVII mRNA were prepared via the above methodologies using the following sequences:

FVII sense: 5‘-gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)-3‘ (SEQ ID NO:35)

FVII anti-sense: 5‘-UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu-3‘ (SEQ ID NO:26), linked via the endonuclease cleavable linkers dCdA and the reductively cleavable linker DTME as follows:

Table 18: Oligonucleotides in Examples 28-36

Sequence ID	Configuration/Strand
X18789	Monomer Sense

Sequence ID	Configuration/Strand
X18795	Monomer Anti-sense
XD-09794	ds Monomer
X30833	Dimer Sense
X18795	Monomer Anti-sense
XD-10635	ds Dimer
X34003	Trimer Sense
X18795	Monomer Anti-sense
XD-10636	ds Trimer
X30836	Tetramer Sense
X18795	Monomer Anti-sense
XD-10637	ds Tetramer
X-34004	Pentamer Sense
X18795	Monomer Anti-sense
XD-10638	ds Pentamer
X34005	Hexamer Sense
X18795	Monomer Anti-sense
XD-10639	ds Hexamer
X30837	Tetramer Sense thiol
X30834	Dimer sense thiol
X30835	Dimer sense-S-DTME
X30838	Hexamer Sense
X18795	Monomer Anti-sense
XD-09795	ds Hexamer
X34006	Pentamer Sense thiol
X30834	Dimer sense thiol
X30835	Dimer sense-S-DTME
X34009	Heptamer Sense
X18795	Monomer Anti-sense
XD-10640	ds Heptamer
X34007	Hexamer Sense thiol
X30834	Dimer sense thiol
X30835	Dimer sense-S-DTME
X34010	Octamer Sense
X18795	Monomer Anti-sense
XD-10641	ds Octamer

Table 19: FVII siRNA homo-multimers XD-10635, XD-10636, XD-06386, XD-10635

Duplex ID	SEQ ID NO:	Single Strand ID	Sequence (5'-3')	Configuration
XD-10635	149	X30833	(C6SSC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)C6NH2)	Dimer
	26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	
XD-10636	150	X34003	(C6SSC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C6NH2)	Trimer
	26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	
XD-06386	151	X30836	(C6SSC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C6NH2)	Tetramer
	26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	
XD-10635	152	X34004	(C6SSC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)C6NH2)	Pentamer
	26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	
XD-06728	153	X34005	(C6SSC6)gcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)dCdAgcAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)C6NH2)	Hexamer
	26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	

[00699] Example 28: Synthesis of FVII Monomer XD-09794

[00700] Monomeric sense strand X18789 of FVII siRNA with amino function at the 5'-terminus on the sense strand was synthesized and purified as shown in FIGS. 27A and 27B. Yield, 48.3 mg, 6.694 mmol, 18.6%. The corresponding antisense strand X18795 was likewise synthesized to yield 46.3mg, 6.35 mmol, 31.9%. 5.35 mg (747.3 nmol) of sense strand and 5.45 mg (747.3 nmol) of anti-sense strand were then annealed to yield 10.8 mg (747.4 nmol) the corresponding double-stranded FVII monomer (XD-09794).

[00701] Example 29: Synthesis of FVII Dimer XD-10635

[00702] Homodimeric sense-strand of FVII siRNA X30833 with amino and di-sulfide groups at the 3'- and 5'- termini respectively and containing a dCdA cleavable linker was synthesized and purified as shown in FIGS. 28A and 28B. Yield, 35.8 mg, 6.694 mmol, 18.6%.

[00703] 5.51 mg (362.6 nmol) of sense strand X30833 and 5.29 mg (725.2 nmol) of anti-sense strand X18795 were then annealed to yield 10.8 mg (362.6 nmol) of the corresponding double-stranded FVII homo-dimer (XD-10635).

[00704] Example 30: Synthesis of FVII Trimer XD-10636

[00705] Homo-trimeric sense-strand of FVII siRNA X34003 with amino and di-sulfide groups at the 3'- and 5'- termini respectively and containing two dCdA cleavable linkers was synthesized and purified as shown in FIGS. 29A and 29B. Yield,19.6 mg (857.9 nmol, 19.3%).

[00706] 5.16 mg (225.5 nmol) of sense strand X34003 and 4.93 mg (676.5 nmol) of anti-sense strand X18795 were then annealed to yield 10.1 mg (225.5 nmol) of the corresponding double-stranded FVII homo-trimer (XD-10636).

[00707] Example 31: Synthesis of FVII Tetramer XD-10637

[00708] Homo-tetrameric sense-strand of FVII siRNA X30836 with amino and di-sulfide groups at the 3'- and 5'-termini respectively and containing three dCdA cleavable linkers was synthesized and purified as shown in FIGS. 30A and 30B. Yield, 53.1 mg (1734.5 nmol, 13%).

[00709] 5.53mg (180.8 nmol) of sense strand X30836 and 5.27 mg (723.2 nmol) of anti-sense strand X18795 were then annealed to yield 10.8 mg (180.8 nmol) of the corresponding double-stranded FVII homo-tetramer (XD-10637).

[00710] Example 32: Synthesis of FVII Pentamer XD-10638

[00711] Homo-pentameric sense-strand of FVII siRNA X34004 with amino and di-sulfide groups at the 3'- and 5'- termini respectively and containing four dCdA cleavable linkers was synthesized and purified as shown in FIGS. 31A and 31B. Yield, 35.9 mg (938 nmol, 10.6%).

[00712] 5.53mg (144.5 nmol) of sense strand X34004 and 5.27 mg (723.2 nmol) of anti-sense strand X18795 were then annealed to yield 10.8 mg (144.5 nmol) of the corresponding double-stranded FVII homo-pentamer (XD-10638).

[00713] Example 33: Synthesis of FVII Hexamer XD-10639

[00714] Homo-hexameric sense-strand of FVII siRNA X34005 with amino and di-sulfide groups at the 3'- and 5'- termini respectively and containing five dCdA cleavable linkers was synthesized and purified as shown in FIGS. 32A and 32B. Yield, 21.4 mg (466.1 nmol, 5.3%).

[00715] 5.15mg (144.5 nmol) of sense strand X34005 and 4.89 mg (723.2 nmol) of anti-sense strand X18795 were then annealed to yield 10.04 mg (111.9 nmol) of the corresponding double-stranded FVII homo-hexamer (XD-10639).

[00716] Example 34: Synthesis of FVII Hexamer XD-09795

[00717] As shown in FIGS. 33A-33B, homo-hexameric sense-strand of FVII siRNA X30838 with amino groups at both of the 3' termini and containing four dCdA cleavable linkers and one reductively cleavable DTME linker was synthesized and purified via the homo-dimeric sense-strand of FVII siRNA X30833 and the homo-tetrameric sense-strand of FVII siRNA X30836 prepared in Examples 28 and 30. Disulfide group was cleaved from X30833 and X30836 using DTT to give the corresponding 5-thiol derivatives X30834 and X30837 in 97.6% and 91.9% yield respectively. Using the procedure described above 14.9 mg (986.7 nmol) of X30834 was then converted to 10.6 mg (700.5 nmol, 71.0%) of the corresponding mono-DTME derivative X30835 which was reacted with one equivalent of X30837 to give 4.2mg (90.7 nmol, 64%) of the single-stranded homo-hexamer X30838. 3.8mg (83 nmol) of sense strand X30838 and 3.7 mg (502 nmol, 6 mol. equiv) of anti-sense strand X18795 were then annealed to yield 7.5 mg (83.7 nmol) of the corresponding double-stranded FVII homo-hexamer (XD-09795).

[00718] Example 35: Synthesis of FVII Heptamer XD-10640

[00719] As shown in FIGS. 34A-34B, homo-heptameric sense-strand of FVII siRNA X34009 with amino groups at both of the 3' termini and containing five dCdA cleavable linkers was synthesized and purified via the homo-dimeric sense-strand of FVII siRNA X30833 and the homo-pentameric sense-strand of FVII siRNA X34004. Disulfide group was cleaved from X30833 and X34004 using DTT to give the corresponding 5-thiol derivatives X30834 (28.3mg, 1877.9 nmol, 86.7%) and X34006 (21.8 mg, 572.2 nmol), respectively. Using the procedure described above X30834 was then converted to

the corresponding mono-DTME derivative X30835 (22.6 mg, 1465.2 nmol, 78.1%). 8.8 mg (572.2 nmol) of X30835 was reacted with X34006 (21.8mg, 572.2 nmol) to give the single-stranded homo-heptamer X34009 (8.96 mg, 167.3 nmol, 29.2%). 5.53 mg, (103.3 nmol) of sense strand X34009 and 5.27 mg (723.1 nmol) of anti-sense strand X18795 were then annealed to yield 10.8 mg (103.3 nmol) of the corresponding double-stranded FVII homo-heptamer (XD-10640).

[00720] Example 36: Synthesis of FVII Octamer XD-10641

[00721] As shown in FIGS. 35A-35B, homo-octameric sense-strand of FVII siRNA X34010 with amino groups at both of the 3' termini and containing six dCdA cleavable linkers was synthesized and purified via the homo-dimeric sense-strand of FVII siRNA X30833 and the homo-hexameric sense-strand of FVII siRNA X34005. Disulfide group was cleaved from X34005 using DTT to give the corresponding 5-thiol derivative X34007 (11.5mg, 251nmol, 99.7%) which was reacted with the previously obtained mono-DTME homo-dimer derivative X30835 (3.85mg, 250.2 nmol) to give the single-stranded homo-octamer X34010 (5.2 mg, 85.0 nmol, 34.0%). 4.92 mg (80.33 nmol) of sense strand X34010 and 4.68 mg (642.4 nmol) of anti-sense strand X18795 were then annealed to yield 9.6 mg (80.3 nmol) of the corresponding double-stranded FVII homo-octamer (XD-10641).

[00722] Example 37: Animal Experiments

[00723] The serum half-lives of the homo-multimers XD-10635, XD-10636, XD-10637, XD-10638, XD-10639, XD-10640 and XD-10641 and the corresponding monomer XD-09794 were determined by iv bolus injection of test material at a concentration of 1ng/ml in x1 PBS via tail vein into 3 cohorts of 4 C57/BL6N female mice aged approx. 11 weeks per cohort. Dosage was 20mg/kg for both FVII monomer and FVII multimers and blood samples were drawn at 5, 30, 60, 120 and 360 minutes. The serum samples were digested with proteinase K and a specific complementary Atto425-Peptide Nucleic Acid-fluorescent probe was hybridized to the antisense strand. Subsequent AEX-HPLC analysis enabled discrimination of intact antisense strand from metabolites leading to high specificity of the method. Only values for the intact parent compound are shown in Table 17, below and illustrated in FIGS. 36A and 36B as smooth

line scatter plot and straight marked scatter plot of FVII siRNA levels in serum for FVII multimers over time, respectively.

Table 20: FVII siRNA levels in serum for FVII homo-multimers over time.

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
Saline	1 ng/mL	S1	25	0 mg/kg	F	7 days	BLOQ
Saline	1 ng/mL	S2	25	0 mg/kg	F	7 days	BLOQ
Saline	1 ng/mL	S3	25	0 mg/kg	F	7 days	BLOQ
Saline	1 ng/mL	S4	25	0 mg/kg	F	7 days	BLOQ
						Mean	BLOQ
						SD	n.a.
Monomer XD-09794	1 ng/mL	A1	1	20 mg/kg	F	5 min	30,988.3
Monomer XD-09794	1 ng/mL	A2	1	20 mg/kg	F	5 min	32,628.0
Monomer XD-09794	1 ng/mL	A3	1	20 mg/kg	F	5 min	37,508.9
Monomer XD-09794	1 ng/mL	A4	1	20 mg/kg	F	5 min	35,858.3
						Mean	34,245.9
						SD	2,970.8
Monomer XD-09794	1 ng/mL	A5	2	20 mg/kg	F	30 min	3,107.0
Monomer XD-09794	1 ng/mL	A6	2	20 mg/kg	F	30 min	3,520.2
Monomer XD-09794	1 ng/mL	A7	2	20 mg/kg	F	30 min	3,371.1
Monomer XD-09794	1 ng/mL	A8	2	20 mg/kg	F	30 min	2,664.5
						Mean	3,165.7
						SD	375.3
Monomer XD-09794	1 ng/mL	A1	1	20 mg/kg	F	1 h	1,339.8
Monomer XD-09794	1 ng/mL	A2	1	20 mg/kg	F	1 h	953.0
Monomer XD-09794	1 ng/mL	A3	1	20 mg/kg	F	1 h	1,435.8
Monomer XD-09794	1 ng/mL	A4	1	20 mg/kg	F	1 h	1,730.9
						Mean	1,364.9
						SD	321.1
Monomer XD-09794	1 ng/mL	A5	2	20 mg/kg	F	2 h	598.8
Monomer XD-09794	1 ng/mL	A6	2	20 mg/kg	F	2 h	202.7
Monomer XD-09794	1 ng/mL	A7	2	20 mg/kg	F	2 h	302.5
Monomer XD-09794	1 ng/mL	A8	2	20 mg/kg	F	2 h	124.6

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
						Mean	307.2
						SD	207.6
Monomer XD-09794	1 ng/mL	A9	3	20 mg/kg	F	6 h	4.2
Monomer XD-09794	1 ng/mL	A10	3	20 mg/kg	F	6 h	4.0
Monomer XD-09794	1 ng/mL	A11	3	20 mg/kg	F	6 h	3.5
Monomer XD-09794	1 ng/mL	A12	3	20 mg/kg	F	6 h	14.5
						Mean	6.6
						SD	5.3
Monomer XD-09794	1 ng/mL	A9	3	20 mg/kg	F	7 days	BLOQ
Monomer XD-09794	1 ng/mL	A10	3	20 mg/kg	F	7 days	BLOQ
Monomer XD-09794	1 ng/mL	A11	3	20 mg/kg	F	7 days	BLOQ
Monomer XD-09794	1 ng/mL	A12	3	20 mg/kg	F	7 days	BLOQ
						Mean	BLOQ
						SD	n.a.
						SD	
Dimer XD-10635	1 ng/mL	B1	4	20 mg/kg	F	5 min	82,272.1
Dimer XD-10635	1 ng/mL	B2	4	20 mg/kg	F	5 min	90,574.4
Dimer XD-10635	1 ng/mL	B3	4	20 mg/kg	F	5 min	94,213.6
Dimer XD-10635	1 ng/mL	B4	4	20 mg/kg	F	5 min	92,612.6
						Mean	89,918.2
						SD	5,310.5
Dimer XD-10635	1 ng/mL	B5	5	20 mg/kg	F	30 min	6,107.7
Dimer XD-10635	1 ng/mL	B6	5	20 mg/kg	F	30 min	5,204.0
Dimer XD-10635	1 ng/mL	B7	5	20 mg/kg	F	30 min	7,221.8
Dimer XD-10635	1 ng/mL	B8	5	20 mg/kg	F	30 min	6,677.9
						Mean	6,302.9
						SD	862.3
Dimer XD-10635	1 ng/mL	B1	4	20 mg/kg	F	1 h	2,114.2
Dimer XD-10635	1 ng/mL	B2	4	20 mg/kg	F	1 h	2,911.0
Dimer XD-10635	1 ng/mL	B3	4	20 mg/kg	F	1 h	2,722.5
Dimer XD-10635	1 ng/mL	B4	4	20 mg/kg	F	1 h	2,092.7
						Mean	2,460.1

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
						SD	419.0
Dimer XD-10635	1 ng/mL	B5	5	20 mg/kg	F	2 h	558.0
Dimer XD-10635	1 ng/mL	B6	5	20 mg/kg	F	2 h	348.9
Dimer XD-10635	1 ng/mL	B7	5	20 mg/kg	F	2 h	2,718.7
Dimer XD-10635	1 ng/mL	B8	5	20 mg/kg	F	2 h	549.0
						Mean	1,043.7
						SD	1,120.9
Dimer XD-10635	1 ng/mL	B9	6	20 mg/kg	F	6 h	16.9
Dimer XD-10635	1 ng/mL	B10	6	20 mg/kg	F	6 h	19.6
Dimer XD-10635	1 ng/mL	B11	6	20 mg/kg	F	6 h	30.3
Dimer XD-10635	1 ng/mL	B12	6	20 mg/kg	F	6 h	1,273.8
						Mean	335.2
						SD	625.8
Dimer XD-10635	1 ng/mL	B9	6	20 mg/kg	F	7 days	BLOQ
Dimer XD-10635	1 ng/mL	B10	6	20 mg/kg	F	7 days	BLOQ
Dimer XD-10635	1 ng/mL	B11	6	20 mg/kg	F	7 days	BLOQ
Dimer XD-10635	1 ng/mL	B12	6	20 mg/kg	F	7 days	BLOQ
						Mean	BLOQ
						SD	n.a.
Trimer XD-10636	1 ng/mL	C1	7	20 mg/kg	F	5 min	144,194.8
Trimer XD-10636	1 ng/mL	C2	7	20 mg/kg	F	5 min	172,691.1
Trimer XD-10636	1 ng/mL	C3	7	20 mg/kg	F	5 min	155,857.5
Trimer XD-10636	1 ng/mL	C4	7	20 mg/kg	F	5 min	147,988.0
						Mean	155,182.9
						SD	12,642.5
Trimer XD-10636	1 ng/mL	C5	8	20 mg/kg	F	30 min	15,887.3
Trimer XD-10636	1 ng/mL	C6	8	20 mg/kg	F	30 min	16,202.8
Trimer XD-10636	1 ng/mL	C7	8	20 mg/kg	F	30 min	17,932.3
Trimer XD-10636	1 ng/mL	C8	8	20 mg/kg	F	30 min	17,537.3
						Mean	16,889.9
						SD	997.2

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
Trimer XD-10636	1 ng/mL	C1	7	20 mg/kg	F	1 h	6,276.1
Trimer XD-10636	1 ng/mL	C2	7	20 mg/kg	F	1 h	3,939.7
Trimer XD-10636	1 ng/mL	C3	7	20 mg/kg	F	1 h	4,018.8
Trimer XD-10636	1 ng/mL	C4	7	20 mg/kg	F	1 h	4,884.4
						Mean	4,779.8
						SD	1,085.5
Trimer XD-10636	1 ng/mL	C5	8	20 mg/kg	F	2 h	102.7
Trimer XD-10636	1 ng/mL	C6	8	20 mg/kg	F	2 h	197.9
Trimer XD-10636	1 ng/mL	C7	8	20 mg/kg	F	2 h	1,680.9
Trimer XD-10636	1 ng/mL	C8	8	20 mg/kg	F	2 h	469.5
						Mean	612.8
						SD	728.9
Trimer XD-10636	1 ng/mL	C9	9	20 mg/kg	F	6 h	32.7
Trimer XD-10636	1 ng/mL	C10	9	20 mg/kg	F	6 h	8.0
Trimer XD-10636	1 ng/mL	C11	9	20 mg/kg	F	6 h	27.5
Trimer XD-10636	1 ng/mL	C12	9	20 mg/kg	F	6 h	12.1
						Mean	20.1
						SD	11.9
Trimer XD-10636	1 ng/mL	C9	9	20 mg/kg	F	7 days	BLOQ
Trimer XD-10636	1 ng/mL	C10	9	20 mg/kg	F	7 days	BLOQ
Trimer XD-10636	1 ng/mL	C11	9	20 mg/kg	F	7 days	BLOQ
Trimer XD-10636	1 ng/mL	C12	9	20 mg/kg	F	7 days	BLOQ
						Mean	BLOQ
						SD	n.a.
Tetramer XD-10637	1 ng/mL	D1	10	20 mg/kg	F	5 min	174,506.7
Tetramer XD-10637	1 ng/mL	D2	10	20 mg/kg	F	5 min	184,149.5
Tetramer XD-10637	1 ng/mL	D3	10	20 mg/kg	F	5 min	180,077.0
Tetramer XD-10637	1 ng/mL	D4	10	20 mg/kg	F	5 min	204,796.1
						Mean	185,882.3
						SD	13,214.1

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
Tetramer XD-10637	1 ng/mL	D5	11	20 mg/kg	F	30 min	89,104.1
Tetramer XD-10637	1 ng/mL	D6	11	20 mg/kg	F	30 min	88,408.8
Tetramer XD-10637	1 ng/mL	D7	11	20 mg/kg	F	30 min	79,389.4
Tetramer XD-10637	1 ng/mL	D8	11	20 mg/kg	F	30 min	83,820.0
						Mean	85,180.6
						SD	4,516.8
Tetramer XD-10637	1 ng/mL	D1	10	20 mg/kg	F	1 h	25,278.6
Tetramer XD-10637	1 ng/mL	D2	10	20 mg/kg	F	1 h	24,494.1
Tetramer XD-10637	1 ng/mL	D3	10	20 mg/kg	F	1 h	23,070.4
Tetramer XD-10637	1 ng/mL	D4	10	20 mg/kg	F	1 h	31,567.0
						Mean	26,102.5
						SD	3,755.9
Tetramer XD-10637	1 ng/mL	D5	11	20 mg/kg	F	2 h	9,191.5
Tetramer XD-10637	1 ng/mL	D6	11	20 mg/kg	F	2 h	8,969.4
Tetramer XD-10637	1 ng/mL	D7	11	20 mg/kg	F	2 h	5,059.5
Tetramer XD-10637	1 ng/mL	D8	11	20 mg/kg	F	2 h	14,666.2
						Mean	9,471.7
						SD	3,948.9
Tetramer XD-10637	1 ng/mL	D9	12	20 mg/kg	F	6 h	15.4
Tetramer XD-10637	1 ng/mL	D10	12	20 mg/kg	F	6 h	254.3
Tetramer XD-10637	1 ng/mL	D11	12	20 mg/kg	F	6 h	58.3
Tetramer XD-10637	1 ng/mL	D12	12	20 mg/kg	F	6 h	17.5
						Mean	86.4
						SD	113.7
Tetramer XD-10637	1 ng/mL	D9	12	20 mg/kg	F	7 days	BLOQ
Tetramer XD-10637	1 ng/mL	D10	12	20 mg/kg	F	7 days	BLOQ
Tetramer XD-10637	1 ng/mL	D11	12	20 mg/kg	F	7 days	BLOQ
Tetramer XD-10637	1 ng/mL	D12	12	20 mg/kg	F	7 days	BLOQ
						Mean	BLOQ
						SD	n.a.
Pentamer	1 ng/mL	E1	13	20 mg/kg	F	5 min	201,669.6

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
XD-10638							
Pentamer XD-10638	1 ng/mL	E2	13	20 mg/kg	F	5 min	214,525.8
Pentamer XD-10638	1 ng/mL	E3	13	20 mg/kg	F	5 min	247,544.7
Pentamer XD-10638	1 ng/mL	E4	13	20 mg/kg	F	5 min	207,872.5
						Mean	217,903.2
						SD	20,446.4
Pentamer XD-10638	1 ng/mL	E5	14	20 mg/kg	F	30 min	112,318.2
Pentamer XD-10638	1 ng/mL	E6	14	20 mg/kg	F	30 min	110,786.0
Pentamer XD-10638	1 ng/mL	E7	14	20 mg/kg	F	30 min	94,714.7
Pentamer XD-10638	1 ng/mL	E8	14	20 mg/kg	F	30 min	47,610.6
						Mean	91,357.4
						SD	30,231.8
Pentamer XD-10638	1 ng/mL	E1	13	20 mg/kg	F	1 h	48,800.2
Pentamer XD-10638	1 ng/mL	E2	13	20 mg/kg	F	1 h	46,770.8
Pentamer XD-10638	1 ng/mL	E3	13	20 mg/kg	F	1 h	57,711.0
Pentamer XD-10638	1 ng/mL	E4	13	20 mg/kg	F	1 h	42,458.4
						Mean	48,935.1
						SD	6,420.4
Pentamer XD-10638	1 ng/mL	E5	14	20 mg/kg	F	2 h	19,806.0
Pentamer XD-10638	1 ng/mL	E6	14	20 mg/kg	F	2 h	20,633.6
Pentamer XD-10638	1 ng/mL	E7	14	20 mg/kg	F	2 h	18,214.2
Pentamer XD-10638	1 ng/mL	E8	14	20 mg/kg	F	2 h	27,970.4
						Mean	21,656.1
						SD	4,327.6
Pentamer XD-10638	1 ng/mL	E9	15	20 mg/kg	F	6 h	16.4
Pentamer XD-10638	1 ng/mL	E10	15	20 mg/kg	F	6 h	15.7
Pentamer XD-10638	1 ng/mL	E11	15	20 mg/kg	F	6 h	14.2
Pentamer XD-10638	1 ng/mL	E12	15	20 mg/kg	F	6 h	49.4
						Mean	23.9
						SD	17.0
Pentamer XD-10638	1 ng/mL	E9	15	20 mg/kg	F	7 days	BLOQ
Pentamer XD-10638	1 ng/mL	E10	15	20 mg/kg	F	7 days	BLOQ

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
Pentamer XD-10638	1 ng/mL	E11	15	20 mg/kg	F	7 days	BLOQ
Pentamer XD-10638	1 ng/mL	E12	15	20 mg/kg	F	7 days	BLOQ
						Mean	BLOQ
						SD	n.a.
Hexamer XD-10639	1 ng/mL	F1	16	20 mg/kg	F	5 min	221,882.0
Hexamer XD-10639	1 ng/mL	F2	16	20 mg/kg	F	5 min	227,901.1
Hexamer XD-10639	1 ng/mL	F3	16	20 mg/kg	F	5 min	230,969.3
Hexamer XD-10639	1 ng/mL	F4	16	20 mg/kg	F	5 min	229,232.9
						Mean	227,496.3
						SD	3,948.1
Hexamer XD-10639	1 ng/mL	F5	17	20 mg/kg	F	30 min	125,871.8
Hexamer XD-10639	1 ng/mL	F6	17	20 mg/kg	F	30 min	145,598.8
Hexamer XD-10639	1 ng/mL	F7	17	20 mg/kg	F	30 min	76,775.7
Hexamer XD-10639	1 ng/mL	F8	17	20 mg/kg	F	30 min	107,085.3
						Mean	113,832.9
						SD	29,284.7
Hexamer XD-10639	1 ng/mL	F1	16	20 mg/kg	F	1 h	69,114.9
Hexamer XD-10639	1 ng/mL	F2	16	20 mg/kg	F	1 h	76,580.8
Hexamer XD-10639	1 ng/mL	F3	16	20 mg/kg	F	1 h	68,920.5
Hexamer XD-10639	1 ng/mL	F4	16	20 mg/kg	F	1 h	78,412.0
						Mean	73,257.1
						SD	4,952.6
Hexamer XD-10639	1 ng/mL	F5	17	20 mg/kg	F	2 h	25,963.0
Hexamer XD-10639	1 ng/mL	F6	17	20 mg/kg	F	2 h	33,380.4
Hexamer XD-10639	1 ng/mL	F7	17	20 mg/kg	F	2 h	19,372.0
Hexamer XD-10639	1 ng/mL	F8	17	20 mg/kg	F	2 h	31,798.1
						Mean	27,628.4
						SD	6,361.7
Hexamer XD-10639	1 ng/mL	F9	18	20 mg/kg	F	6 h	52.7
Hexamer XD-10639	1 ng/mL	F10	18	20 mg/kg	F	6 h	33.1
Hexamer	1 ng/mL	F11	18	20 mg/kg	F	6 h	69.4

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
XD-10639							
Hexamer XD-10639	1 ng/mL	F12	18	20 mg/kg	F	6 h	47.3
						Mean	50.6
						SD	15.0
Hexamer XD-10639	1 ng/mL	F9	18	20 mg/kg	F	7 days	BLOQ
Hexamer XD-10639	1 ng/mL	F10	18	20 mg/kg	F	7 days	BLOQ
Hexamer XD-10639	1 ng/mL	F11	18	20 mg/kg	F	7 days	BLOQ
Hexamer XD-10639	1 ng/mL	F12	18	20 mg/kg	F	7 days	3.4
						Mean	BLOQ
						SD	n.a.
Heptamer XD-10640	1 ng/mL	G1	19	20 mg/kg	F	5 min	189,155.8
Heptamer XD-10640	1 ng/mL	G2	19	20 mg/kg	F	5 min	203,092.8
Heptamer XD-10640	1 ng/mL	G3	19	20 mg/kg	F	5 min	227,234.0
Heptamer XD-10640	1 ng/mL	G4	19	20 mg/kg	F	5 min	266,250.0
						Mean	221,433.2
						SD	33,765.8
Heptamer XD-10640	1 ng/mL	G5	20	20 mg/kg	F	30 min	123,590.6
Heptamer XD-10640	1 ng/mL	G6	20	20 mg/kg	F	30 min	119,556.1
Heptamer XD-10640	1 ng/mL	G7	20	20 mg/kg	F	30 min	120,686.6
Heptamer XD-10640	1 ng/mL	G8	20	20 mg/kg	F	30 min	142,606.4
						Mean	126,609.9
						SD	10,798.9
Heptamer XD-10640	1 ng/mL	G1	19	20 mg/kg	F	1 h	73,022.3
Heptamer XD-10640	1 ng/mL	G2	19	20 mg/kg	F	1 h	58,856.0
Heptamer XD-10640	1 ng/mL	G3	19	20 mg/kg	F	1 h	64,204.3
Heptamer XD-10640	1 ng/mL	G4	19	20 mg/kg	F	1 h	74,596.0
						Mean	67,669.7
						SD	7,445.7
Heptamer XD-10640	1 ng/mL	G5	20	20 mg/kg	F	2 h	13,907.3
Heptamer XD-10640	1 ng/mL	G6	20	20 mg/kg	F	2 h	12,667.2
Heptamer XD-10640	1 ng/mL	G7	20	20 mg/kg	F	2 h	17,123.0

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
Heptamer XD-10640	1 ng/mL	G8	20	20 mg/kg	F	2 h	24,537.2
						Mean	17,058.7
						SD	5,327.6
Heptamer XD-10640	1 ng/mL	G9	21	20 mg/kg	F	6 h	45.5
Heptamer XD-10640	1 ng/mL	G10	21	20 mg/kg	F	6 h	151.3
Heptamer XD-10640	1 ng/mL	G11	21	20 mg/kg	F	6 h	56.1
Heptamer XD-10640	1 ng/mL	G12	21	20 mg/kg	F	6 h	58.7
						Mean	77.9
						SD	49.3
Heptamer XD-10640	1 ng/mL	G9	21	20 mg/kg	F	7 days	BLOQ
Heptamer XD-10640	1 ng/mL	G10	21	20 mg/kg	F	7 days	BLOQ
Heptamer XD-10640	1 ng/mL	G11	21	20 mg/kg	F	7 days	BLOQ
Heptamer XD-10640	1 ng/mL	G12	21	20 mg/kg	F	7 days	BLOQ
						Mean	BLOQ
						SD	n.a.
Octamer XD-10641	1 ng/mL	H1	22	20 mg/kg	F	5 min	203,428.1
Octamer XD-10641	1 ng/mL	H2	22	20 mg/kg	F	5 min	231,234.5
Octamer XD-10641	1 ng/mL	H3	22	20 mg/kg	F	5 min	243,057.5
Octamer XD-10641	1 ng/mL	H4	22	20 mg/kg	F	5 min	246,973.6
						Mean	231,173.4
						SD	19,669.6
Octamer XD-10641	1 ng/mL	H5	23	20 mg/kg	F	30 min	152,545.2
Octamer XD-10641	1 ng/mL	H6	23	20 mg/kg	F	30 min	116,917.0
Octamer XD-10641	1 ng/mL	H7	23	20 mg/kg	F	30 min	127,392.7
Octamer XD-10641	1 ng/mL	H8	23	20 mg/kg	F	30 min	119,659.9
						Mean	129,128.7
						SD	16,228.9
Octamer XD-10641	1 ng/mL	H1	22	20 mg/kg	F	1 h	59,270.6
Octamer XD-10641	1 ng/mL	H2	22	20 mg/kg	F	1 h	67,819.6
Octamer XD-10641	1 ng/mL	H3	22	20 mg/kg	F	1 h	74,942.8
Octamer	1 ng/mL	H4	22	20 mg/kg	F	1 h	75,228.9

Analyte ID	LLOQ	Animal ID	Group	Dose Level	Sex	Time point	[FVII] ng/mL
XD-10641							
						Mean	69,315.5
						SD	7,522.7
Octamer XD-10641	1 ng/mL	H5	23	20 mg/kg	F	2 h	37,353.7
Octamer XD-10641	1 ng/mL	H6	23	20 mg/kg	F	2 h	16,390.7
Octamer XD-10641	1 ng/mL	H7	23	20 mg/kg	F	2 h	27,527.3
Octamer XD-10641	1 ng/mL	H8	23	20 mg/kg	F	2 h	20,359.7
						Mean	25,407.9
						SD	9,201.2
Octamer XD-10641	1 ng/mL	H9	24	20 mg/kg	F	6 h	81.2
Octamer XD-10641	1 ng/mL	H10	24	20 mg/kg	F	6 h	76.9
Octamer XD-10641	1 ng/mL	H11	24	20 mg/kg	F	6 h	162.5
Octamer XD-10641	1 ng/mL	H12	24	20 mg/kg	F	6 h	138.5
						Mean	114.8
						SD	42.4
Octamer XD-10641	1 ng/mL	H9	24	20 mg/kg	F	7 days	BLOQ
Octamer XD-10641	1 ng/mL	H10	24	20 mg/kg	F	7 days	BLOQ
Octamer XD-10641	1 ng/mL	H11	24	20 mg/kg	F	7 days	BLOQ
Octamer XD-10641	1 ng/mL	H12	24	20 mg/kg	F	7 days	BLOQ
						Mean	BLOQ
						SD	n.a.

[00724] FIGS. 37A, 37B, 37C, and 37D show bar chart graphs of FVII siRNA levels in serum for FVII multimers at 5 minutes, 30 minutes, 60 minutes, and 120 minutes, respectively.

[00725] FIGS. 38A and 38B show total FVII siRNA levels in serum, as represented by area under the curve, for FVII multimers, in ng*min/mL and normalized to monomer AUC value.

Table 21: Area under the curve values for FVII siRNA monomer and multimers.

	Monomer	Dimer	3-mer	4-mer	5-mer	6-mer	7-mer	8-mer
5	34,245.0	82272	155182	185882	217903	227496	221433	231173
30	3,165.0	6302	16889	85180	91357	133832	126609	129128
60	1,364.0	2460	4779	26102	48935	73257	67669	69315

	Monomer	Dimer	3-mer	4-mer	5-mer	6-mer	7-mer	8-mer
120	307.0	1043	612	9471	21656	27268	17058	25407
360	6.6	625	20	86	23.9	50.6	77	115
Total AUC (min * ng / mL)	621727	1604630	2713490	7271583	10689448	13420917	11862813	13384588
AUC, Normalized to Monomer	1.0	2.6	4.4	11.7	17.2	21.6	19.1	21.5

[00726] The serum half-lives of the multimers were calculated from FVII concentration data using non-linear one phase decay according to the following equation:

$$Y = (Y0 - Plateau) * \exp(-k * x) + Plateau$$

wherein Y is the concentration at time X and the half-life is the natural log of 2/k. 4 different assumptions concerning the initial and final conditions were applied as follows:

1: No assumptions made about the data

2: All siRNAs were injected at the same initial concentration (i.e., the Conc at t=0 is the same for all).

3: All siRNA concentrations all decay to 0 at t=infinity.

4: All siRNAs were injected at the same initial concentration (i.e., the Conc at t=0 is the same for all) and all siRNA concentrations all decay to 0 at t=infinity.

Table 22: Calculated serum half-lives of FVII siRNA homo-multimers.

	Monomer	Dimer	3-mer	4-mer	5-mer	6-mer	7-mer	8-mer
½ life, no assumptions (min)	7.10	6.10	7.63	20.82	20.65	29.1	31.66	30.81
½ life, all samples plateau to 0 (min)	7.37	6.59	7.93	21.23	23.38	31.50	31.62	31.92
½ life, all samples start with same initial value = 231173	1.80	3.61	8.39	19.29	24.21	33.65	35.07	36.63
½ life, all samples start with same initial value = 123173, and plateau to 0 (min)	1.81	3.68	8.45	19.88	25.83	34.72	34.39	36.49

[00727] Example 38: Calculation of Time Taken for FVII siRNA Homo-Multimers to Reach Same FVII siRNA Concentration as Monomer at 5 Minutes.

[00728] Because the FVII concentration of the monomer was already significantly less than 50% of that injected at the first sample time (5 minutes) the time taken for the

serum FVII levels of the multimers to equal that of the monomer at 5 minutes were also calculated using the following equation:

$$Y = (Y0 - Plateau) * \exp(-k * x) + Plateau$$

wherein Plateau was set at the concentration of monomer at 5 minutes (34,245 ng/ml) and shown in FIG. 39.

[00729] The following calculations were performed to determine the time in minutes for FVII siRNA homo-multimers to reach concentration of FVII siRNA monomer at 5 minutes:

Y = (Y0 - Plateau) * exp (-k * x) + Plateau, where x is time in minutes
34245 = (231173 - 0) * e^(-kx) + 0, where x is minutes
34245 = 231173* ^e(-kx)
0.14813453 = e^-kx
In (0.14813453) = kx
-1.909625779 = kx

Table 23: Calculated times for FVII siRNA homo-multimers to reach concentration of FVII siRNA monomer at 5 minutes.

	Monomer	Dimer	3-mer	4-mer	5-mer	6-mer	7-mer	8-mer
k values for different constructs	0.3819	0.1882	0.08203	0.03487	0.02683	0.01996	0.02015	0.019
Time (min)	5.0	10.1	23.3	54.8	71.2	95.7	94.8	100.5

Example 39: Preparation of GalNAc 4:1:1 FVII:ApoB:TTR Hetero-hexamer of siRNA

[00730] A GalNAc 4:1:1 FVII:ApoB:TTR Hetero-hexamer was prepared via the reaction sequence shown in Figure 42. Oligos in the gray boxes were prepared on the synthesizer according to the methods above under "Additional General Procedure 1: Single Chain Oligonucleotide Synthesis", the remainder being prepared according to the procedures in Examples 1-6. Sequences prepared were as shown in the Key wherein "X", "x", and "Xf" represent a ribonucleotide, 2'-O-methylribonucleotide and 2'-fluoro-2'-deoxyribonucleotide, respectively. "InvdT" represents inverted deoxythymidine

residues and "s" represents phosphorothioate linkages. "(SHC_6)" and "(C_6SSC_6)" represent thiohexyl and dihexyldisulfide linkers, respectively. "C_6NH_2" and "C_6NH" are used interchangeably to represent the aminohexyl linker. "(DTME)" represents dithiobismaleimidoethane. The specific siRNA sequences are outlined below in Table 24.

[00731] Table 24: Sequences of oligonucleotides in Example 39

SEQ ID NO:	ss-ID	Sequence (5'-3')	Target(s)
156	X39850	(GalNAc3)(NHC6)gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(SHC6)(DTME)	FVII sense strand with trivalent GalNAc cluster at 5'-end and 3'-thiol DTME functionality
157	X39851	(NH2C6)gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(SHC6)	FVII sense strand with free 5'-amino group and 3'-thiol group
26	X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu	FVII antisense strand
158	X39855	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C6SSC6)UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu	FVII antisense linked to ApoB antisense via a disulfide linkage
159	X39852	gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C6SSC6) AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)	FVII sense linked to TTR sense via a disulfide linkage
160	X39854	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C6SSC6)usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu	FVII antisense linked to TTR antisense via a disulfide linkage
161	X39853	gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C6SSC6)csusAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)	FVII sense linked to ApoB sense via a disulfide linkage

[00732] As described above, the general reaction scheme for the generation of X39850, a GalNAc-conjugated FVII sense strand, and X39851, a FVII sense strand with a free 5'-amino group and a 3' thiol group, are shown in Fig. 43. Reverse-phase HPLC (RP-HPLC) and mass spectrometry (MS) were used to confirm the purity of X39850, X39851, X18795, and X39855 (Fig. 44 - Fig. 47).

[00733] Stepwise annealing was performed to obtain the desired heterodimeric duplex with an ApoB antisense overhang. First, the GalNAc-conjugated FVII sense strand X39850 was annealed with 1 mole equivalent of antisense X18795 to form the duplex X39850-X18795. RP-HPLC confirmed a duplex purity of 78.2% (Fig. 48). The FVII duplex X39850-X18795 was then conjugated with the FVII sense strand X39851 followed by ion exchange purification to form X39850-X18795- X39851. RP-HPLC confirmed a duplex purity of 87.0% (Fig. 49). Next, 1 mole equivalent of the dimeric antisense strand X39855 was added to the X39850-X18795-X39851 duplex to form X39850-X18795-X39851-X39855. RP-HPLC confirmed a duplex purity of 97.6% (Fig. 50).

[00734] X39852, a FVII sense strand linked to TTR sense strand via a disulfide linkage, X39854, a FVII antisense strand linked to TTR antisense strand via a disulfide linkage, and X39853, a FVII sense strand linked to ApoB sense strand via a disulfide linkage, were then generated. RP-HPLC and MS were used to confirm the purity of X39852, X39854, and X39853 (Fig. 51 - Fig. 53).

[00735] Stepwise annealing was performed to obtain the desired heterotrimeric duplex with an ApoB sense overhang. First, the dimeric sense strand X39852 was annealed with 1 mole equivalent of antisense X18795 to form the duplex X39852-X18795. RP-HPLC confirmed a duplex purity of 99.5% (Fig. 54). Next, 1 mole equivalent of the dimeric antisense strand X39854 was added to the X39852-X18795 duplex to form X39852-X18795-X39854. RP-HPLC confirmed a duplex purity of 94.9% (Fig. 55). Finally, 1 mole equivalent of the dimeric sense strand X39853 was added to the X39852-X18795-X39854 duplex followed by ion exchange purification to form X39852-X18795-X39854- X39853. RP-HPLC confirmed a duplex purity of 98.3% (Fig. 56).

[00736] The final GalNAc-conjugated hetero-hexameric siRNA was formed by annealing equimolar amounts of each of the above duplexes. RP-HPLC confirmed a duplex purity of 96.7% (Fig. 57A) and MS confirmed the presence of the correct species (Fig. 57B).

Example 40: Determination of Target Knockdown by a 4:1:1 FVII:ApoB:TTR Hetero-hexamer of siRNA

[00737] It is a feature of the disclosure that multimeric oligonucleotides have superior bioactivity when administered intravenously to the corresponding monomers administered subcutaneously.

[00738] This was demonstrated as follows: our GalNAc-directed 4:1:1 FVII:ApoB:TTR hetero-hexamer of siRNA (four units of siFVII, one unit of siApoB, and one unit of siTTR) was administered intravenously at a dose of 6 mg/kg (equivalent to 4 mg/kg for siFVII, 1 mg/kg for siApoB, and 1 mg/kg for siTTR) to a cohort of 5 mice according to the methods in "General Procedure 4: Animal Experiments" above. Blood samples were taken on days -1, 1, 3 and 7 and analyzed according to the methods and procedures described in "General Procedure 5: Measurement of Gene Knockdown" above.

[00739] The knockdowns at the various time points of TTR protein by the 4:1:1 hetero-hexamer administered by intravenous injection is shown in Figure 58.

[00740] It can be seen in FIG. 58 that the knockdown of TTR protein by the effective dose of 1 mg/kg siTTR via intravenous administration of 6 mg/kg GalNAc-directed 4:1:1 hetero-hexamer is approximately 60%. This exceeds the approximately 50% knockdown by 1 mg/kg of GalNAc-directed monomeric siTTR (with a virtually identical siRNA sequence) administered subcutaneously in Nair, J.K., *et al*; *J. Am. Chem. Soc.*, 2014, *136* (49), pp 16958–16961 (Fig. 5B). Accordingly, one subunit of siTTR within a GalNAc-directed hetero-hexameric siRNA delivered intravenously demonstrated the same or even increased *in vivo* activity when compared to the same dosage level of GalNAc-directed siTTR monomer delivered subcutaneously.

Example 41: Determination of Stability of Disulfide linkers

[00741] The stability of disulfide linkers was evaluated as summarized in FIG. 59. Those skilled in the art will appreciate that the dimers exemplified in FIG. 59 include a C_6SSC_6 linker that is an example of a disulfide linker of the general formula $-(CH_2)_n-S-S-(CH_2)_m-$ in which n and m are both 6. Other disulfide linkers of this general formula may be used in the manner described elsewhere herein, where n and m are each individually integers in the range of about 2 to about 8. For example, an embodiment provides a C_3SSC_3 disulfide linker in which n and m are each 3. Other linkers that may be used include nucleobase cleavable linkers such as UUU and dTdTdTdT. Those skilled in the art will also appreciate that linkers as described herein may be used to link combinations of sense and anti-sense strands.

CLAIMS

1. A multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and

the increase in activity of one or more subunits within the multimeric oligonucleotide is independent of phosphorothioate content in the multimeric oligonucleotide.

2. A multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and

at least one subunit within the multimeric oligonucleotide is a double-stranded oligonucleotide.

3. A multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and

the multimeric oligonucleotide comprises 4 or more subunits.

4. A multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and

the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

5. The multimeric oligonucleotide of claim 1, wherein at least one subunit within the multimeric oligonucleotide is a double-stranded oligonucleotide.

6. The multimeric oligonucleotide of claim 5, wherein the multimeric oligonucleotide comprises 4 or more subunits.

7. The multimeric oligonucleotide of claim 6, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

8. The multimeric oligonucleotide of claim 1, wherein the multimeric oligonucleotide comprises 4 or more subunits.

9. The multimeric oligonucleotide of claim 8, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

10. The multimeric oligonucleotide of claim 1, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

11. The multimeric oligonucleotide of claim 2, wherein the multimeric oligonucleotide comprises 4 or more subunits.

12. The multimeric oligonucleotide of claim 11, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

13. The multimeric oligonucleotide of claim 3, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

14. A multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to decrease its clearance due to glomerular filtration; and

the molecular weight of the multimeric oligonucleotide is at least about 45 kD,

wherein the multimeric oligonucleotide comprises a hetero-multimer of six or more subunits -------, wherein at least two subunits ------- are substantially different.

15. A multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide comprises five or more subunits -------; and

wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

16. A multimeric oligonucleotide comprising subunits ------ forming Structure 117:

------•------•------•------, wherein:

each subunit is independently a single or double-stranded oligonucleotide;

each of the subunits is joined to another subunit by a covalent linker •; and

wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

17. A multimeric oligonucleotide comprising two subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded

oligonucleotide, and each of the subunits ------- is joined to the other subunit by a covalent linker •;

the molecular weight of the compound is at least about 45 kD; and

at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

18. The multimeric oligonucleotide as in any one of claims 1-13 and 15-17, wherein at least two subunits ------- are substantially different.

19. The multimeric oligonucleotide of claim 18, wherein all of the subunits are substantially different.

20. The multimeric oligonucleotide as in any one of claims 1-17, wherein at least two subunits ------- are substantially the same or are identical.

21. The multimeric oligonucleotide as in any one of claims 1-13 and 15-17, wherein all of the subunits ------- are substantially the same or are identical.

22. The multimeric oligonucleotide as in any one of claims 1-13 and 15-17, wherein the multimeric oligonucleotide comprises five, six, seven, eight, nine, or ten subunits -------.

23. The multimeric oligonucleotide as in any one of claims 1-13 and 15-17, wherein the multimeric oligonucleotide comprises six subunits -------.

24. The multimeric oligonucleotide as in any one of claims 1-17, wherein the multimeric oligonucleotide comprises seven, eight, nine, or ten subunits -------.

25. The multimeric oligonucleotide as in any one of claims 1-24, wherein each subunit ------- is independently 10-30, 17-27, 19-26, or 20-25 nucleotides in length.

26. The multimeric oligonucleotide as in any one of claims 1-25, wherein one or more subunits are double-stranded.

27. The multimeric oligonucleotide as in any one of claims 1-26, wherein one or more subunits are single-stranded.

28. The multimeric oligonucleotide as in any one of claims 1-27, wherein the subunits comprise a combination of single-stranded and double-stranded oligonucleotides.

29. The multimeric oligonucleotide as in any one of claims 1-28, wherein one or more nucleotides within an oligonucleotide is an RNA, a DNA, or an artificial or non-natural nucleic acid analog.

30. The multimeric oligonucleotide as in any one of claims 1-29, wherein at least one of the subunits is RNA.

31. The multimeric oligonucleotide as in any one of claims 1-30, wherein at least one of the subunits is a siRNA, a saRNA, or a miRNA.

32. The multimeric oligonucleotide of claim 31, wherein at least one of the subunits is a siRNA.

33. The multimeric oligonucleotide of claim 31, wherein at least one of the subunits is a miRNA.

34. The multimeric oligonucleotide as in any one of claims 1-33, wherein at least one of the subunits is an antisense oligonucleotide.

35. The multimeric oligonucleotide as in any one of claims 1-34, wherein at least one of the subunits is a double-stranded siRNA.

36. The multimeric oligonucleotide of claim 35, wherein two or more siRNA subunits are joined by covalent linkers attached to the sense strands of the siRNA.

37. The multimeric oligonucleotide of claim 35, wherein two or more siRNA subunits are joined by covalent linkers attached to the antisense strands of the siRNA.

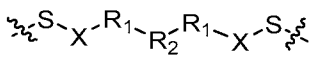
38. The multimeric oligonucleotide of claim 35, wherein two or more siRNA subunits are joined by covalent linkers attached to the sense strand of a first siRNA and the antisense strand of a second siRNA.

39. The multimeric oligonucleotide as in any one of claims 1-38, wherein one or more of the covalent linkers • comprise a cleavable covalent linker.

40. The multimeric oligonucleotide of claim 39, wherein the cleavable covalent linker contains an acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond.

41. The multimeric oligonucleotide as in any one of claims 39 and 40, in which the cleavable covalent linker is cleavable under intracellular conditions.

42. The multimeric oligonucleotide as in any one of claims 1-41, wherein at least one covalent linker comprises a disulfide bond or a compound of Formula (I):



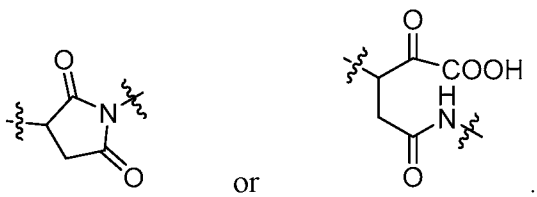
wherein:

S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit;

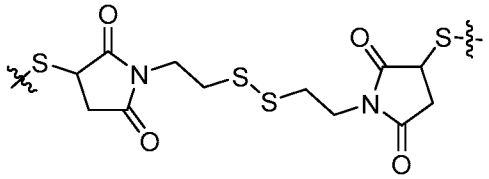
each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group;

R_2 is a thiopropionate or disulfide group; and

each X is independently selected from:

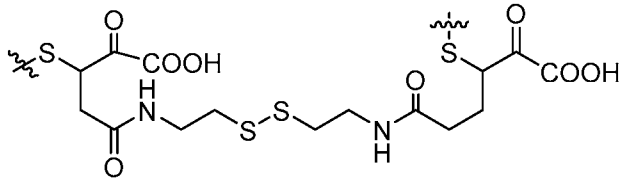


43. The multimeric oligonucleotide of claim 42, wherein the compound of Formula (I) is



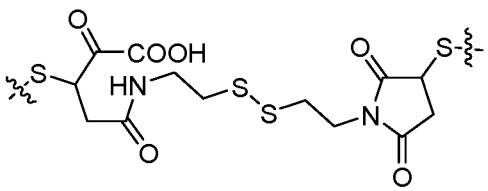
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

44. The multimeric oligonucleotide of claim 42, wherein the compound of Formula (I) is



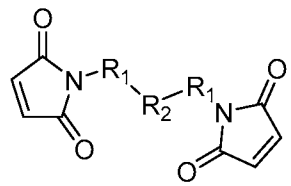
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

45. The multimeric oligonucleotide of claim 42, wherein the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

46. The multimeric oligonucleotide of any one of claims 42-45, wherein the covalent linker of Formula (I) is formed from a covalent linking precursor of Formula (II):



wherein:

each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and

R_2 is a thiopropionate or disulfide group.

47. The multimeric oligonucleotide as in any one of claims 1-46, wherein one or more of the covalent linkers • comprise a nucleotide linker.

48. The multimeric oligonucleotide of claim 47, wherein the nucleotide linker is between 2-6 nucleotides in length.

49. The multimeric oligonucleotide of claim 48, wherein the nucleotide linker is a dinucleotide linker.

50. The multimeric oligonucleotide as in any one of claims 1-49, wherein each covalent linker • is the same.

51. The multimeric oligonucleotide as in any one of claims 1-49, wherein the covalent linkers • comprise two or more different covalent linkers.

52. The multimeric oligonucleotide as in any one of claims 1-51, wherein at least two subunits are joined by covalent linkers • between the 3' end of a first subunit and the 3' end of a second subunit.

53. The multimeric oligonucleotide as in any one of claims 1-51, wherein at least two subunits are joined by covalent linkers • between the 3' end of a first subunit and the 5' end of a second subunit.

54. The multimeric oligonucleotide as in any one of claims 1-51, wherein at least two subunits are joined by covalent linkers • between the 5' end of a first subunit and the 3' end of a second subunit.

55. The multimeric oligonucleotide as in any one of claims 1-51, wherein at least two subunits are joined by covalent linkers • between the 5' end of a first subunit and the 5' end of a second subunit.

56. The multimeric oligonucleotide as in any one of claims 1-55, wherein the multimeric oligonucleotide further comprises one or more targeting ligands.

57. The multimeric oligonucleotide as in any one of claims 1-55, wherein at least one of the subunits is a targeting ligand.

58. The multimeric oligonucleotide as in any one of claims 56 and 57, wherein the targeting ligand is an aptamer.

59. The multimeric oligonucleotide of claim 56, wherein the targeting ligand comprises N-Acetylgalactosamine (GalNAc).

60. The multimeric oligonucleotide as in any one of claims 56 and 57, wherein the targeting ligand comprises an immunostimulant.

61. The multimeric oligonucleotide as in any one of claims 56 and 57, wherein the targeting ligand comprises a CpG oligonucleotide.

62. The multimeric oligonucleotide of claim 61, wherein the CpG oligonucleotide comprises the sequence TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 162).

63. The multimeric oligonucleotide of claim 61, wherein the CpG oligonucleotide comprises the sequence GGTGCATCGATGCAGGGGG (SEQ ID NO: 163).

64. The multimeric oligonucleotide as in any one of claims 1-63, wherein the multimeric oligonucleotide is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% pure.

65. The multimeric oligonucleotide as in any one of claims 1-64, wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

66. The multimeric oligonucleotide of claim 65, wherein the subunit with complementarity to TTR mRNA comprises increased activity *in vivo* relative to a monomeric oligonucleotide with complementarity to TTR mRNA.

67. The multimeric oligonucleotide of any one of claims 65 and 66, wherein the oligonucleotide with complementarity to TTR mRNA comprises UUAUAGAGCAAGAACACUGUUUU (SEQ ID NO: 164).

68. The multimeric oligonucleotide as in any one of claims 1-67, wherein the multimeric oligonucleotide is administered *in vivo* by intravenous injection.

69. The multimeric oligonucleotide of any one of claims 1-13, wherein the multimeric oligonucleotide is administered *in vivo* by intravenous injection and has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered subcutaneously in monomeric form.

70. The multimeric oligonucleotide of any one of claims 1-13 and 69, wherein the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide at least a 2-fold increase relative to in vivo activity of the same subunit when administered in monomeric form.

71. The multimeric oligonucleotide of claim 70, wherein the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide at least a 5-fold increase relative to in vivo activity of the same subunit when administered in monomeric form.

72. The multimeric oligonucleotide of claim 71, wherein the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide at least a 10-fold increase relative to in vivo activity of the same subunit when administered in monomeric form.

73. A method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and

the increase in activity of one or more subunits within the multimeric oligonucleotide is independent of phosphorothioate content in the multimeric oligonucleotide.

74. A method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and

at least one subunit within the multimeric oligonucleotide is a double-stranded oligonucleotide.

75. A method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and

the multimeric oligonucleotide comprises 4 or more subunits.

76. A method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form; and

the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

77. The method of claim 73, wherein at least one subunit within the multimeric oligonucleotide is a double-stranded oligonucleotide

78. The method of claim 77, wherein the multimeric oligonucleotide comprises 4 or more subunits.

79. The method of claim 78, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

80. The method of claim 73, wherein the multimeric oligonucleotide comprises 4 or more subunits.

81. The method of claim 80, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

82. The method of claim 73, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

83. The method of claim 74, wherein the multimeric oligonucleotide comprises 4 or more subunits.

84. The method of claim 83, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

85. The method of claim 75, wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD.

86. A method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form;

wherein the molecular weight of the multimeric oligonucleotide is at least about 45 kD; and

wherein the multimeric oligonucleotide comprises a hetero-multimer of six or more subunits -------, wherein at least two subunits ------- are substantially different.

87. A method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric

oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form;

the multimeric oligonucleotide comprises five or more subunits -------; and

wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

88. A method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form;

wherein the multimeric oligonucleotide comprises Structure 119:

------•------•------•------; and

wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

89. A method of administering a multimeric oligonucleotide to a subject in need thereof, the method comprising administering an effective amount of the multimeric oligonucleotide to the subject, the multimeric oligonucleotide comprising subunits -------, wherein:

each of the subunits ------- is independently a single or double-stranded

oligonucleotide, and each of the subunits ------- is joined to another subunit by a covalent linker •;

the multimeric oligonucleotide has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form;

wherein the multimeric oligonucleotide comprises two subunits -------;

wherein the molecular weight of the compound is at least about 45 kD; and

wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

90. The method as in any one of claims 73-89, wherein the administering comprises intravenous injection.

91. The method as in any one of claims 73-88, wherein the number of subunits contained in the multimeric oligonucleotide is m, m being an integer selected to enable the multimeric oligonucleotide to have the molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered in monomeric form.

92. The method as in any one of claims 73-85 and 87-89, wherein at least two subunits ------- are substantially different.

93. The method of claim 92, wherein all of the subunits are substantially different.

94. The method as in any one of claims 73-89, wherein at least two subunits ------- are substantially the same or are identical.

95. The method as in any one of claims 73-85 and 87-89, wherein all of the subunits ------- are substantially the same or are identical.

96. The method as in any one of claims 73-85 and 87-89, wherein the multimeric oligonucleotide comprises five, six, seven, eight, nine, or ten subunits -------.

97. The method as in any one of claims 73-85 and 87-89, wherein the multimeric oligonucleotide comprises six subunits -------.

98. The method as in any one of claims 73-89, wherein the multimeric oligonucleotide comprises seven, eight, nine, or ten subunits -------.

99. The method as in any one of claims 73-98, wherein each subunit ------- is independently 10-30, 17-27, 19-26, or 20-25 nucleotides in length.

100. The method as in any one of claims 73-99, wherein one or more subunits are double-stranded.

101. The method as in any one of claims 73-100, wherein one or more subunits are single-stranded.

102. The method as in any one of claims 73-101, wherein the subunits comprise a combination of single-stranded and double-stranded oligonucleotides.

103. The method as in any one of claims 73-102, wherein one or more nucleotides within an oligonucleotide is an RNA, a DNA, or an artificial or non-natural nucleic acid analog.

104. The method as in any one of claims 73-103, wherein at least one of the subunits is RNA.

105. The method as in any one of claims 73-104, wherein at least one of the subunits is a siRNA, a saRNA, or a miRNA.

106. The method of claim 105, wherein at least one of the subunits is a siRNA.

107. The method of claim 105, wherein at least one of the subunits is a miRNA.

108. The method as in any one of claims 73-107, wherein at least one of the subunits is an antisense oligonucleotide.

109. The method as in any one of claims 73-108, wherein at least one of the subunits is a double-stranded siRNA.

110. The method of claim 109, wherein two or more siRNA subunits are joined by covalent linkers attached to the sense strands of the siRNA.

111. The method of claim 109, wherein two or more siRNA subunits are joined by covalent linkers attached to the antisense strands of the siRNA.

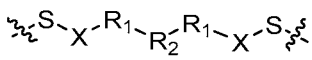
112. The method of claim 109, wherein two or more siRNA subunits are joined by covalent linkers attached to the sense strand of a first siRNA and the antisense strand of a second siRNA.

113. The method as in any one of claims 73-112, wherein one or more of the covalent linkers • comprise a cleavable covalent linker.

114. The method of claim 113, wherein the cleavable covalent linker contains an acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond.

115. The method as in any one of claims 113 and 114, in which the cleavable covalent linker is cleavable under intracellular conditions.

116. The method as in any one of claims 73-115, wherein at least one covalent linker comprises a disulfide bond or a compound of Formula (I):



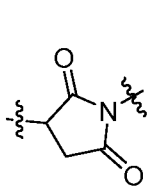
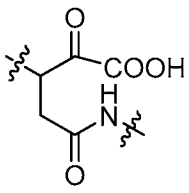
wherein:

S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit;

each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group;

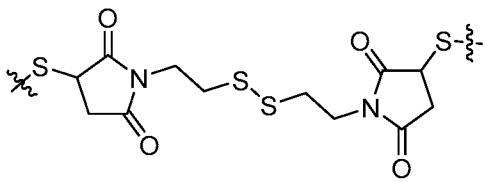
R_2 is a thiopropionate or disulfide group; and

each X is independently selected from:

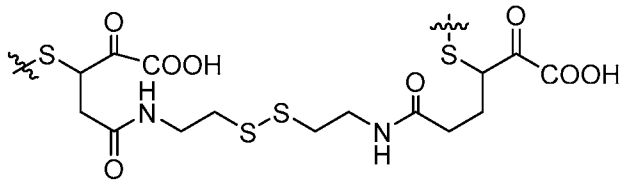
or .

117. The method of claim 116, wherein the compound of Formula (I) is



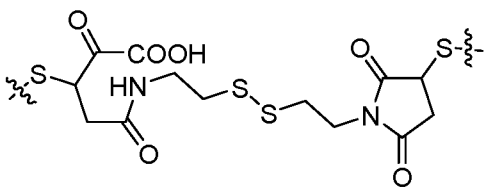
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

118. The method of claim 116, wherein the compound of Formula (I) is



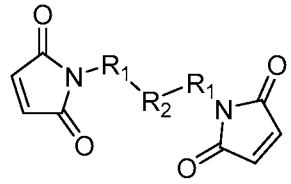
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

119. The method of claim 116, wherein the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of a subunit.

120. The method of any one of claims 116-119, wherein the covalent linker of Formula (I) is formed from a covalent linking precursor of Formula (II):



wherein:

each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and

R_2 is a thiopropionate or disulfide group.

121. The method as in any one of claims 73-120, wherein one or more of the covalent linkers • comprise a nucleotide linker.

122. The method of claim 121, wherein the nucleotide linker is between 2-6 nucleotides in length.

123. The method of claim 122, wherein the nucleotide linker is a dinucleotide linker.

124. The method as in any one of claims 73-123, wherein each covalent linker • is the same.

125. The method as in any one of claims 73-123, wherein the covalent linkers • comprise two or more different covalent linkers.

126. The method as in any one of claims 73-125, wherein at least two subunits are joined by covalent linkers • between the 3' end of a first subunit and the 3' end of a second subunit.

127. The method as in any one of claims 73-125, wherein at least two subunits are joined by covalent linkers • between the 3' end of a first subunit and the 5' end of a second subunit.

128. The method as in any one of claims 73-125, wherein at least two subunits are joined by covalent linkers • between the 5' end of a first subunit and the 3' end of a second subunit.

129. The method as in any one of claims 73-125, wherein at least two subunits are joined by covalent linkers • between the 5' end of a first subunit and the 5' end of a second subunit.

130. The method as in any one of claims 73-129, wherein the multimeric oligonucleotide further comprises one or more targeting ligands.

131. The method as in any one of claims 73-129, wherein at least one of the subunits is a targeting ligand.

132. The method as in any one of claims 130 and 131, wherein the targeting ligand is an aptamer.

133. The method as in claim 130, wherein the targeting ligand comprises N-Acetylgalactosamine (GalNAc).

134. The method as in any one of claims 130 and 131, wherein the targeting ligand comprises an immunostimulant.

135. The method as in any one of claims 130 and 131, wherein the targeting ligand comprises a CpG oligonucleotide.

136. The method of claim 135, wherein the CpG oligonucleotide comprises the sequence TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 162).

137. The method of claim 135, wherein the CpG oligonucleotide comprises the sequence GGTGCATCGATGCAGGGGG (SEQ ID NO: 163).

138. The method as in any one of claims 73-137, wherein the multimeric oligonucleotide is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% pure.

139. The method as in any one of claims 73-138, wherein at least one subunit comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

140. The method of claim 139, wherein the subunit with complementarity to TTR mRNA comprises increased activity *in vivo* relative to a monomeric oligonucleotide with complementarity to TTR mRNA.

141. The method of any one of claims 139 and 140, wherein the oligonucleotide with complementarity to TTR mRNA comprises UUAUAGAGCAAGAACACUGUUUU (SEQ ID NO: 164).

142. The method as in any one of claims 73-141, wherein the multimeric oligonucleotide is administered *in vivo* by intravenous injection.

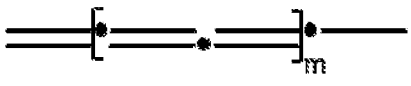
143. The method of any one of claims 73-85, wherein the multimeric oligonucleotide is administered *in vivo* by intravenous injection and has a molecular weight and/or size configured to increase *in vivo* activity of one or more subunits within the multimeric oligonucleotide relative to *in vivo* activity of the same subunit when administered subcutaneously in monomeric form.

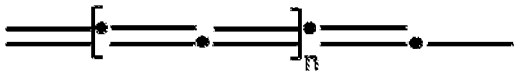
144. The method of any one of claims 73-85 and 143, wherein the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide at least a 2-fold increase relative to in vivo activity of the same subunit when administered in monomeric form.

145. The method of claim 144, wherein the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide at least a 5-fold increase relative to in vivo activity of the same subunit when administered in monomeric form.

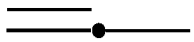
146. The method of claim 145, wherein the increase in *in vivo* activity of one or more subunits within the multimeric oligonucleotide at least a 10-fold increase relative to in vivo activity of the same subunit when administered in monomeric form.

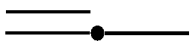
147. A method of synthesizing a multimeric oligonucleotide comprising Structure 92 or Structure 93:

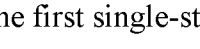
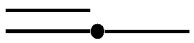

(Structure 92); or

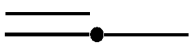
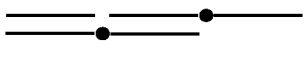

(Structure 93),

wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each • is a covalent linker joining adjacent oligonucleotides, and m is an integer ≥ 0 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 a heterodimer , and annealing the first single-stranded oligonucleotide ——— and the heterodimer , thereby forming ;

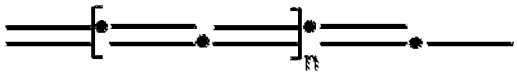
(ii) optionally annealing and a single-stranded dimer , thereby forming ; and

(iii) optionally annealing one or more additional single-stranded dimers ,

thereby forming Structure 92 or Structure 93.

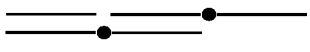
148. A method of synthesizing a multimeric oligonucleotide comprising Structure 92 or Structure 93:

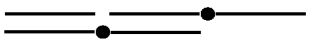

(Structure 92); or

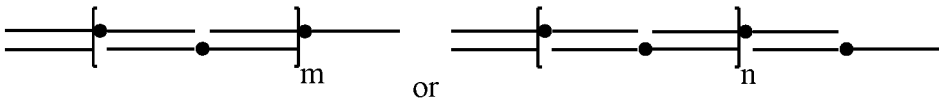

(Structure 93),

wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each • is a covalent linker joining adjacent oligonucleotides, and m is an integer ≥ 0 and n is an integer ≥ 0, the method comprising:

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

(ii) optionally annealing ═══•——— and a second single-stranded dimer ———•———, thereby forming  ; and

(iii) optionally annealing one or more additional single-stranded dimers ———•——— to , thereby forming


,

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

149. A method of synthesizing a multimeric oligonucleotide comprising:

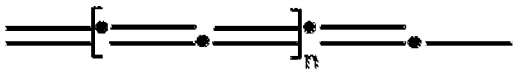

(Structure 94);


(Structure 95); or


(Structure 96),

wherein each——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each • is a covalent linker joining adjacent oligonucleotides, and p is an integer ≥ 0, q is an integer ≥ 0, and r is an integer ≥ 0, the method comprising:

(i) annealing Structure 92 and Structure 93: 

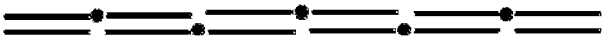
(Structure 92)  (Structure 93);

(ii) annealing a first Structure 92 with a second Structure 92; or

(iii) annealing a first Structure 93 and a second Structure 93,

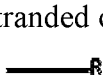
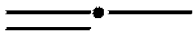
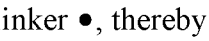
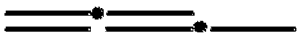
thereby forming Structure 94, Structure 95, or Structure 96, wherein m is an integer ≥ 0 and n is an integer ≥ 0.

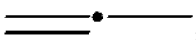
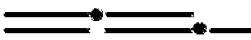
150. A method of synthesizing a multimeric oligonucleotide comprising Structure 97:

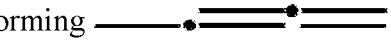
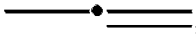
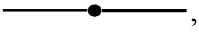
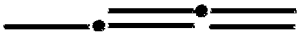
 (Structure 97),

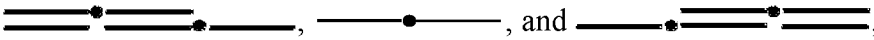
wherein each ——— is independently a single-stranded oligonucleotide, each  is independently a double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the steps of:

(i) forming  by:

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

(b) reacting the second single-stranded oligonucleotide ———R1 and the third single-stranded oligonucleotide ———R2, thereby forming a heterodimer ———•———, annealing the first single-stranded oligonucleotide ——— and the heterodimer ———•———, thereby forming , and annealing ———•——— with , thereby forming ;

(ii) forming  by annealing the first single-stranded oligonucleotide ——— and ———•———, thereby forming , and annealing ———•——— and , thereby forming ; and

(iii) forming  by annealing , thereby forming .

151. The method as in any one of claims 147-150, wherein a terminus of the multimeric oligonucleotide is conjugated to a targeting ligand.

152. The method as in any one of claims 147-151, wherein each ——— and ═══ is independently 10-30, 17-27, 19-26, or 20-25 nucleotides in length.

153. The method as in any one of claims 147-152, wherein one or more nucleotides within ——— and ═══ is an RNA, a DNA, or an artificial or non-natural nucleic acid analog.

154. The method as in any one of claims 147-153, wherein at least one of ——— and ═══is a RNA.

155. The method as in any one of claims 147-154, wherein at least one of ——— and ═══ is a siRNA, a saRNA, or a miRNA.

156. The method of claim 155, wherein at least one of ——— and ═══ is a siRNA.

157. The method of claim 155, wherein at least one ——— and ═══ is a miRNA.

158. The method as in any one of claims 147-153, wherein at least one of ———is an antisense oligonucleotide.

159. The method of claim 156, wherein two or more siRNA are joined by covalent linkers attached to the sense strands of the siRNA.

160. The method of claim 156, wherein two or more siRNA are joined by covalent linkers attached to the antisense strands of the siRNA.

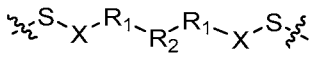
161. The method of claim 156, wherein two or more siRNA are joined by covalent linkers attached to the sense strand of a first siRNA and the antisense strand of a second siRNA.

162. The method as in any one of claims 147-161, wherein one or more of the covalent linkers • comprise a cleavable covalent linker.

163. The method of claim 162, wherein the cleavable covalent linker contains an acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond.

164. The method as in any one of claims 162 and 163, in which the cleavable covalent linker is cleavable under intracellular conditions.

165. The method as in any one of claims 147-164, wherein at least one covalent linker comprises a disulfide bond or a compound of Formula (I):



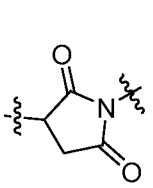
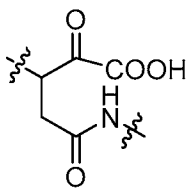
wherein:

S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══;

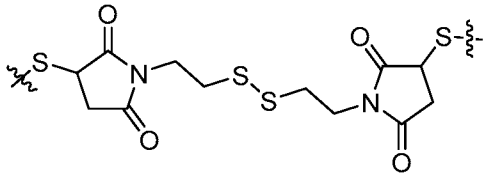
each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group;

R_2 is a thiopropionate or disulfide group; and

each X is independently selected from:

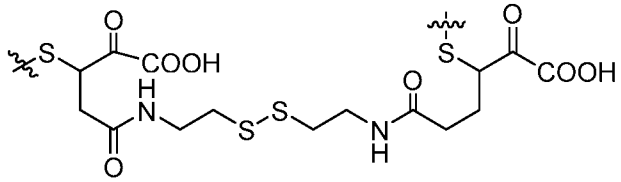
 or .

166. The method of claim 165, wherein the compound of Formula (I) is



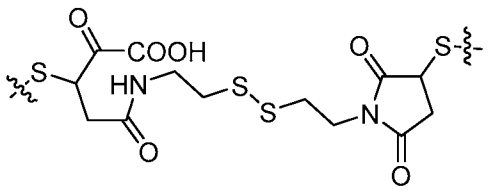
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══.

167. The method of claim 165, wherein the compound of Formula (I) is



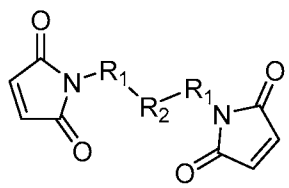
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══.

168. The method of claim 165, wherein the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——— or ═══.

169. The method as in any one of claims 164-168, wherein the covalent linker of Formula (I) is formed from a covalent linking precursor of Formula (II):



wherein:

each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and

R_2 is a thiopropionate or disulfide group.

170. The method as in any one of claims 147-169, wherein one or more of the covalent linkers • comprise a nucleotide linker.

171. The method of claim 170, wherein the nucleotide linker is between 2-6 nucleotides in length.

172. The method of claim 171, wherein the nucleotide linker is a dinucleotide linker.

173. The method as in any one of claims 147-172, wherein each covalent linker • is the same.

174. The method as in any one of claims 147-172, wherein the covalent linkers • comprise two or more different covalent linkers.

175. The method as in any one of claims 147-174, wherein two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 3' end of a first subunit and the 3' end of a second subunit.

176. The method as in any one of claims 147-174, wherein two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 3' end of a first subunit and the 5' end of a second subunit.

177. The method as in any one of claims 147-174, wherein two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 5' end of a first subunit and the 3' end of a subunit.

178. The method as in any one of claims 147-174, wherein two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 5' end of a first subunit and the 5' end of a second subunit.

179. The method as in any one of claims 147-174, wherein the multimeric oligonucleotide further comprises one or more targeting ligands.

180. The method as in any one of claims 147-179, wherein at least one of the oligonucleotide subunits is a targeting ligand.

181. The method as in any one of claims 179 and 180, wherein the targeting ligand is an aptamer.

182. The method as in any one of claims 147-179, wherein a terminus of the multimeric oligonucleotide is conjugated to a targeting ligand.

183. The method of claim179, wherein the targeting ligand comprises N-Acetylgalactosamine (GalNAc).

184. The method as in any one of claims 147-183, wherein the multimeric oligonucleotide is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% pure.

185. The method as in any one of claims 147-184, wherein at least one of the oligonucleotide subunits comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

186. The method of claim 185, wherein the oligonucleotide with complementarity to TTR mRNA comprises UUAUAGAGCAAGAACACUGUUUU (SEQ ID NO: 164).

187. The multimeric oligonucleotide of any one of claims 1-72, wherein one or more subunits comprise one or more phosphorothioate modifications.

188. The multimeric oligonucleotide of any one of claims 1-72, wherein one or more subunits comprise 1-3 phosphorothioate modifications at the 5' and/or 3' end.

189. The multimeric oligonucleotide of any one of claims 1-72, wherein each subunit comprises 1-10 phosphorothioate modifications.

190. The method of any one of claims 73-146, wherein one or more subunits comprise one or more phosphorothioate modifications.

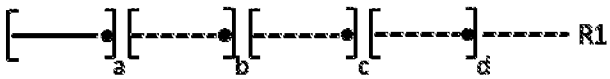
191. The method of any one of claims 73-146, wherein one or more subunits comprise 1-3 phosphorothioate modifications at the 5' and/or 3' end.

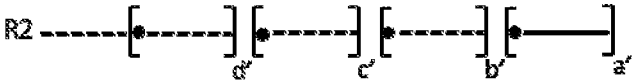
192. The method of any one of claims 73-146, wherein each subunit comprises 1-10 phosphorothioate modifications.

193. A method of synthesizing a multimeric oligonucleotide comprising Structure 100



(Structure 100), wherein each ——— is a single-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the steps of:

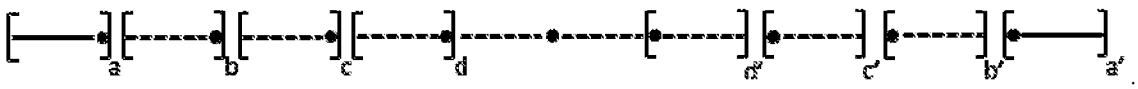
a) reacting Structure 98 

(Structure 98) with Structure 99 

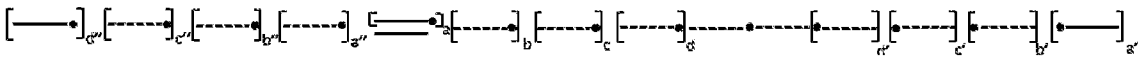
(Structure 99),

wherein:

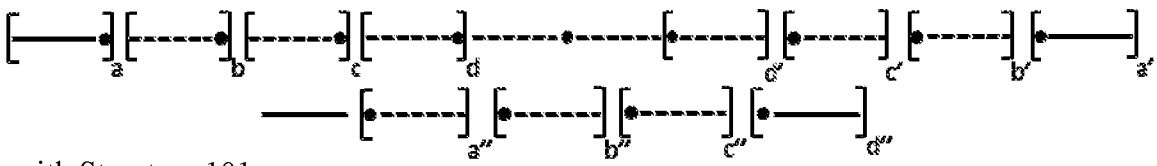
a, a', b, b', c, c', d and d' are each independently 0 or 1, and R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker •, thereby forming Structure 100



194. A method of synthesizing a multimeric oligonucleotide comprising Structure 102



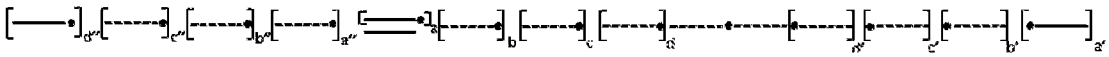
(Structure 102), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 100



with Structure 101 ,

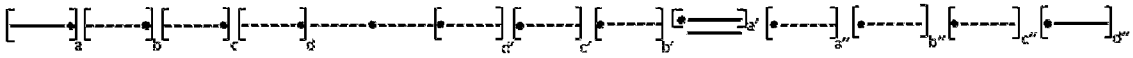
wherein:

a is 1, and a', a'', b, b', b'', c, c', c'', d, d', and d'' are each independently 0 or 1, thereby forming Structure 102

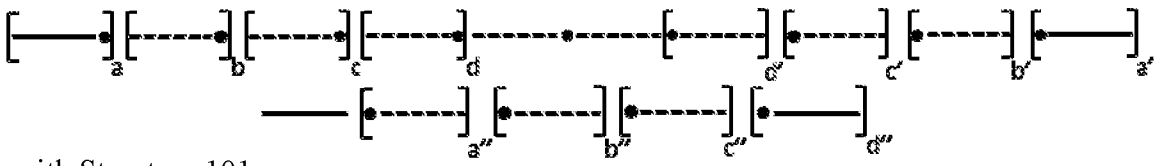


.

195. A method of synthesizing a multimeric oligonucleotide comprising Structure 103



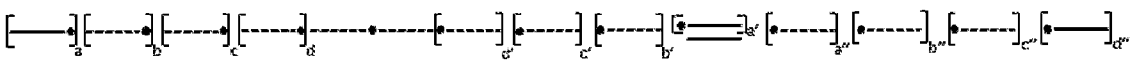
(Structure 103), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 100



with Structure 101 ,

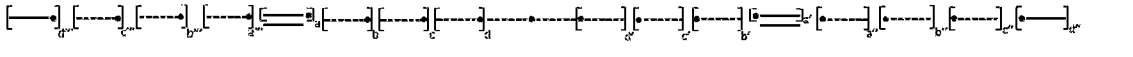
wherein:

a' is 1, and a, a'', b, b', b'', c, c', c'', d, d', and d'' are each independently 0 or 1, thereby forming Structure 103



.

196. A method of synthesizing a multimeric oligonucleotide comprising Structure 104



(Structure 104), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 103



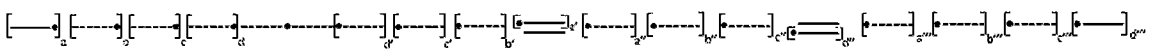
with Structure 105 ,

wherein:

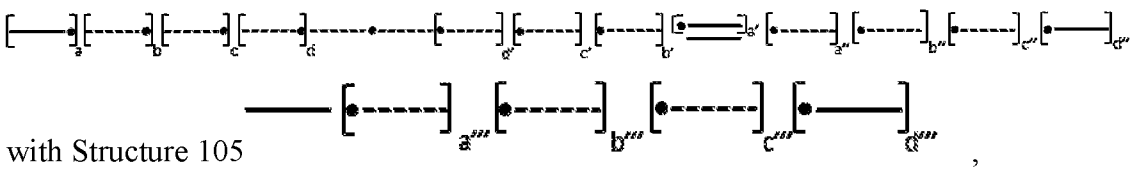
a and a' are 1, and a'', a''', b, b', b'', b''', c, c', c'', c''', d, d', d'', and d''' are each independently 0 or 1, thereby forming Structure 104



197. A method of synthesizing a multimeric oligonucleotide comprising Structure 107



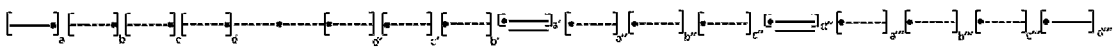
(Structure 107), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 103



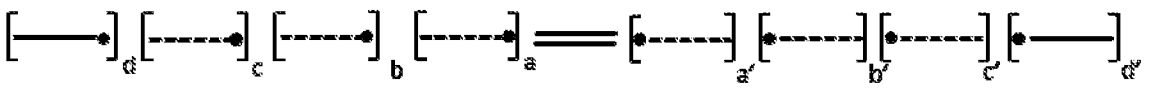
with Structure 105 ,

wherein:

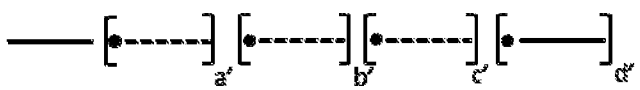
a and d'' are 1, and a', a'', a''', b, b', b'', b''', c, c', c'', c''', d, d', and d''' are each independently 0 or 1, thereby forming Structure 107



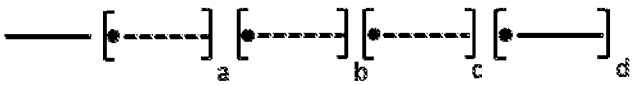
198. A method of synthesizing a multimeric oligonucleotide comprising Structure 108



(Structure 108), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure



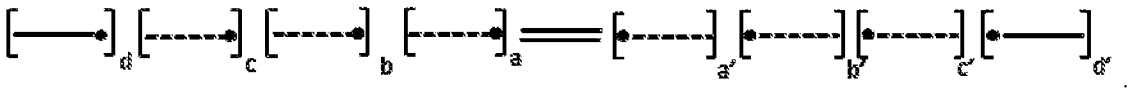
109 with Structure 110



,

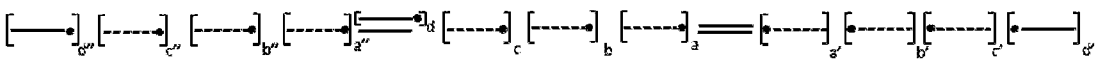
wherein:

a, a', b, b', c, c', d, and d' are each independently 0 or 1, thereby forming Structure 108

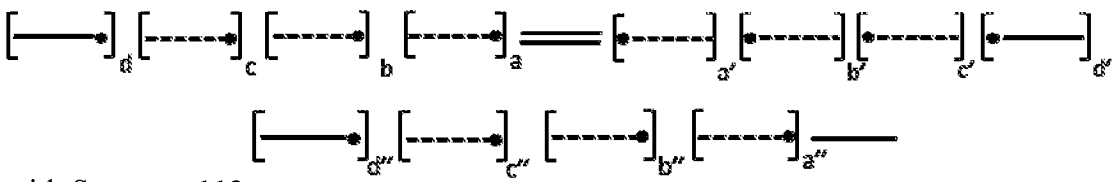


.

199. A method of synthesizing a multimeric oligonucleotide comprising Structure 111



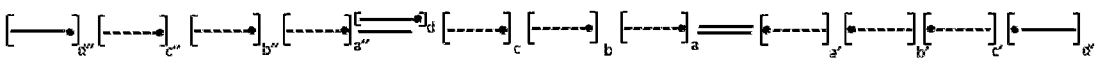
(Structure 111), wherein each ——— is independently a single-stranded oligonucleotide, each ═══ is independently a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure 108



with Structure 112 ,

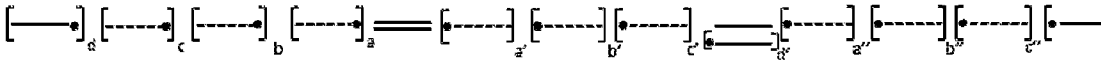
wherein:

d is 1, and a, a', a'', b, b', b'', c, c', c'', d' and d'' are each independently 0 or 1, thereby forming Structure 111



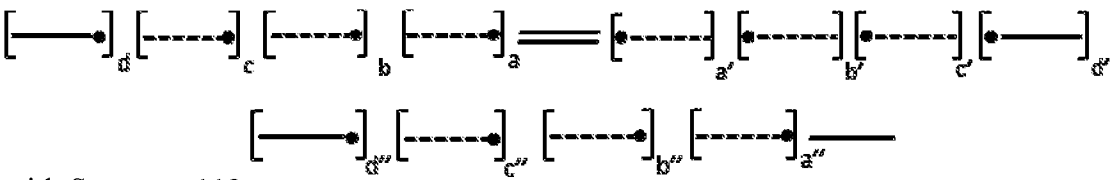
.

200. A method of synthesizing a multimeric oligonucleotide comprising Structure 113



(Structure 113), wherein each ——— is a single-stranded oligonucleotide, each ═══ is a double-stranded oligonucleotide, each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising the step of annealing Structure

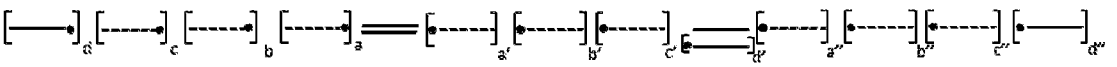
108



with Structure 112 ,

wherein:

d' is 1, and a, a', a'', b, b', b'', c, c', c'', d and d'' are each independently 0 or 1, thereby forming Structure 113



.

201. The method of any one of claims 193-200, further comprising annealing one or more single-stranded oligonucleotides ——— with a complementary single-stranded oligonucleotide ——— in Structure 100 to Structure 113, thereby forming a double-stranded oligonucleotide ═══.

202. The method of any one of claims 193-201, wherein each single-stranded oligonucleotide ——— and each single or double strand oligonucleotide ------ comprises 0-10 phosphorothioate modifications.

203. The method of any one of claims 193-202, wherein at least one ------ is a double-stranded oligonucleotide.

204. The method of any one of claims 193-203, wherein the total number of ——— and ------ in the multimeric oligonucleotide is at least 4.

205. The method of any one of claims 193-204, wherein the multimeric oligonucleotide is at least about 45 kD.

206. The method of any one of claims 193-205, wherein the multimeric oligonucleotide is selected from the group consisting of ------●------, ------●------●------, and ------●------●------●------, wherein each ------ is substanially the same or different.

207. The method of any one of claims 193-205, wherein the multimeric oligonucleotide is selected from the group consisting of

,

,

, and

, wherein each is substantially the same.

208. The method as in any one of claims 193-207, wherein a terminus of the multimeric oligonucleotide is conjugated to a targeting ligand.209. The method as in any one of claims 193-208, wherein each , , and is independently 10-30, 17-27, 19-26, or 20-25 nucleotides in length.

210. The method as in any one of claims 193-209, wherein one or more nucleotides within , , and is an RNA, a DNA, or an artificial or non-natural nucleic acid analog.

211. The method as in any one of claims 193-210, wherein at least one of , , and is a RNA.

212. The method as in any one of claims 193-211, wherein at least one of , , and is a siRNA, a saRNA, or a miRNA.

213. The method of claim 212, wherein at least one of , , and is a siRNA.

214. The method of claim 212, wherein at least one of , , and is a miRNA.

215. The method as in any one of claims 193-209, wherein at least one of and is an antisense oligonucleotide.

216. The method of claim 213, wherein two or more siRNA are joined by covalent linkers attached to the sense strands of the siRNA.

217. The method of claim 213, wherein two or more siRNA are joined by covalent linkers attached to the antisense strands of the siRNA.

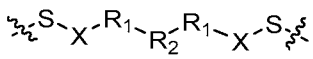
218. The method of claim 213, wherein two or more siRNA are joined by covalent linkers attached to the sense strand of a first siRNA and the antisense strand of a second siRNA.

219. The method as in any one of claims 193-218, wherein one or more of the covalent linkers • comprise a cleavable covalent linker.

220. The method of claim 219, wherein the cleavable covalent linker contains an acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond.

221. The method as in any one of claims 219 and 220, in which the cleavable covalent linker is cleavable under intracellular conditions.

222. The method as in any one of claims 193-221, wherein at least one covalent linker comprises a disulfide bond or a compound of Formula (I):



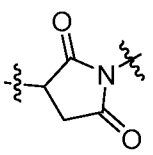
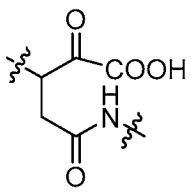
wherein:

S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——, ═══, or ------;

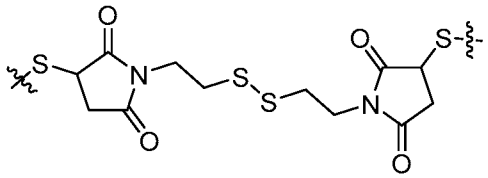
each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group;

R_2 is a thiopropionate or disulfide group; and

each X is independently selected from:

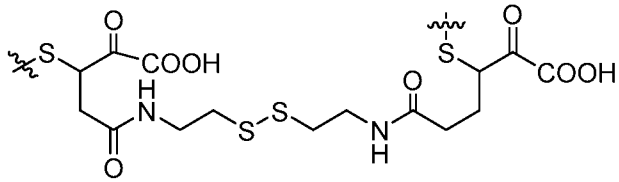
 or .

223. The method of claim 222, wherein the compound of Formula (I) is



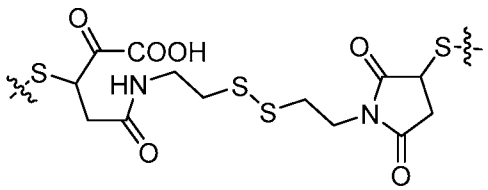
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——, ═══, or - - - - - -.

224. The method of claim 222, wherein the compound of Formula (I) is



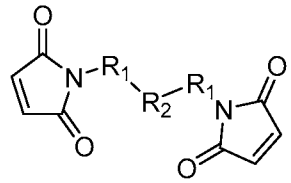
and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——, ═══, - - - - - -.

225. The method of claim 222, wherein the compound of Formula (I) is



and wherein S is attached by a covalent bond or by a linker to the 3' or 5' terminus of ——, ═══, - - - - - -.

226. The method as in any one of claims 193-225, wherein the covalent linker of Formula (I) is formed from a covalent linking precursor of Formula (II):



wherein:

each R_1 is independently a C_2-C_{10} alkyl, alkoxy, or aryl group; and

R_2 is a thiopropionate or disulfide group.

227. The method as in any one of claims 193-226, wherein one or more of the covalent linkers • comprise a nucleotide linker.

228. The method of claim 227, wherein the nucleotide linker is between 2-6 nucleotides in length.

229. The method of claim 228, wherein the nucleotide linker is a dinucleotide linker.

230. The method as in any one of claims 193-229, wherein each covalent linker • is the same.

231. The method as in any one of claims 193-229, wherein the covalent linkers • comprise two or more different covalent linkers.

232. The method as in any one of claims 193-231, wherein two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 3' end of a first subunit and the 3' end of a second subunit.

233. The method as in any one of claims 193-231, wherein two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 3' end of a first subunit and the 5' end of a second subunit.

234. The method as in any one of claims 193-231, wherein two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 5' end of a first subunit and the 3' end of a subunit.

235. The method as in any one of claims 193-231, wherein two or more adjacent oligonucleotide subunits are joined by covalent linkers • between the 5' end of a first subunit and the 5' end of a second subunit.

236. The method as in any one of claims 193-235, wherein the multimeric oligonucleotide further comprises one or more targeting ligands.

237. The method as in any one of claims 193-236, wherein at least one of the oligonucleotide subunits is a targeting ligand.

238. The method as in any one of claims 236 and 237, wherein the targeting ligand is an aptamer.

239. The method as in any one of claims 193-238, wherein a terminus of the multimeric oligonucleotide is conjugated to a targeting ligand.

240. The method of claim 236, wherein the targeting ligand comprises N-Acetylgalactosamine (GalNAc).

241. The method as in any one of claims 193-240, wherein the multimeric oligonucleotide is at least 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% pure.

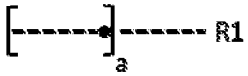
242. The method as in any one of claims 193-241, wherein at least one of the oligonucleotide subunits comprises an oligonucleotide with complementarity to transthyretin (TTR) mRNA.

243. The method of claim 242, wherein the oligonucleotide with complementarity to TTR mRNA comprises UUAUAGAGCAAGAACACUGUUUU (SEQ ID NO: 164).

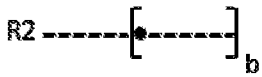
244. A method of synthesizing a multimeric oligonucleotide comprising Structure 114



(Structure 114), wherein each ------ is independently a single or double-stranded oligonucleotide, and each • is a covalent linker joining adjacent oligonucleotides, the method comprising reacting Structure 115



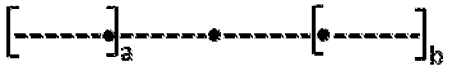
(Structure 115) with Structure 116



(Structure 116),

wherein:

R1 and R2 are chemical moieties capable of reacting directly or indirectly to form a covalent linker ●, a and b are each independently an integer ≥ 0, with the proviso that the sum of a and b is ≥ 4, thereby forming Structure 114



245. The method of claim 244, wherein Structure 115 and/or Structure 116 further comprise one or more targeting ligands.

246. The method of claim 245, wherein the targeting ligand is a terminal targeting ligand.

247. The method as in any one of claims 244-246, wherein a is an integer of 4, 5, 6, 7, 8, 9, or 10.

248. The method as in any one of claims 244-247, wherein b is an integer of 4, 5, 6, 7, 8, 9, or 10.

249. The method as in any one of claims 151, 182, 208, and 239, wherein the multimeric oligonucleotide is conjugated via a covalent bond.

250. The multimeric oligonucleotide of claim 14, wherein the at least two subunits that are substantially different are siRNAs, miRNAs or antisense oligonucleotides targeting different molecular targets *in vivo*.

251. The multimeric oligonucleotide of claim 250, wherein the at least two subunits that are substantially different are siRNAs.

252. The multimeric oligonucleotide of claim 250, wherein the at least two subunits that are substantially different are miRNAs.

253. The multimeric oligonucleotide of claim 250, wherein the at least two subunits that are substantially different are antisense oligonucleotides.

254. The multimeric oligonucleotide of any one of claims 250-253, further comprising a targeting ligand.

255. The multimeric oligonucleotide of claim 254, wherein the targeting ligand comprises an aptamer, N-Acetylgalactosamine (GalNAc), an immunostimulant or a CpG oligonucleotide.

256. The multimeric oligonucleotide of any one of claims 250-255, wherein one or more of the covalent linkers • comprise a cleavable covalent linker.

257. The multimeric oligonucleotide of claim 256, wherein the cleavable covalent linker contains an acid cleavable bond, a reductant cleavable bond, a bio-cleavable bond, or an enzyme cleavable bond.

258. The multimeric oligonucleotide of claim 256 or 257, wherein the cleavable covalent linker comprises a disulfide bond.

259. The multimeric oligonucleotide of any one of claims 256-258, wherein the cleavable covalent linker is cleavable under intracellular conditions.

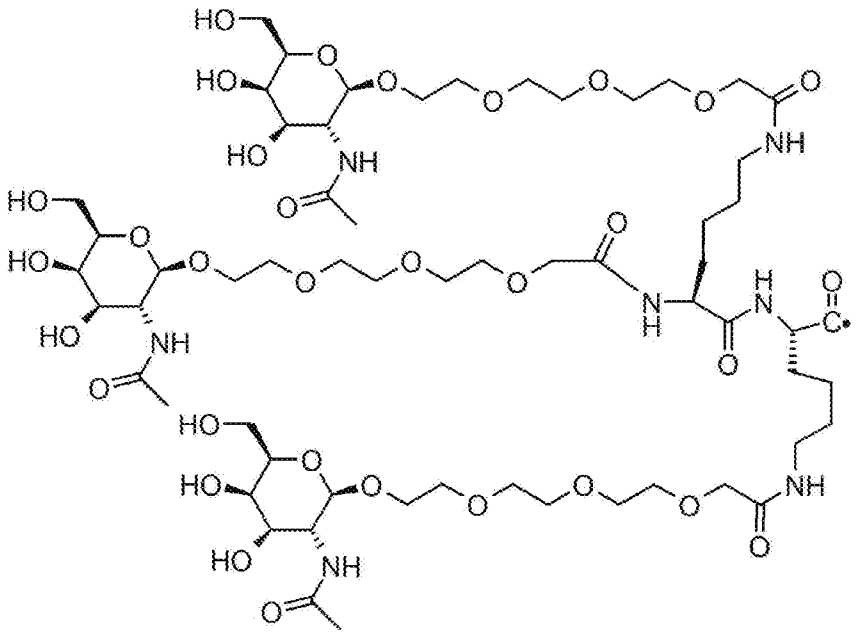


FIG. 1A

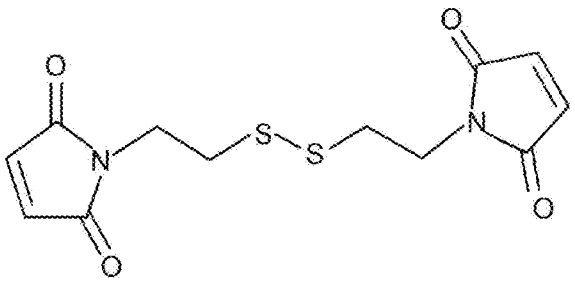


FIG. 1B

FIG. 2

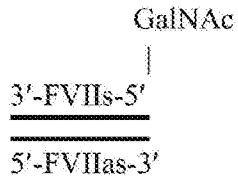

GalNAc
3′-FVIIs-5′
5′-FVIIas-3′

FIG. 3

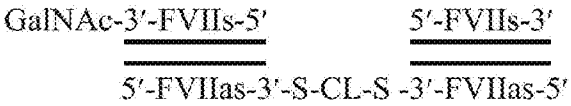

GalNAc-3′-FVIIs-5′
5′-FVIIs-3′
5′-FVIIas-3′-S-CL-S -3′-FVIIas-5′

FIG. 4

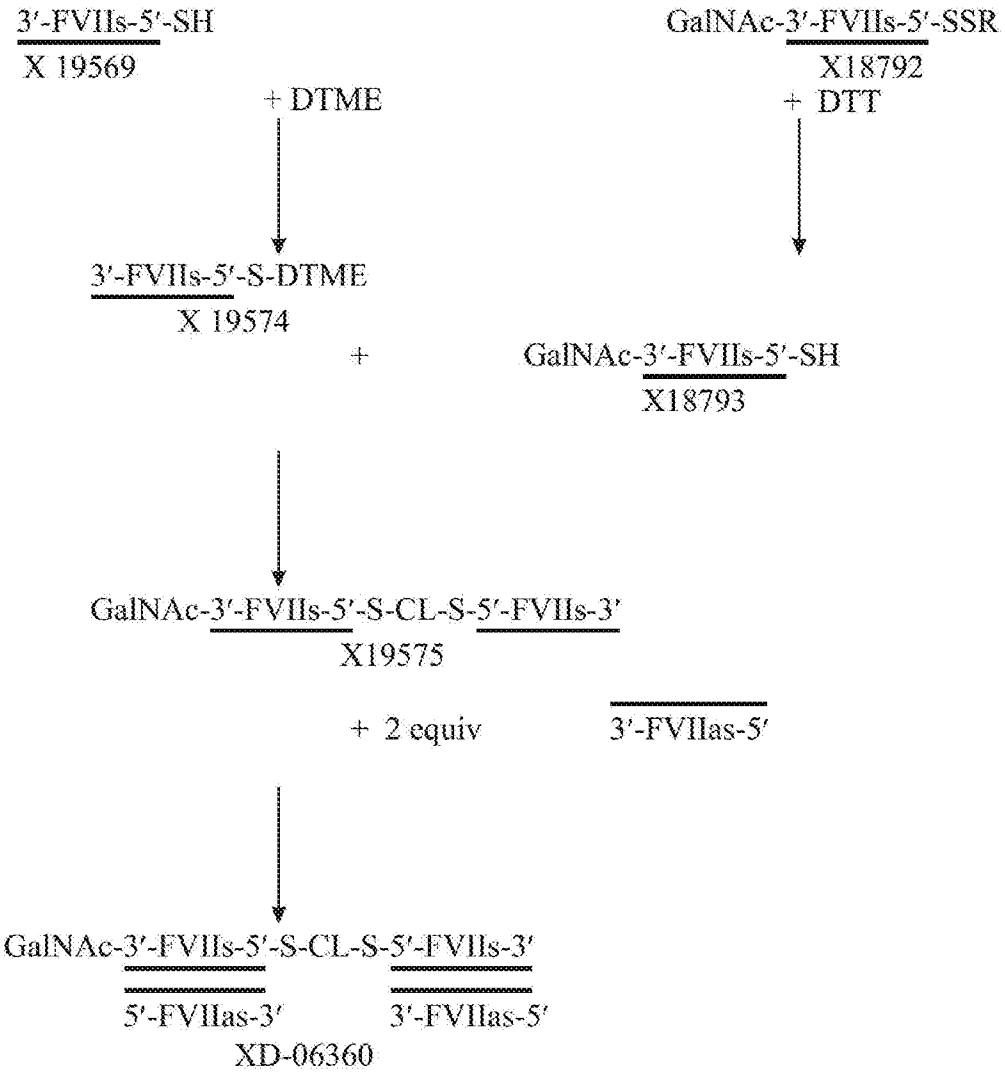
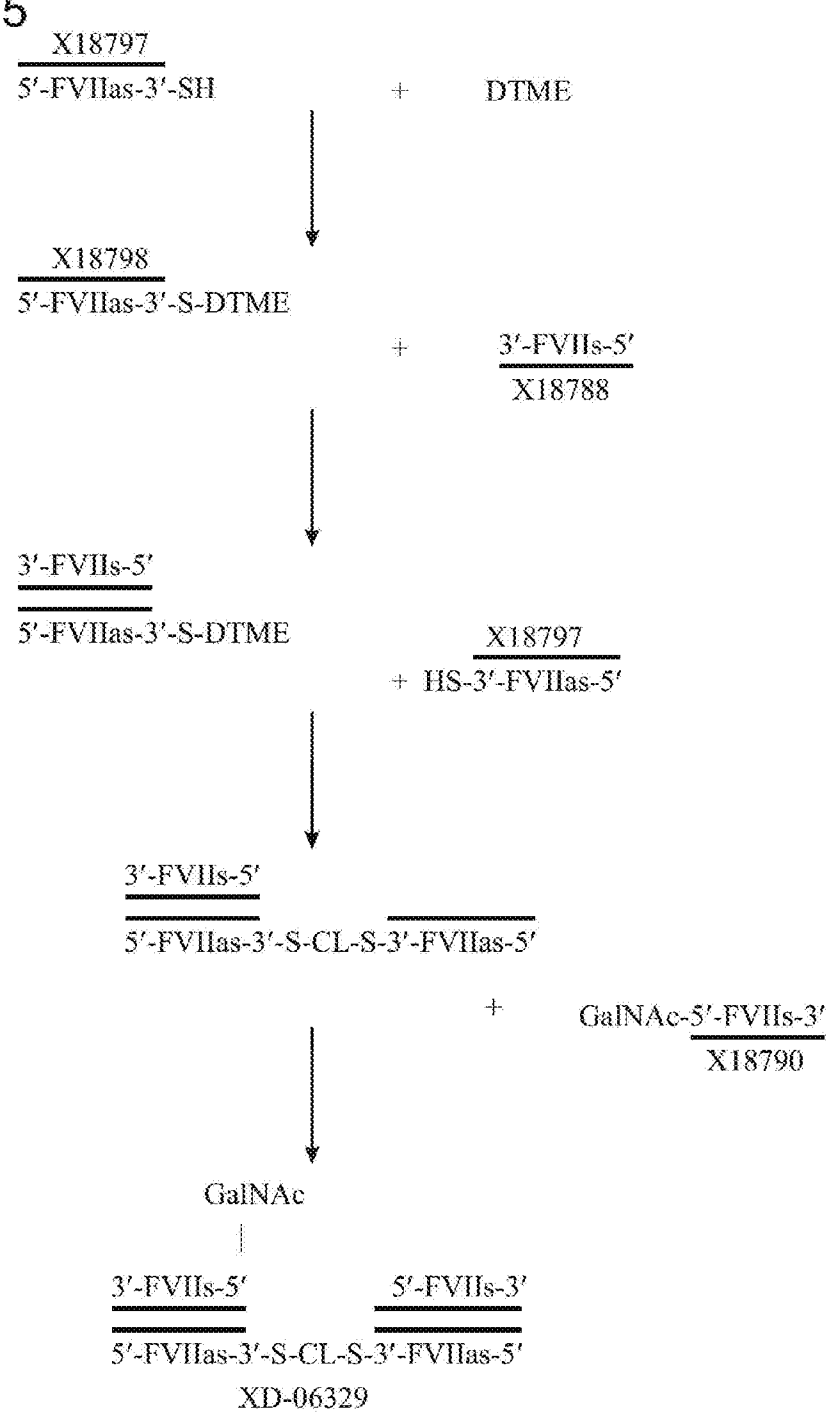

3′-FVIIs-5′-SH
X 19569
+ DTME
GalNAc-3′-FVIIs-5′-SSR
X18792
+ DTT
3′-FVIIs-5′-S-DTME
X 19574
+
GalNAc-3′-FVIIs-5′-SH
X18793
GalNAc-3′-FVIIs-5′-S-CL-S-5′-FVIIs-3′
X19575
+ 2 equiv
3′-FVIIas-5′
GalNAc-3′-FVIIs-5′-S-CL-S-5′-FVIIs-3′
5′-FVIIas-3′
3′-FVIIas-5′
XD-06360

FIG. 5


X18797
5′-FVIIas-3′-SH
+
DTME
X18798
5′-FVIIas-3′-S-DTME
+
3′-FVIIs-5′
X18788
3′-FVIIs-5′
5′-FVIIas-3′-S-DTME
X18797
+ HS-3′-FVIIas-5′
3′-FVIIs-5′
5′-FVIIas-3′-S-CL-S-3′-FVIIas-5′
+
GalNAc-5′-FVIIs-3′
X18790
GalNAc
3′-FVIIs-5′
5′-FVIIas-3′-S-CL-S-3′-FVIIas-5′
5′-FVIIs-3′
XD-06329

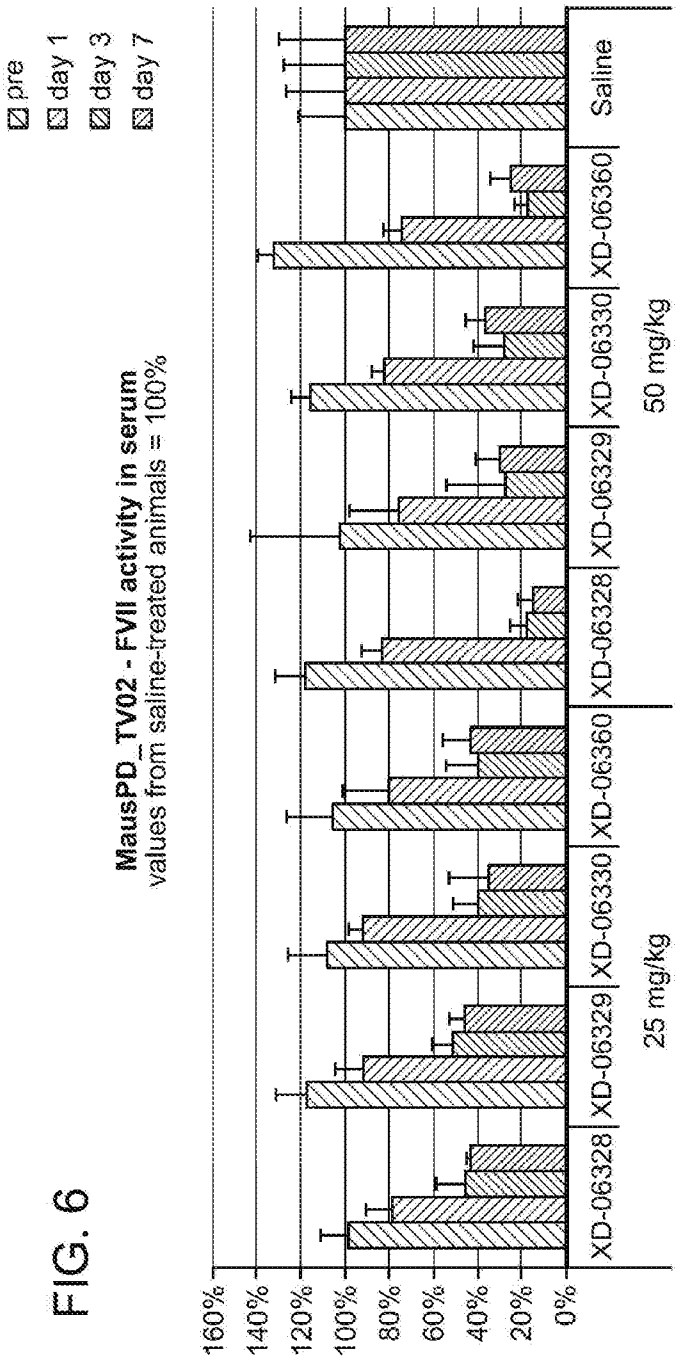

pre
day 1
day 3
day 7
MausPD_TV02 - FVII activity in serum
values from saline-treated animals = 100%
160%
140%
120%
100%
80%
60%
40%
20%
0%
XD-06328
XD-06329
XD-06330
XD-06360
XD-06328
XD-06329
XD-06330
XD-06360
Saline
25 mg/kg
50 mg/kg

FIG. 6

FIG. 7A

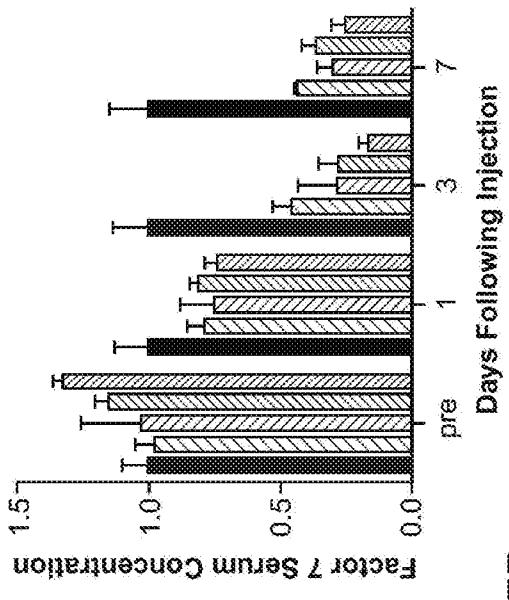


FIG. 7B

FIG. 7C

FIG. 8

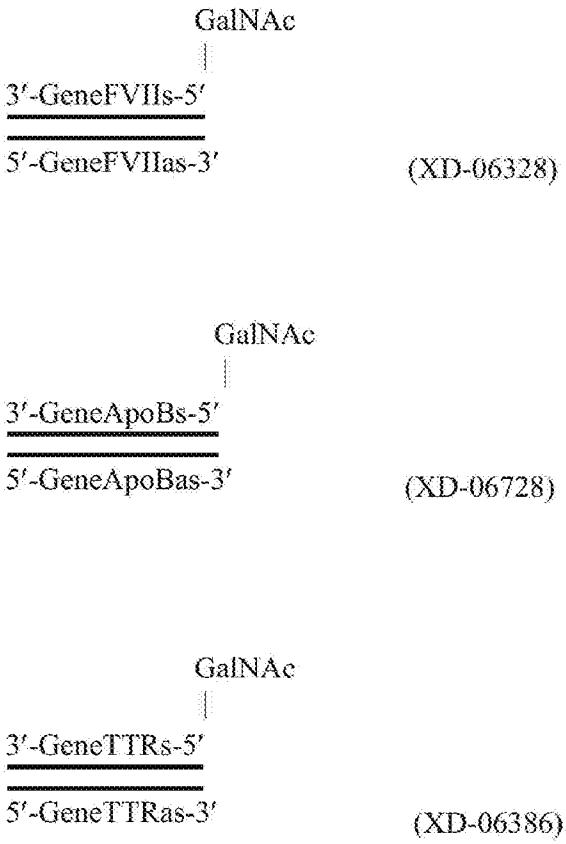

GalNAc
3′-GeneFVIIs-5′
5′-GeneFVIIas-3′
(XD-06328)
GalNAc
3′-GeneApoBs-5′
5′-GeneApoBas-3′
(XD-06728)
GalNAc
3′-GeneTTRs-5′
5′-GeneTTRas-3′
(XD-06386)

FIG. 9

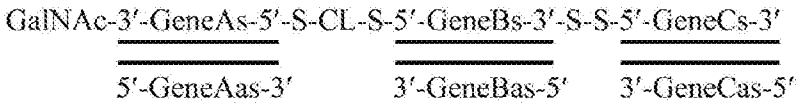

GalNAc-3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′-S-S-5′-GeneCs-3′
5′-GeneAas-3′
3′-GeneBas-5′
3′-GeneCas-5′

FIG. 10

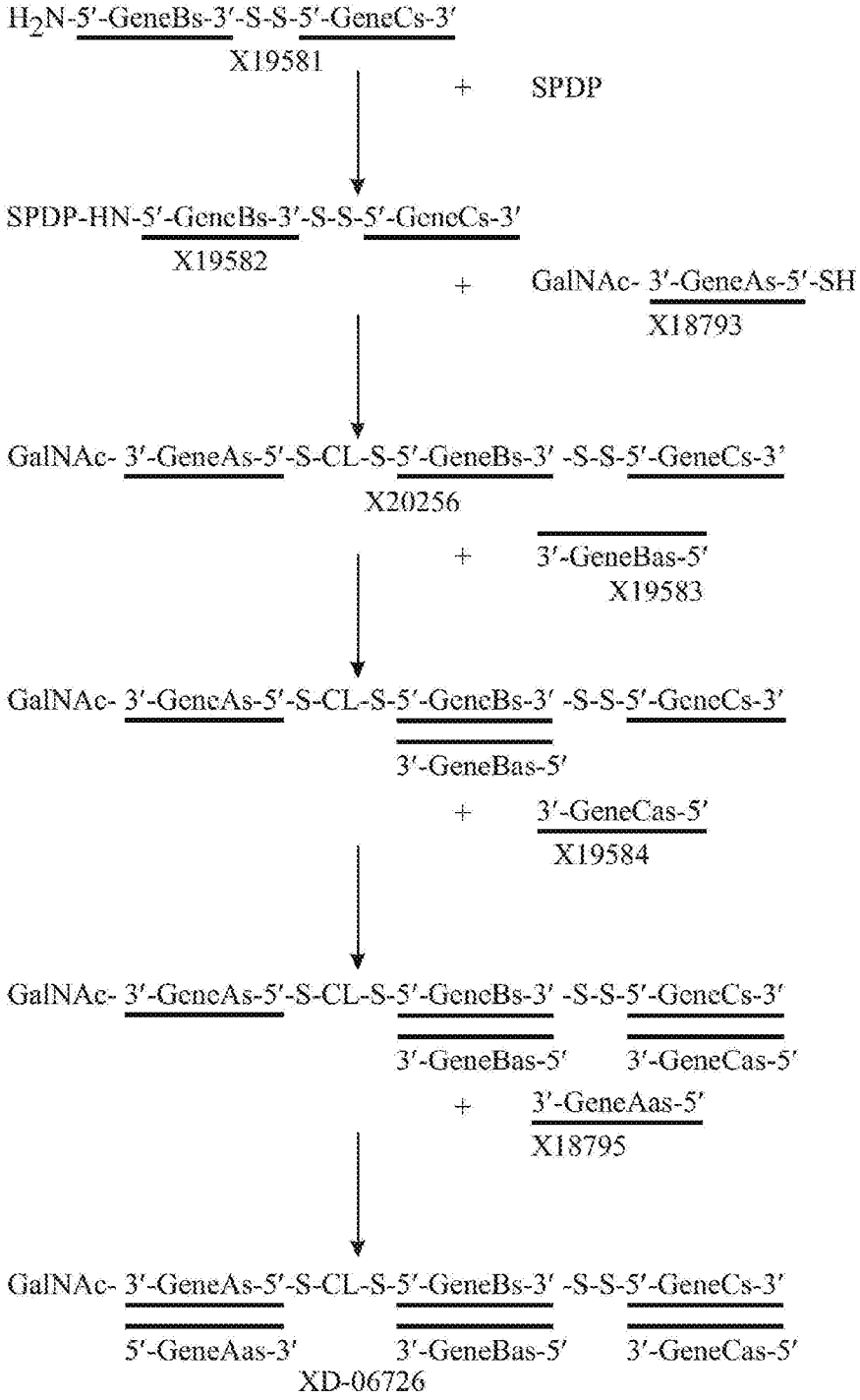

H2N-5′-GeneBs-3′-S-S-5′-GeneCs-3′
X19581
+ SPDP
SPDP-HN-5′-GeneBs-3′-S-S-5′-GeneCs-3′
X19582
+ GalNAc- 3′-GeneAs-5′-SH
X18793
GalNAc- 3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′ -S-S-5′-GeneCs-3′
X20256
+ 3′-GeneBas-5′
X19583
GalNAc- 3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′ -S-S-5′-GeneCs-3′
3′-GeneBas-5′
+ 3′-GeneCas-5′
X19584
GalNAc- 3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′ -S-S-5′-GeneCs-3′
3′-GeneBas-5′ 3′-GeneCas-5′
+ 3′-GeneAas-5′
X18795
GalNAc- 3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′ -S-S-5′-GeneCs-3′
5′-GeneAas-3′ 3′-GeneBas-5′ 3′-GeneCas-5′
XD-06726

FIG. 11

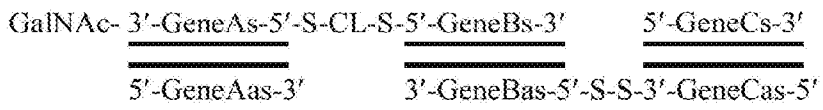

GalNAc- 3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′ 5′-GeneCs-3′
5′-GeneAas-3′ 3′-GeneBas-5′-S-S-3′-GeneCas-5′

FIG. 12

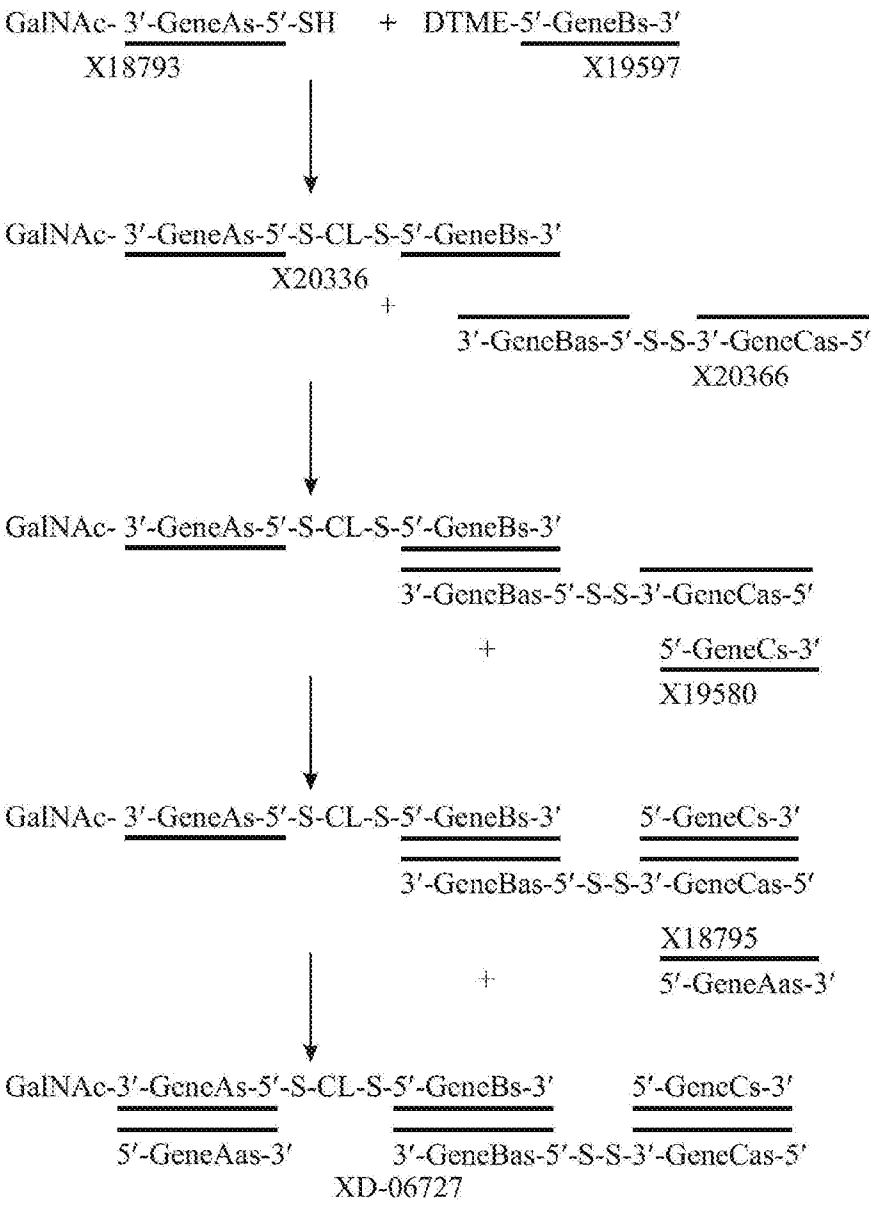

GalNAc- 3′-GeneAs-5′-SH + DTME-5′-GeneBs-3′
X18793 X19597
GalNAc- 3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′
X20336
+
3′-GeneBas-5′-S-S-3′-GeneCas-5′
X20366
GalNAc- 3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′
3′-GeneBas-5′-S-S-3′-GeneCas-5′
+
5′-GeneCs-3′
X19580
GalNAc- 3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′ 5′-GeneCs-3′
3′-GeneBas-5′-S-S-3′-GeneCas-5′
X18795
+
5′-GeneAas-3′
GalNAc-3′-GeneAs-5′-S-CL-S-5′-GeneBs-3′ 5′-GeneCs-3′
5′-GeneAas-3′ 3′-GeneBas-5′-S-S-3′-GeneCas-5′
XD-06727

FIG. 13

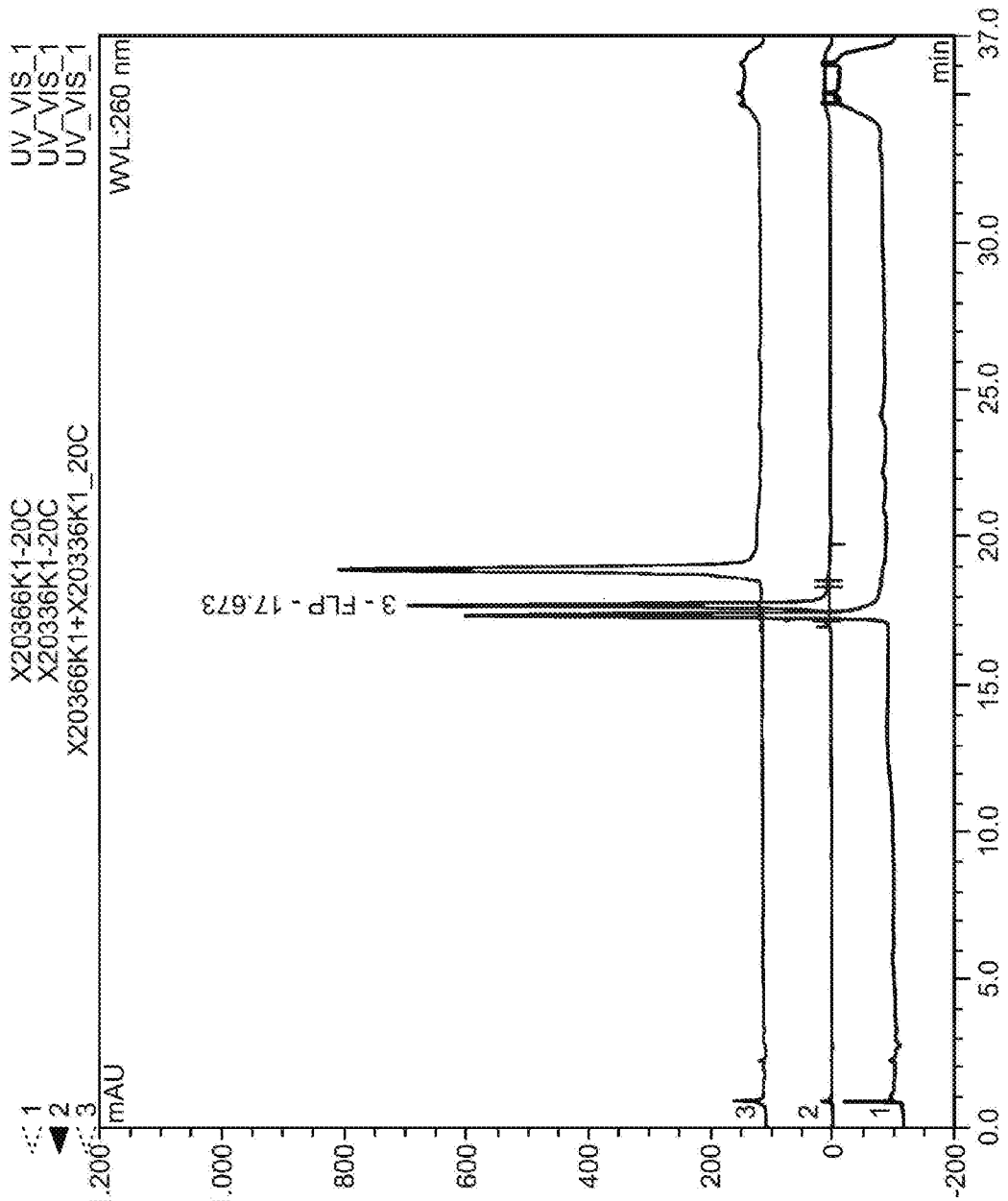

X20366K1-20C
X20336K1-20C
X20366K1+X20336K1_20C
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL:260 nm
mAU
min
3 - FLP - 17.673
1
2
3
1.200
1.000
800
600
400
200
0
-200
0.0
5.0
10.0
15.0
20.0
25.0
30.0
37.0

FIG. 14

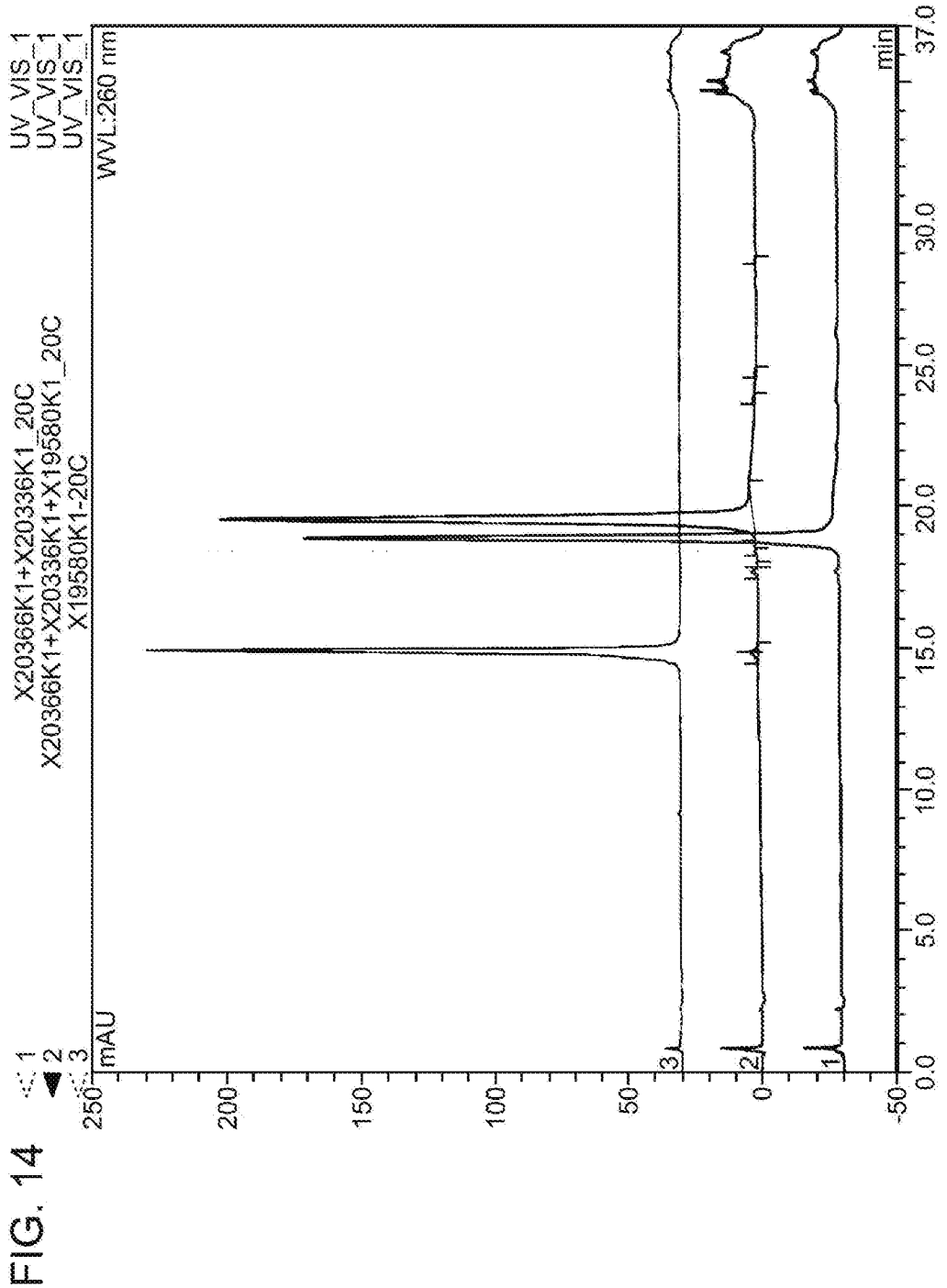

X20366K1+X20336K1_20C
X20366K1+X20336K1+X19580K1_20C
X19580K1-20C
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL:260 nm
mAU
min
250
200
150
100
50
0
-50
0.0
5.0
10.0
15.0
20.0
25.0
30.0
37.0
1
2
3

FIG. 15

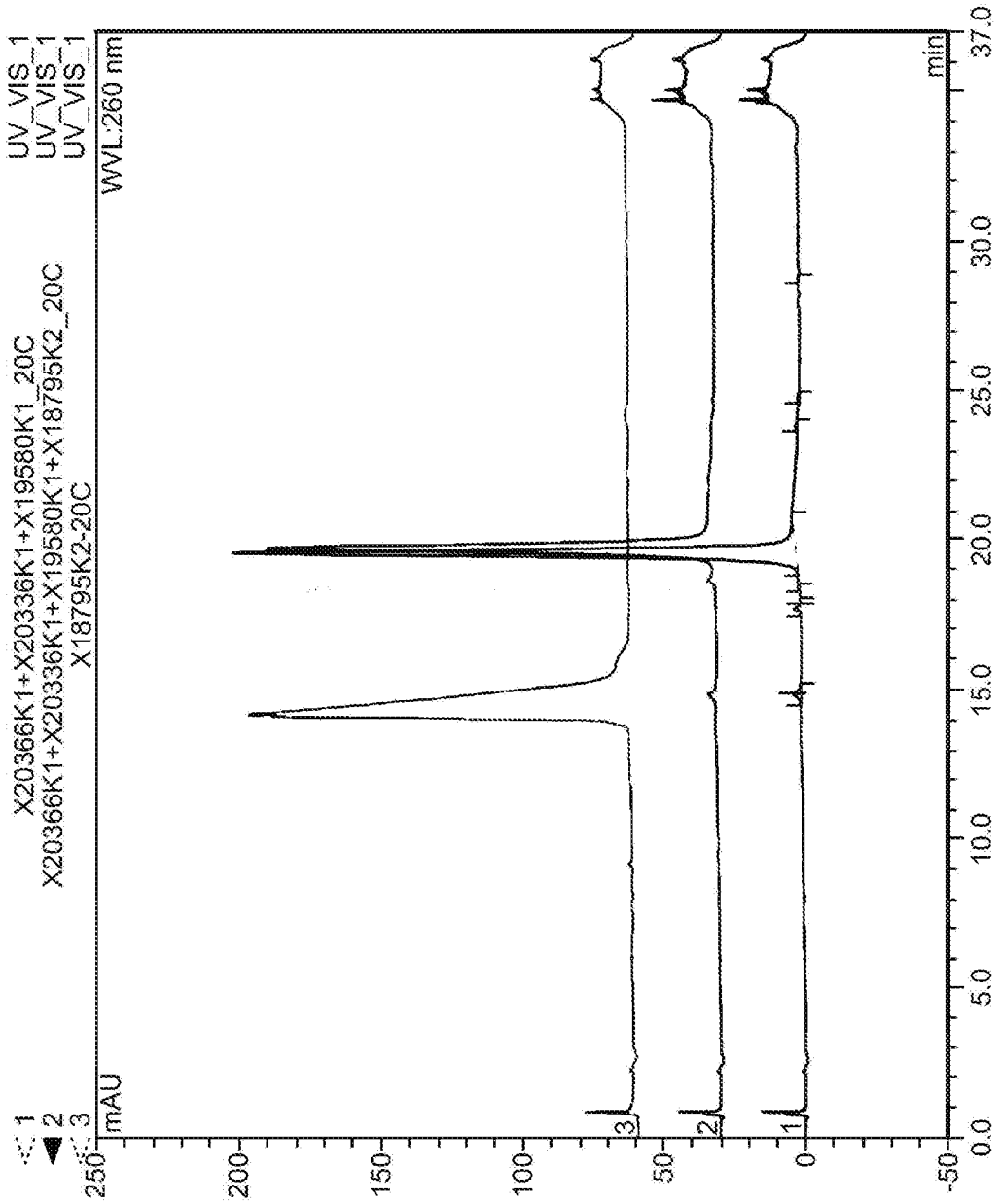

X20366K1+X20336K1+X19580K1_20C
X20366K1+X20336K1+X19580K1+X18795K2_20C
X18795K2-20C
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL:260 nm
mAU
min
250
200
150
100
50
0
-50
0.0
5.0
10.0
15.0
20.0
25.0
30.0
37.0
1
2
3

FIG. 16A

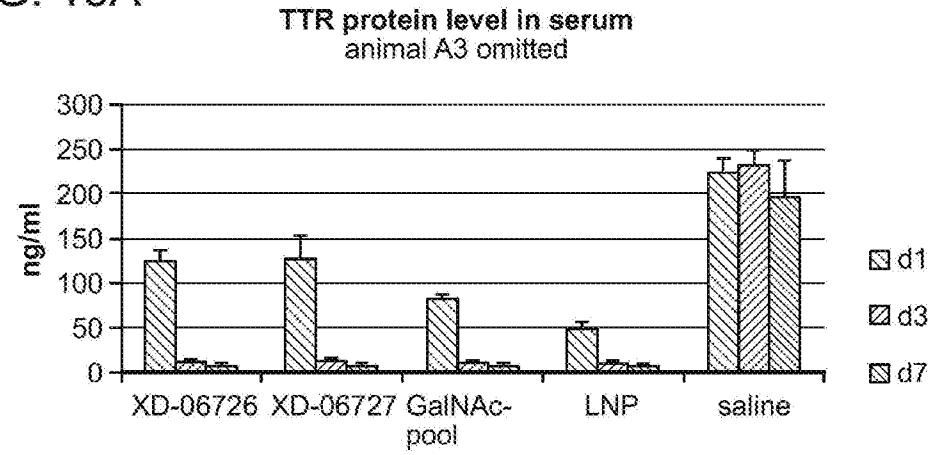


FIG. 16B

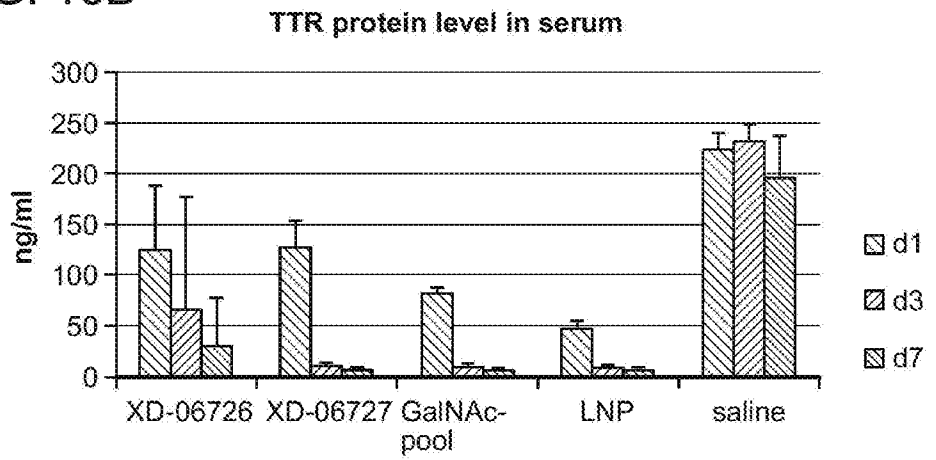


FIG. 17A

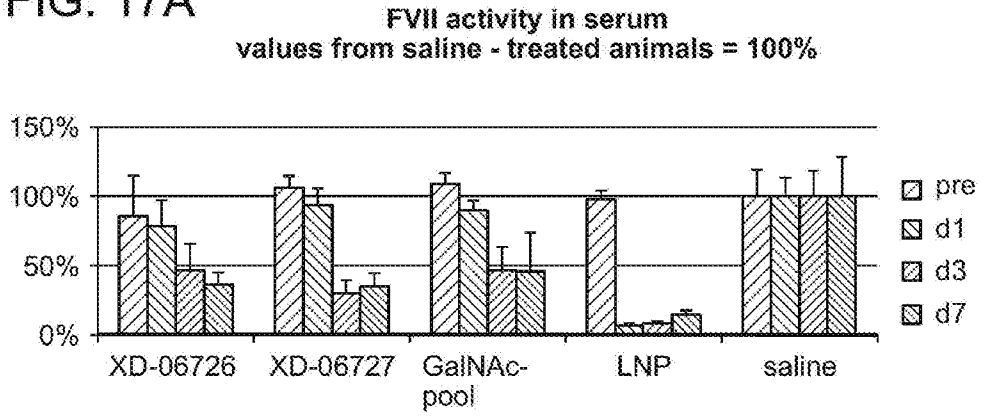


FIG. 17B

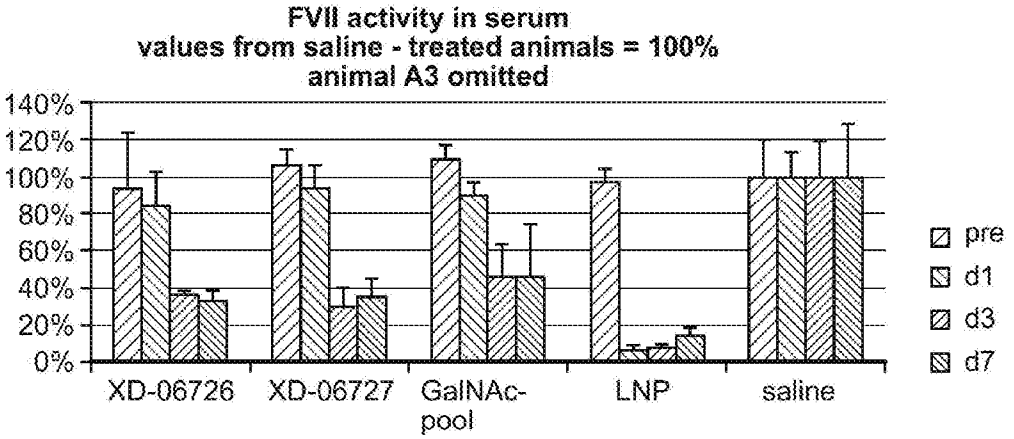


FIG. 18A

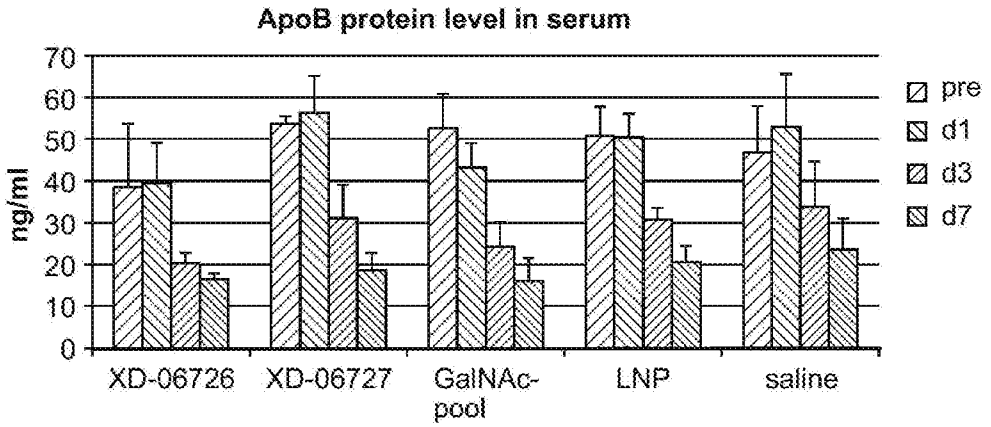


FIG. 18B

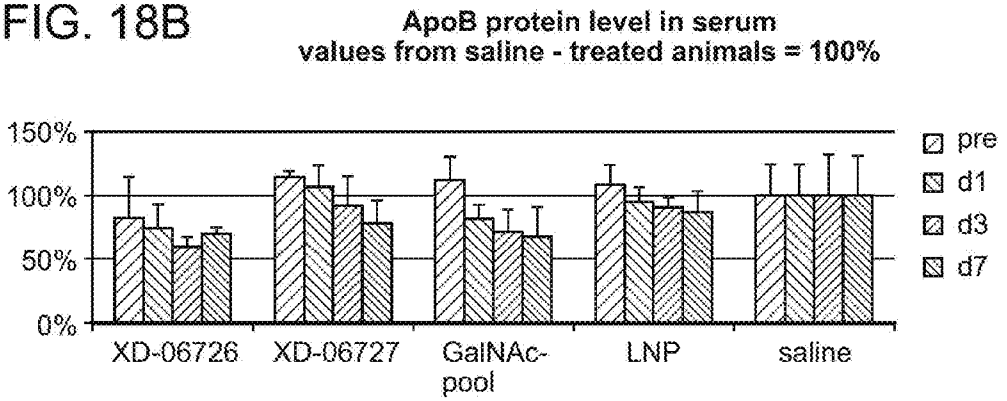


FIG. 19A

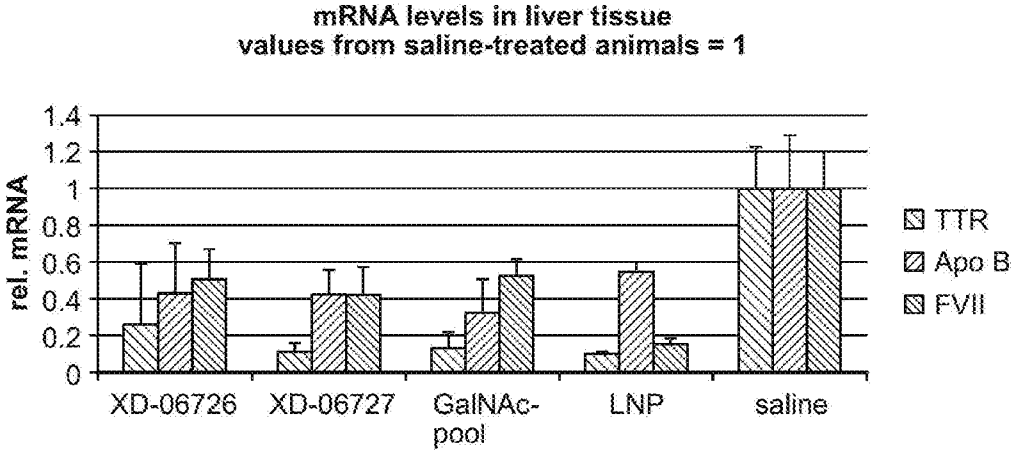


FIG. 19B

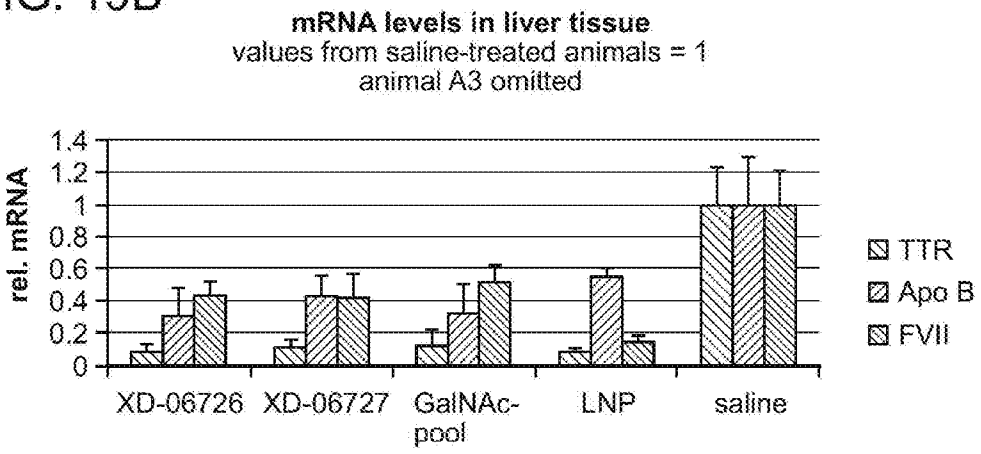


FIG. 20

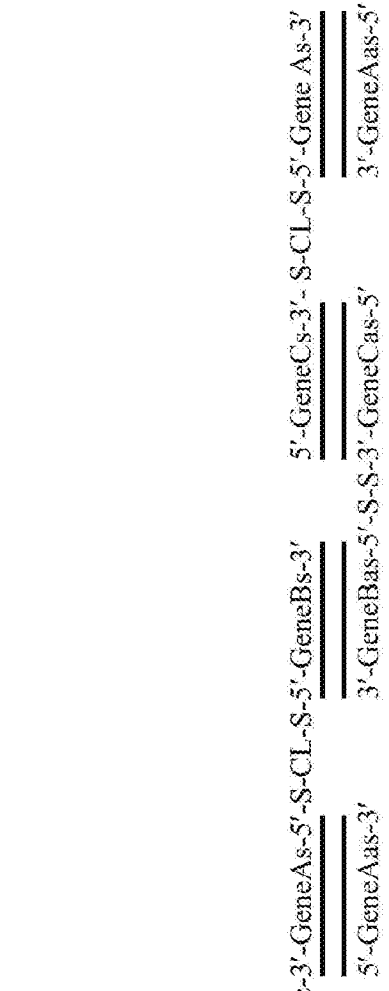

GalNAc-3'-GeneAs-5'-S-CL-S-5'-GeneBs-3'
5'-GeneCs-3'- S-CL-S-5'-Gene As-3'
5'-GeneAas-3'
3'-GeneBas-5'-S-S-3'-GeneCas-5'
3'-GeneAas-5'

FIG. 21

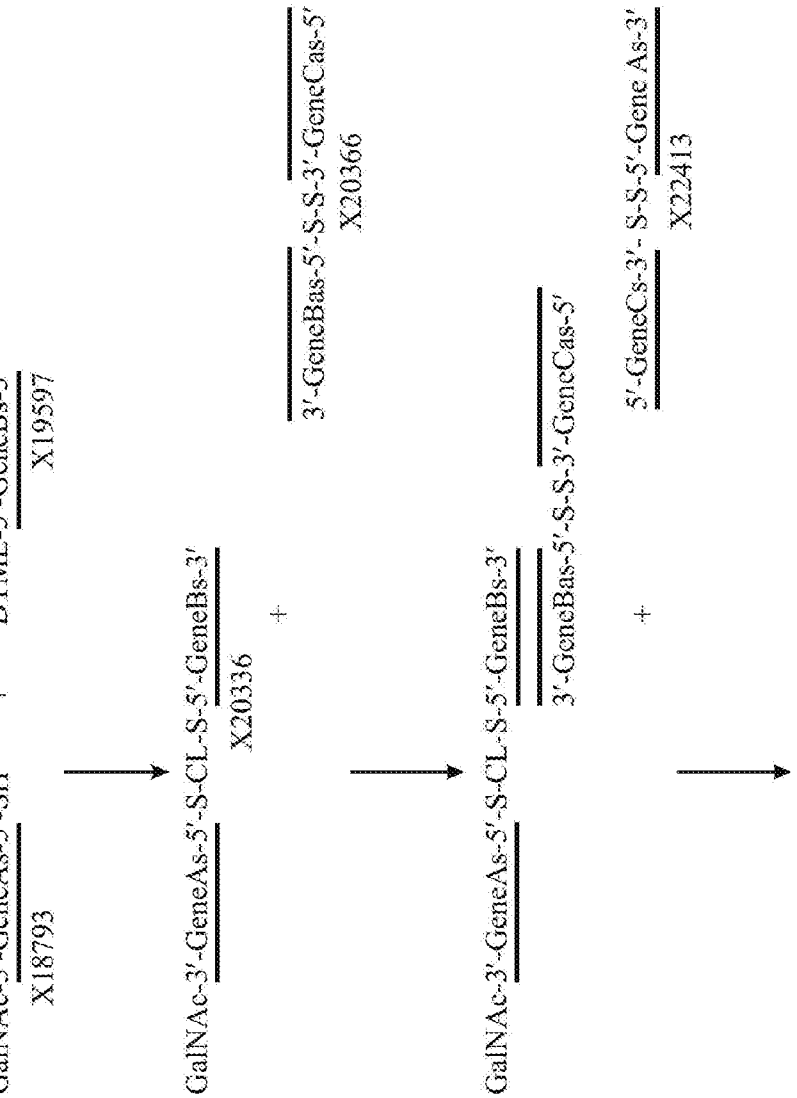
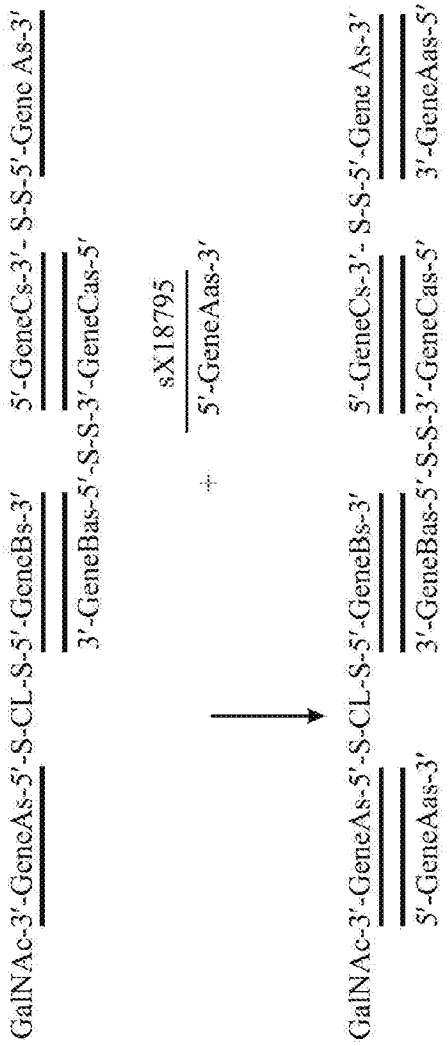

GalNAc-3'-GeneAs-5'-SH
X18793
+
DTME-5'-GeneBs-3'
X19597
GalNAc-3'-GeneAs-5'-S-CL-S-5'-GeneBs-3'
X20336
+
3'-GeneBas-5'-S-S-3'-GeneCas-5'
X20366
GalNAc-3'-GeneAs-5'-S-CL-S-5'-GeneBs-3'
3'-GeneBas-5'-S-S-3'-GeneCas-5'
+
5'-GeneCs-3'- S-S-5'-Gene As-3'
X22413

FIG. 21 (continued)



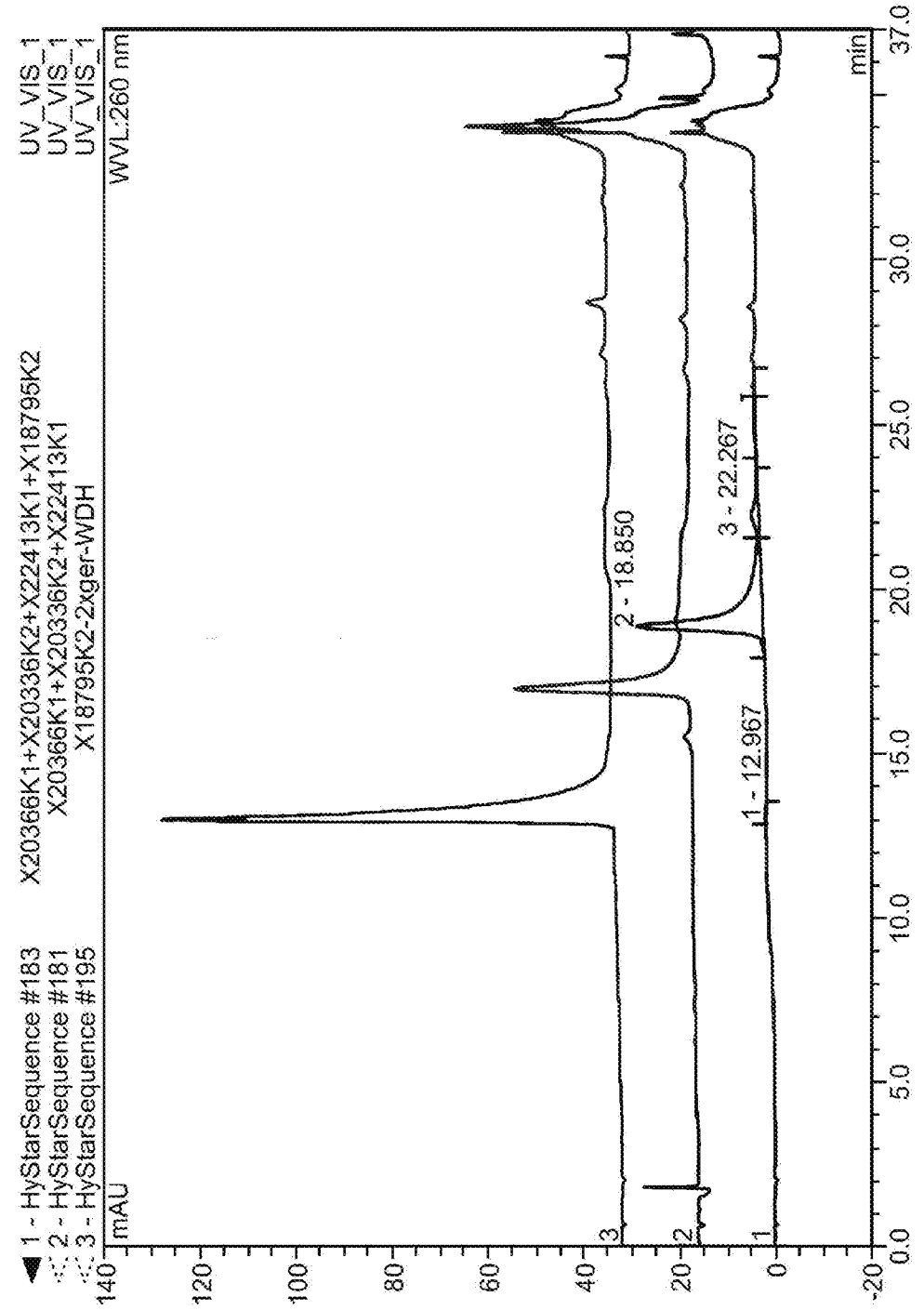

1 - HyStarSequence #183
2 - HyStarSequence #181
3 - HyStarSequence #195
X20366K1+X20336K2+X22413K1+X18795K2
X20366K1+X20336K2+X22413K1
X18795K2-2xger-WDH
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL:260 nm
mAU
min
2 - 18.850
3 - 22.267
1 - 12.967

FIG. 22

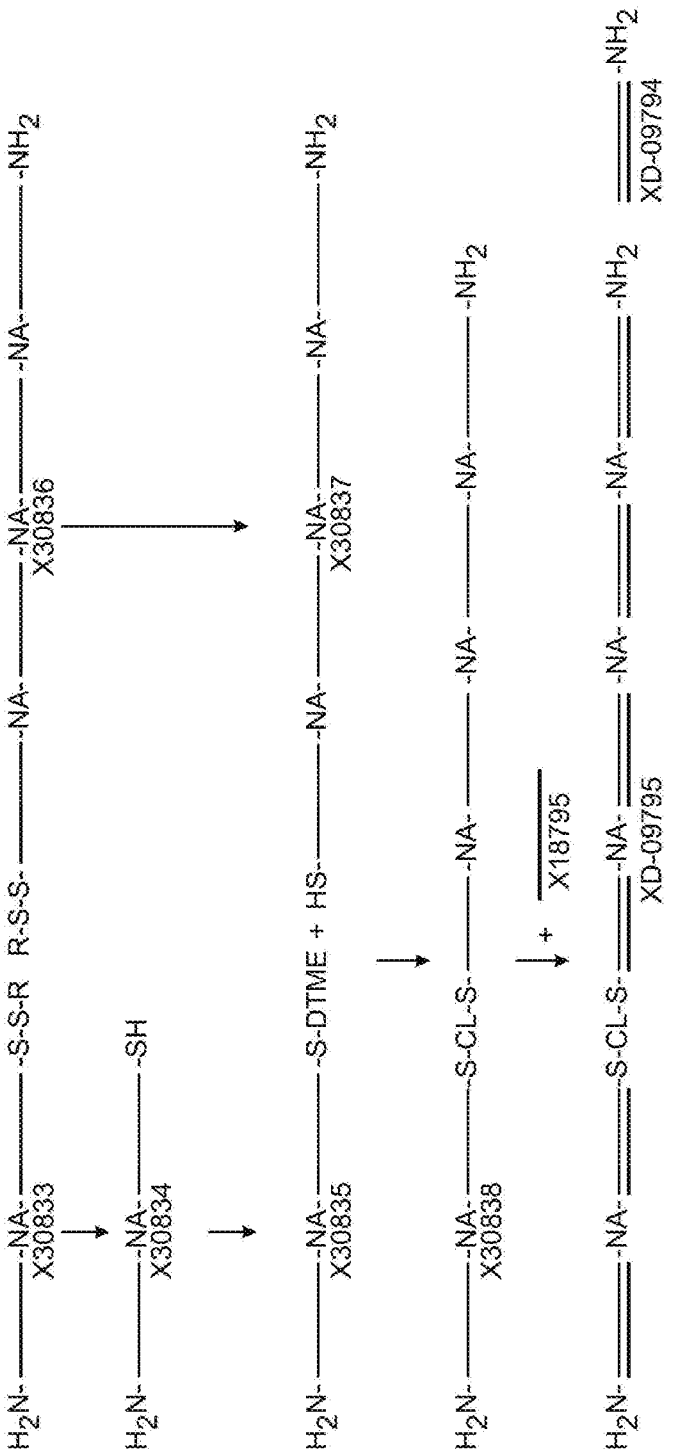

H2N-NA-X30833-S-S-R
R-S-S-NA-NA-X30836-NH2
H2N-NA-X30834-SH
H2N-NA-X30835-S-DTME + HS-NA-NA-X30837-NH2
H2N-NA-X30838-S-CL-S-NA-NA-NH2
+ X18795
H2N-NA-S-CL-S-NA-NA-NA-NH2
XD-09795
XD-09794

FIG. 23

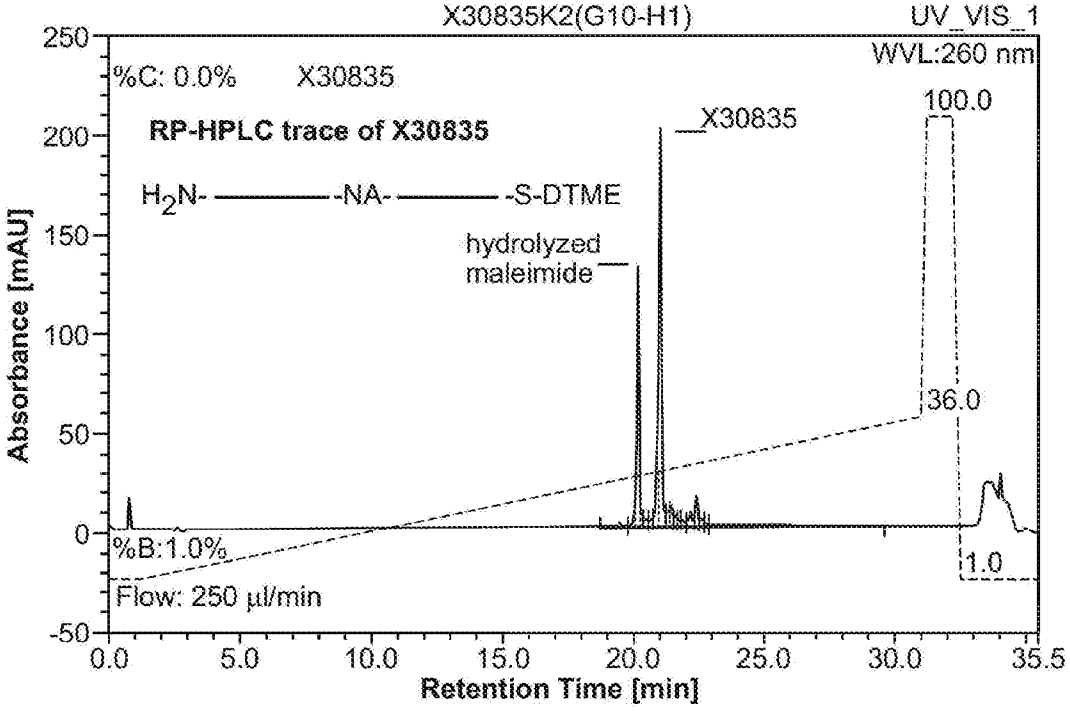


FIG. 24A

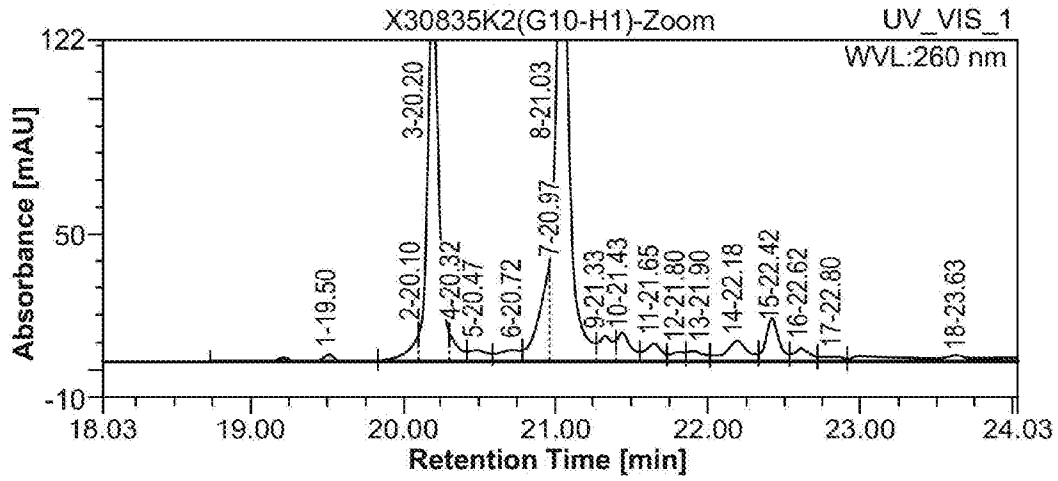


FIG. 24B

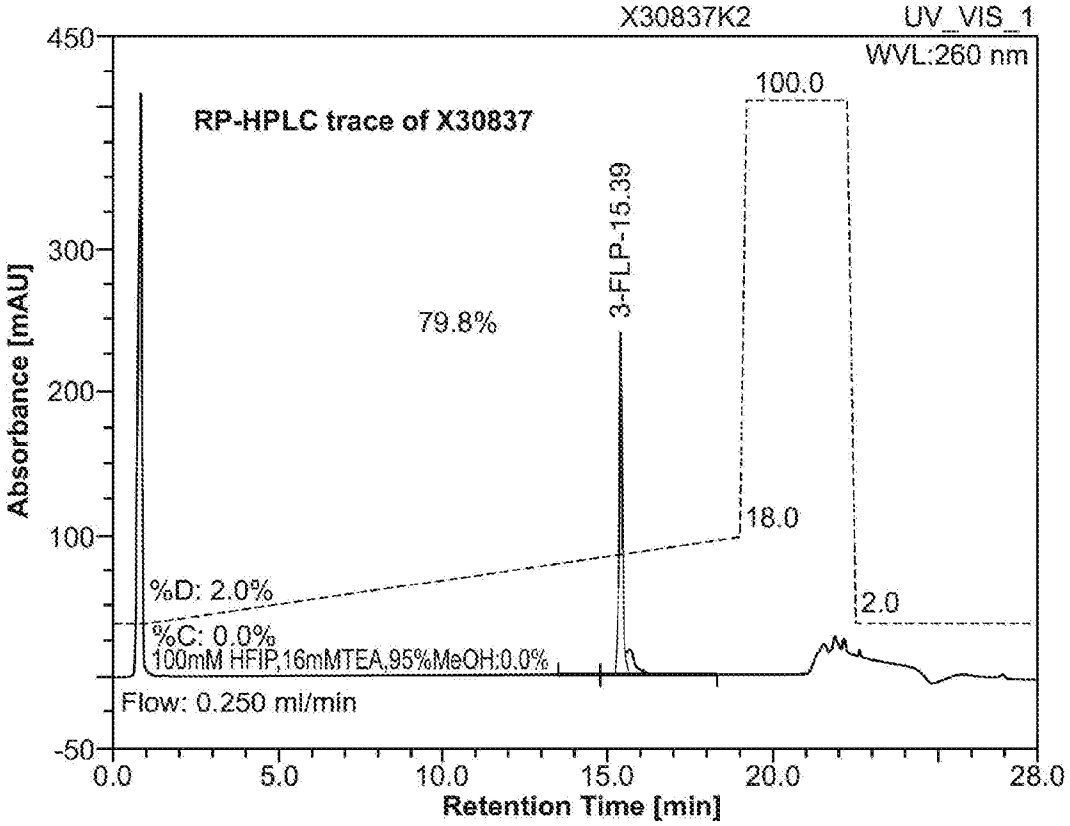


FIG. 24C

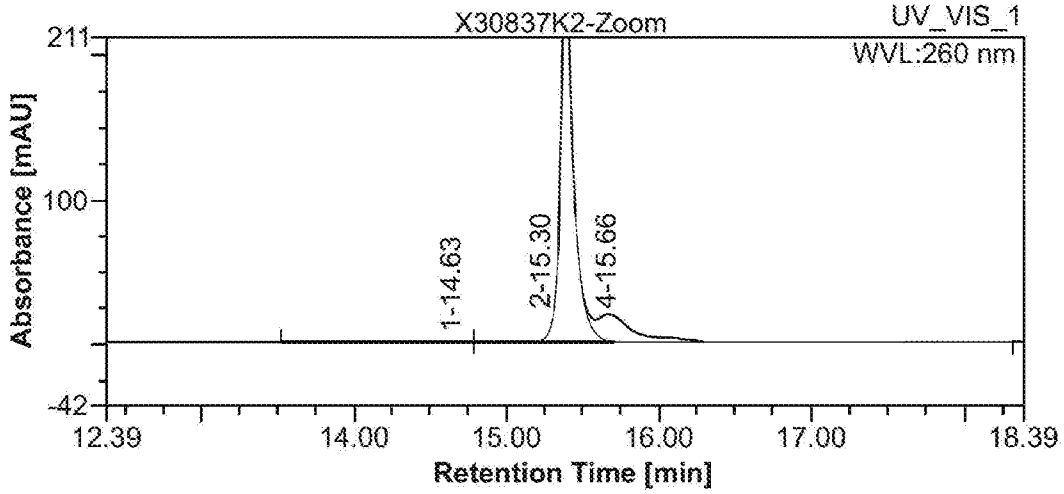


FIG. 24D

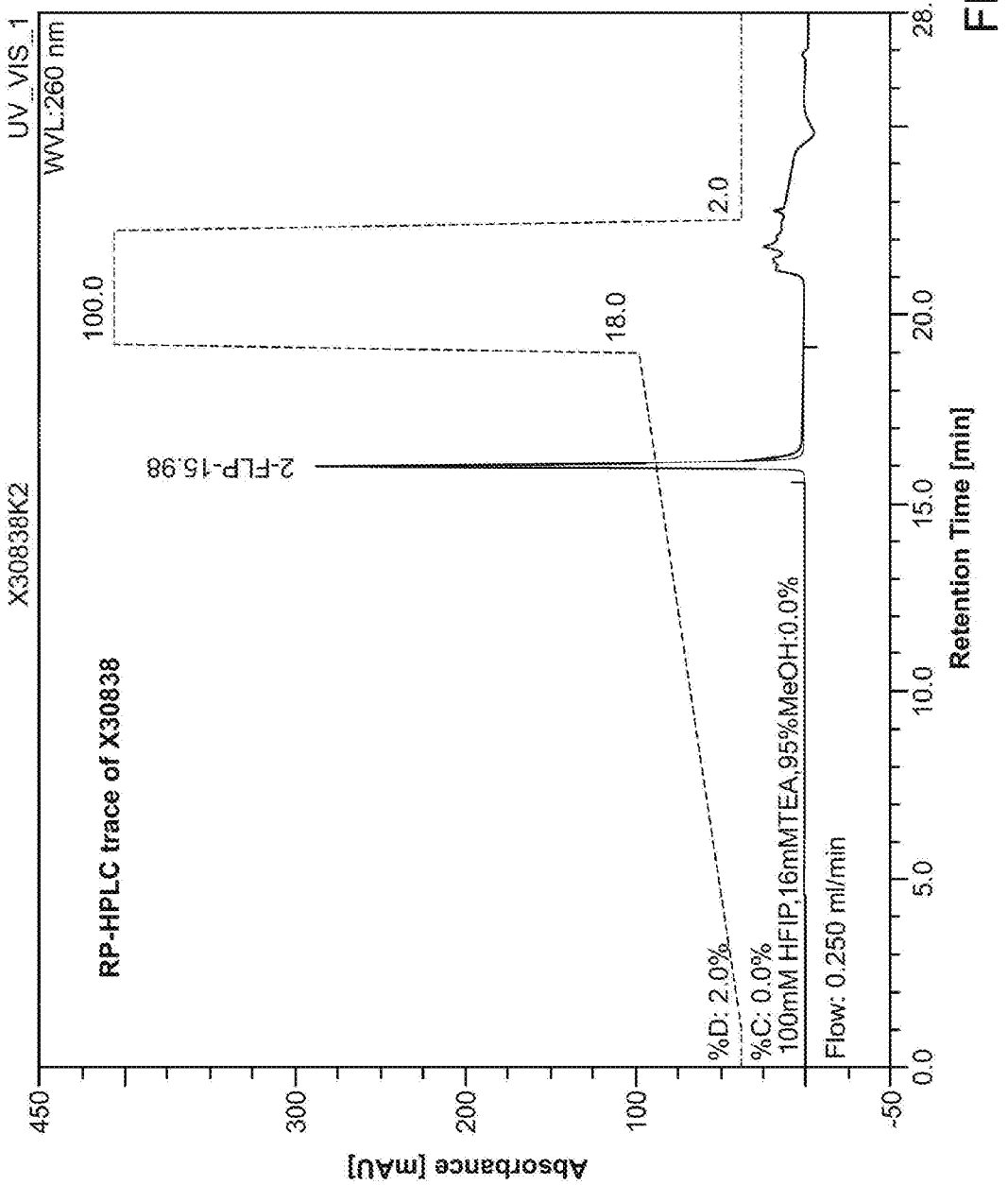

UV_VIS_1
WVL:260 nm
X30838K2
RP-HPLC trace of X30838
2-FLP-15.98
100.0
18.0
2.0
%D: 2.0%
%C: 0.0%
100mM HFIP,16mMTEA,95%MeOH:0.0%
Flow: 0.250 ml/min
Absorbance [mAU]
Retention Time [min]
450
300
200
100
-50
0.0
5.0
10.0
15.0
20.0
28.0

FIG. 24E

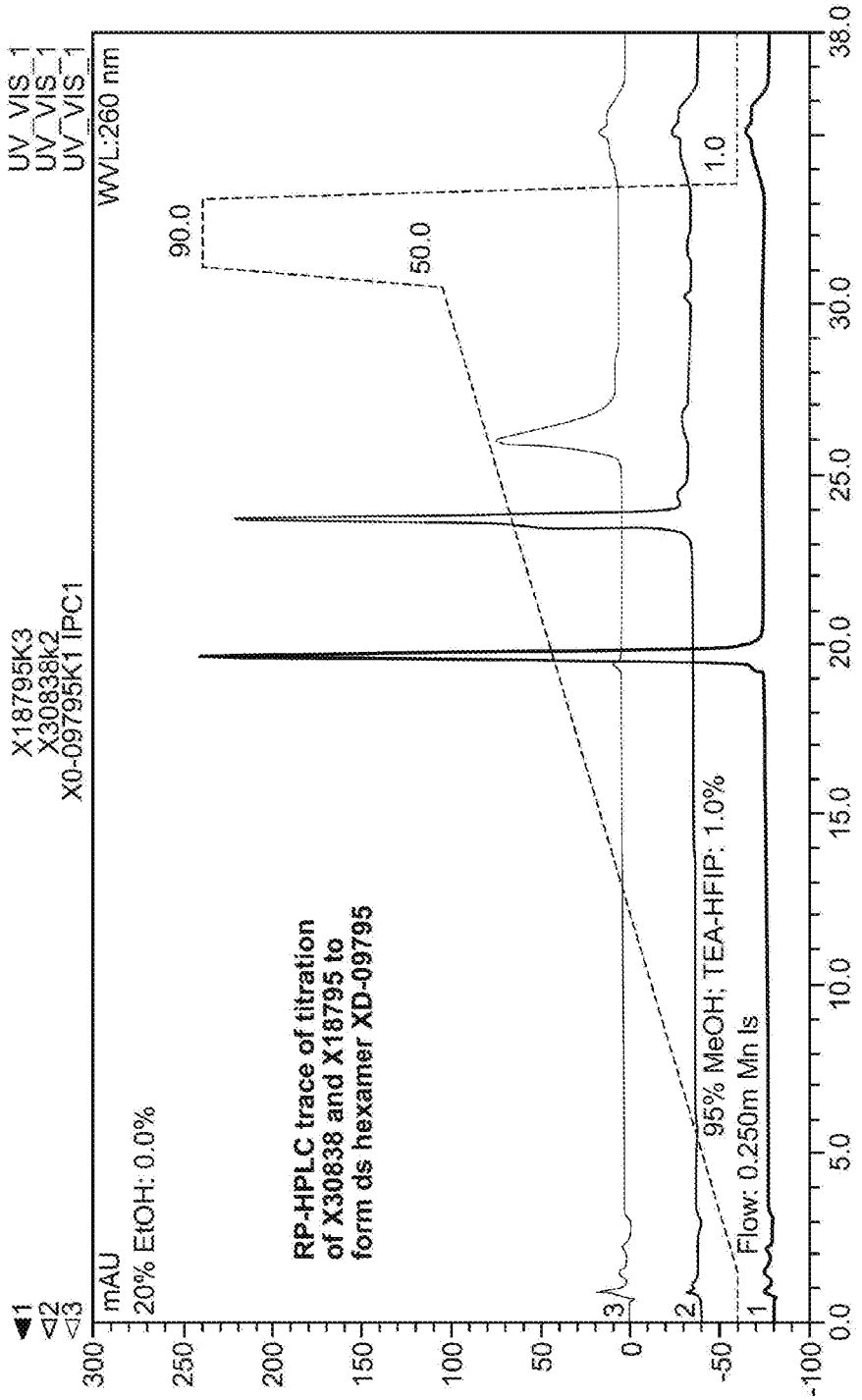

RP-HPLC trace of titration
of X30838 and X18795 to
form ds hexamer XD-09795
X18795K3
X30838K2
X0-09795K1 IPC1
UV_VIS_1
UV_VIS_1
UV_VIS_1
WVL:260 nm
mAU
20% EtOH: 0.0%
95% MeOH; TEA-HFIP: 1.0%
Flow: 0.250m Mn ls
90.0
50.0
1.0
300
250
200
150
100
50
0
-50
-100
0.0
5.0
10.0
15.0
20.0
25.0
30.0
38.0

FIG. 24F

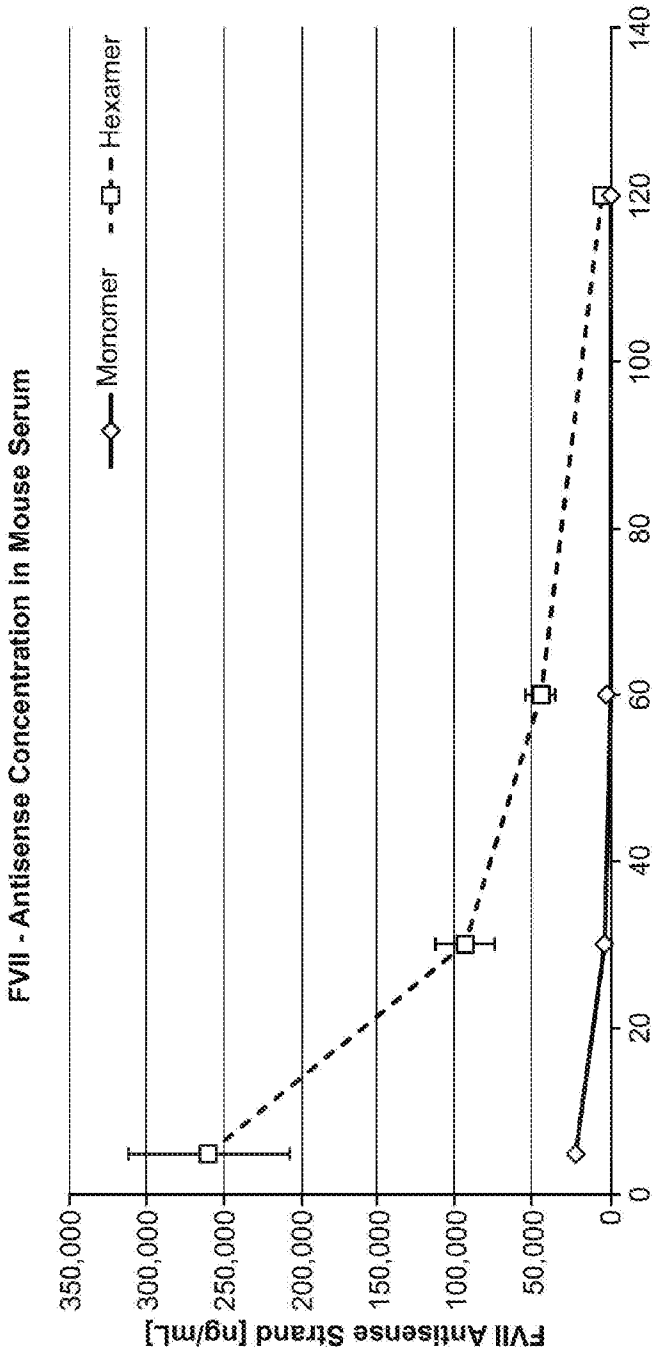

FVII - Antisense Concentration in Mouse Serum
Monomer
Hexamer
FVII Antisense Strand [ng/mL]
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
Time Point [min]
0
20
40
60
80
100
120
140

FIG. 25

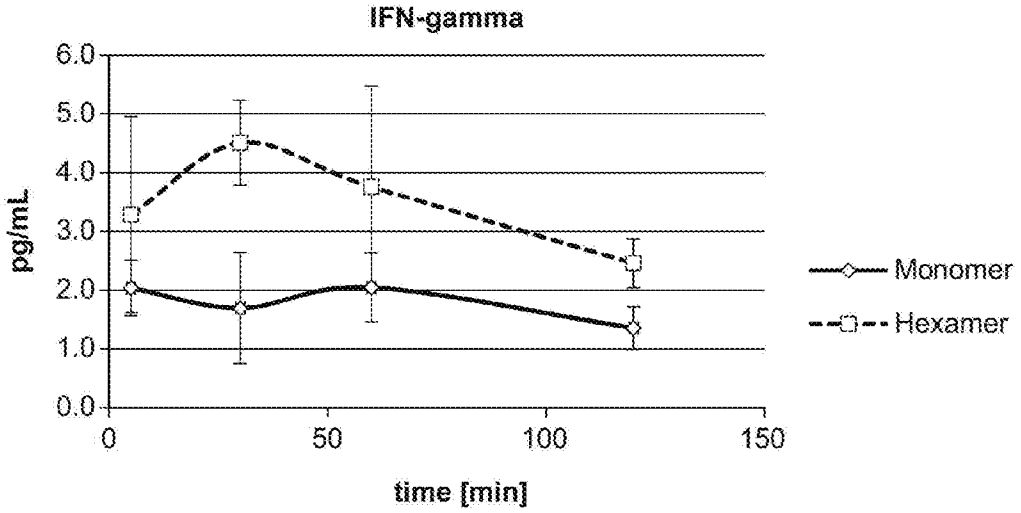


FIG. 26A

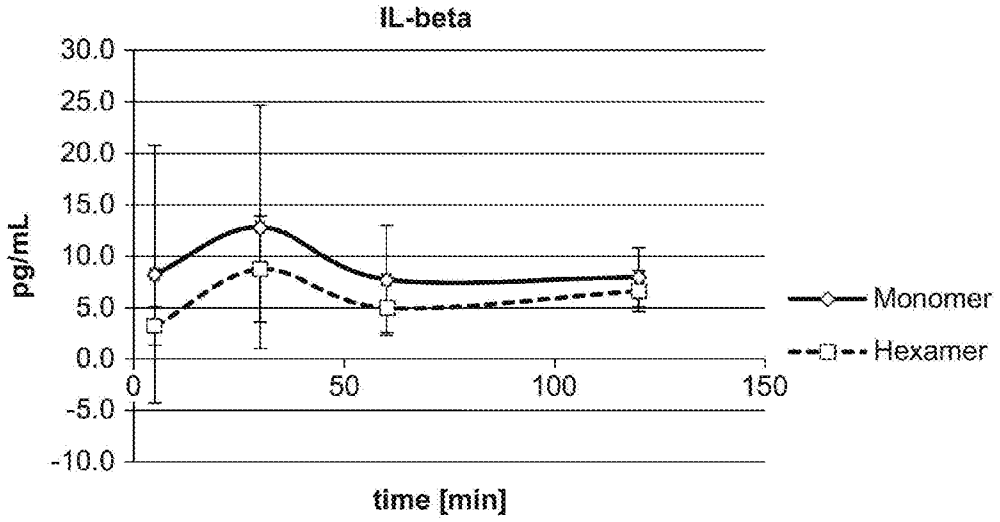


FIG. 26B

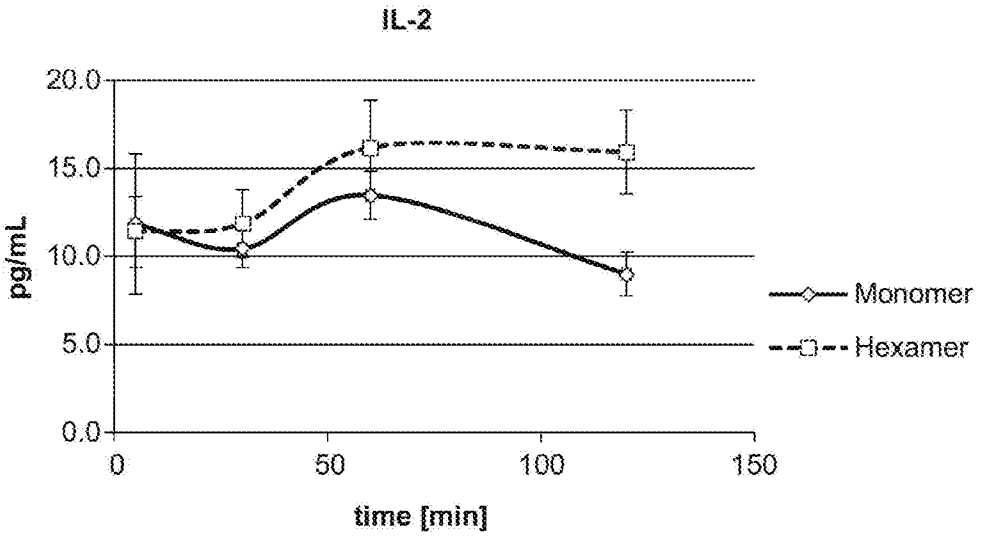


FIG. 26C

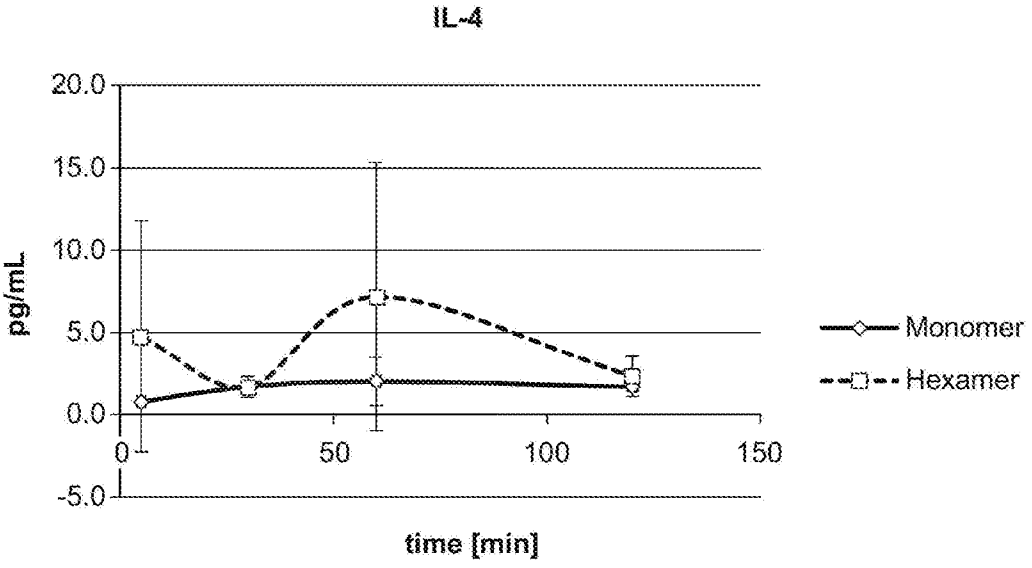


FIG. 26D

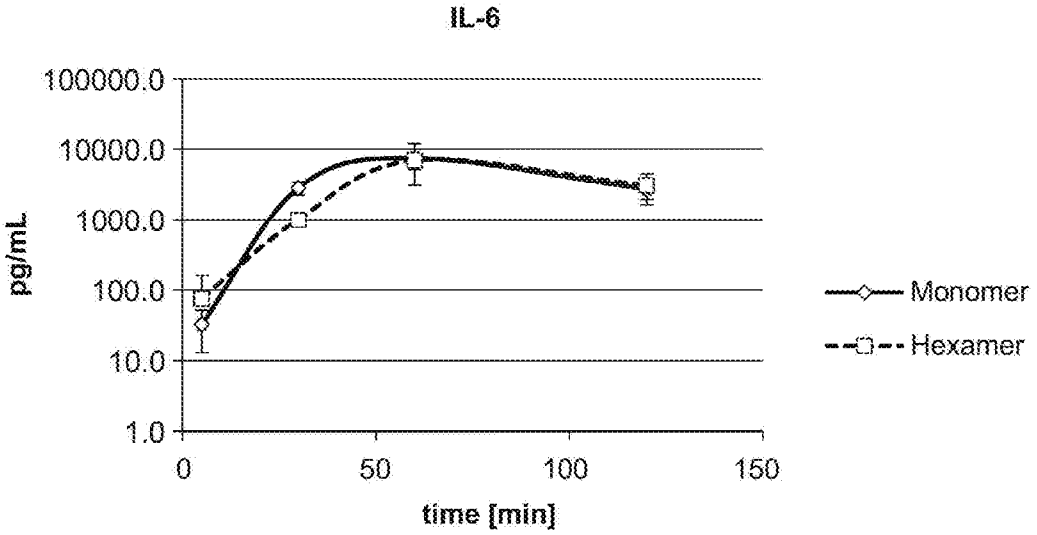


FIG. 26E

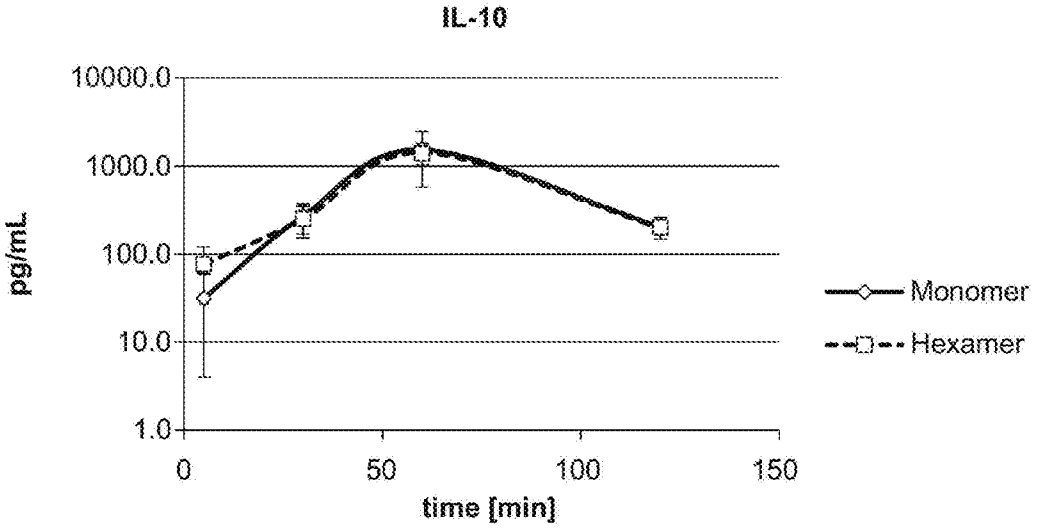


FIG. 26F

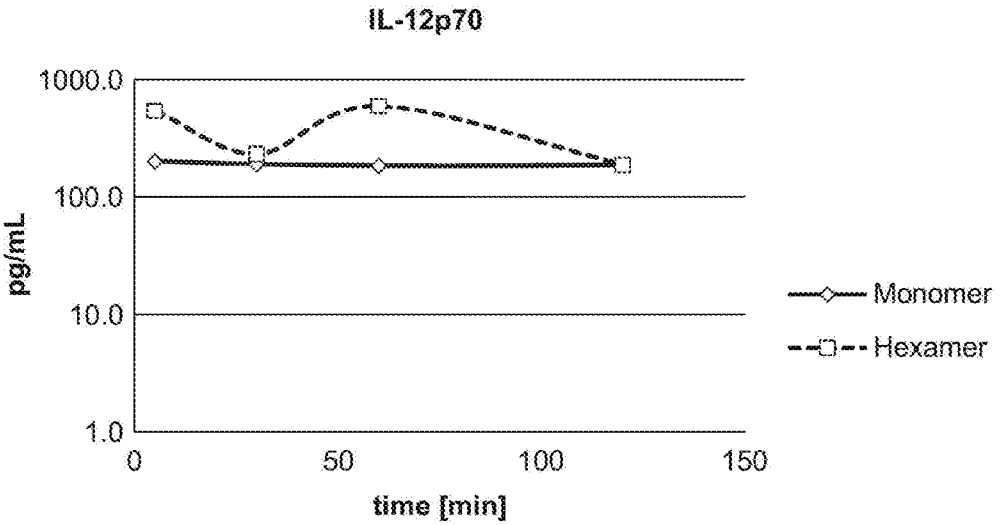


FIG. 26G

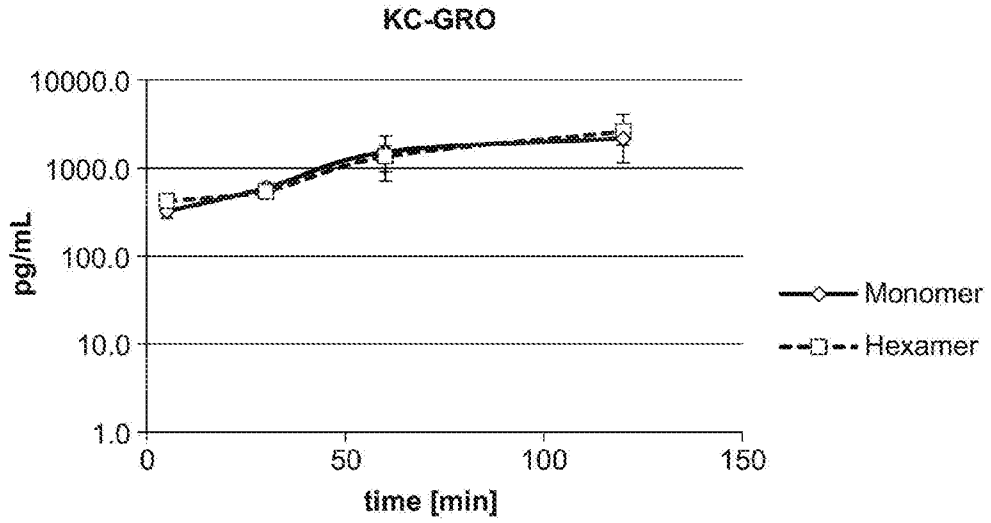


FIG. 26H

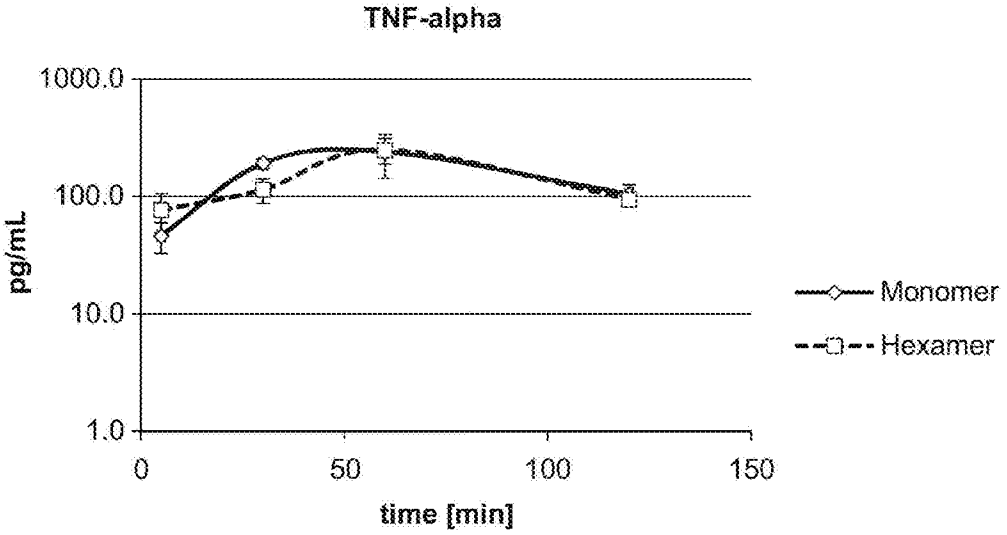


FIG. 26I

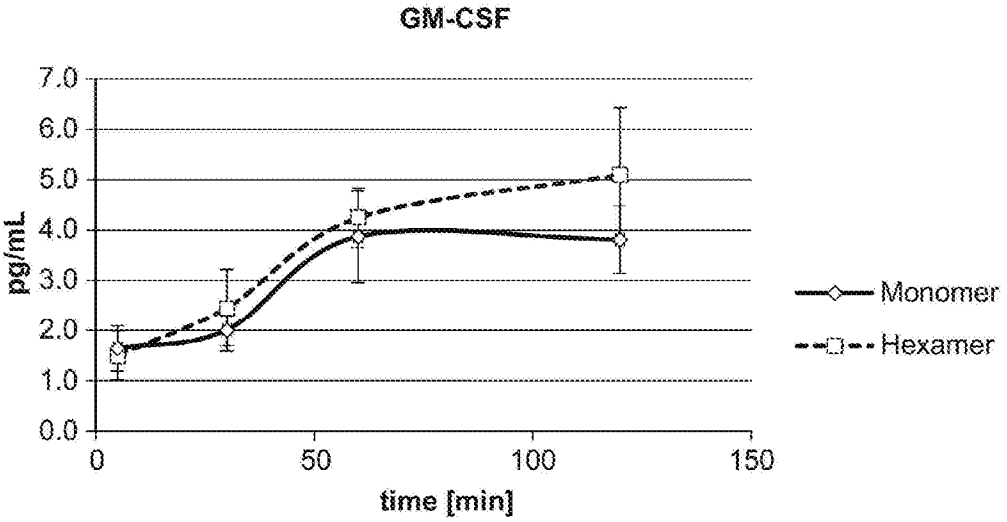


FIG. 26J

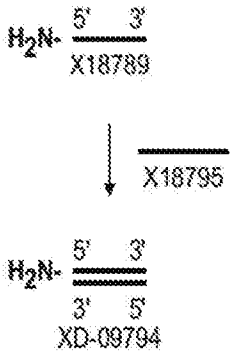


FIG. 27A

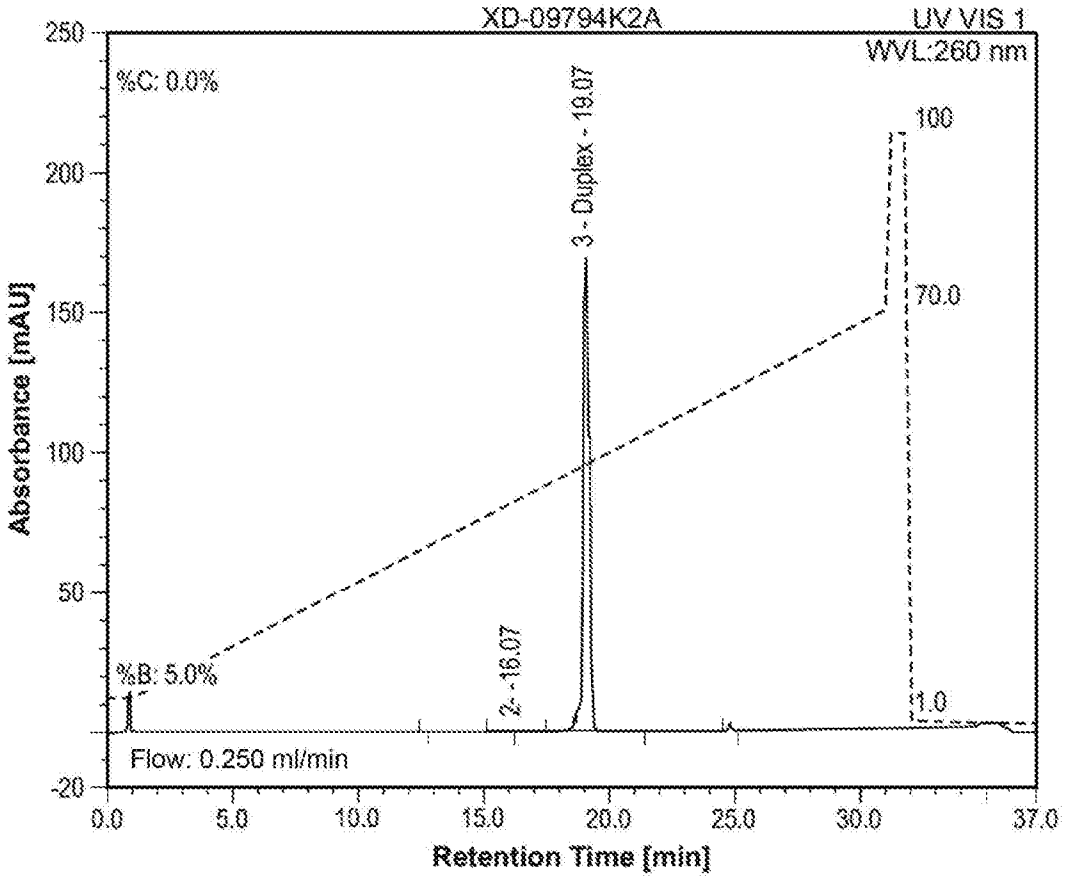


FIG. 27B

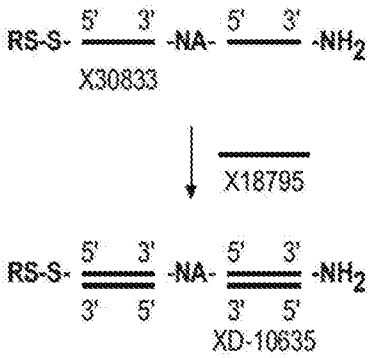


FIG. 28A

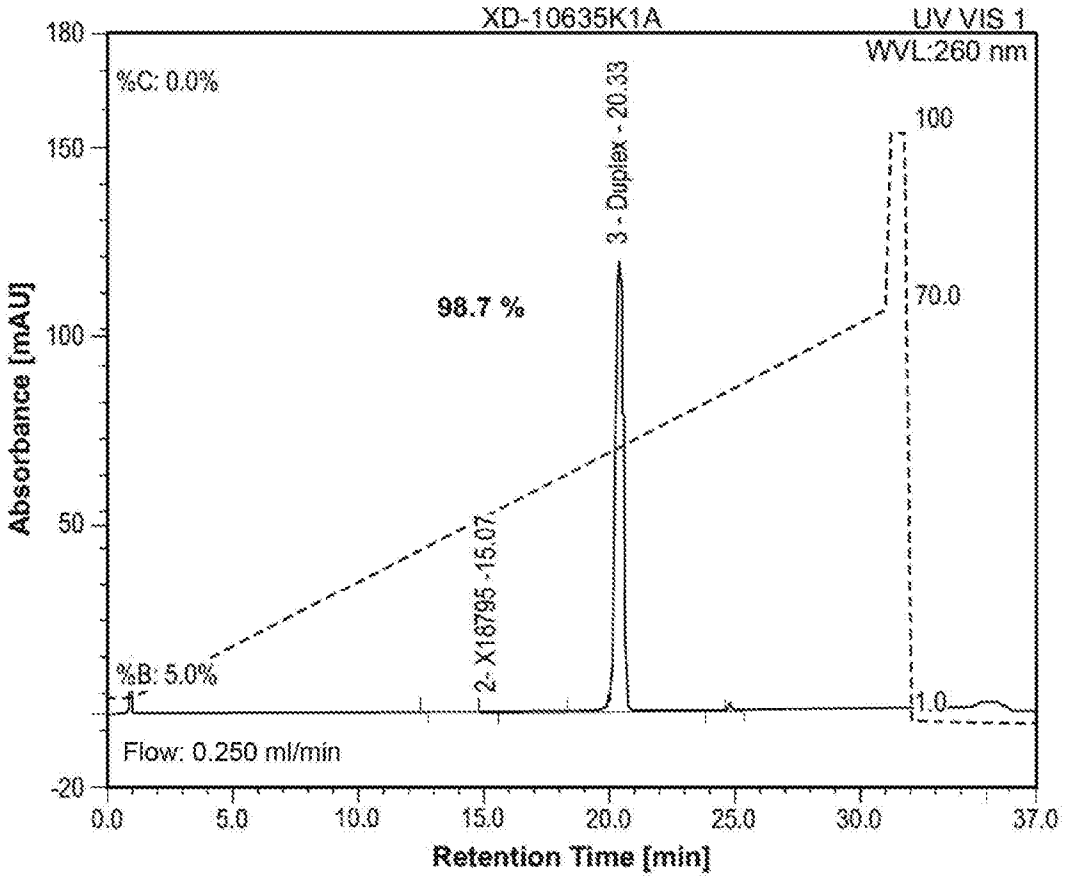


FIG. 28B

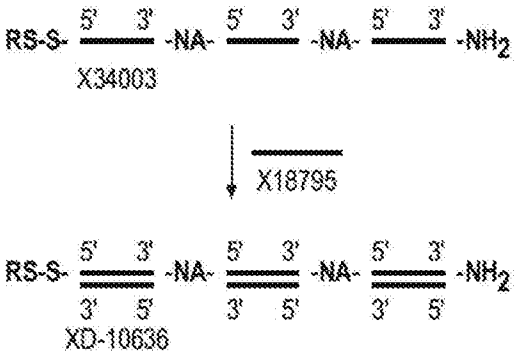


FIG. 29A

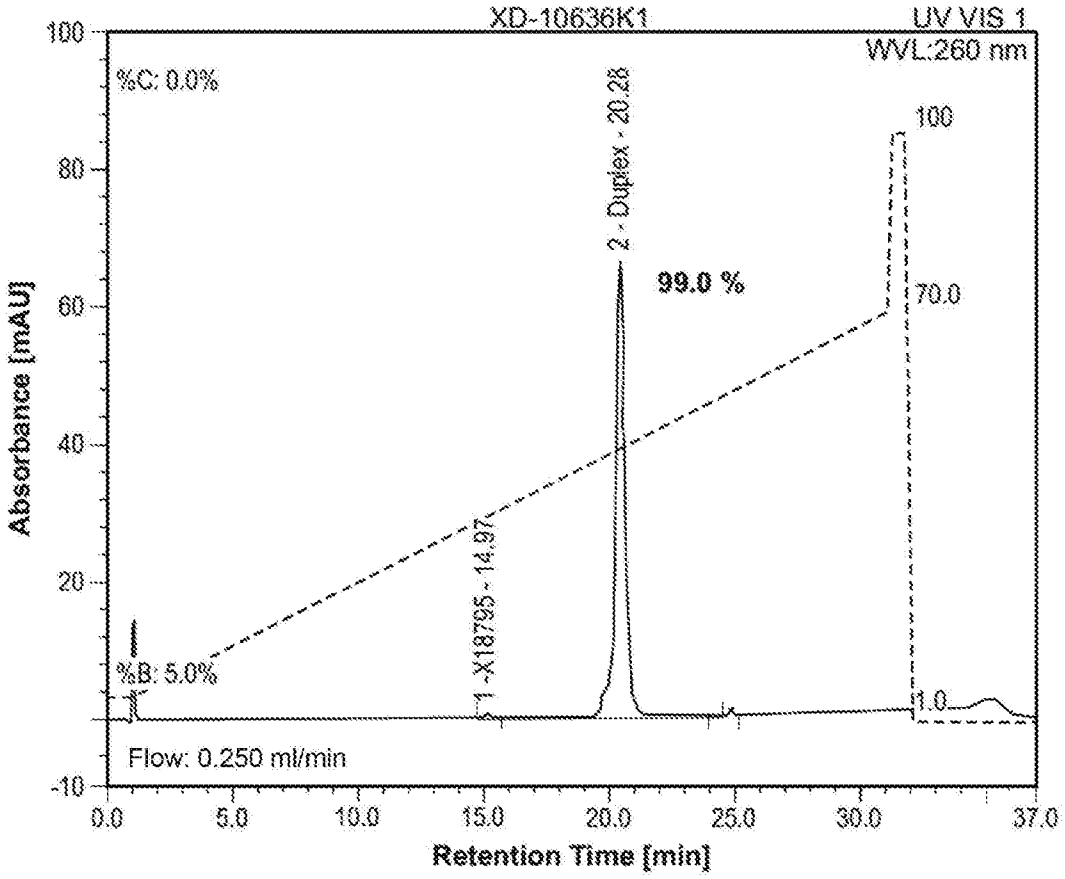


FIG. 29B

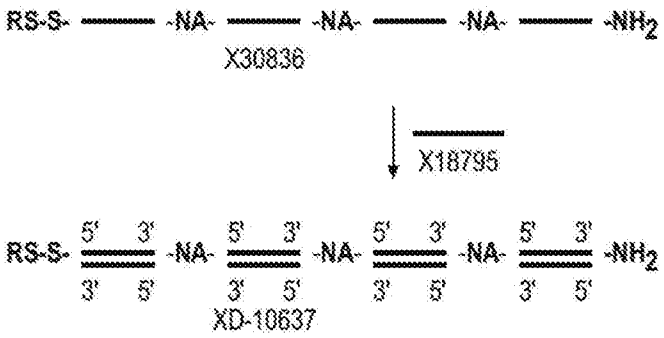


FIG. 30A

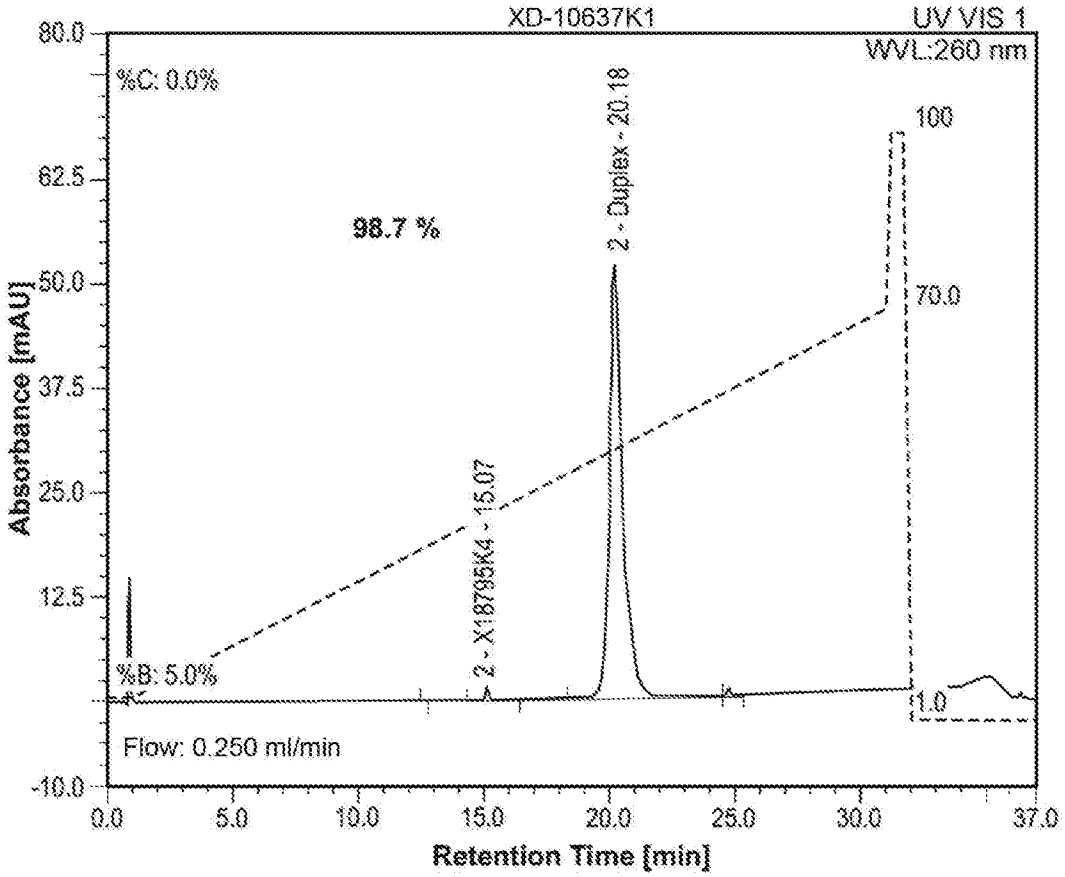


FIG. 30B

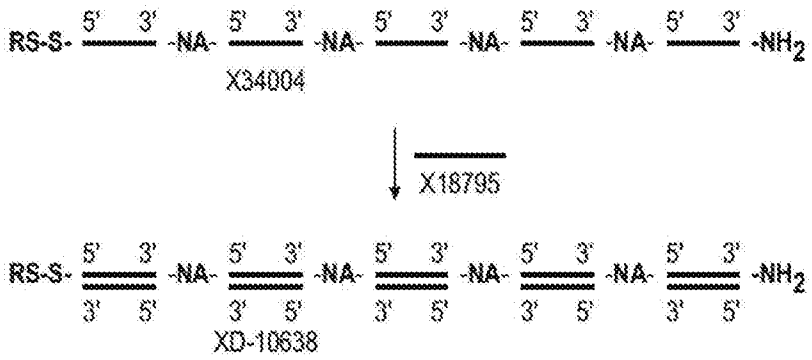


FIG. 31A

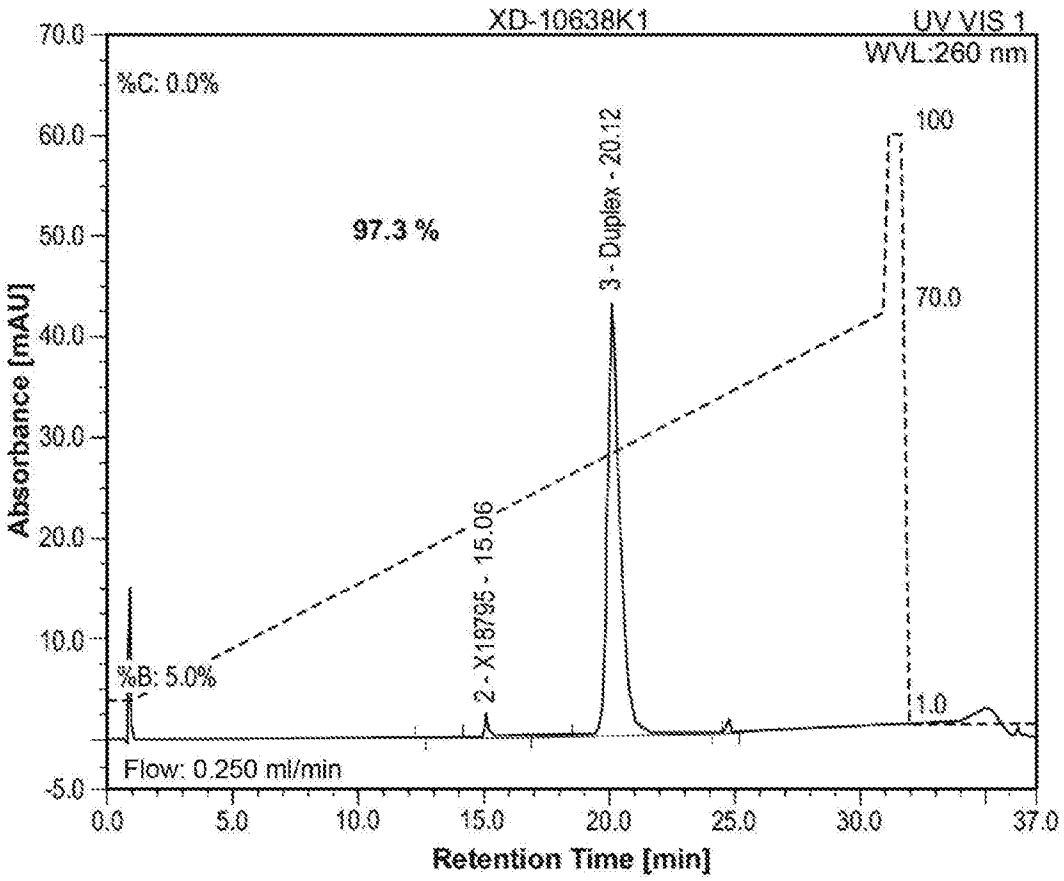


FIG. 31B

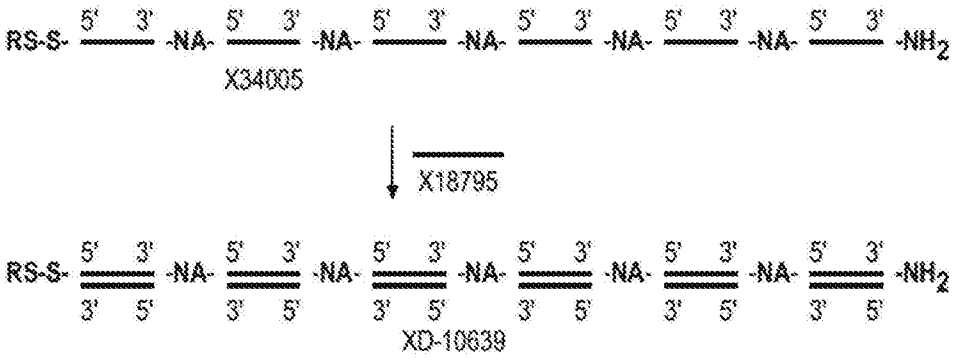


FIG. 32A

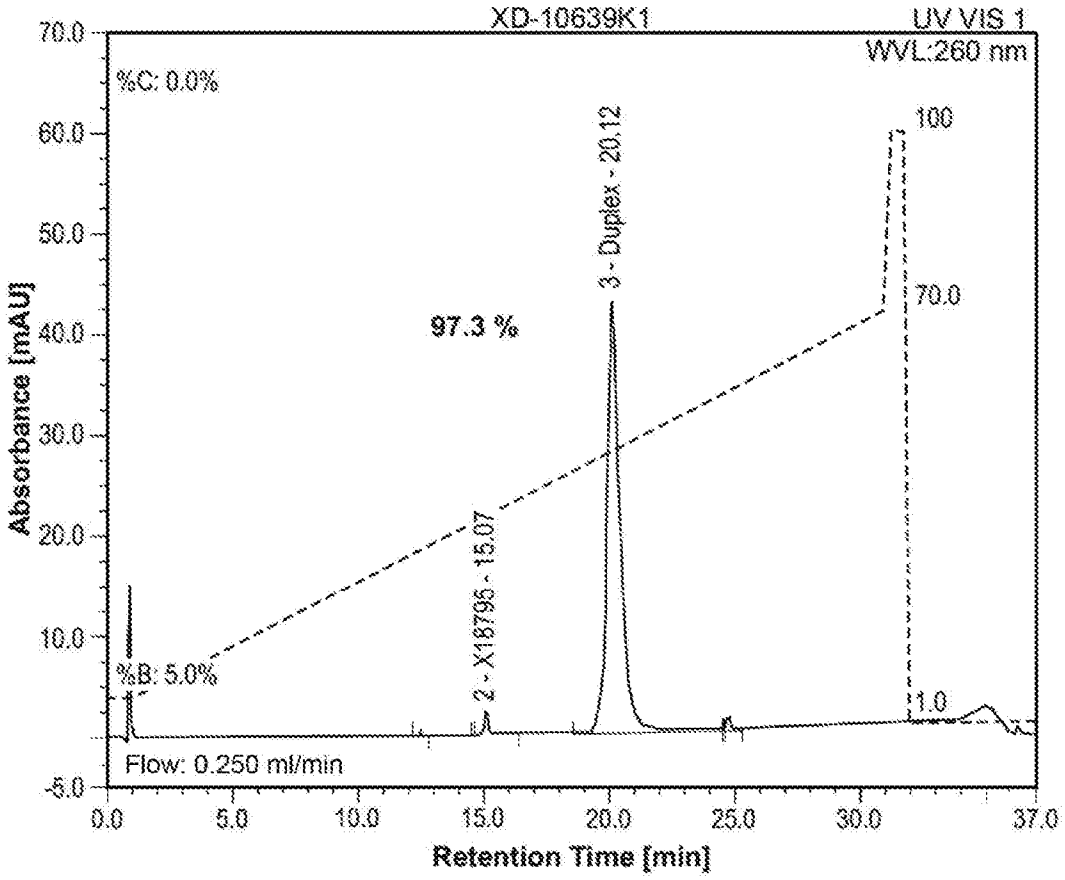


FIG. 32B

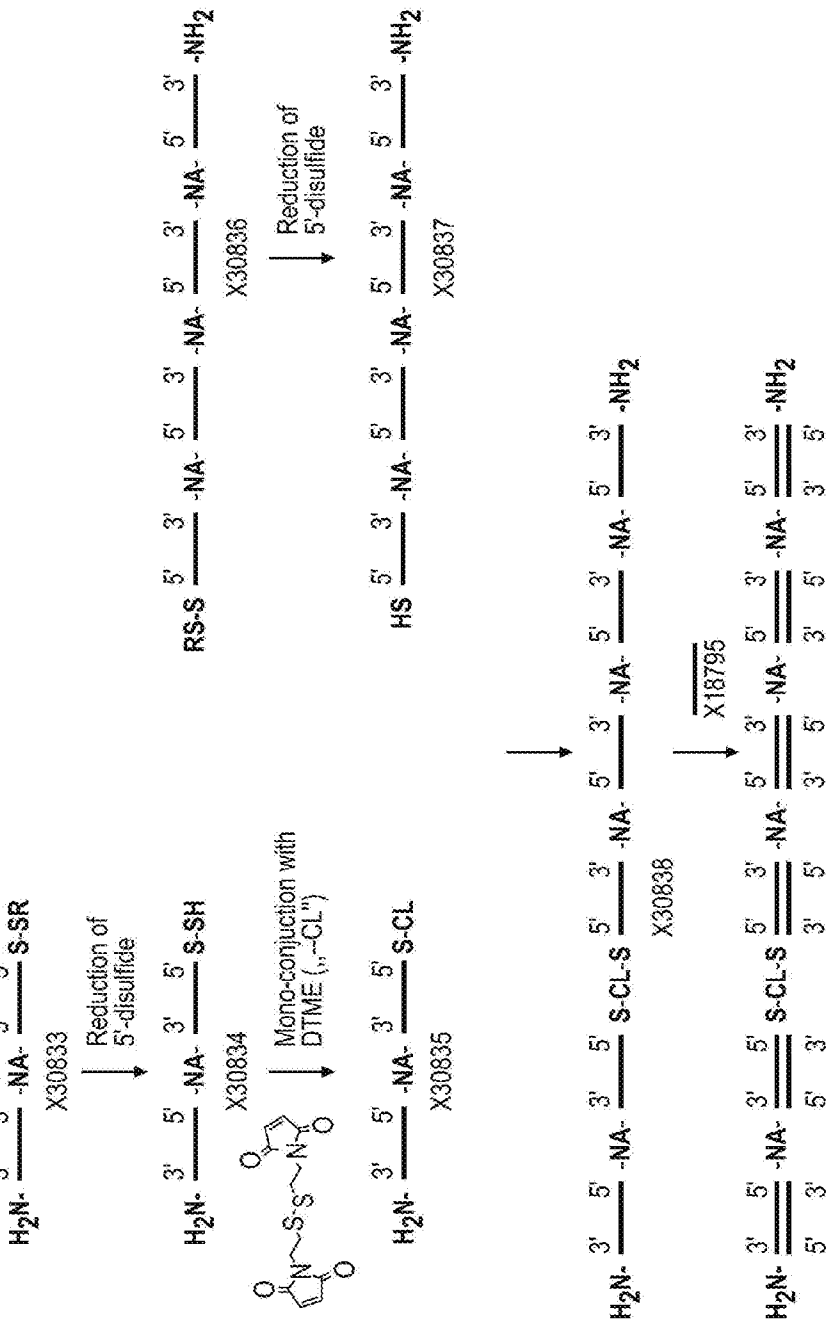

H2N- 3' 5' -NA- 3' 5' S-SR
X30833
Reduction of 5'-disulfide
H2N- 3' 5' -NA- 3' 5' S-SH
X30834
Mono-conjuction with DTME (,,-CL")
H2N- 3' 5' -NA- 3' 5' S-CL
X30835
RS-S 5' 3' -NA- 5' 3' -NA- 5' 3' -NA- 5' 3' -NH2
X30836
Reduction of 5'-disulfide
HS 5' 3' -NA- 5' 3' -NA- 5' 3' -NA- 5' 3' -NH2
X30837
H2N- 3' 5' -NA- 3' 5' S-CL-S 5' 3' -NA- 5' 3' -NA- 5' 3' -NA- 5' 3' -NH2
X30838
X18795
H2N- 3' 5' -NA- 3' 5' S-CL-S 5' 3' -NA- 5' 3' -NA- 5' 3' -NA- 5' 3' -NH2
XD-09795

FIG. 33A

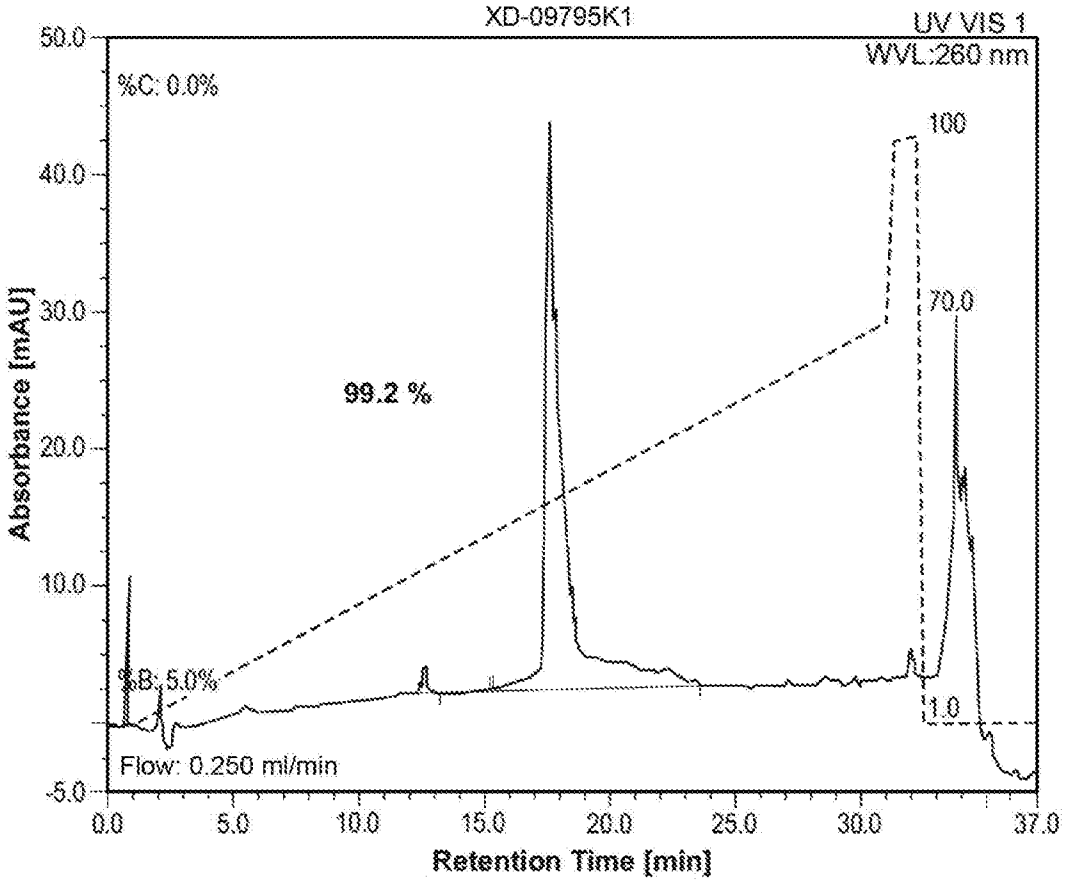

XD-09795K1
UV VIS 1
WVL:260 nm
%C: 0.0%
100
70.0
99.2 %
%B: 5.0%
1.0
Flow: 0.250 ml/min
Absorbance [mAU]
Retention Time [min]
50.0
40.0
30.0
20.0
10.0
-5.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
37.0

FIG. 33B

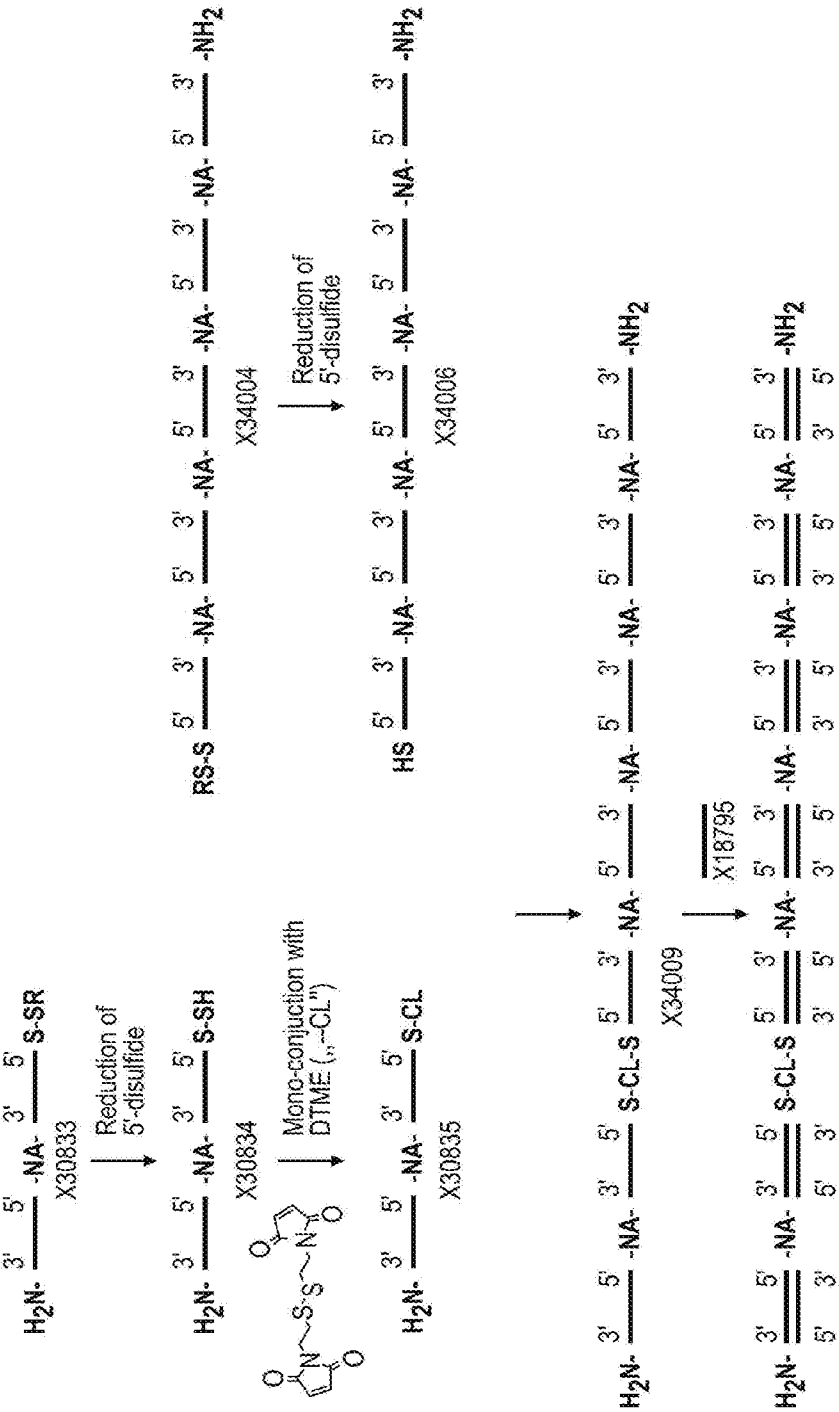

H2N- 3' 5' -NA- 3' 5' S-SR
X30833
Reduction of 5'-disulfide
H2N- 3' 5' -NA- 3' 5' S-SH
X30834
Mono-conjuction with DTME (,,-CL")
H2N- 3' 5' -NA- 3' 5' S-CL
X30835
RS-S 5' 3' -NA- 5' 3' -NA- 5' 3' -NA- 5' 3' -NH2
X34004
Reduction of 5'-disulfide
HS 5' 3' -NA- 5' 3' -NA- 5' 3' -NA- 5' 3' -NH2
X34006
H2N- 3' 5' -NA- 3' 5' S-CL-S 5' 3' -NA- 5' 3' -NA- 5' 3' -NA- 5' 3' -NH2
X34009
X18795
H2N- 3' 5' -NA- 3' 5' S-CL-S 5' 3' -NA- 5' 3' -NA- 5' 3' -NA- 5' 3' -NH2
5' 3' 3' 5'
XD-10640

FIG. 34A

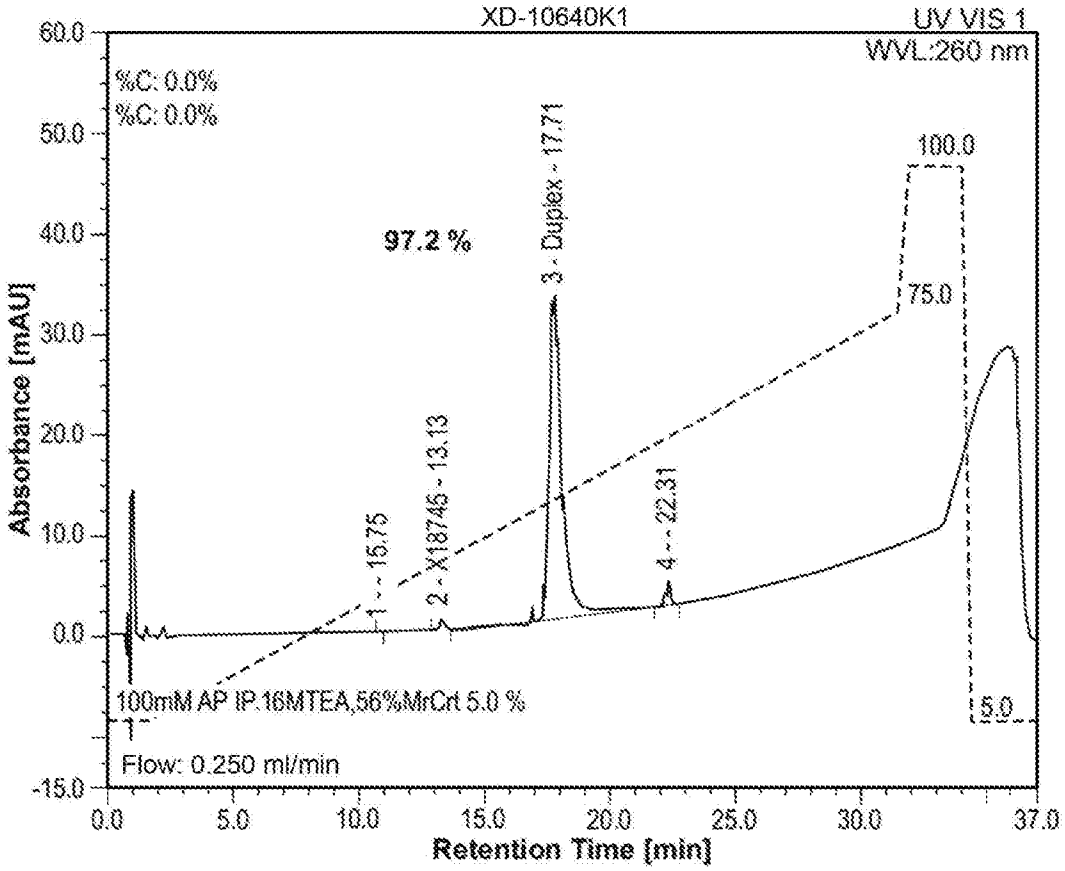

XD-10640K1
UV VIS 1
WVL:260 nm
%C: 0.0%
%C: 0.0%
97.2 %
3 - Duplex - 17.71
100.0
75.0
1 - - 16.75
2 - X18746 - 13.13
4 - - 22.31
5.0
100mM AP IP.16MTEA,56%MrCn 5.0 %
Flow: 0.250 ml/min
Absorbance [mAU]
Retention Time [min]
60.0
50.0
40.0
30.0
20.0
10.0
0.0
-15.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
37.0

FIG. 34B

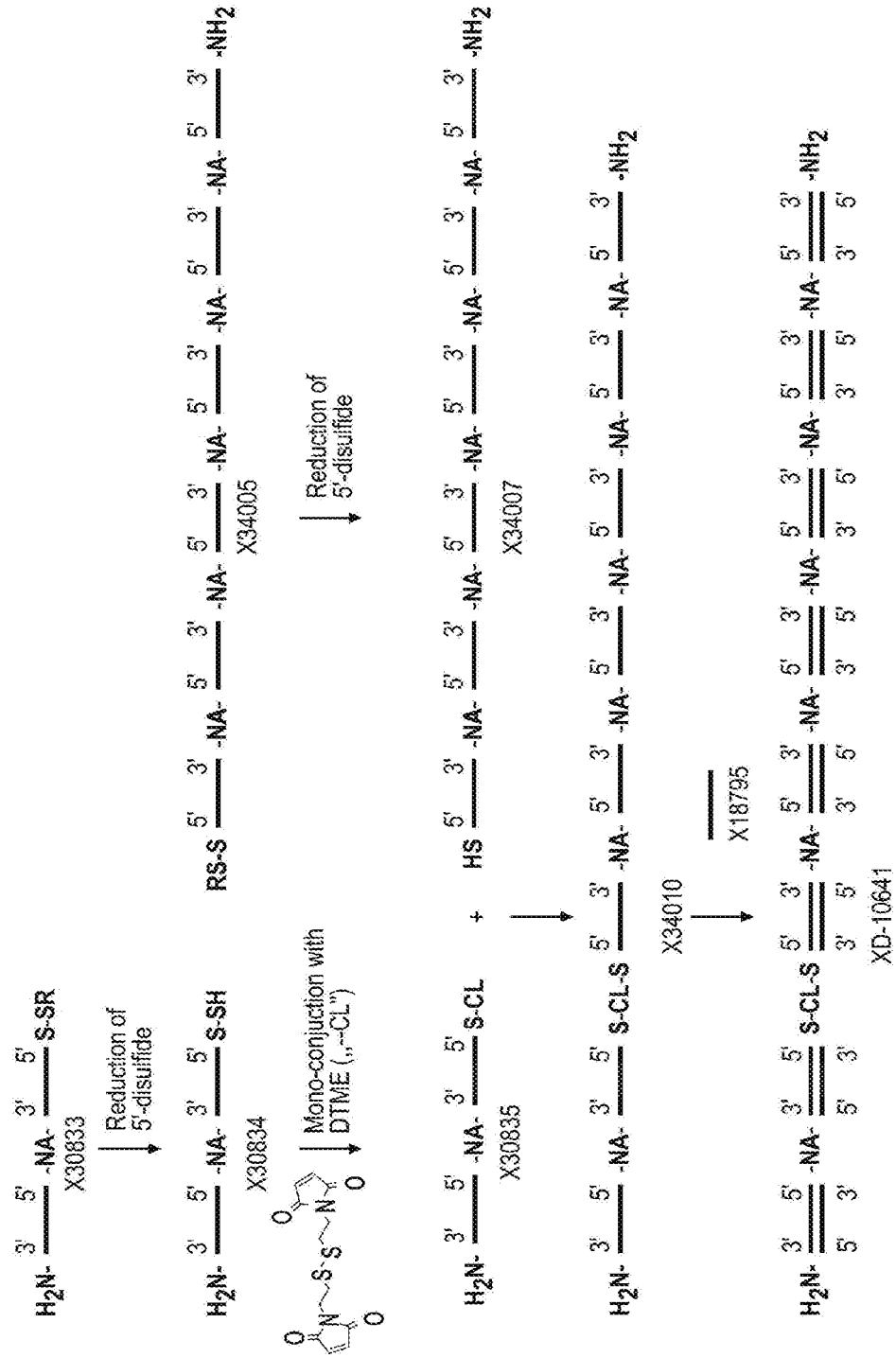

H2N-
S-SR
X30833
Reduction of 5'-disulfide
S-SH
X30834
Mono-conjuction with DTME („-CL")
S-CL
X30835
RS-S
X34005
Reduction of 5'-disulfide
HS
X34007
S-CL-S
X34010
X18795
XD-10641
-NA-
-NH2

FIG. 35A

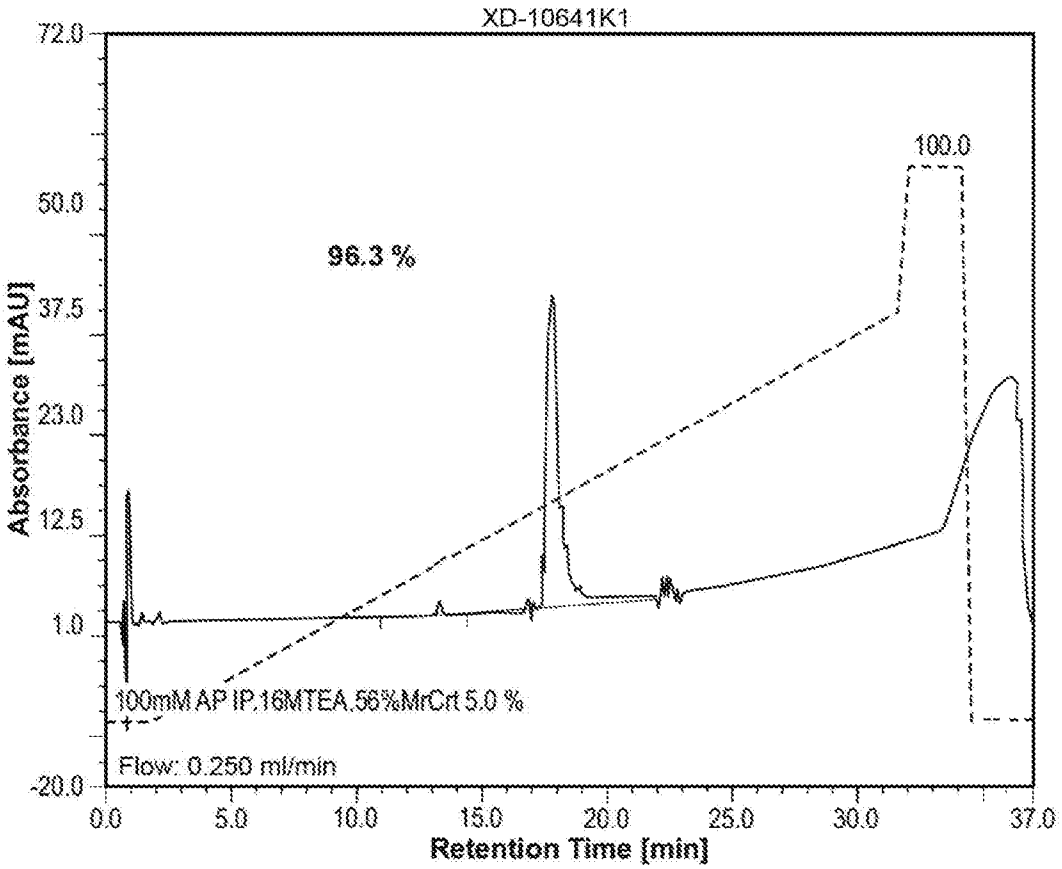

XD-10641K1
72.0
50.0
37.5
23.0
12.5
1.0
-20.0
Absorbance [mAU]
96.3 %
100.0
100mM AP IP,16MTEA,56%MrCrt 5.0 %
Flow: 0.250 ml/min
0.0
5.0
10.0
15.0
20.0
25.0
30.0
37.0
Retention Time [min]

FIG. 35B

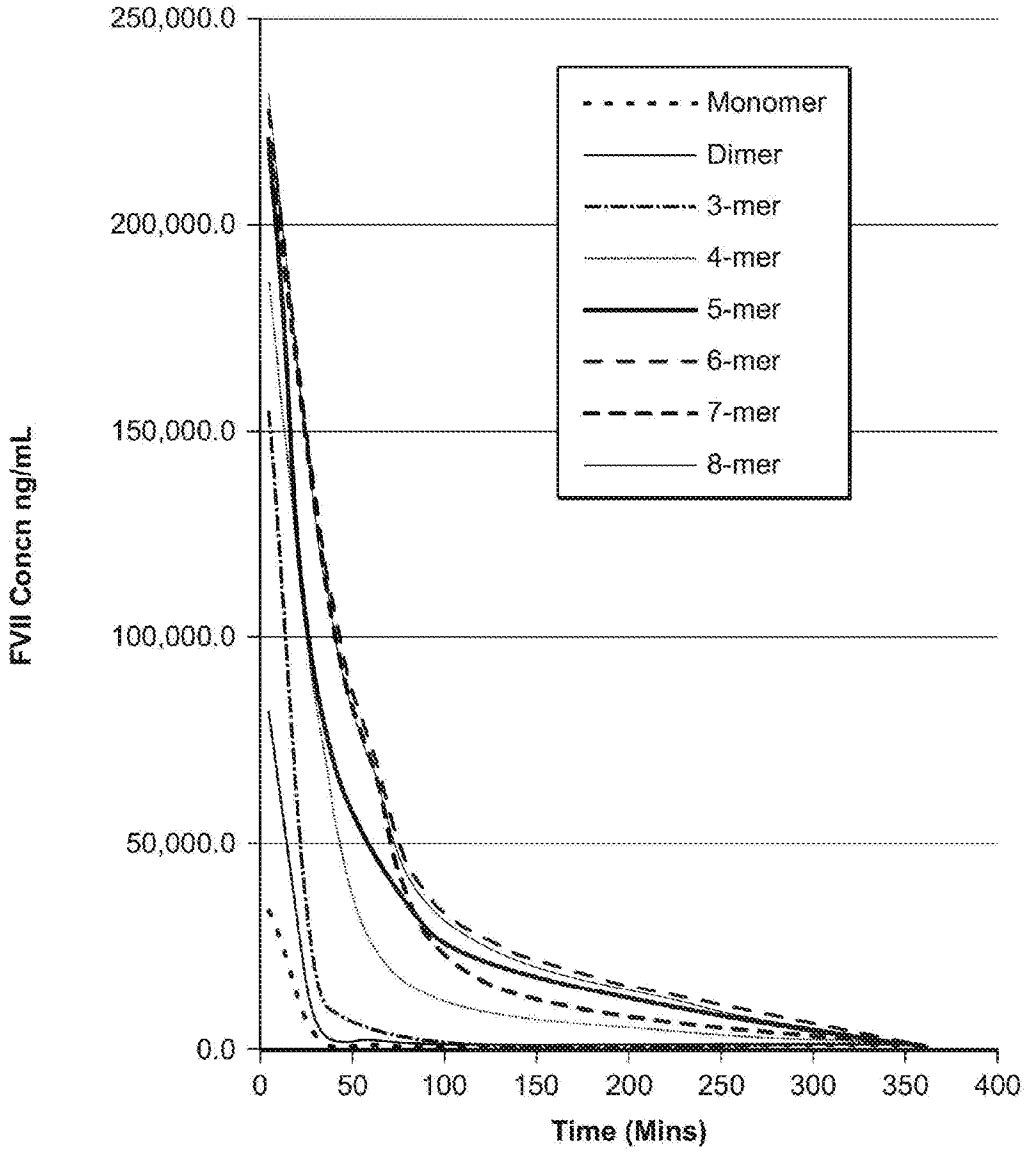

250,000.0
200,000.0
150,000.0
100,000.0
50,000.0
0.0
FVII Concn ng/mL
0
50
100
150
200
250
300
350
400
Time (Mins)
Monomer
Dimer
3-mer
4-mer
5-mer
6-mer
7-mer
8-mer

FIG. 36A

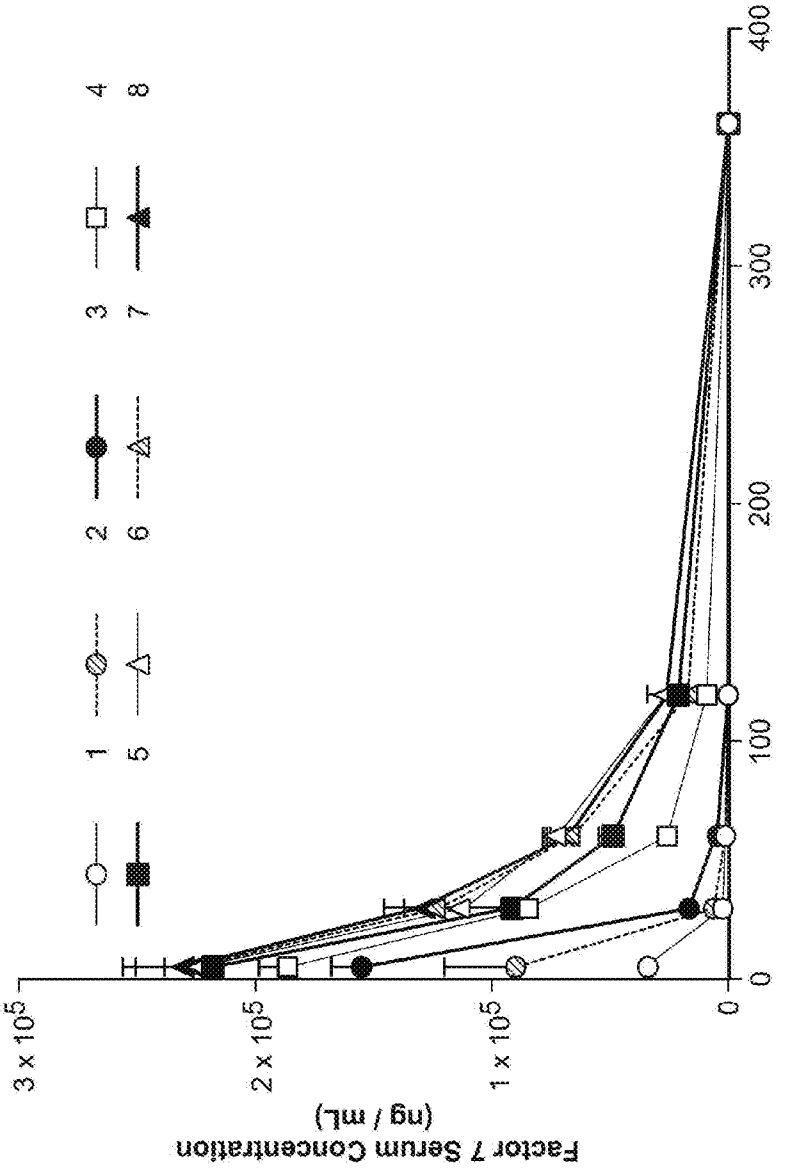

Factor 7 Serum Concentration
(ng / mL)
3 x 10^5
2 x 10^5
1 x 10^5
0
0
100
200
300
400
Time (Mins)
1
2
3
4
5
6
7
8

FIG. 36B

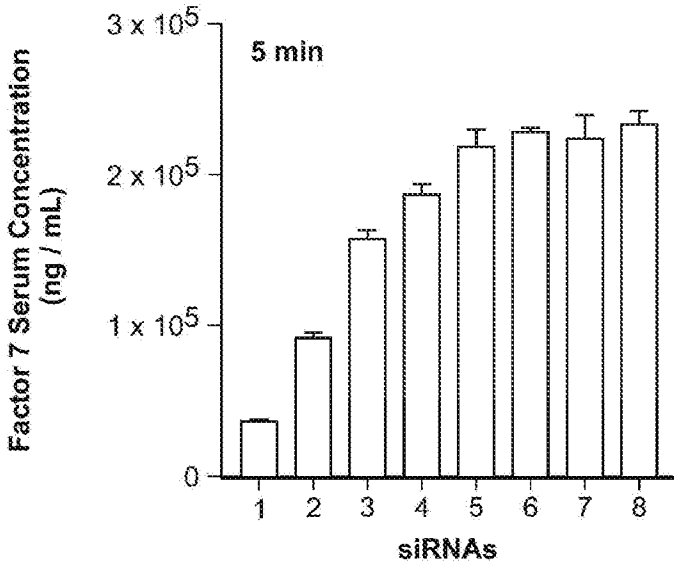


FIG. 37A

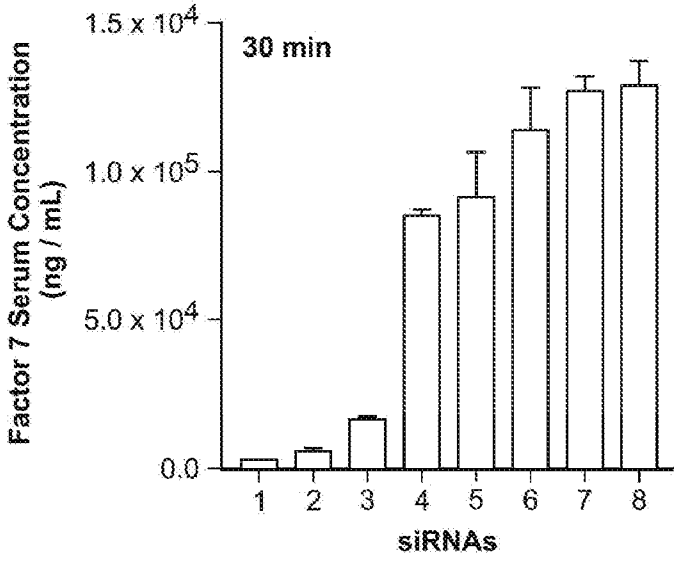


FIG. 37B

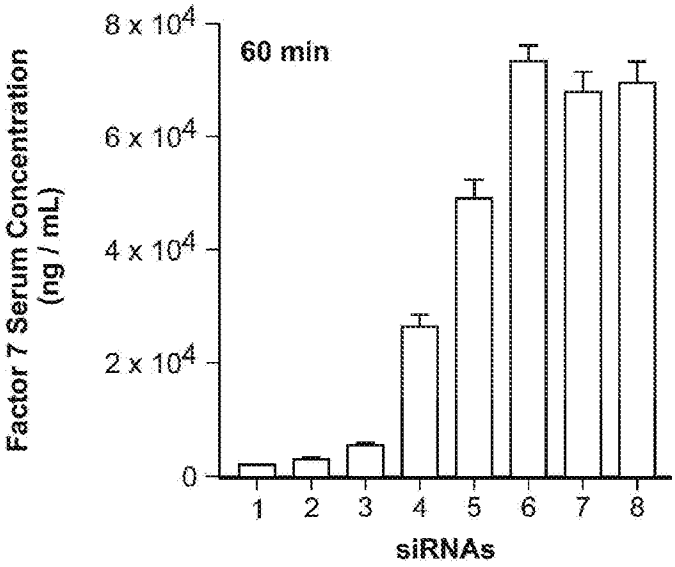


FIG. 37C

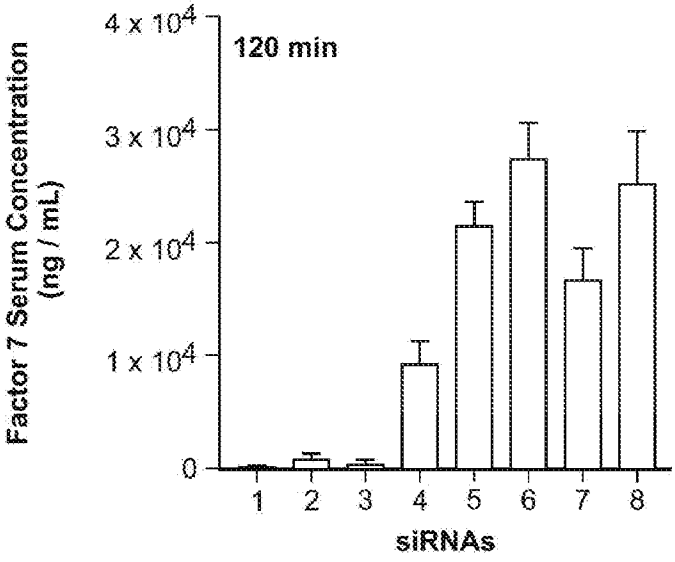


FIG. 37D

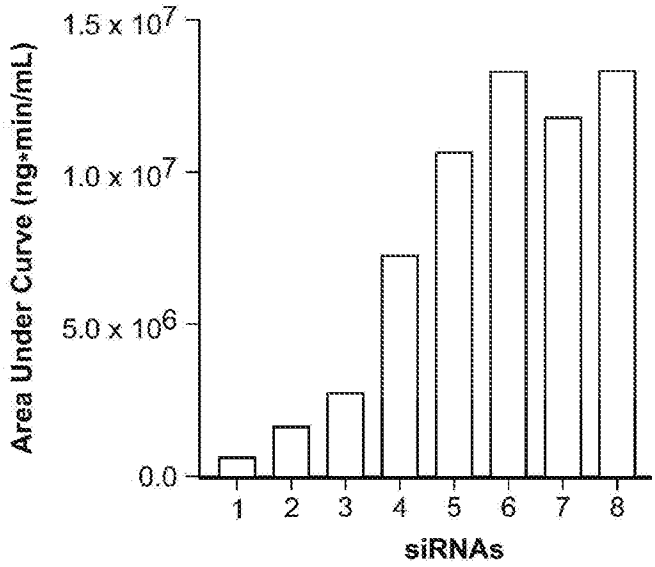


FIG. 38A

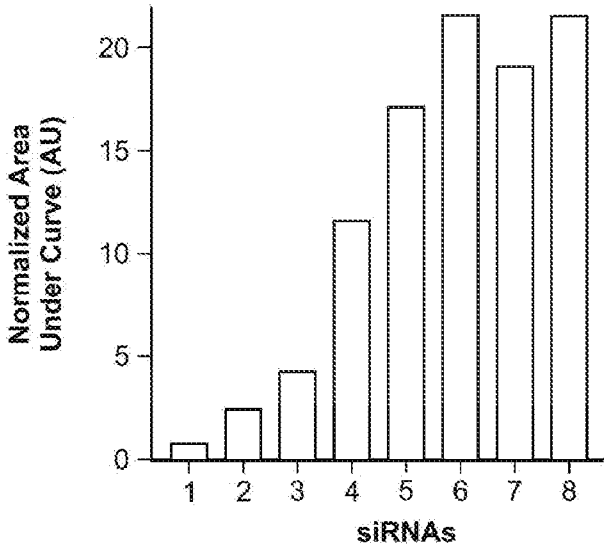


FIG. 38B

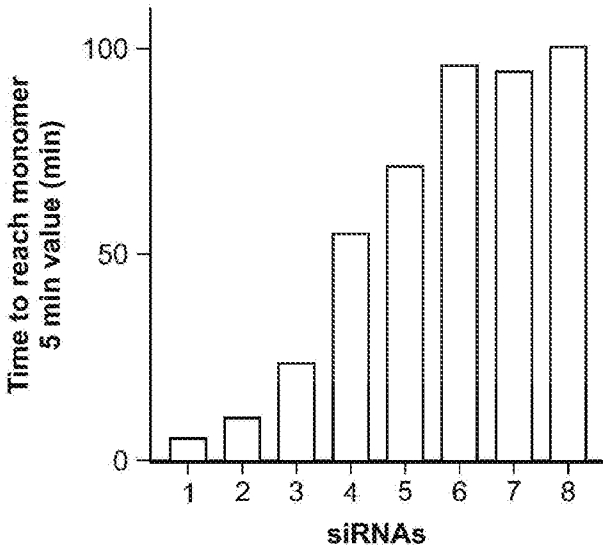

Time to reach monomer
5 min value (min)
100
50
0
1
2
3
4
5
6
7
8
siRNAs

FIG. 39

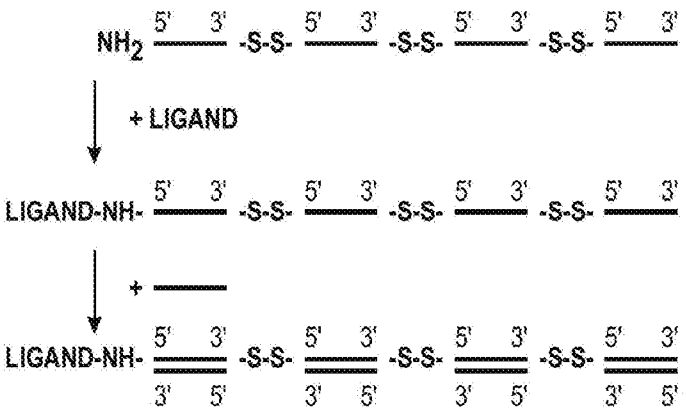

NH2
5' 3'
-S-S-
+ LIGAND
LIGAND-NH-
+
3' 5'

FIG. 40

FIG. 41

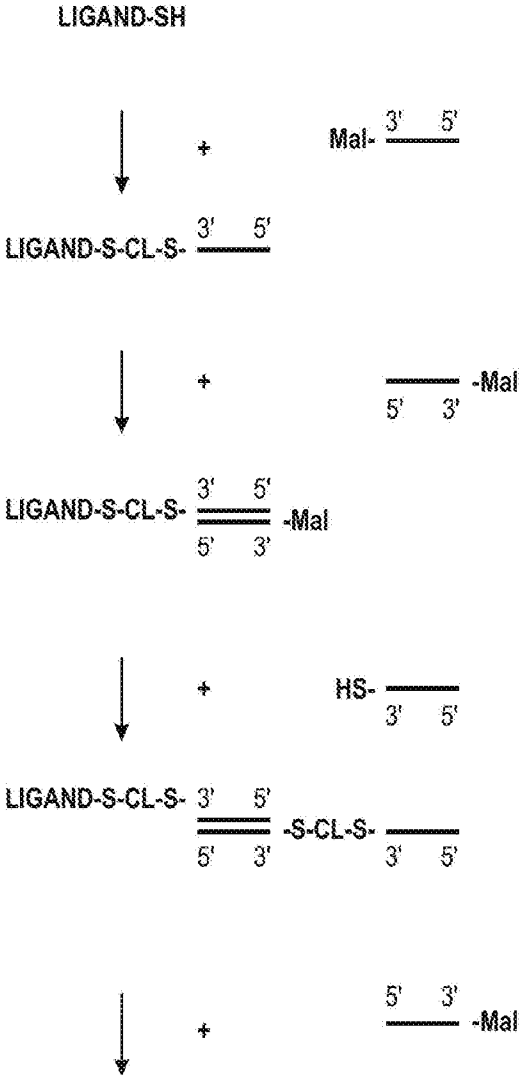
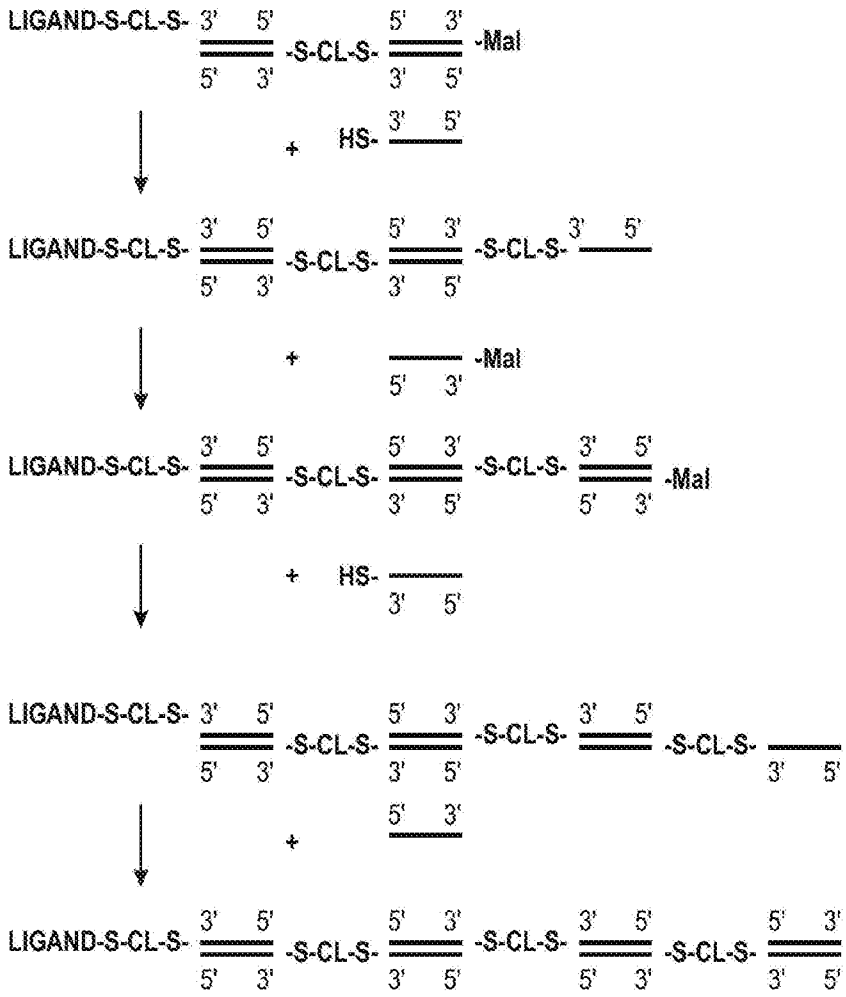
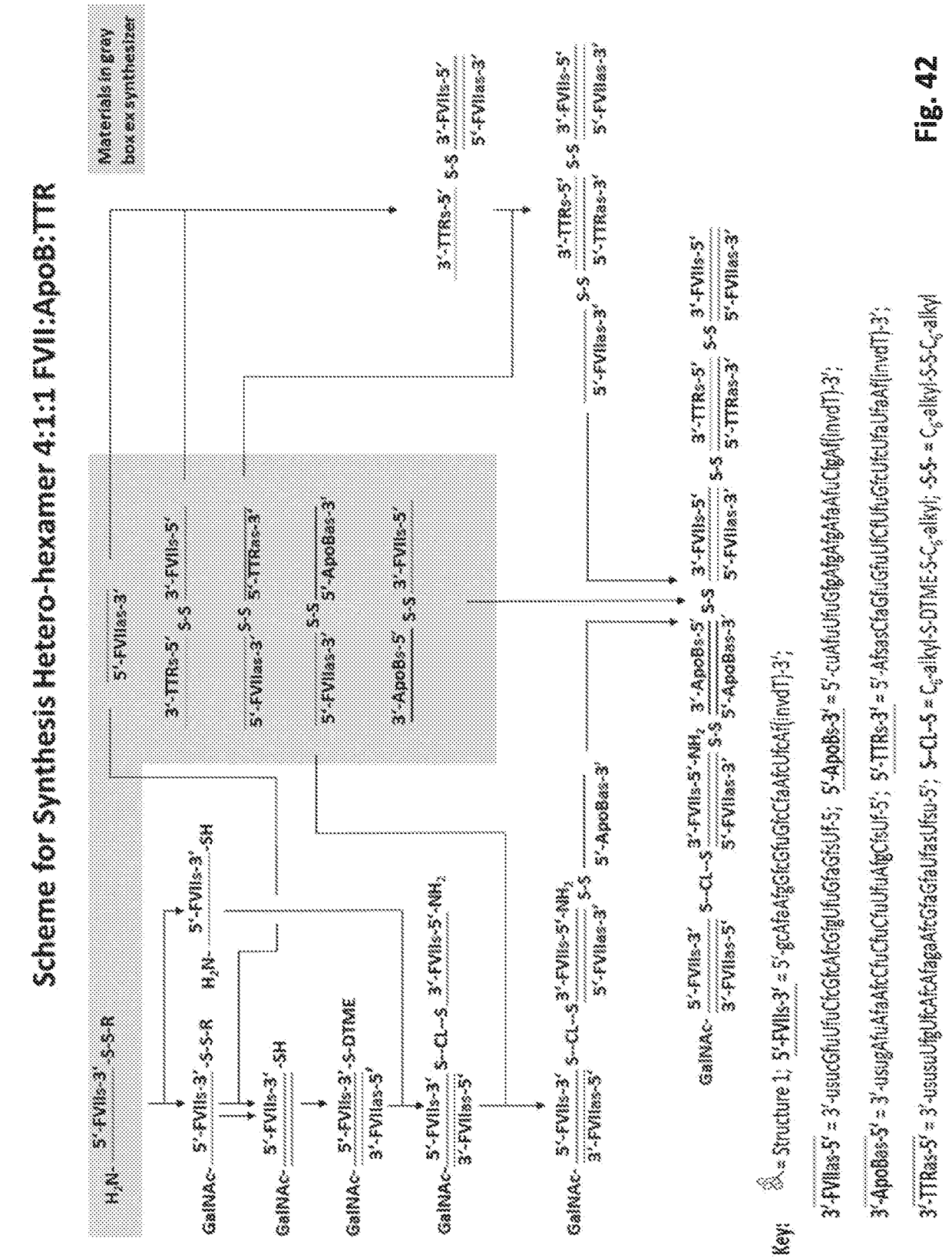

LIGAND-SH
+
Mal-
3'
5'
LIGAND-S-CL-S-
3'
5'
+
-Mal
5'
3'
LIGAND-S-CL-S-
3'
5'
-Mal
5'
3'
+
HS-
3'
5'
LIGAND-S-CL-S-
3'
5'
-S-CL-S-
5'
3'
3'
5'
+
5'
3'
-Mal

FIG. 41 (continued)

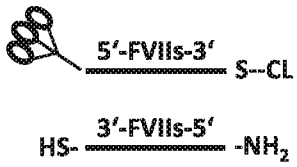



Scheme for Synthesis Hetero-hexamer 4:1:1 FVII:ApoB:TTR
Materials in gray box ex synthesizer
Key:
= Structure 1; 5'-FVIIs-3' = 5'-gcAfeAfgsGfcGfuGfcCfaAfcUfcAf(invdT)-3';
3'-FVIIas-5' = 3'-usucGfuUfuCfcGfcAfcGfgUfuGfaGfsUf-5; 5'-ApoBs-3' = 5'-cuAfuUfuGfgAfgAfgAfaAfuCfgAf(invdT)-3';
3'-ApoBas-5' = 3'-usugAfuAfaAfcCfuCfuUfuAfgCfsUf-5'; 5'-TTRs-3' = 5'-AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)-3';
3'-TTRas-5' = 3'-ususUfgUfcAfcAfagaAfcGfaGfaUfasUfsu-5'; 5'-CL-5 = C6-alkyl-S-DTME-5'-C6-alkyl; 5'-S- = C6-alkyl-S-5'-C6-alkyl
Fig. 42

Preparation of FVII Sense Strands

X39850 and X39851

- **X39850** = FVII sense strand with trivalent GalNAc cluster at 5'-end and 3'-thiol DTME functionality.
- **X39851** = FVII sense strand with free 5'-amino group and 3'-thiol group.



ssRNA ID	Sequence (5'--3')
X39850	(GalNAc3)(NHC6) gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT) (SHC6)(DTME)
X39851	(NH2C6) gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(SHC6)

Reaction scheme:

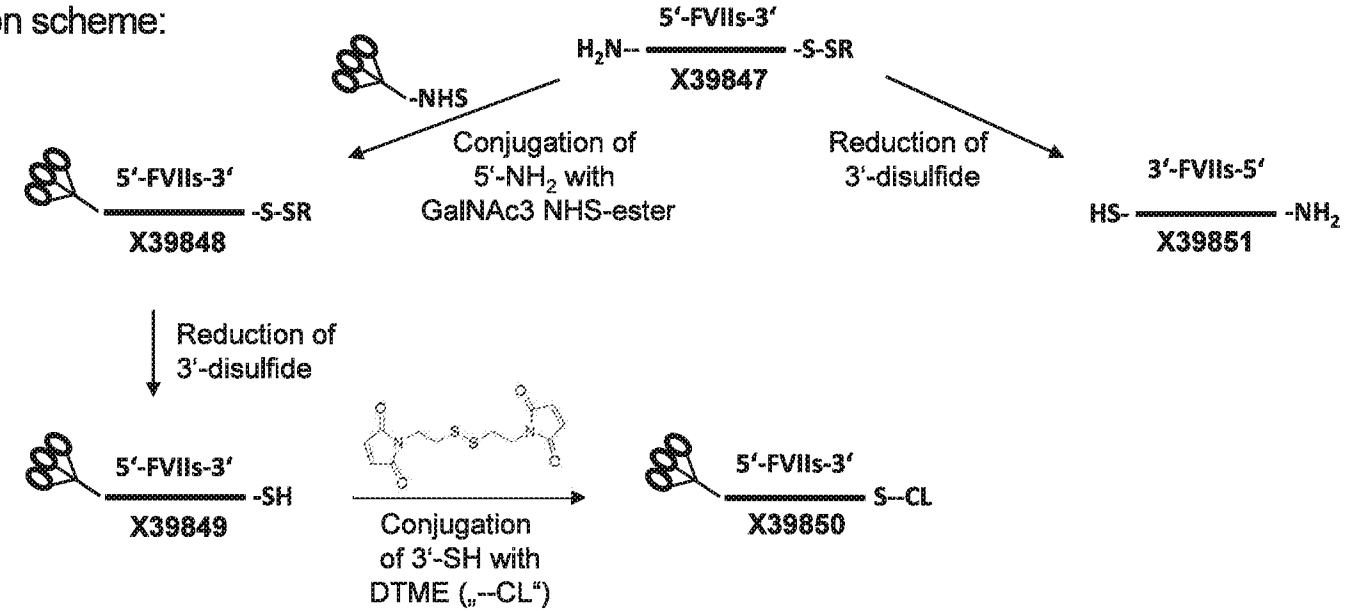


Fig. 43

QC Data for Heterohexamer Constituents (1)

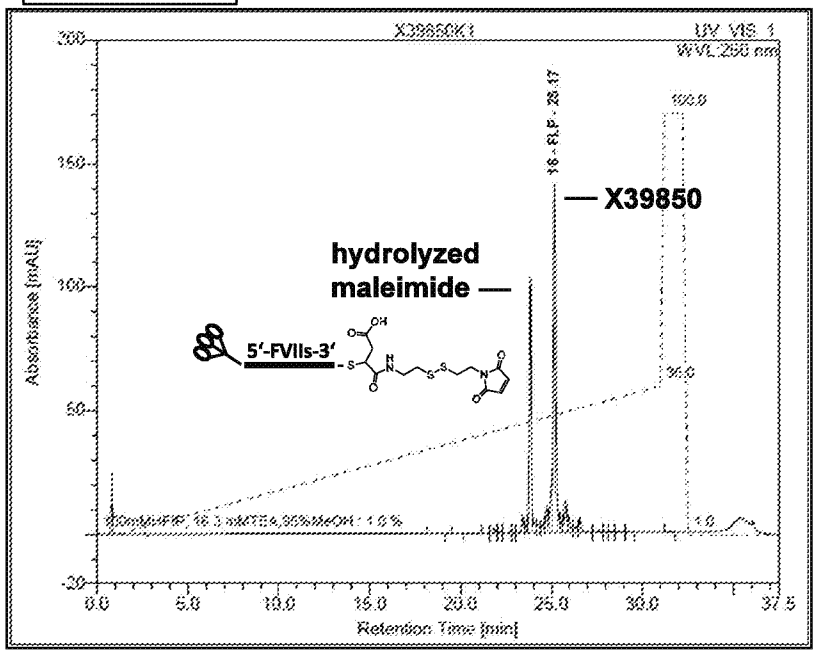
RP-HPLC-MS Analysis for X39850

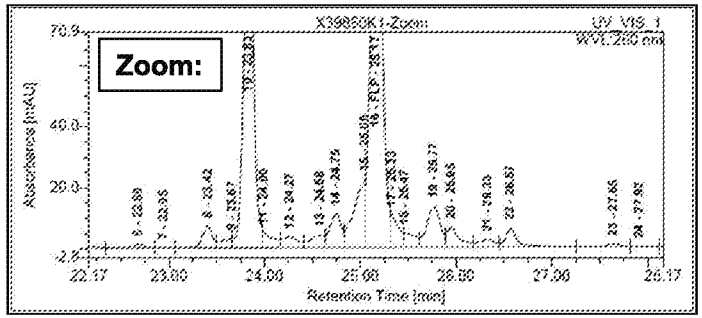
- **X39850** = FVII sense strand with trivalent GalNAc cluster at 5'-end and 3'-thiol DTME functionality.



ssRNAID	Sequence (5'--3')
X39850	(GalNAc3)(NHC6) gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT) (SHC6)(DTME)

RP-HPLC:





ESI-MS:

MW (calcd)	MW (obsd)
8703.48	8700.5534

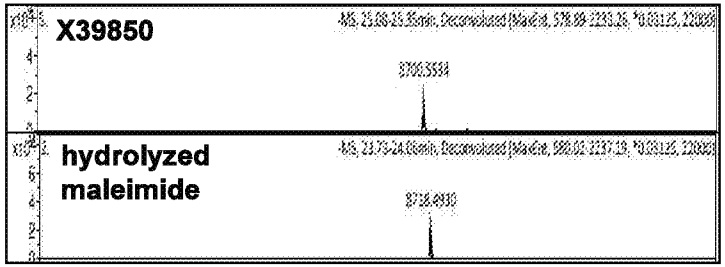


Fig. 44

QC Data for Heterohexamer Constituents (1)

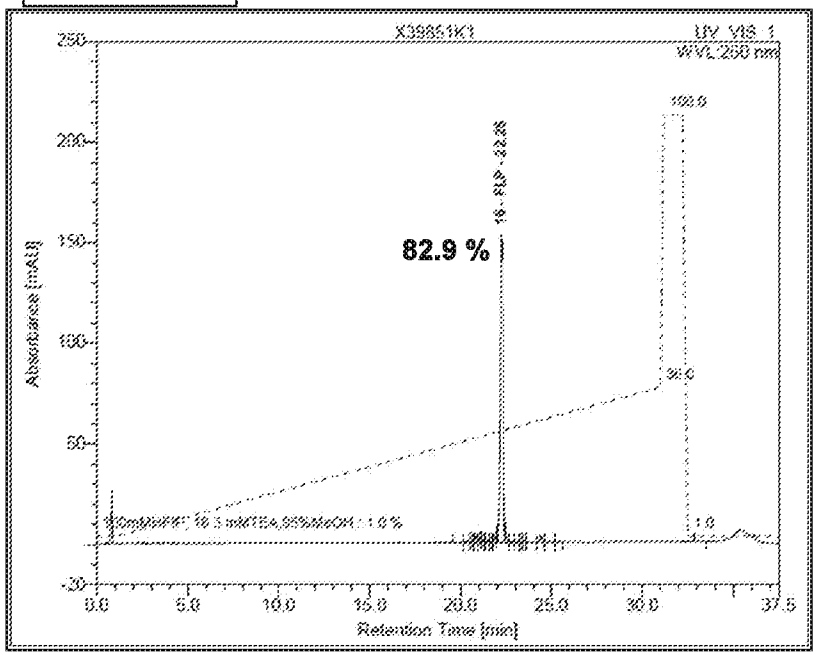
RP-HPLC-MS Analysis for X39851

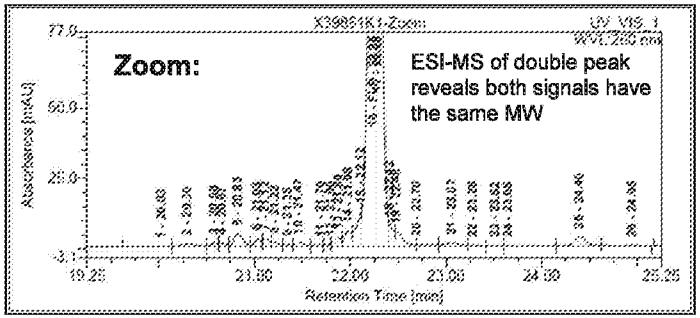
- **X39851** = FVII sense strand with free 5'-amino group and 3'-thiol group.

HS- 3'-FVIIs-5' -NH_2

ssRNAID	Sequence (5'–3')
X39851	(NH2C6) gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT) (SHC6)

RP-HPLC:





ESI-MS:

MW (calcd)	MW (obsd)
6952.59	6951.9325

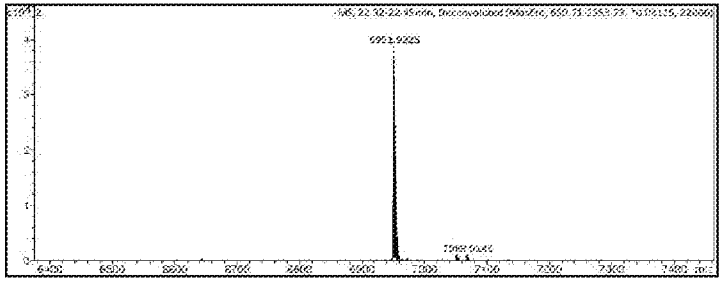


Fig. 45

QC Data for Heterohexamer Constituents (1)

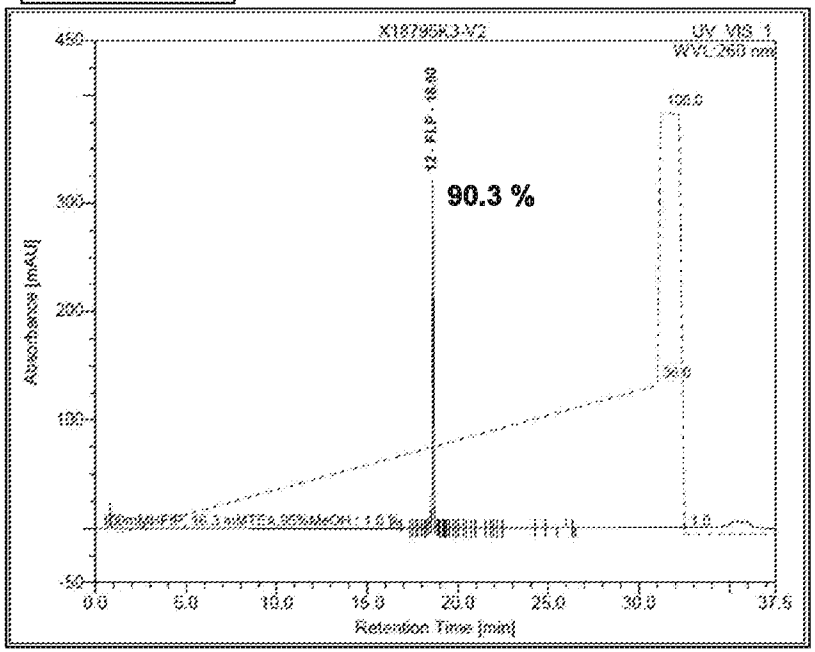
RP-HPLC-MS Analysis for X18795

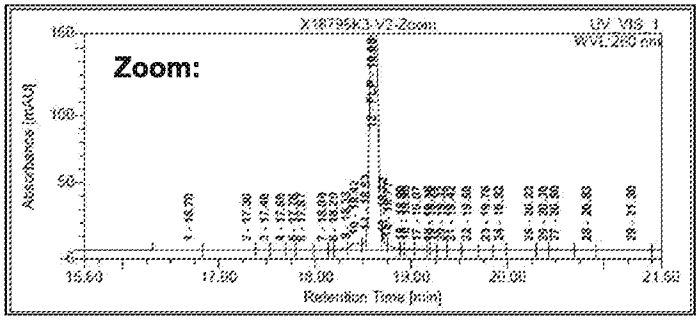
- **X18795** = FVII antisense strand.

5'-FVIIas-3'

ssRNAID	Sequence (5'--3')
X18795	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu

RP-HPLC:





ESI-MS:

MW (calcd)	MW (obsd)
6849.25	6848.7035

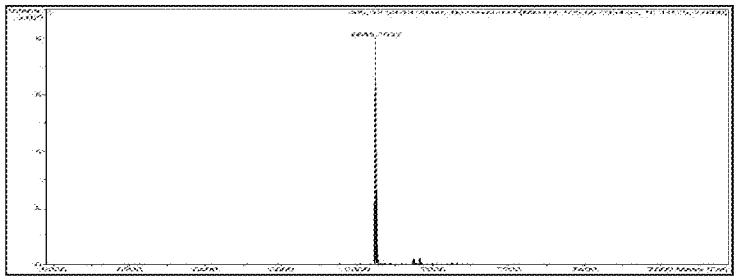


Fig. 46

QC Data for Heterohexamer Constituents (1)

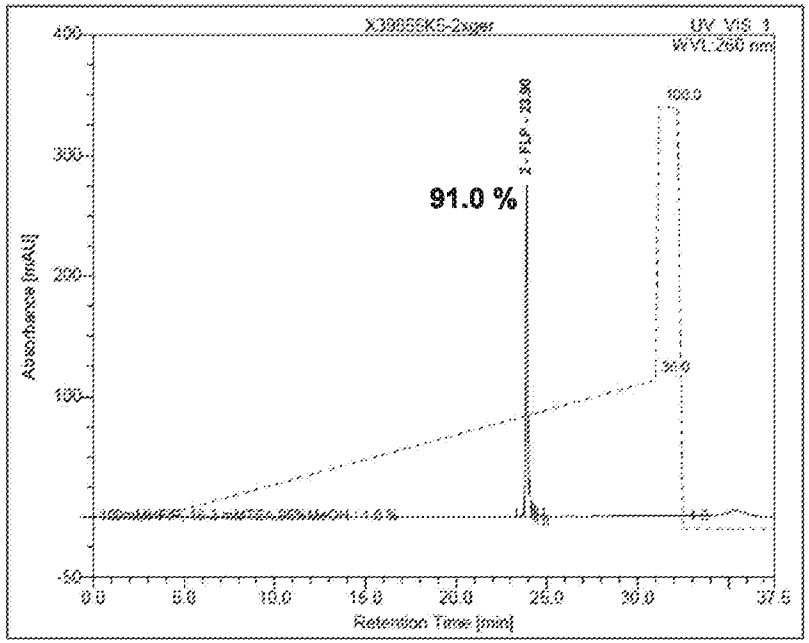
RP-HPLC-MS Analysis for X39855

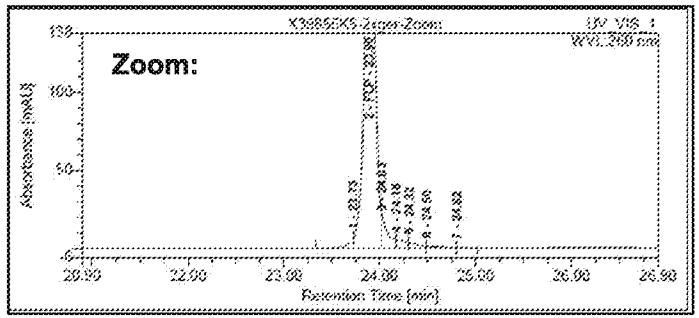
- **X39855** consists of FVII antisense linked to ApoB antisense via a disulfide linkage.

5′-FVIIas-3′ S-S 5′-ApoBas-3′

ssRNAID	Sequence (5′–3′)
X39855	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C6SSC6) UfsCfgAfuUfuCfuCfuCfcAfaAfuAfgusu

RP-HPLC:





ESI-MS:

MW (calcd)	MW (obsd)
14001.87	14001.5292

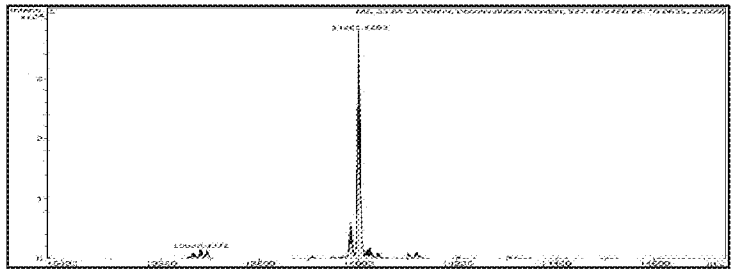


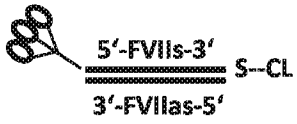
Fig. 47

QC Data for Heterodimeric Duplex

Native RP-HPLC of 1st Annealing

- Annealings are performed stepwise to obtain the desired heterodimeric duplex with ApoB antisense overhang.

- First, the GalNAc-conjugated FVII sense strand **X39850** is annealed with 1 mole equivalent of antisense **X18795**.



Duplex purity: **78.2 %**

RP-HPLC:

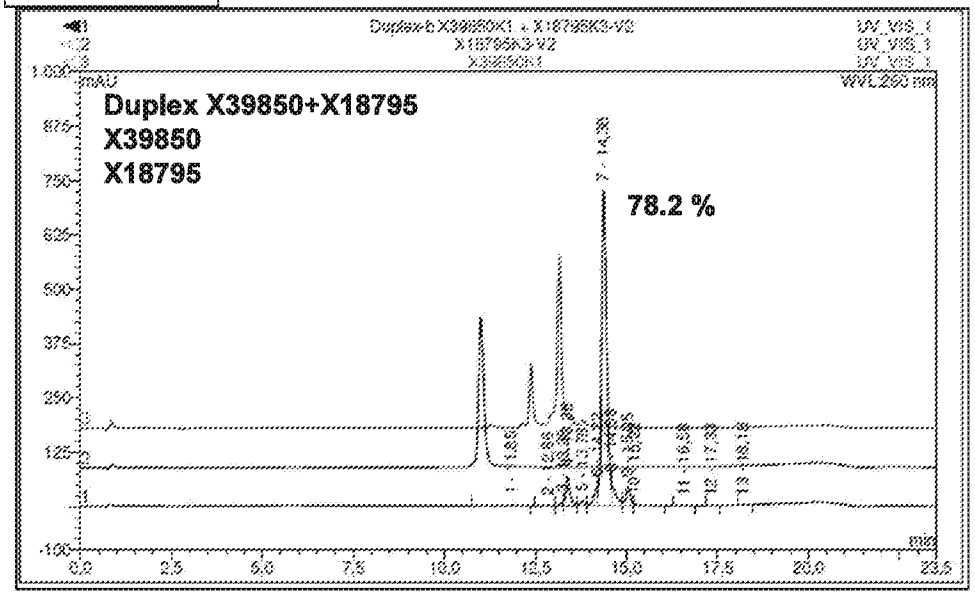


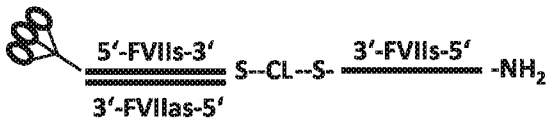
Fig. 48

QC Data for Heterodimeric Duplex

Native RP-HPLC After Conjugation

- Annealings are performed stepwise to obtain the desired heterodimeric duplex with ApoB antisense overhang.

- The FVII duplex X39850-X18795 is conjugated with the FVII sense strand **X39851** followed by IEX purification.



Duplex purity: **87.0 %**
X39851 excess: 0.8 %

RP-HPLC:

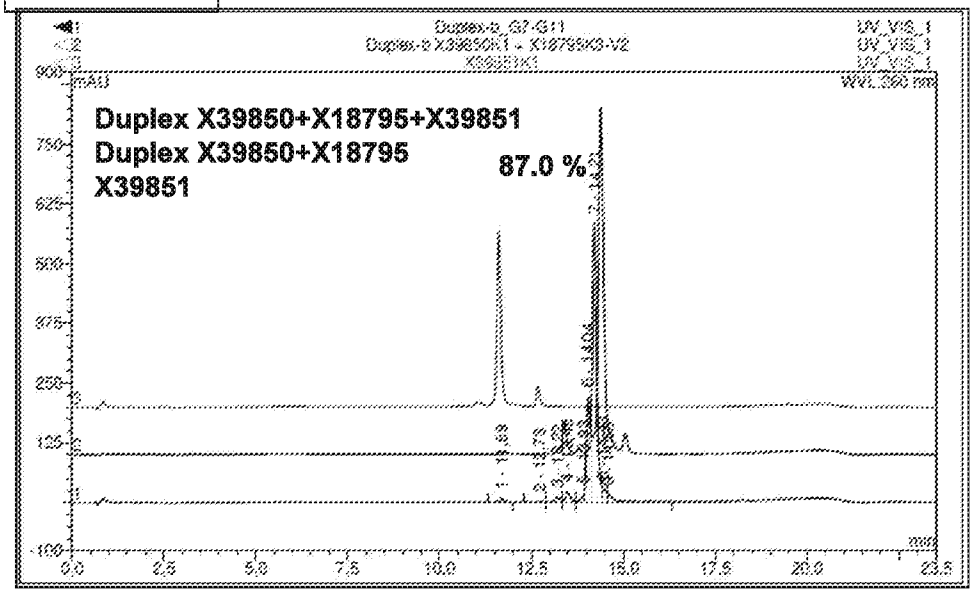


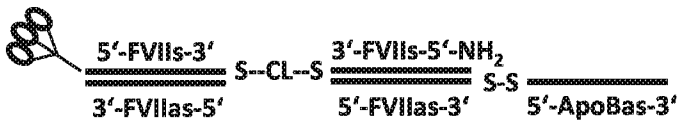
Fig. 49

QC Data for Heterodimeric Duplex

Native RP-HPLC of 2nd Annealing

- Annealings are performed stepwise to obtain the desired heterodimeric duplex with ApoB antisense overhang.

- Next, 1 mole equivalent of the dimeric antisense strand **X39855** is added to the X39850-X18795-X39851 duplex.



Duplex purity: **97.6 %**
Antisense strand excess: 0.6 %

RP-HPLC:

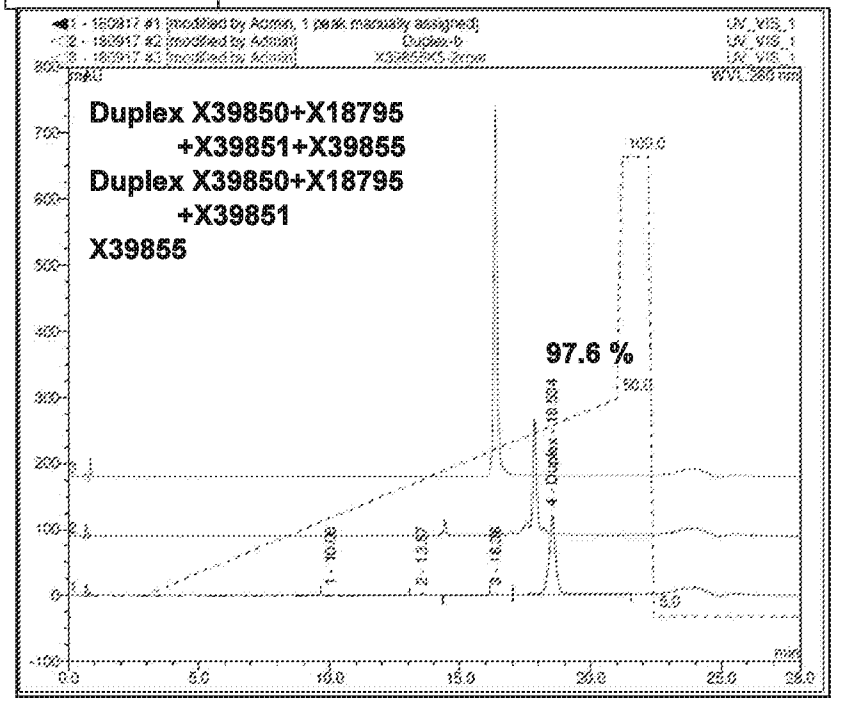


Fig. 50

QC Data for Heterohexamer Constituents (2)

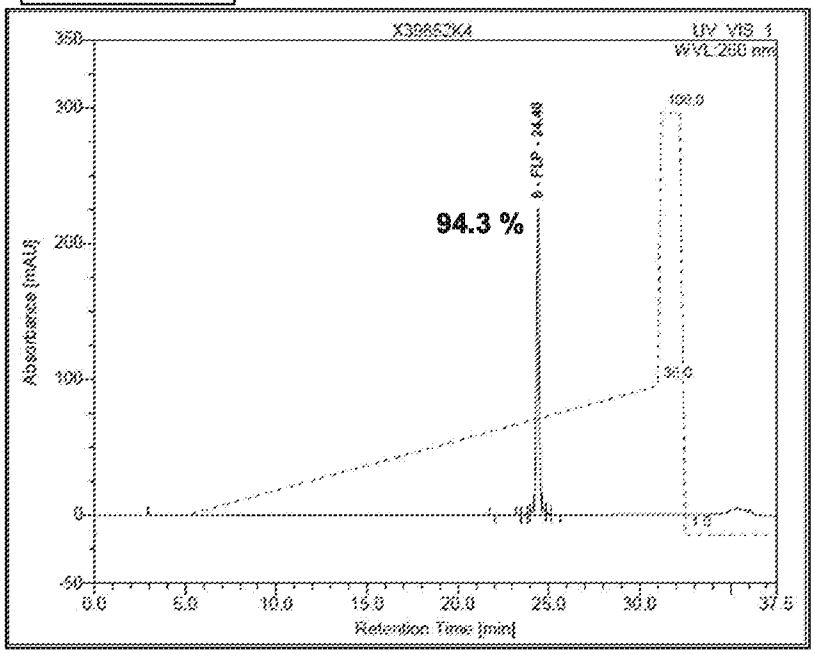
RP-HPLC-MS Analysis for X39852

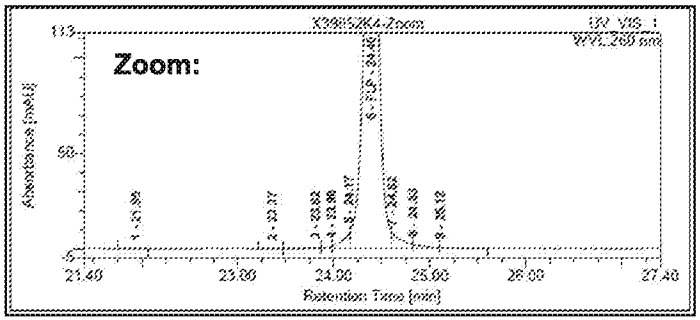
- **X39852** consists of FVII sense linked to TTR sense via a disulfide linkage.

3'-TTRs-5' S-S 3'-FVIIs-5'

ssRNAID	Sequence (5'–3')
X39852	gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C6SSC6) AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(invdT)

RP-HPLC:





ESI-MS:

MW (calcd)	MW (obsd)
14073.03	14071.5142

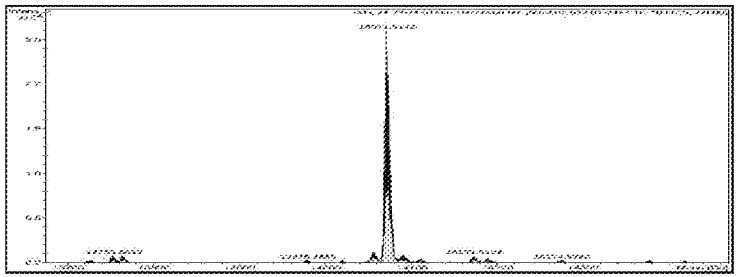
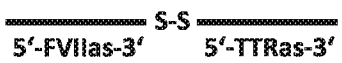


Fig. 51

QC Data for Heterohexamer Constituents (2)

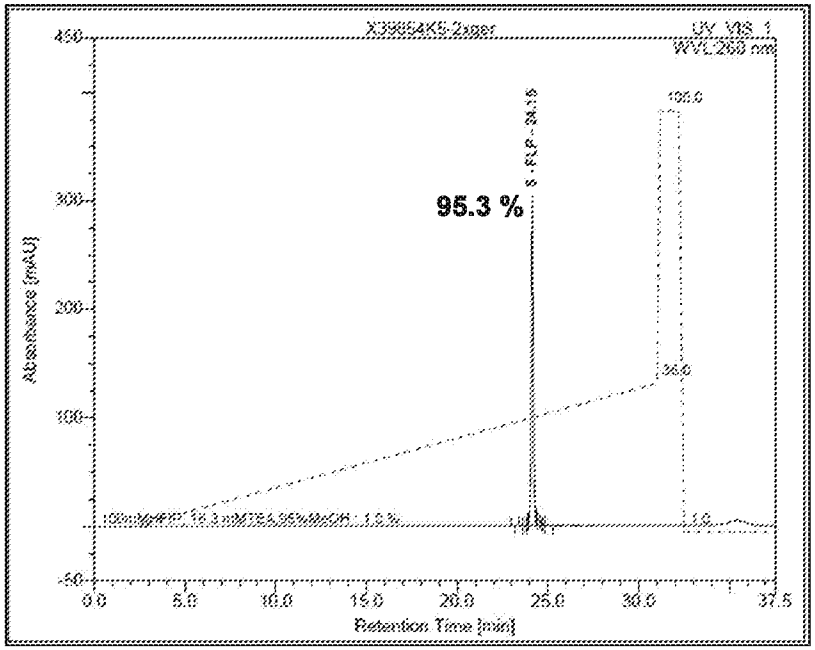
RP-HPLC-MS Analysis for X39854

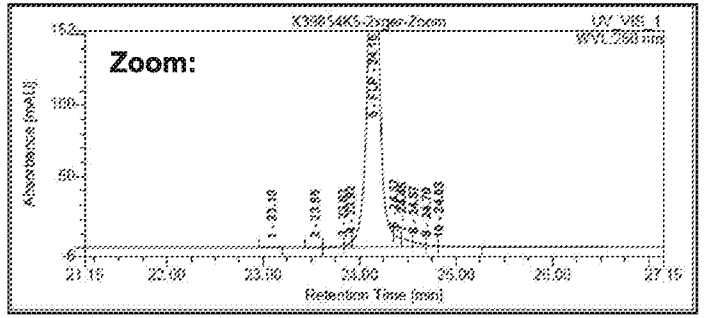
- **X39854** consists of FVII antisense linked to TTR antisense via a disulfide linkage.



ssRNA ID	Sequence (5'–3')
X39854	UfsGfaGfuUfgGfcAfcGfcCfuUfuGfcusu(C6SSC6) usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu

RP-HPLC:





ESI-MS:

MW (calcd)	MW (obsd)
14835.59	14834.6257

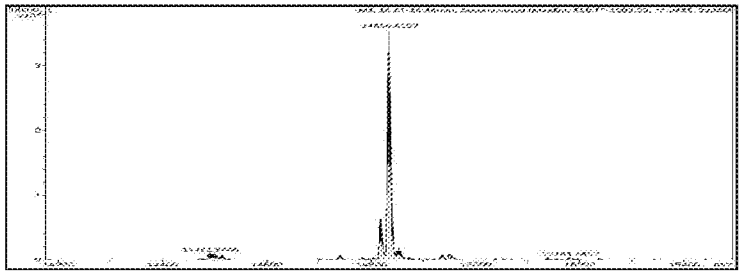


Fig. 52

QC Data for Heterohexamer Constituents (2)

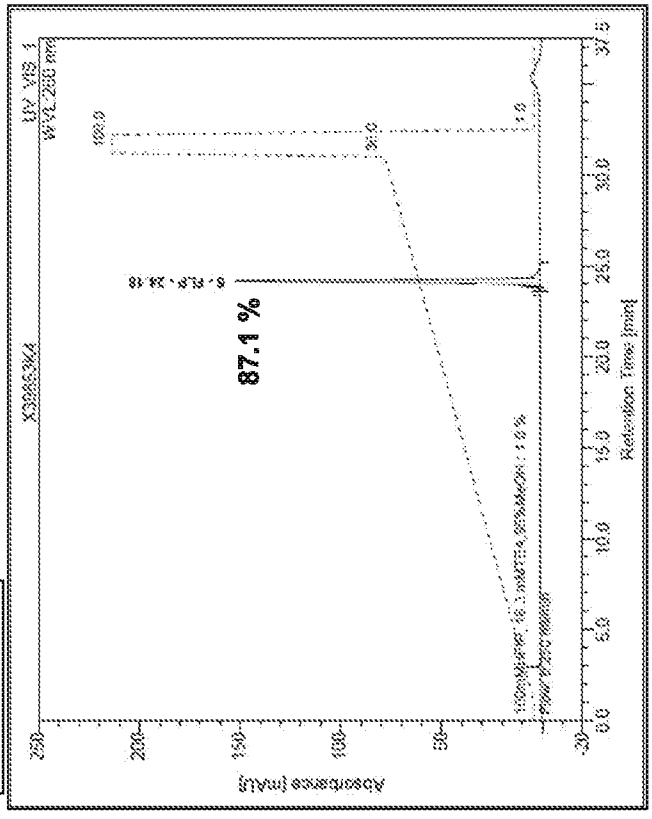
RP-HPLC-MS Analysis for X39853

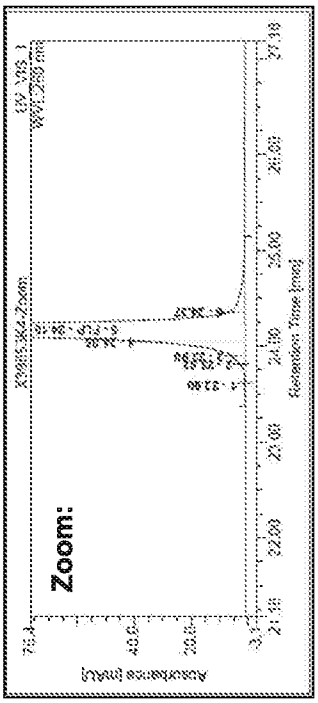
- **X39853** consists of FVII sense linked to ApoB sense via a disulfide linkage.

3′-ApoBs-5′ S-S 3′-FVIIs-5′

ssRNA ID	Sequence (5′–3′)
X39853	gscsAfaAfgGfcGfuGfcCfaAfcUfcAf(invdT)(C6SSC6) csusAfuUfuGfgAfgAfgAfaAfuCfgAf (invdT)

RP-HPLC:





ESI-MS:

MW (calcd)	MW (obsd)
13571.84	13570.5278

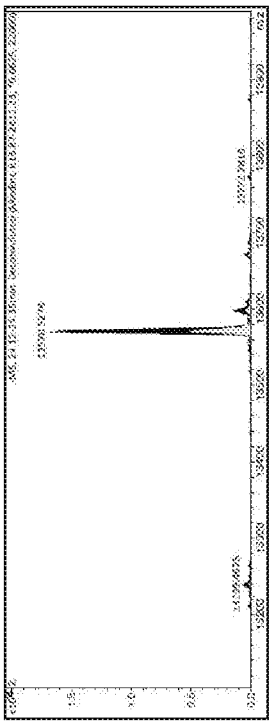


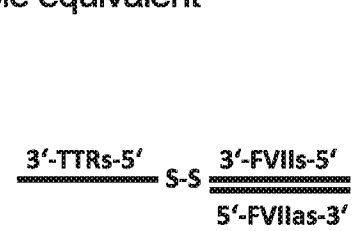
Fig. 53

QC Data for Heterotrimeric Duplex

Native RP-HPLC of 1st Annealing

- Annealings are performed stepwise to obtain the desired heterotrimeric duplex with ApoB sense overhang.

- First, the dimeric sense strand **X39852** is annealed with 1 mole equivalent of antisense **X18795**.



Duplex purity: **99.5 %**
Antisense strand excess: 0.5 %

RP-HPLC:

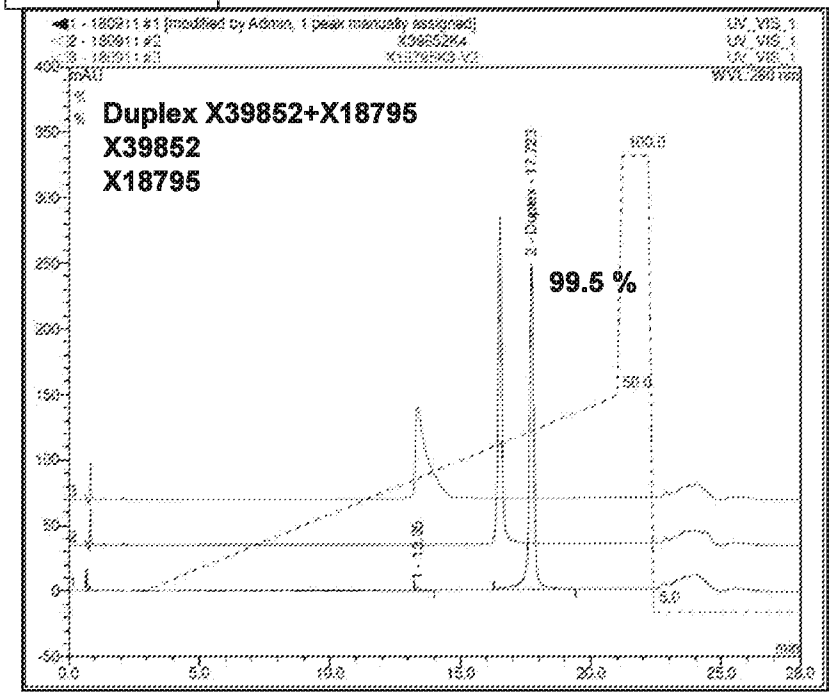


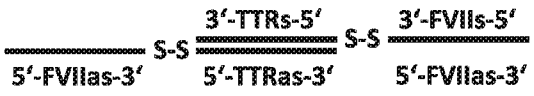
Fig. 54

QC Data for Heterotrimeric Duplex

Native RP-HPLC of 2nd Annealing

- Annealings are performed stepwise to obtain the desired heterotrimeric duplex with ApoB sense overhang.

- Next, 1 mole equivalent of the dimeric antisense strand **X39854** is added to the X39852-X18795 duplex.



Duplex purity: **94.9 %**
Antisense strand excess: 2.0 %

RP-HPLC:

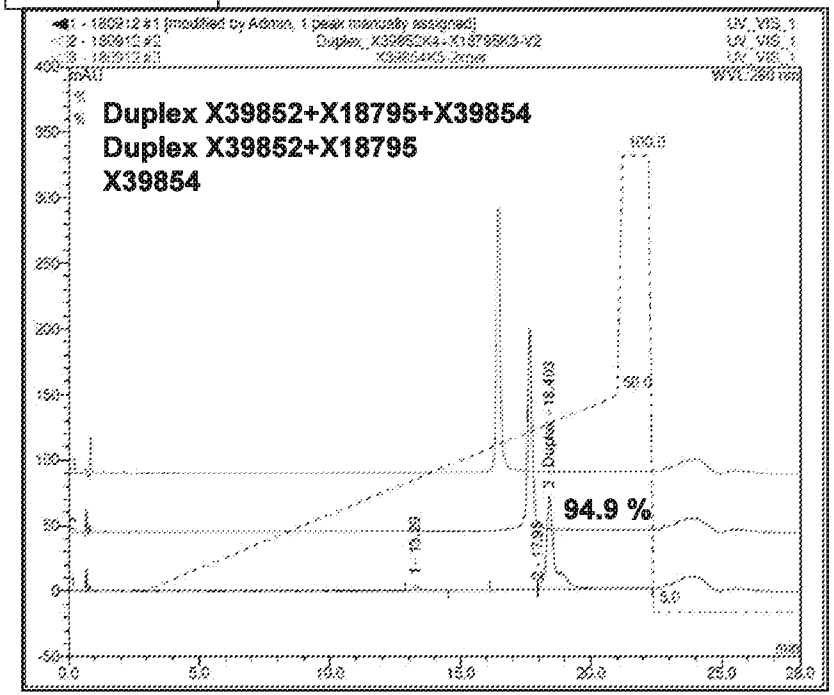


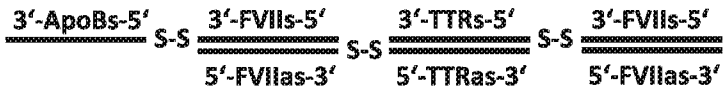
Fig. 55

QC Data for Heterotrimeric Duplex

Native RP-HPLC of 3rd Annealing

- Annealings are performed stepwise to obtain the desired heterotrimeric duplex with ApoB sense overhang.

- Last, 1 mole equivalent of the dimeric sense strand **X39853** is added to the X39852-X18795-X39854 duplex followed by IEX purification.



Duplex purity: **98.3 %**

RP-HPLC:

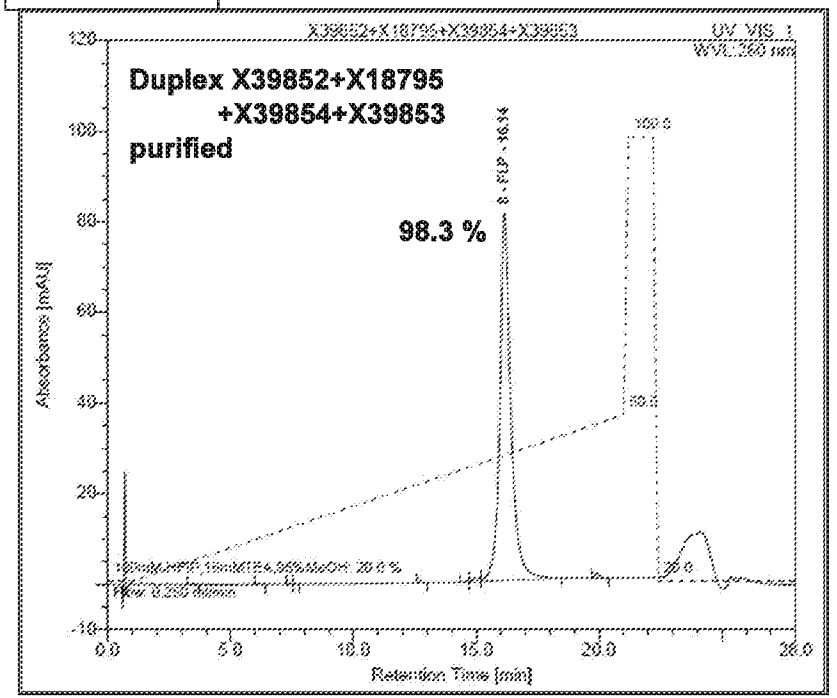
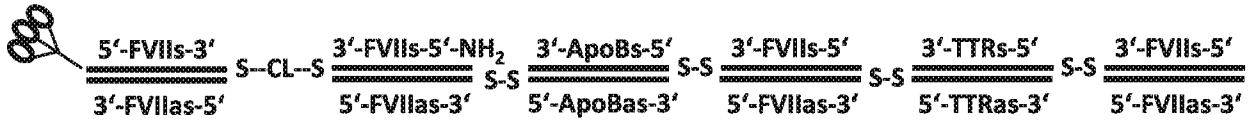


Fig. 56

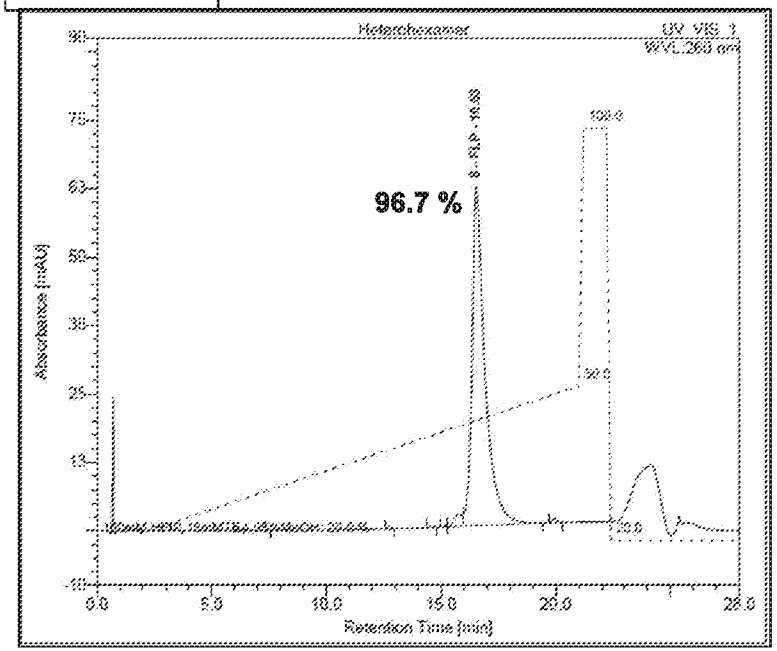
QC Data for Heterohexameric Duplex

Native RP-HPLC After Final Annealing

GalNAc-conjugated heterohexameric siRNA:



RP-HPLC:



Annealing is performed with equimolar amounts of each duplex.

Yield: **4.4 mg**

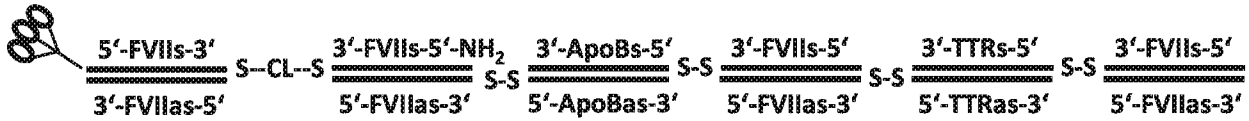
Duplex purity: **96.7 %**

Fig. 57A

QC Data for Heterohexameric Duplex

ESI-MS Spectrum

GalNAc-conjugated heterohexameric siRNA:



ESI-MS:

ssRNA ID	MW (calcd)	MW (obsd)
X39850-X39851	15656.07	15651.2871
X18795	6849.25	6848.2744
X39855	14001.87	14000.4507
X39852	14073.03	14070.5658
X39854	14835.59	14833.5073
X39853	13571.84	13569.6524

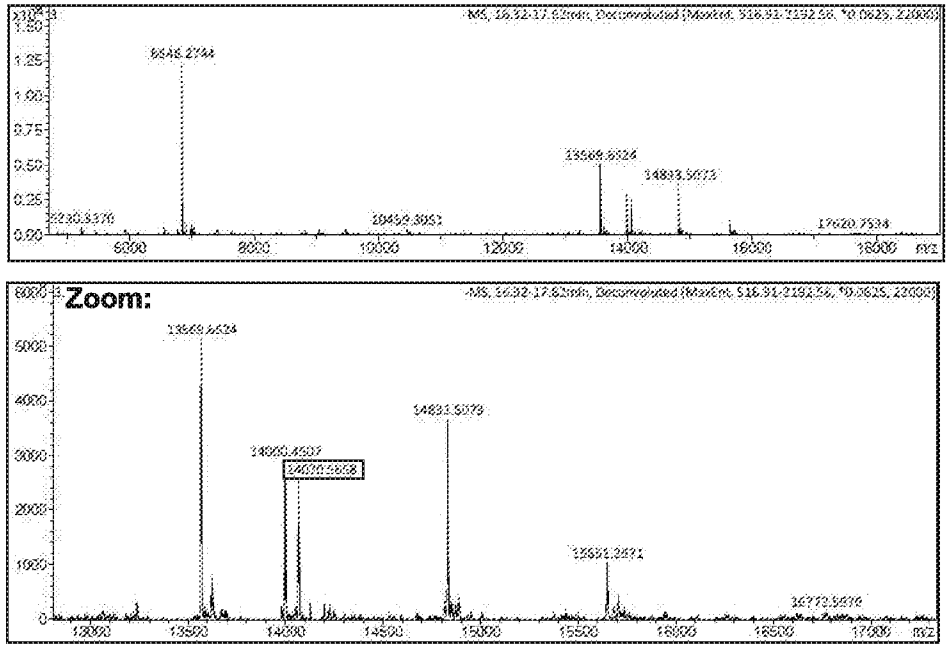


Fig. 57B

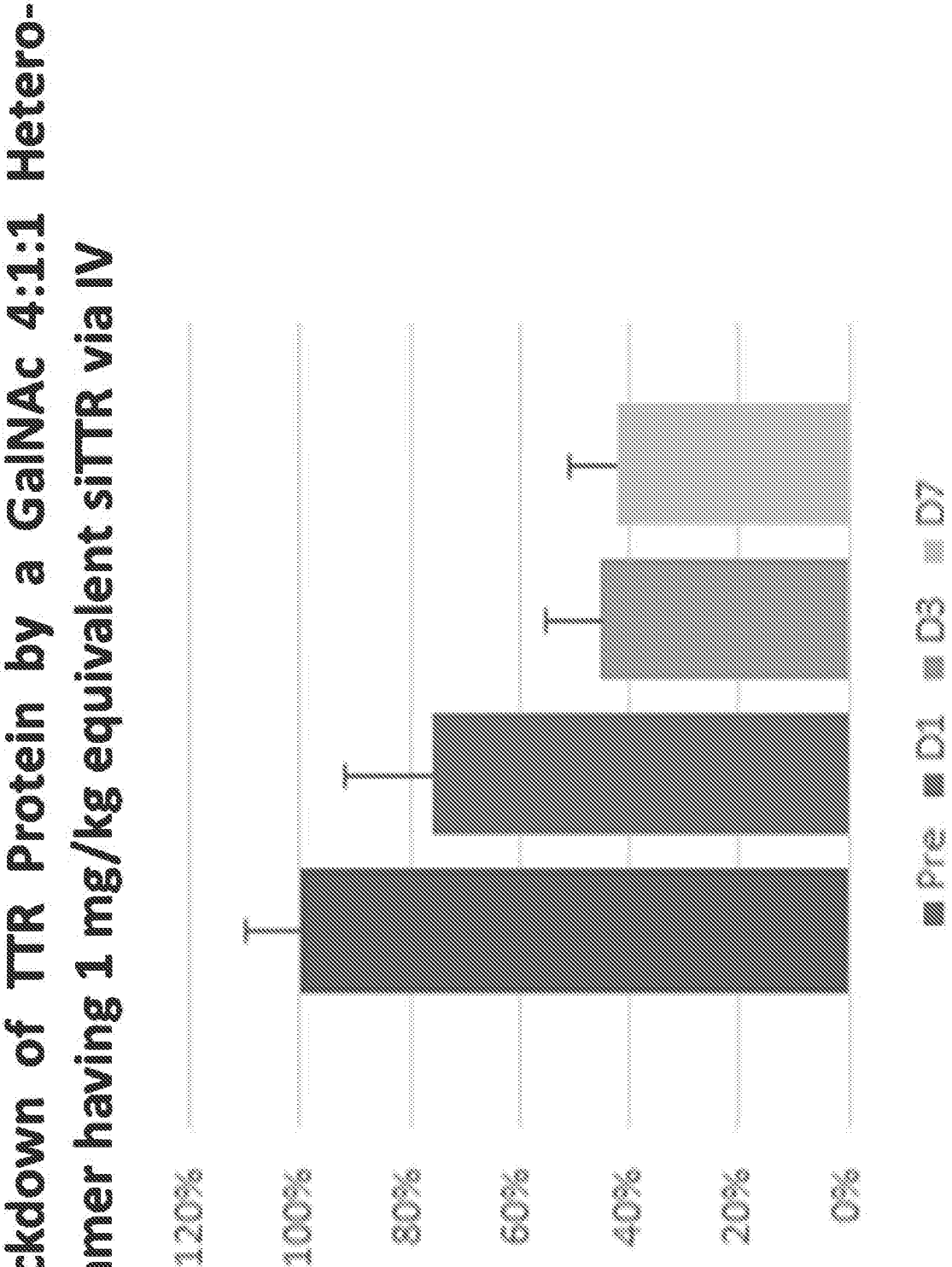

Knockdown of TTR Protein by a GalNAc 4:1:1 Hetero-hexamer having 1 mg/kg equivalent siTTR via IV
120%
100%
80%
60%
40%
20%
0%
Pre
D1
D3
D7

Fig. 58

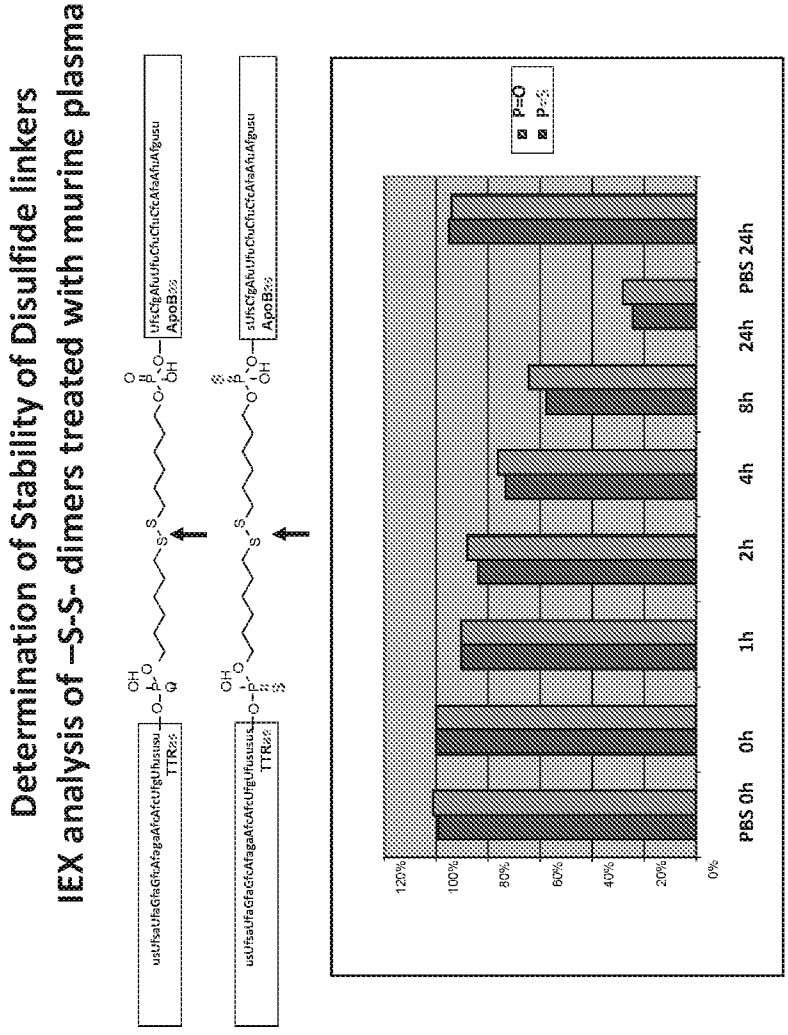

Determination of Stability of Disulfide linkers
IEX analysis of –S-S- dimers treated with murine plasma
ApoB
TTR
P=O
PBS 0h
0h
1h
2h
4h
8h
24h
PBS 24h
120%
100%
80%
60%
40%
20%
0%
Samples (50 µM) were incubated at 37°C for 0, 1, 2, 4, 8, and 24h in 90% mouse plasma
Incubations were stopped by addition of DTT-free proteinase K, followed by heat inactivation and samples were diluted with water for subsequent IEX HPLC analysis

Fig. 59