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(54) Title: SPLIT SYNTHESIS OF LONG RNAS

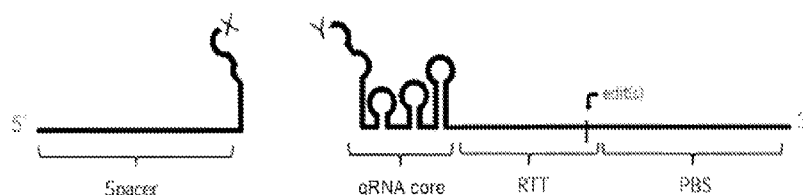


FIG. 3A

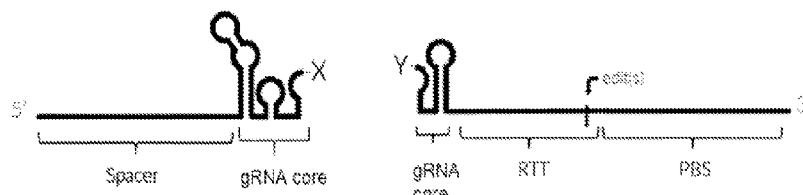


FIG. 3B

(57) Abstract: Provided herein are methods for preparing long RNAs, such as prime editing guide RNAs (PEgRNAs).

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GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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upon receipt of that report (Rule 48.2(g))*
- *with sequence listing part of description (Rule 5.2(a))*

SPLIT SYNTHESIS OF LONG RNAs**CROSS REFERENCE TO RELATED APPLICATIONS**

[1] The present application claims priority to and the benefits of U.S. Provisional Application No. 63/384,872, filed November 23, 2022, and U.S. Provisional Application No. 63/586,548, filed September 29, 2023, the contents of each of which are herein incorporated by reference in their entirety.

SEQUENCE LISTING

[2] This application includes and incorporates by reference in its entirety a Sequence Listing XML in the required .xml format. The Sequence Listing XML file that has been electronically filed contains the information of the nucleotide and/or amino acid sequences disclosed in the patent application using the symbols and format in accordance with the requirements of 37 C.F.R. §§1.832 through 1.834.

[3] The Sequence Listing XML filed herewith serves as the electronic copy required by §1.834(b)(1).

[4] The Sequence Listing XML is identified as follows: “272059-538182_Sequence-listing.xml” (196,614 bytes in size), which was created on November 21, 2023.

BACKGROUND

[5] Prime editing is a gene editing technology that allows researchers to make nucleotide substitutions, insertions, deletions, or combinations thereof in the DNA of cells. Prime editing can be used to correct disease associated gene mutations, and can be used for treating disease with a genetic component. However, technical challenges remain, limiting the rapid growth of gene editing technology in therapeutic areas. One of the major challenges is the production of high-quality long RNA oligonucleotides.

[6] In general, the synthesis of RNA/DNA oligonucleotides is achieved by solid phase synthesis on automated synthesizers. However, the solid phase synthesis of long RNA oligonucleotides suffers from low yield and low purity of the final product. The coupling efficiency is not quantitative, limiting the yield for long-RNA oligonucleotides. These non-quantitative couplings generate shorter and longer impurities which are difficult to remove, especially in long RNAs. There is a need for improved synthetic and purification methods for making long RNAs.

SUMMARY

[7] Provided herein are methods for synthesizing long RNAs such as full-length prime editing guide RNAs (PEgRNAs) useful in prime editing, as well as methods of using such PEgRNAs.

[8] In certain aspects, the disclosure provides methods comprising, contacting a first RNA sequence with a second RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end and the second RNA sequence comprises a second chemical moiety at its 5' end; and

ligating the first RNA sequence and the second RNA sequence to form a synthesized RNA by reacting the first chemical moiety of the first RNA sequence with the second chemical moiety of the second RNA sequence;

wherein the ligation forms a linker in a stem loop structure, a tetraloop structure, and/or an extension arm of the synthesized RNA.

[9] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the methods comprising,

contacting a first RNA sequence with a second RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end and the second RNA sequence comprises a second chemical moiety at its 5' end; and

ligating the first RNA sequence and the second RNA sequence to obtain the synthesized full-length PEgRNA,

wherein the ligation forms a linker between the first chemical moiety of the first RNA sequence and the second chemical moiety of the second RNA sequence; and

wherein the linker is present in the gRNA core of the synthesized full-length PEgRNA.

[10] In some embodiments, the ligation occurs via a chemical ligation. In some embodiments, the ligation occurs via an enzymatic ligation.

[11] In some embodiments, the gRNA core is a modified gRNA core. In some embodiments, the gRNA core is a chemically-modified gRNA core.

[12] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

the methods comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end;

providing a second RNA sequence, wherein the second RNA sequence comprises a second chemical moiety at its 5' end;

providing a third RNA sequence, wherein the third RNA sequence comprises a third chemical moiety at its 5' end and a fourth chemical moiety at its 3' end;

ligating the first, second, and third RNA sequences to obtain the synthesized full-length PEgRNA,

wherein the ligation forms (i) a first linker between the first chemical moiety of the first RNA sequence and the third chemical moiety of the third RNA sequence, and (ii) a second linker between the second chemical moiety of the second RNA sequence and the fourth chemical moiety of the third RNA sequence;

wherein the first linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA, and

wherein the second linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA or in a junction of the synthesized full-length PEgRNA.

[13] In some embodiments, the second linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA. In some embodiments, the second linker is present in the junction of the synthesized full-length PEgRNA.

[14] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

the methods comprising,

providing a first RNA sequence,

wherein the first RNA sequence comprises an amino modified phosphodiester backbone at its 3' end;

functionalizing the 3' amino group of the first RNA sequence with a first chemical moiety;

providing a second RNA sequence;

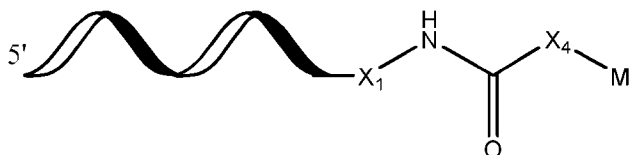
wherein the second RNA sequence comprises an amino modified phosphodiester backbone at its 5' end;
functionalizing the 5' amino group of the second RNA sequence with a second chemical moiety;
reacting the first chemical moiety of the first RNA sequence with the second chemical moiety of the second RNA sequence to form a linker;
wherein forming the linker results in the formation of the synthesized full-length PEgRNA; and
wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[15] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the methods comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises an amino modified phosphodiester backbone at its 3' end;
providing a second RNA sequence; wherein the second RNA sequence comprises an amino modified phosphodiester backbone at its 5' end;
annealing the first and second RNA sequences to form a pre-annealed duplex;
providing a reagent;
contacting the pre-annealed duplex with the reagent to form a linker,
wherein forming the linker results in the formation of the synthesized full-length PEgRNA; and
wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

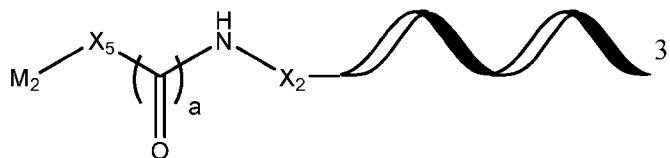
[16] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the methods comprising,

providing a first RNA sequence of Formula A:



Formula A;

providing a second RNA sequence of Formula B:



Formula B;

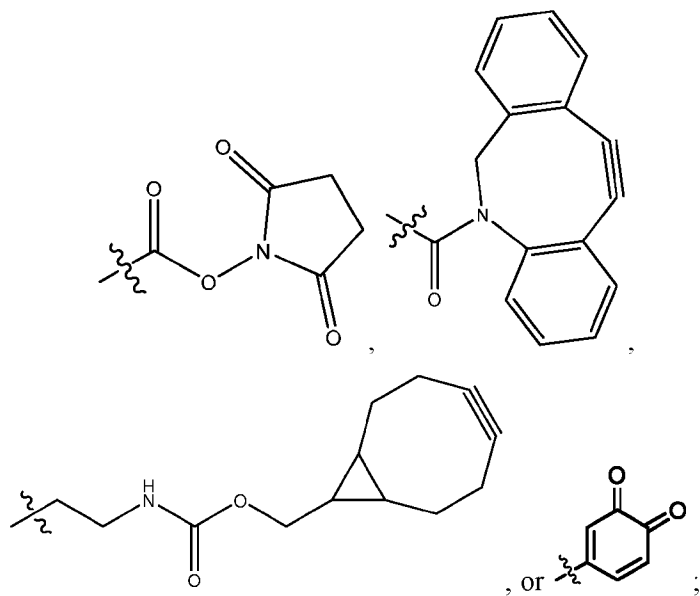
reacting M_1 with M_2 to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA;

wherein:

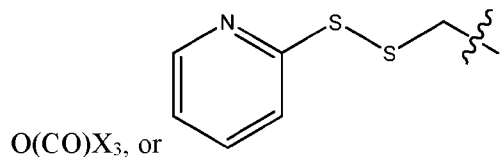
each X_1 , X_2 , X_4 , and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more of $-OH$ or $-O-$, or $-O-$;

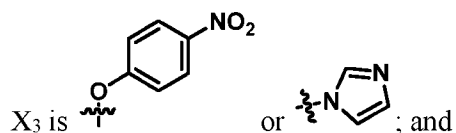
“n” is an integer between 1 and 12;

M_1 is an amino, a halogen, a hydroxyl, a phosphate ion, a maleimide, an aldehyde-substituted phenyl, an aniline, $-O(CO)X_3$,



M_2 is an amino, a hydroxyl, a phosphate ion, a thiol, an azido, an O-aminophenyl, $-$





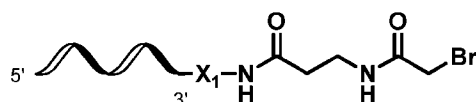
“a” is an integer of 0 or 1;

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[17] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

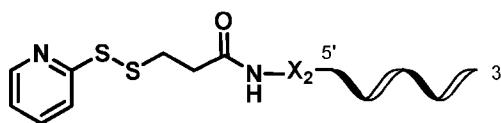
the methods comprising,

providing a first RNA sequence of Formula C:



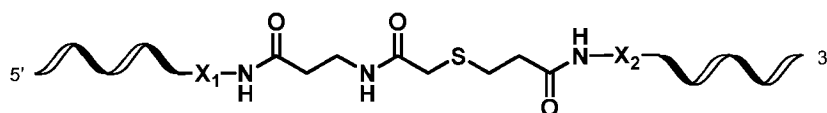
Formula C;

providing a second RNA sequence of Formula D:



Formula D;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula E:



Formula E;

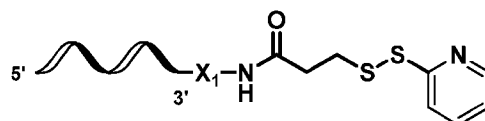
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$,

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

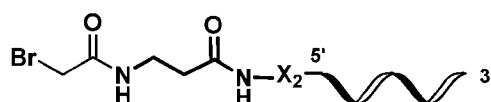
[18] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the methods comprising,

providing a first RNA sequence of Formula C-1:



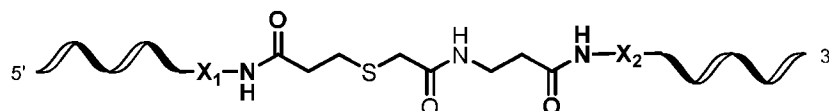
Formula C-1;

providing a second RNA sequence of Formula D-1:



Formula D-1;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula E-1:



Formula E-1;

wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$,

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

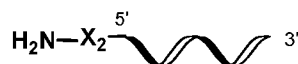
[19] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the methods comprising,

providing a first RNA sequence of Formula F:



Formula F;

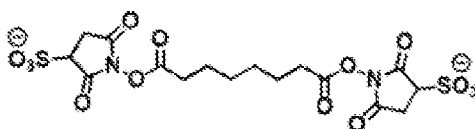
providing a second RNA sequence of Formula G:



Formula G;

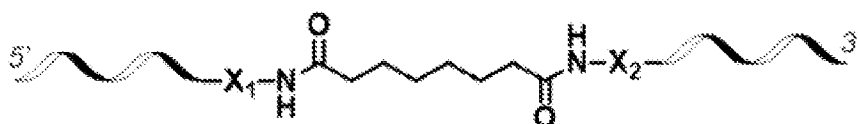
annealing the first and second RNA sequences to form a pre-annealed duplex;

providing a BS3 reagent of the formula:



BS3;

contacting the pre-annealed duplex with the BS3 reagent to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula H:



Formula H;

wherein each X_1 and X_2 is independently absent or $(\text{O}-\text{CH}_2-\text{CH}_2)_n$, $(\text{CH}_2-\text{CH}_2-\text{O})_n$, $((\text{CH}_2)_n-\text{C}(\text{O})\text{NH}-\text{CH}_2)$, or an alkylene optionally substituted with one or more $-\text{OH}$ or $-\text{O}-$, or $-\text{O}-$,

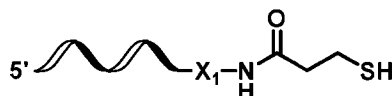
“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[20] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

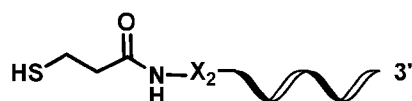
the methods comprising,

providing a first RNA sequence of Formula I:



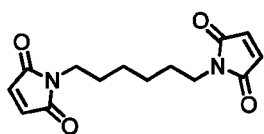
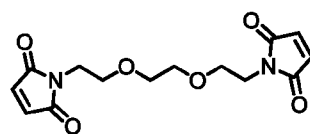
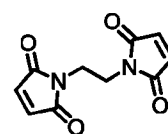
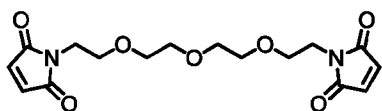
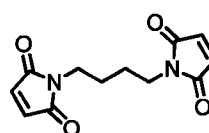
Formula I;

providing a second RNA sequence of Formula J:

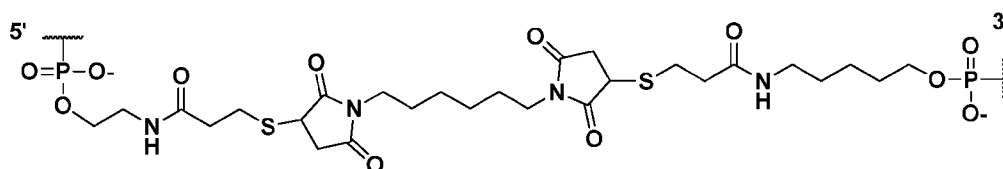
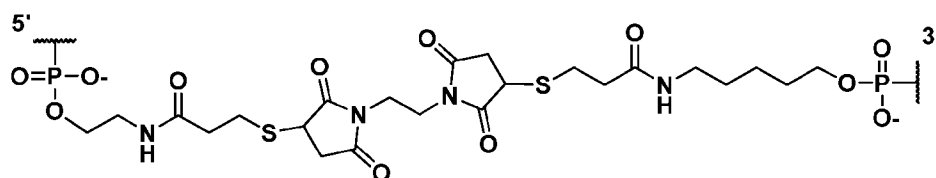
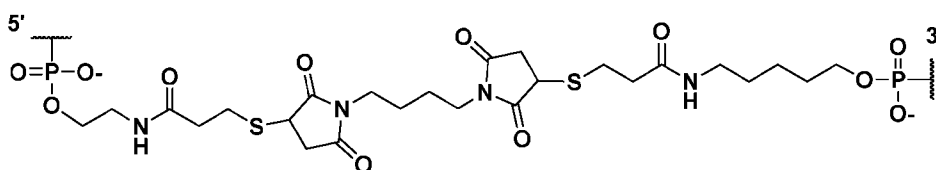
**Formula J;**

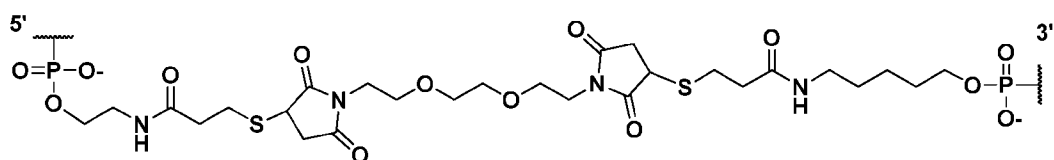
annealing the first and second RNA sequences to form a pre-annealed duplex;

providing a BMH, BM(PEG)₂, BMOE, BM(PEG)₃, or BMB reagent of the formulas:

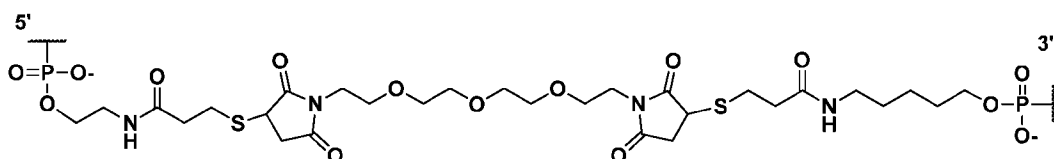
**BMH**
bismaleimidoethane**BM(PEG)₂**
1,8-bisamleimidodiethyleneglycol**BMOE**
bismaleimidoethane**BM(PEG)₃**
1,11-bismaleimidotriethyleneglycol**BMB**
1,4-bismaleimidobutane

contacting the pre-annealed duplex with the BMH, BM(PEG)₂, BMOE, BM(PEG)₃, or BMB reagent to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEGRNA of Formulas K-O:

**Formula K;****Formula L;****Formula M;**



Formula N; or



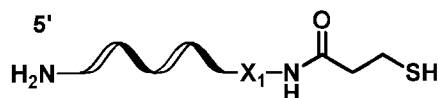
Formula O;

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[21] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

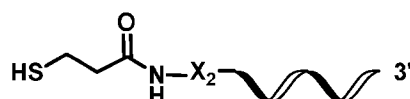
the methods comprising,

providing a first RNA sequence of Formula I:



Formula I-1;

providing a second RNA sequence of Formula J:

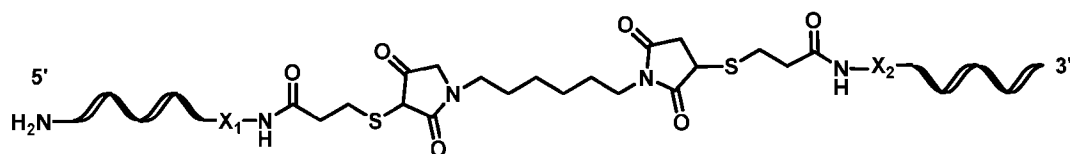


Formula J;

annealing the first and second RNA sequences to form a pre-annealed duplex;

providing a BMH reagent;

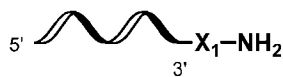
contacting the pre-annealed duplex with the BMH reagent to form a linker, wherein forming the linker results in the formation of Formula P:



Formula P;

wherein Formula P comprises an amine at its 5' end;

providing a third RNA sequence of Formula F:

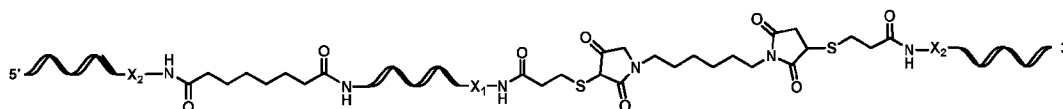
**Formula F;**

contacting Formula P with Formula F to form a second pre-annealed duplex;

providing a BS3 reagent;

contacting the second pre-annealed duplex with the BS3 reagent to form a linker,
wherein forming the linker results in the formation of the synthesized full-length PEgRNA of

Formula Q:

**Formula Q;**

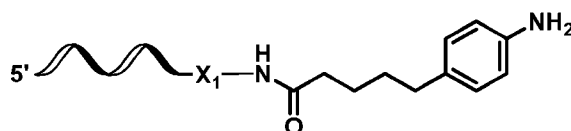
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“n” is an integer between 1 and 12.

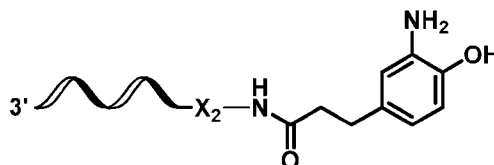
[22] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

the methods comprising,

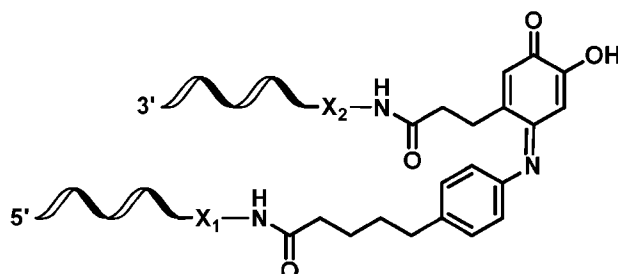
providing a first RNA sequence of Formula R:

**Formula R;**

providing a second RNA sequence of Formula S:

**Formula S;**

contacting the first RNA sequence with the second RNA sequence to form a linker,
wherein forming the linker results in the formation of the synthesized full-length PEgRNA of
Formula T:

**Formula T;**

wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

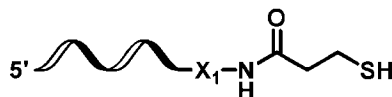
“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEGRNA.

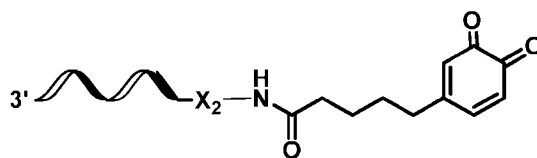
[23] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

the methods comprising,

providing a first RNA sequence of Formula I:

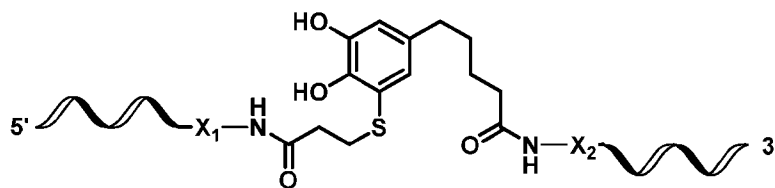
**Formula I;**

providing a second RNA sequence of Formula U:

**Formula U;**

annealing the first and second RNA sequences to form a pre-annealed duplex;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula W:



Formula W;

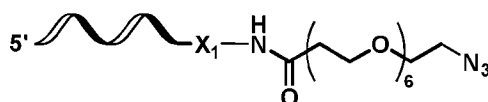
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

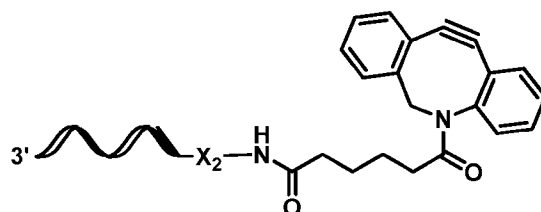
[24] In certain aspects, the disclosure provides methods for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the methods comprising,

providing a first RNA sequence of Formula X:



Formula X;

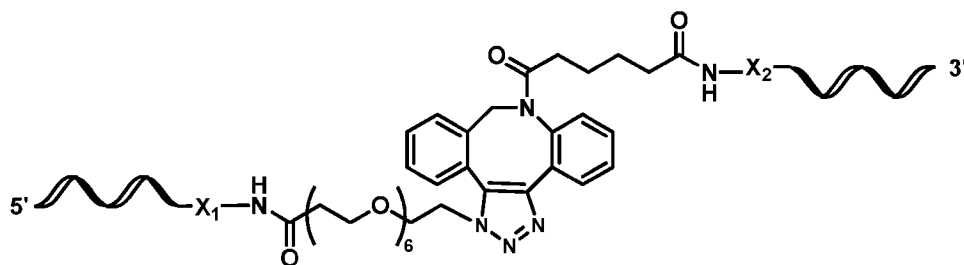
providing a second RNA sequence of Formula Y:



Formula Y;

annealing the first and second RNA sequences to form a pre-annealed duplex;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula Z:

**Formula Z;**

wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[25] In certain aspects, the disclosure provides a full-length PEgRNA comprising a chemically-modified gRNA core, wherein the full-length PEgRNA is produced by:

contacting a first RNA sequence with a second RNA sequence,

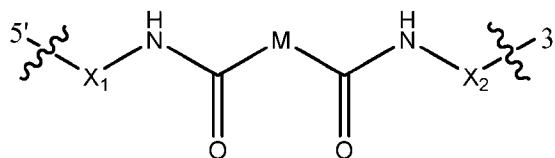
wherein the first RNA sequence comprises a first chemical moiety at its 3' end and the second RNA sequence comprises a second chemical moiety at its 5' end; and;

ligating the first RNA sequence and the second RNA sequence to obtain the full-length PEgRNA,

wherein the ligation forms a linker between the first chemical moiety of the first RNA sequence and the second chemical moiety of the second RNA sequence; and

wherein the linker is present in the chemically-modified gRNA core of the full-length PEgRNA.

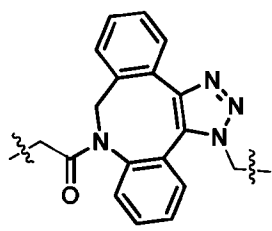
[26] In certain aspects, the disclosure provides a full-length PEgRNA comprising a chemically-modified gRNA core, wherein the chemically-modified gRNA core comprises one or more linkers of Formula AA:

**Formula AA**

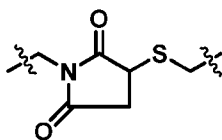
wherein:

each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$,
 “n” is an integer between 1 and 12;

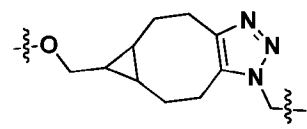
M is a linker, wherein the linker comprises an alkylene optionally substituted with one or more of $-OH$, $-O-$, $-S-$, or $(O-CH_2-CH_2)_n$; or a structure of any one of Formulas AA-1 to AA-10:



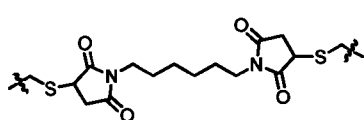
Formula AA-1



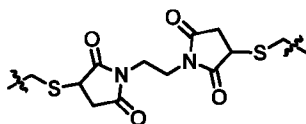
Formula AA-2



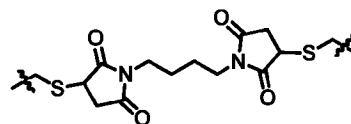
Formula AA-3



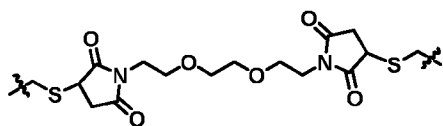
Formula AA-4



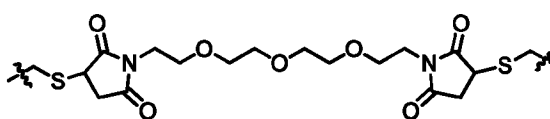
Formula AA-5



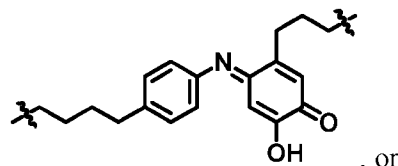
Formula AA-6



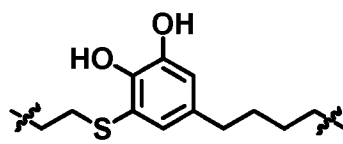
Formula AA-7



Formula AA-8



Formula AA-9



Formula AA-10

[27] In certain aspects, the disclosure provides a prime editing complex comprising: (i) the full-length PEGRNA prepared by the methods described herein, and (ii) a prime editor comprising a DNA binding domain and a DNA polymerase domain.

[28] In certain aspects, the disclosure provides a lipid nanoparticle (LNP) or ribonucleoprotein (RNP) comprising the prime editing complex disclosed herein, or a component thereof.

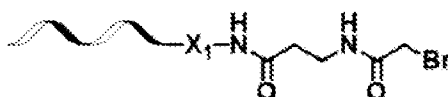
[29] In certain aspects, the disclosure provides a cell comprising the full-length PEGRNA prepared by the methods described herein, the prime editing complex disclosed herein, or the LNP or RNP disclosed herein.

[30] In certain aspects, the disclosure provides a pharmaceutical composition comprising (i) the full-length PEGRNA prepared by the methods described herein, the prime editing complex disclosed herein, or the LNP or RNP disclosed herein; and (ii) a pharmaceutically acceptable carrier.

[31] In certain aspects, the disclosure provides methods for editing a gene, the method comprising contacting the gene with (i) the full-length PEGRNA prepared by the methods described herein and (ii) a prime editor comprising a DNA binding domain and a DNA polymerase domain, wherein the PEGRNA directs the prime editor to incorporate the intended nucleotide edit in the gene, thereby editing the gene.

[32] In certain aspects, the disclosure provides methods for editing a gene, the method comprising contacting the gene with the prime editing complex disclosed herein, wherein the PEGRNA directs the prime editor to incorporate the intended nucleotide edit in the gene, thereby editing the gene.

[33] In certain aspects, the disclosure provides an RNA sequence of Formula C:

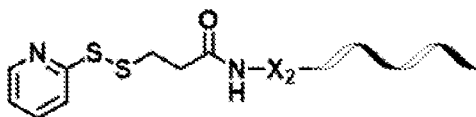


Formula C;

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“n” is an integer between 1 and 12.

[34] In certain aspects, the disclosure provides an RNA sequence of Formula D:

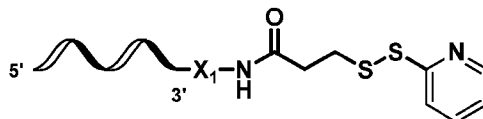


Formula D;

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“n” is an integer between 1 and 12.

[35] In certain aspects, the disclosure provides an RNA sequence of Formula C-1:

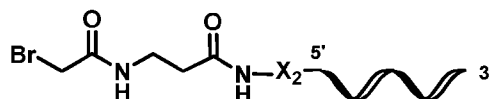


Formula C-1;

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

[36] In certain aspects, the disclosure provides an RNA sequence of Formula D-1:



Formula D-1;

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

[37] In certain aspects, the disclosure provides an RNA sequence of Formula G:



Formula F,

wherein X_1 is absent or $(-O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

[38] In certain aspects, the disclosure provides an RNA sequence of Formula G:

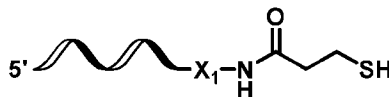


Formula G;

wherein X_2 is absent or $(-O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

[39] In certain aspects, the disclosure provides an RNA sequence of Formula I:

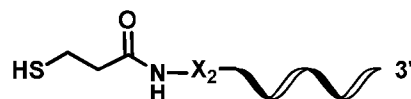


Formula I,

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

"n" is an integer between 1 and 12.

[40] In certain aspects, the disclosure provides an RNA sequence of Formula J:

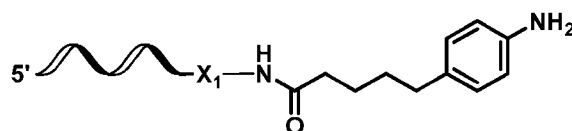


Formula J;

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

"n" is an integer between 1 and 12.

[41] In certain aspects, the disclosure provides an RNA sequence of Formula R:

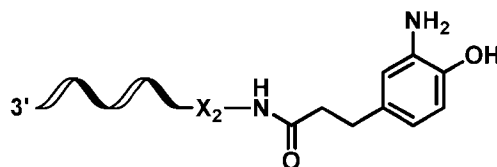


Formula R,

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

"n" is an integer between 1 and 12.

[42] In certain aspects, the disclosure provides an RNA sequence of Formula S:

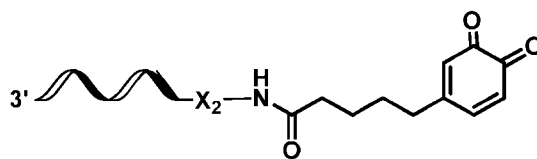


Formula S;

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

"n" is an integer between 1 and 12.

[43] In certain aspects, the disclosure provides an RNA sequence of Formula U:

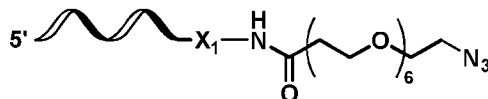


Formula U;

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“n” is an integer between 1 and 12.

[44] In certain aspects, the disclosure provides an RNA sequence of Formula X:

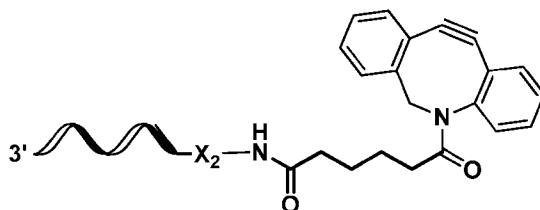


Formula X,

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“n” is an integer between 1 and 12.

[45] In certain aspects, the disclosure provides an RNA sequence of Formula Y:



Formula Y;

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“n” is an integer between 1 and 12.

[46] In certain aspects, the disclosure provides a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the method comprising,

contacting a first RNA sequence with a second RNA sequence,

wherein the first RNA sequence comprises a free 3' OH and the second RNA sequence comprises a 5'-monophosphate;

ligating the first RNA sequence and the second RNA sequence with a nucleic acid ligase to obtain the synthesized full-length PEgRNA,

wherein the ligation forms a linker between the 3' OH and the 5'-monophosphate; and

wherein the linker is present in the spacer, gRNA core, reverse transcription editing template (RTT), or primer binding site (PBS) of the synthesized full-length PEgRNA.

[47] In certain aspects, the disclosure provides a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the method comprising,

contacting a first RNA sequence with a second RNA sequence and a splint sequence,
wherein the first RNA sequence comprises a free 3' OH, the second RNA sequence comprises a 5'-monophosphate, and the splint sequence comprises a sequence partially complementary to both the first and second RNA sequences;

ligating the first RNA sequence and the second RNA sequence with a nucleic acid ligase to obtain the synthesized full-length PEGRNA,

wherein the ligation forms a linker between the 3' OH and the 5'-monophosphate; and

wherein the linker is present in the spacer, gRNA core, RTT, or PBS of the synthesized full-length PEGRNA.

[48] In certain aspects, the disclosure provides a method for synthesizing a full-length prime editing guide RNA (PEGRNA) comprising a guide RNA (gRNA) core, the method comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end;

providing a second RNA sequence, wherein the second RNA sequence comprises a second chemical moiety at its 5' end;

providing a third RNA sequence, wherein the third RNA sequence comprises a third chemical moiety at its 5' end and a fourth chemical moiety at its 3' end;

ligating the first, second, and third RNA sequences to obtain the synthesized full-length PEGRNA,

wherein the ligation forms (i) a first linker between the first chemical moiety of the first RNA sequence and the third chemical moiety of the third RNA sequence, and (ii) a second linker between the second chemical moiety of the second RNA sequence and the fourth chemical moiety of the third RNA sequence;

wherein the first linker is present in the chemically-modified gRNA core of the synthesized full-length PEGRNA, and

wherein the second linker is present in the chemically-modified gRNA core of the synthesized full-length PEGRNA or in a junction of the synthesized full-length PEGRNA.

[49] In certain aspects, the disclosure provides a method for synthesizing a full-length prime editing guide RNA (PEGRNA) comprising a guide RNA (gRNA) core, the method comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end;

providing a second RNA sequence, wherein the second RNA sequence comprises a second chemical moiety at its 5' end and a free 3' OH;

providing a third RNA sequence, wherein the third RNA sequence comprises a 5'-monophosphate;

ligating the first, second, and third RNA sequences to obtain the synthesized full-length PEgRNA,

wherein the ligation forms (i) a first linker between the first chemical moiety of the first RNA sequence and the second chemical moiety of the second RNA sequence, and (ii) a second linker between the 3' OH of the second RNA sequence and the 5'-monophosphate of the third RNA sequence;

wherein the first linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA, and

wherein the second linker is present in the spacer, gRNA core, RTT, or PBS of the synthesized full-length PEgRNA.

[50] In some embodiments, the method further comprises providing a splint sequence, wherein the splint sequence comprises a sequence partially complementary to both the second and third RNA sequences.

INCORPORATION BY REFERENCE

[51] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[52] The novel features of the methods and compositions provided herein are set forth with particularity in the appended claims. A better understanding of the features and advantages of the methods and compositions provided herein will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the methods and compositions provided herein are utilized, and the accompanying drawings of which:

[53] **FIG. 1** shows an exemplary schematic of a PEgRNA.

- [54] **FIG. 2** shows an exemplary PEgRNA gRNA core.
- [55] **FIG. 3A** shows an exemplary PEgRNA with the split sites at the TL in the gRNA core for use in a two-part ligation method: “X” and “Y” represent chemical moieties.
- [56] **FIG. 3B** shows an exemplary PEgRNA with the split sites at the SL2 in the gRNA core for use in a two-part ligation method: “X” and “Y” represent chemical moieties.
- [57] **FIG. 4A** shows an exemplary PEgRNA with the split sites in the PEgRNA for use in a three-part ligation method. The figure shows a split site at the TL and a split site at the SL2 of the gRNA core and “X,” “Y,” “M,” and “N” represent chemical moieties.
- [58] **FIG. 4B** shows an exemplary PEgRNA with the split sites in the PEgRNA for use in a three-part ligation method. The figure shows split sites at the TL of the gRNA core and at the junction between the gRNA core and the extension arm (RTT and PBS).
- [59] **FIG. 5A** shows an exemplary gRNA core extension at the TL position. An original TL is shown as “native,” and the extended TL is shown as “extension.” The loop structure is shown in gray with underline font, the original base pairs are shown in gray, the extended 11-base pairs are shown in black with bold font. The base pair sequences shown in the figure are arbitrary and meant to function mainly as an example. The split site is shown in dash line. The rest of the sequences are abbreviated as gray dots.
- [60] **FIG. 5B** shows an exemplary gRNA core extension at the SL2 position. An original SL2 is shown as “native,” and the extended SL2 is shown as “extension.” The loop structure is shown in gray with underline font, the original base pairs are shown in gray, the extended 11-base pairs are shown in black with bold font. The base pair sequences shown in the figure are arbitrary and meant to function mainly as an example. The split site is shown in dash line. The rest of the sequences are abbreviated as gray dots.
- [61] **FIG. 6** shows a denaturing PAGE (15%) analysis of the thioether ligation of Example 1. Lane 1: oligonucleotide ladder for length; Lane 2: RNA010; Lane 3: RNA011; Lane 4: Thioether ligation of RNA010 and RNA011 to generate the ligation product RNA010_thioether_RNA011 at 65 % yield.
- [62] **FIG. 7** shows a RP-HPLC analysis of the thioether ligation. The bottom chromatogram is the RNA010 functionalized by SBAP. The middle chromatogram is the RNA011 functionalized by SPDP. The top chromatogram is the thioether ligation reaction.
- [63] **FIG. 8** shows a LC-MS analysis of the thioether ligation. For the main peak, the observed mass was 36085.8 Da, the expected mass for the ligation product is 36083.9 Da, $\Delta = +1.9$ Da.

[64] **FIG. 9A** shows split synthesis of PEGRNAL1001 using thioether ligation. The figure is a denaturing PAGE (15%) analysis of the purified ligation product with different loading amounts (Lane 1: oligonucleotide length ladder; Lane 2: 1.8 pmol loading; Lane 3: 0.9 pmol loading; Lane 4: 0.5 pmol loading; Lane 5: 0.2 pmol loading).

[65] **FIG. 9B** shows split synthesis of PEGRNAL1001 using thioether ligation. The figure is a RP-HPLC analysis of the purified ligation product.

[66] **FIG. 10A** shows split synthesis of PEGRNAL1005 using thioether ligation. The figure shows a denaturing PAGE (15%) analysis of purified ligation product with different loading amounts (Lane 1: oligonucleotide length ladder; Lane 2: 1.6 pmol loading; Lane 3: 0.8 pmol loading; Lane 4: 0.5 pmol loading; Lane 5: 0.2 pmol loading).

[67] **FIG. 10B** shows split synthesis of PEGRNAL1005 using thioether ligation. The figure shows an RP-HPLC analysis of purified ligation product.

[68] **FIG. 11** shows an LC-MS analysis of PEGRNAL1005 purified ligation product.

[69] **FIG. 12** shows *In vitro* prime editing of split synthesized PEgRNAs in primary human hepatocytes.

[70] **FIG. 13** shows a denaturing PAGE (15%) analysis of the bisamide ligation. Lane 1: oligonucleotide ladder for length; Lane 2-4: Bisamide ligation of RNA010 and RNA011 to generate the ligation product RNA010_bisamide_RNA011 using different BS3 reagent equivalents (from high to low equivalents). The quantified yield is up to 70% (Lane 3).

[71] **FIG. 14** shows a RP-HPLC analysis of the bisamide ligation. The bottom chromatogram is the RNA010. The middle chromatogram is the RNA011. The top chromatogram is the bisamide ligation reaction.

[72] **FIG. 15** shows a LC-MS analysis of the bisamide ligation. For the main peak, the observed mass was 36025.1 Da, the expected mass for the ligation product is 36022.9 Da, $\Delta = +2.2$ Da.

[73] **FIG. 16** shows a denaturing PAGE (10%) result of a self-templated ligation reaction. Lane 1) RNA B with ligase; Lane 2) RNA's A & B without ligase; Lane 3) RNA's A & B with ligase; Lane 4) pegRNA C full-length control; Lane 5) ssRNA size standard (300-50mer). In Lane 3 the ligation efficiency of RNA A to RNA B to yield a full-length pegRNA is 75%.

[74] **FIG. 17** shows a denaturing PAGE (10%) result of a templated ligation reaction. Lane 1) ssRNA size standard (300-50mer); Lane 2) RNA's X & Y and Splint L without ligase; Lane 3) RNA's X & Y and splint M without ligase; Lane 4) RNA's X & Y and Splint N without ligase; Lane 5) RNA's X & Y and splint L with ligase; Lane 6) RNA's X & Y and

splint M with ligase; Lane 7) RNA's X & Y and splint N with ligase; Lane 8) RNA's X & Y with ligase; Lane 9) pegRNA Z full-length control. In Lane 8 the self-templated ligation efficiency of RNA X to RNA Y to yield a full-length pegRNA is 37%; in Lanes 5-7, the templated ligation efficiency with Splints L, M, & N increases to 47, 50, & 51%, respectively.

[75] **FIG. 18** shows analytical UPLC traces of LC-purified acceptor A1 (Upper panel) and donor D1 (Middle panel) RNA oligonucleotides along with the enzymatically ligated full-length product FLP1-T4 with T4 RNA Ligase II (Bottom panel). The enzymatically ligated RNA FLP1-T4 shows good purity in absence of LC-based purification with <1% of A1 and D1 remaining.

[76] **FIG. 19** shows analytical UPLC traces of LC-purified acceptor A2 (Upper panel) and donor D2 (Middle panel) RNA oligonucleotides along with the enzymatically ligated full-length product FLP2-T4 with T4 RNA Ligase II (Bottom panel). The enzymatically ligated RNA FLP2-T4 shows good purity in absence of LC-based purification with <3% of A2+D2 remaining.

[77] **FIG. 20** shows analytical UPLC traces of the full-length product synthesized through linear solid-phase synthesis FLP1-LS (after LC purification) (top panel) and the enzymatically, ligated full-length product FLP1-T4 with T4 RNA Ligase II (in absence of LC purification) (Bottom panel).

[78] **FIG. 21** shows analytical UPLC traces of the full-length product synthesized through linear solid-phase synthesis FLP1-LS (after LC purification) (top panel) and the enzymatically, ligated full-length product FLP1-T4 with T4 RNA Ligase II (in absence of LC purification) (Bottom panel).

[79] **FIG. 22** shows dose-dependent editing of enzymatically ligated, linearly synthesized and control PEGRNAs in primary human hepatocytes. ngRNA001 was used.

[80] **FIG. 23** shows a polyacrylamide gel analysis of the reaction products of SPAAC chemical ligation.

[81] **FIG. 24** shows a polyacrylamide gel analysis of 3-part ligation products.

[82] **FIG. 25** shows a RP-HPLC analysis of 3-part ligation products. The bottom line of the graph corresponds to rOLI052_BS3_rOLI055, the second line from the bottom corresponds to rOLI055_BMH_rOLI051+OLI399; the third line from the bottom corresponds to rOLI052 + rOLI055_BMH_rOLI051+OLI399, the top line corresponds to rOLI052_BS3_rOLI055_BMH_rOLI051+OLI399.

[83] **FIG. 26** shows a PAGE-urea analysis of oxidative ligations of aminophenol-RNAs with aniline-RNAs.

[84] **FIG. 27** shows a PAGE-urea analysis of oxidative ligations of thiol-RNA to catechol-RNA.

DETAILED DESCRIPTION

[85] Provided herein, in some embodiments, are methods for synthesizing long RNAs such as full-length prime editing guide RNAs (PEgRNAs). In certain embodiments, the methods provided herein for synthesizing long RNAs, such as full-length PEgRNAs, involve contacting a first RNA sequence with a second RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end and the second RNA sequence comprises a second chemical moiety at its 5' end. The first and second RNA sequences are ligated to obtain the long RNA, such as a full-length PEgRNA. The ligation forms a linker between the first and second RNA and, in some embodiments, can be present in the gRNA core of the long RNAs, such as full-length PEgRNA.

[86] The following description and examples illustrate embodiments of the present disclosure in detail. It is to be understood that this disclosure is not limited to the particular embodiments described herein and as such can vary. Those of skill in the art will recognize that there are numerous variations and modifications of this disclosure, which are encompassed within its scope. Although various features of the present disclosure can be described in the context of a single embodiment, the features can also be provided separately or in any suitable combination. Conversely, although the present disclosure can be described herein in the context of separate embodiments for clarity, the present disclosure can also be implemented in a single embodiment.

Definitions

[87] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art.

[88] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. As used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms “including”, “includes”, “having”, “has”, “with”, or variants thereof as used herein mean “comprising”.

[89] Unless otherwise specified, the words “comprising”, “comprise”, “comprises”, “having”, “have”, “has”, “including”, “includes”, “include”, “containing”, “contains” and

“contain” are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[90] Reference to “some embodiments”, “an embodiment”, “one embodiment”, or “other embodiments” means that a particular feature or characteristic described in connection with the embodiments is included in at least one or more embodiments, but not necessarily all embodiments, of the present disclosure.

[91] The term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, “about” can mean within 1 standard deviation, per the practice in the art.

Alternatively, “about” can mean a range of up to 20%, up to 10%, up to 5%, or up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated, the term “about” meaning within an acceptable error range for the particular value should be assumed.

[92] The term “substantially” as used herein may refer to a value approaching 100% of a given value. In some embodiments, the term may refer to an amount that may be at least about 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.9%, or 99.99% of a total amount. In some embodiments, the term may refer to an amount that may be about 100% of a total amount.

[93] The terms “protein” and “polypeptide” can be used interchangeably to refer to a polymer of two or more amino acids joined by covalent bonds (*e.g.*, an amide bond) that can adopt a three-dimensional conformation. In some embodiments, a protein or polypeptide comprises at least 10 amino acids, 15 amino acids, 20 amino acids, 30 amino acids or 50 amino acids joined by covalent bonds (*e.g.*, amide bonds). In some embodiments, a protein comprises at least two amide bonds. In some embodiments, a protein comprises multiple amide bonds. In some embodiments, a protein comprises an enzyme, enzyme precursor proteins, regulatory protein, structural protein, receptor, nucleic acid binding protein, a biomarker, a member of a specific binding pair (*e.g.*, a ligand or aptamer), or an antibody. In some embodiments, a protein may be a full-length protein (*e.g.*, a fully processed protein having certain biological function). In some embodiments, a protein may be a variant or a fragment of a full-length protein. For example, in some embodiments, a Cas9 protein domain comprises an H840A amino acid substitution compared to a naturally occurring *S. pyogenes*

Cas9 protein. A variant of a protein or enzyme, for example a variant reverse transcriptase, comprises a polypeptide having an amino acid sequence that is about 60% identical, about 70% identical, about 80% identical, about 90% identical, about 95% identical, about 96% identical, about 97% identical, about 98% identical, about 99% identical, about 99.5% identical, or about 99.9% identical to the amino acid sequence of a reference protein.

[94] In some embodiments, a protein comprises one or more protein domains or subdomains. As used herein, the term “polypeptide domain”, “protein domain”, or “domain” when used in the context of a protein or polypeptide, refers to a polypeptide chain that has one or more biological functions, *e.g.*, a catalytic function, a protein-protein binding function, or a protein-DNA function. In some embodiments, a protein comprises multiple protein domains. In some embodiments, a protein comprises multiple protein domains that are naturally occurring. In some embodiments, a protein comprises multiple protein domains from different naturally occurring proteins. For example, in some embodiments, a prime editor may be a fusion protein comprising a Cas9 protein domain of *S. pyogenes* and a reverse transcriptase protein domain of Moloney murine leukemia virus. A protein that comprises amino acid sequences from different origins or naturally occurring proteins may be referred to as a fusion, or chimeric protein.

[95] The term “polynucleotide,” “oligonucleotide,” or “nucleic acid molecule” can be any polymeric form of nucleotides, including DNA, RNA, a hybridization thereof, or RNA-DNA chimeric molecules. In some embodiments, a polynucleotide comprises cDNA, genomic DNA, mRNA, tRNA, rRNA, or microRNA. In some embodiments, a polynucleotide is double stranded, *e.g.*, a double-stranded DNA in a gene. In some embodiments, a polynucleotide is single-stranded or substantially single-stranded, *e.g.*, single-stranded DNA or an mRNA. In some embodiments, a polynucleotide is a cell-free nucleic acid molecule. In some embodiments, a polynucleotide circulates in blood. In some embodiments, a polynucleotide is a cellular nucleic acid molecule. In some embodiments, a polynucleotide is a cellular nucleic acid molecule in a cell circulating in blood.

[96] Polynucleotides can have any three-dimensional structure. The following are nonlimiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), an exon, an intron, intergenic DNA (including, without limitation, heterochromatic DNA), messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), a ribozyme, cDNA, a recombinant polynucleotide, a branched polynucleotide, a plasmid, a vector, isolated DNA, isolated RNA, sgRNA, guide RNA, a nucleic acid probe, a primer, an snRNA, a long non-coding RNA, a snoRNA, a siRNA, a

miRNA, a tRNA-derived small RNA (tsRNA), an antisense RNA, an shRNA, or a small rDNA-derived RNA (srRNA).

[97] In some embodiments, a polynucleotide comprises deoxyribonucleotides, ribonucleotides or analogs thereof. In some embodiments, a polynucleotide comprises modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component.

[98] In some embodiments, a polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. In some embodiments, the polynucleotide may comprise one or more other nucleotide bases, such as inosine (I), which is read by the translation machinery as guanine (G).

[99] In some embodiments, a polynucleotide (e.g., PEgRNA) may be modified. As used herein, the terms “modified” or “modification” refers to chemical modification with respect to the A, C, G, T and U nucleotides. In some embodiments, modifications may be on the nucleoside base and/or sugar portion of the nucleosides that comprise the polynucleotide. In some embodiments, the modification may be on the internucleoside linkage (e.g., phosphate backbone). In some embodiments, multiple modifications are included in the modified nucleic acid molecule. In some embodiments, a single modification is included in the modified nucleic acid molecule. In some embodiments, a polynucleotide (e.g., PEgRNA) may be chemically modified. A “chemically modified” polynucleotide (e.g., PEgRNA) includes modifications which introduce chemistries which differ from those seen in naturally occurring DNA or RNAs, for example, covalent modifications that introduce a group not naturally found in DNA or RNA molecules. A “chemically modified” polynucleotide may be obtained by a chemical reaction with a polynucleotide precursor and a chemical reagent.

[100] A “modified gRNA core” refers to a gRNA core that has been obtained by a chemical reaction with a gRNA core precursor and a chemical reagent. The modified gRNA core may comprise chemical modifications in the base, sugar, and/or backbone.

[101] In some embodiments, the gRNA core is a chemically-modified gRNA core. A “chemically-modified gRNA core” refers to a gRNA core containing a non-nucleotidic chemical moiety, a modified nucleobase, a modified sugar, and/or a modified phosphate backbone.

[102] The term “moiety” refers to a specific segment or functional group of a molecule. A “chemical moiety” can be a chemical entity embedded in or appended to a molecule. A “chemical moiety” can also be a part or functional group of a molecule that can react with another chemical moiety. In some embodiments, a chemical moiety is a functional group appended to the 3' end and/or the 5' end of a polynucleotide. In some embodiments, a chemical moiety is a hydroxyl (-OH) group. In some embodiments, a chemical moiety is a phosphate (-PO₄) group. In some embodiments, the chemical moiety is not a nucleic acid moiety, or polynucleotide moiety, connected to a sugar moiety of the rest of a polynucleotide as it would do if it is part of a polynucleotide. In some embodiments, the chemical moiety is structurally different from a natural nucleotide, a natural sugar, and/or a natural internucleoside linkage. In some embodiments, the chemical moiety is a chemical modification on the internucleoside linkage (*e.g.*, phosphate backbone) of the first, second, and/or third RNA sequence.

[103] A “linker” refers to a covalent bond or a chemical moiety which connects one chemical moiety to another. In some embodiments, a linker is a covalent bond. In some embodiments, a linker is a chemical linker.

[104] The term “complement,” “complementary,” or “complementarity” as used herein, refers to the ability of two polynucleotide molecules to base pair with each other. Complementary polynucleotides may base pair via hydrogen bonding, which may be Watson Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding. For example, an adenine on one polynucleotide molecule will base pair to a thymine or uracil on a second polynucleotide molecule and a cytosine on one polynucleotide molecule will base pair to a guanine on a second polynucleotide molecule. Two polynucleotide molecules are complementary to each other when a first polynucleotide molecule comprising a first nucleotide sequence can base pair with a second polynucleotide molecule comprising a second nucleotide sequence. For instance, the two DNA molecules 5'-ATGC-3' and 5'-GCAT-3' are complementary, and the complement of the DNA molecule 5'-ATGC-3' is 5'-GCAT-3'. A percentage of complementarity indicates the percentage of nucleotides in a polynucleotide molecule which can base pair with a second polynucleotide molecule (*e.g.*, 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary, respectively). “Perfectly complementary” means that all the contiguous nucleotides of a polynucleotide molecule will base pair with the same number of contiguous nucleotides in a second polynucleotide molecule. “Substantially complementary” as used herein refers to a degree of complementarity that can be 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% over all or

a portion of two polynucleotide molecules. In some embodiments, the portion of complementarity may be a region of 10, 15, 20, 25, 30, 35, 40, 45, 50, or more nucleotides. “Substantial complementary” can also refer to a 100% complementarity over a portion of two polynucleotide molecules. In some embodiments, the portion of complementarity between the two polynucleotide molecules is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% of the length of at least one of the two polynucleotide molecules or a functional or defined portion thereof.

[105] The term “subject” and its grammatical equivalents as used herein may refer to a human or a non-human. A subject may be a mammal. A human subject may be male or female. A human subject may be of any age. A subject may be a human embryo. A human subject may be a newborn, an infant, a child, an adolescent, or an adult. A human subject may be up to about 100 years of age. A human subject may be in need of treatment for a genetic disease or disorder.

[106] The terms “treatment” or “treating” and their grammatical equivalents may refer to the medical management of a subject with an intent to cure, ameliorate, or ameliorate a symptom of, a disease, condition, or disorder. Treatment may include active treatment, that is, treatment directed specifically toward the improvement of a disease, condition, or disorder. Treatment may include causal treatment, that is, treatment directed toward removal of the cause of the associated disease, condition, or disorder. In addition, this treatment may include palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, condition, or disorder. Treatment may include supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the disease, condition, or disorder. In some embodiments, a condition may be pathological. In some embodiments, a treatment may not completely cure or prevent a disease, condition, or disorder. In some embodiments, a treatment ameliorates, but does not completely cure or prevent a disease, condition, or disorder. In some embodiments, a subject may be treated for 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, indefinitely, or life of the subject.

[107] The term “ameliorate” and its grammatical equivalents means to decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

[108] The terms “prevent” or “preventing” means delaying, forestalling, or avoiding the onset or development of a disease, condition, or disorder for a period of time. Prevent also means reducing risk of developing a disease, disorder, or condition. Prevention includes

minimizing or partially or completely inhibiting the development of a disease, condition, or disorder. In some embodiments, a composition, *e.g.*, a pharmaceutical composition, prevents a disorder by delaying the onset of the disorder for 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, indefinitely, or life of a subject.

[109] The term “effective amount” or “therapeutically effective amount” may refer to a quantity of a composition, for example a composition comprising a construct, that can be sufficient to result in a desired activity upon introduction into a subject as disclosed herein. An effective amount of the prime editing compositions can be provided to the target gene or cell, whether the cell is *ex vivo* or *in vivo*.

[110] An effective amount can be the amount to induce, for example, at least about a 2-fold change (increase or decrease) or more in the amount of target nucleic acid modulation (*e.g.*, expression of a gene to produce functional a protein) observed relative to a negative control. An effective amount or dose can induce, for example, about 2-fold increase, about 3-fold increase, about 4-fold increase, about 5-fold increase, about 6-fold increase, about 7-fold increase, about 8-fold increase, about 9-fold increase, about 10-fold increase, about 25-fold increase, about 50-fold increase, about 100-fold increase, about 200-fold increase, about 500-fold increase, about 700-fold increase, about 1000-fold increase, about 5000-fold increase, or about 10,000-fold increase in target gene modulation (*e.g.*, expression of a target gene to produce a functional protein).

[111] The amount of target gene modulation may be measured by any suitable method known in the art. In some embodiments, the “effective amount” or “therapeutically effective amount” is the amount of a composition that is required to ameliorate the symptoms of a disease relative to an untreated patient. In some embodiments, an effective amount is the amount of a composition sufficient to introduce an alteration in a gene of interest in a cell (*e.g.*, a cell *in vitro* or *in vivo*).

[112] For purposes of this disclosure, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 75th Ed. Additionally, general principles of organic chemistry are described in “Organic Chemistry,” Thomas Sorrell, University Science Books, Sausalito: 1999, and “March's Advanced Organic Chemistry,” 5th Ed., Ed.: Smith, M.B. and March, J., John Wiley and Sons, New York: 2001, the entire contents of which are hereby incorporated by reference.

alkoxy), $-C(O)-C_{1-6}$ alkoxy and $-C(O)-C_{1-6}$ alkyl. In other embodiments, an alkyl and/or alkylene can be substituted with one or more substituents independently selected from halo, alkoxy, and hydroxy.

[119] In some embodiments, an alkyl and/or alkylene can be substituted with $-OH$ (“hydroxy-substituted alkyl” or “hydroxy-substituted alkylene”). For example, a hydroxy-substituted alkyl/alkylene can be $-(CH_2)_n-CH(OH)-(CH_2)_m-$, where n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

[120] In some embodiments, an alkyl and/or alkylene can be substituted with $-O-$ to form an alkoxy group. As used herein, an “alkoxy” group refers to an alkyl- $O-$ group where “alkyl” has been defined previously. The alkyl portion of the alkoxy group can be substituted (i.e., optionally substituted) as provided herein. For example, an alkoxy or an $-O-$ -substituted alkyl/alkylene can be $-(CH_2)_n-O-(CH_2)_m-$, where n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

[121] In some embodiments, an alkyl and/or alkylene can be substituted with both $-OH$ and $-O-$. Examples of these instances include, but are not limited to, $-(CH_2)_n-CH(OH)-(CH_2)_m-O-(CH_2)_p-$ or $-(CH_2)_n-CH(OH)-O-(CH_2)_m-$, where n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

[122] As used herein, a “carbonyl” refers to $-C(O)-$.

[123] As used herein, a “halogen” or “halo” group refers to fluorine, chlorine, bromine or iodine.

[124] A “heteroaryl” group, as used herein, refers to a monocyclic, bicyclic, or tricyclic ring system having 4 to 15 ring atoms wherein one or more of the ring atoms is a heteroatom (e.g., N, O, S, or combinations thereof) and in which the monocyclic ring system is aromatic or at least one of the rings in the bicyclic or tricyclic ring systems is aromatic. A heteroaryl group includes a benzofused ring system having 2 to 3 rings. For example, a benzofused group includes benzo fused with one or two 4 to 8 membered heterocycloaliphatic moieties (e.g., indolizyl, indolyl, isoindolyl, 3H-indolyl, indolinyl, benzo[*b*]furyl, benzo[*b*]thiophene-yl, quinolinyl, or isoquinolinyl). Some examples of heteroaryl are azetidiny, pyridyl, 1H-indazolyl, furyl, pyrrolyl, thienyl, thiazolyl, oxazolyl, imidazolyl, tetrazolyl, benzofuryl, isoquinolinyl, benzthiazolyl, xanthene, thioxanthene, phenothiazine, dihydroindole, benzo[1,3]dioxole, benzo[*b*]furyl, benzo[*b*]thiophenyl, indazolyl, benzimidazolyl, benzthiazolyl, puryl, cinnolyl, quinolyl, quinazolyl, cinnolyl, phthalazyl, quinazolyl, quinoxalyl, isoquinolyl, 4H-quinolizyl, benzo-1,2,5-thiadiazolyl, or 1,8-naphthyridyl.

[125] Without limitation, monocyclic heteroaryls include furyl, thiophene-yl, 2H-pyrrolyl, pyrrolyl, oxazolyl, thiazolyl, imidazolyl, pyrazolyl, isoxazolyl, isothiazolyl, 1,3,4-thiadiazolyl,

2H-pyran-2-yl, 4-H-pyran-4-yl, pyridyl, pyridazinyl, pyrimidinyl, pyrazolyl, pyrazolyl, or 1,3,5-triazolyl.

Monocyclic heteroaryls are numbered according to standard chemical nomenclature.

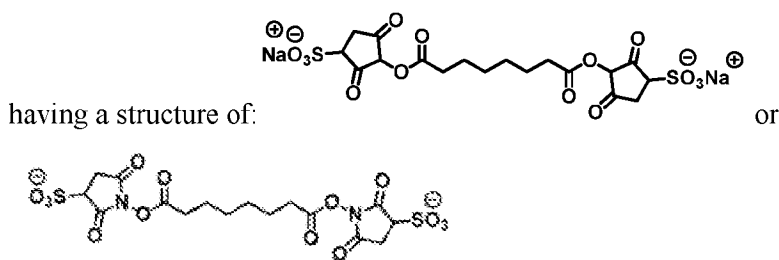
[126] Without limitation, bicyclic heteroaryls include indolizyl, indolyl, isoindolyl, 3H-indolyl, indolinyl, benzo[*b*]furyl, benzo[*b*]thiophenyl, quinolinyl, isoquinolinyl, indolizyl, isoindolyl, indolyl, benzo[*b*]furyl, benzo[*b*]thiophenyl, indazolyl, benzimidazolyl, benzthiazolyl, purinyl, 4H-quinolizyl, quinolyl, isoquinolyl, cinnolyl, phthalazolyl, quinazolyl, quinoxalyl, 1,8-naphthyridyl, or pteridyl. Bicyclic heteroaryls are numbered according to standard chemical nomenclature.

[127] In some embodiments, a heteroaryl group has 1-3 heteroatoms independently selected from N, O, or S.

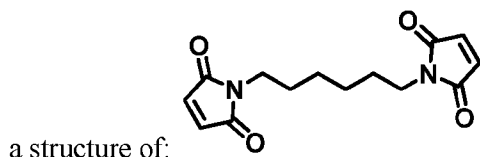
[128] In some embodiments, a heteroaryl group can be substituted (i.e., optionally substituted) with one or more substituents such as aliphatic [e.g., alkyl, alkenyl, or alkynyl]; cycloaliphatic; (cycloaliphatic)aliphatic; heterocycloaliphatic; (heterocycloaliphatic)aliphatic; aryl; heteroaryl; alkoxy; (cycloaliphatic)oxy; (heterocycloaliphatic)oxy; aryloxy; heteroaryloxy; (araliphatic)oxy; (heteroaraliphatic)oxy; aroyl; heteroaroyl; amino; oxo (on a non-aromatic carbocyclic or heterocyclic ring of a bicyclic or tricyclic heteroaryl); carboxy; amido; acyl [e.g., aliphaticcarbonyl; (cycloaliphatic)carbonyl; ((cycloaliphatic)aliphatic)carbonyl; (araliphatic)carbonyl; (heterocycloaliphatic)carbonyl; ((heterocycloaliphatic)aliphatic)carbonyl; or (heteroaraliphatic)carbonyl]; sulfonyl [e.g., aliphaticsulfonyl or aminosulfonyl]; sulfinyl [e.g., aliphaticsulfinyl]; sulfanyl [e.g., aliphatic sulfanyl]; nitro; cyano; halo; hydroxy; mercapto; sulfoxy; urea; thiourea; sulfamoyl; sulfamide; or carbamoyl. Alternatively, a heteroaryl can be unsubstituted.

[129] As used herein, an “oxo” refers to =O.

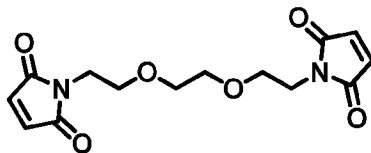
[130] As used herein, a “BS3” reagent refers to bis(sulfosuccinimidyl)suberate, a compound



[131] As used herein, a “BMH” reagent refers to bismaleimido-hexane, a compound having

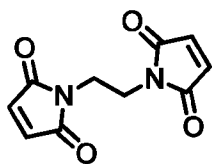


[132] As used herein, a “BM(PEG)₂” reagent refers to 1,8-bisamleimidodiethyleneglycol, a



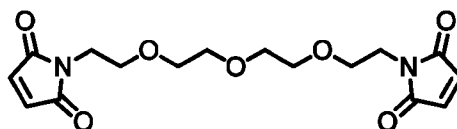
compound having a structure of:

[133] As used herein, a “BMOE” reagent refers to bismaleimidoethane, a compound having



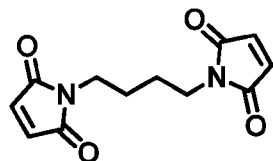
a structure of:

[134] As used herein, a “BM(PEG)₃” reagent refers to 1,11-bismaleimidotriethyleneglycol,



a compound having a structure of:

[135] As used herein, a “BMB” reagent refers to 1,4-bismaleimidobutane, a compound



having a structure of:

[136] As used herein, the term “substituted,” whether preceded by the term “optionally” or not, refers generally to the replacement of hydrogen atoms in a given structure with the radical of a specified substituent. Specific substituents are described above in the definitions and below in the description of compounds and examples thereof. Unless otherwise indicated, an optionally substituted group can have a substituent at each substitutable position of the group, and when more than one position in any given structure can be substituted with more than one substituent selected from a specified group, the substituent can be either the same or different at every position. A ring substituent, such as a heterocycloalkyl, can be bound to another ring, such as a cycloalkyl, to form a spiro-bicyclic ring system, e.g., both rings share one common atom. As one of ordinary skill in the art will recognize, combinations of substituents envisioned by this disclosure are those combinations that result in the formation of stable or chemically feasible compounds.

[137] Unless otherwise stated, structures depicted herein also are meant to include all isomeric (e.g., enantiomeric, diastereomeric, and geometric (or conformational)) forms of the structure; for example, the R and S configurations for each asymmetric center, (Z) and (E) double bond isomers, and (Z) and (E) conformational isomers. Therefore, single

stereochemical isomers as well as enantiomeric, diastereomeric, and geometric (or conformational) mixtures of the present compounds are within the scope of the disclosure. Unless otherwise stated, all tautomeric forms of the compounds of the disclosure are within the scope of the disclosure. Additionally, unless otherwise stated, structures depicted herein also are meant to include compounds that differ only in the presence of one or more isotopically enriched atoms. For example, compounds having the present structures except for the replacement of hydrogen by deuterium or tritium, or the replacement of a carbon by a ^{13}C - or ^{14}C -enriched carbon are within the scope of this disclosure. Such compounds are useful, for example, as analytical tools or probes in biological assays, or as therapeutic agents.

Split Synthesis

[138] In general, the synthesis of RNA/DNA oligonucleotides is achieved by solid phase synthesis on automated synthesizers. However, the solid phase synthesis of long RNA oligonucleotides (100 nucleotides or more in length) suffers from low yield and purity of the final product. For example, the coupling efficiency is not quantitative limiting the synthesis yield for long RNA oligonucleotides. Further, these non-quantitative couplings generate shorter impurities such as N-X species (X nucleotides shorter than the full-length product (FLP; N), X= 1, 2, etc.). During the coupling step, the protecting groups can also be cleaved, resulting in impurities with longer length called N+X species (X nucleotides longer than the FLP, X= 1, 2, etc.). Due to the similarity of the chemical and/or physical properties of these impurities and the FLP, it is difficult to remove said impurities during the purification process. This is particularly problematic for long RNAs having 100-nucleotides or more, because just several nucleotides shorter or longer becomes a trivial difference for long oligonucleotides (100 nucleotides or more in length) compared to much shorter oligonucleotides (i.e., 20 nucleotides in length). Hence, alternate synthesis, and purification methods are required for long-RNA synthesis. The long RNAs, such as PEGRNAs, of this disclosure have a length of about 100 nucleotides, about 110 nucleotides, about 120 nucleotides, about 130 nucleotides, about 140 nucleotides, about 150 nucleotides, about 160 nucleotides, about 170 nucleotides, about 180 nucleotides, about 190 nucleotides, about 200 nucleotides, about 210 nucleotides, about 220 nucleotides, about 230 nucleotides, about 240 nucleotides, about 250 nucleotides, about 275 nucleotides, about 300 nucleotides, about 325 nucleotides, about 350 nucleotides, about 375 nucleotides, about 400 nucleotides, about 425 nucleotides, about 450 nucleotides, about 475 nucleotides, or about 500 nucleotides. In some

embodiments, the long RNA has a length of about 140 nucleotides. In some embodiments, the long RNA has a length of about 150 nucleotides. In some embodiments, the long RNA has a length of about 170 nucleotides. In some embodiments, the long RNA has a length of about 190 nucleotides. In some embodiments, the long RNA has a length of about 200 nucleotides. In some embodiments, the long RNA has a length of about 210 nucleotides. In some embodiments, the long RNA has a length of about 220 nucleotides. In some embodiments, the long RNA has a length of about 230 nucleotides. In some embodiments, the long RNA has a length of about 240 nucleotides. In some embodiments, the long RNA has a length of about 250 nucleotides. In some embodiments, the long RNA has a length of about 300 nucleotides. In some embodiments, the long RNA has a length of about 400 nucleotides. In some embodiments, the long RNA has a length of about 500 nucleotides.

[139] The present disclosure provides an approach termed “split synthesis.” In a split synthesis method, the long RNA is split into shorter fragments, sometimes containing chemical modifications on the 3' and 5' end, and the fragments are ligated (bound) through appropriate ligation methods to obtain a full-length product. In some embodiments, a chemical moiety in the form of a functional group is appended to the 3' end and/or the 5' end. In some embodiments, the chemical moiety is structurally different from a natural nucleotide, a natural sugar, and/or a natural internucleoside linkage. The unreacted shorter fragments can be removed to obtain high purity full-length ligated product. In some embodiments, the purity of the full-length ligated product (e.g., long RNA or PEgRNA) is over 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%. In some embodiments, the purity of the full-length ligated product (e.g., long RNA or PEgRNA) is over 55%. In some embodiments, the purity of the full-length ligated product (e.g., long RNA or PEgRNA) is over 60%. In some embodiments, the purity of the full-length ligated product (e.g., long RNA or PEgRNA) is over 70%. In some embodiments, the purity of the full-length ligated product (e.g., long RNA or PEgRNA) is over 75%. In some embodiments, the purity of the full-length ligated product (e.g., long RNA or PEgRNA) is over 80%. In some embodiments, the purity of the full-length ligated product (e.g., long RNA or PEgRNA) is over 90%. In some embodiments, the purity of the full-length ligated product (e.g., long RNA or PEgRNA) is over 95%. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% of unreacted starting materials (e.g., RNA fragments such as donor D1 and acceptor A1 or RNA sequences containing chemical moieties as described herein). In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 10% of unreacted starting

materials (e.g., RNA fragments such as donor D1 and acceptor A1 or RNA sequences containing chemical moieties as described herein). In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 9% of unreacted starting materials. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 8% of unreacted starting materials. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 7% of unreacted starting materials. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 6% of unreacted starting materials. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 5% of unreacted starting materials. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 4% of unreacted starting materials. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 3% of unreacted starting materials. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 2% of unreacted starting materials. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) contains less than 1% of unreacted starting materials. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) is produced at a yield of about 50%, 55%, 60%, 65%, 70%, 75% or more. In some embodiments, full-length ligated product (e.g., long RNA or PEgRNA) produced at a yield of about 60%. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) is produced at a yield of about 70%. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) is produced at a yield of about 80%. In some embodiments, the full-length ligated product (e.g., long RNA or PEgRNA) is produced at a yield of about 90%.

[140] The methods according to all aspects of this disclosure can equally be methods for synthesizing long guide RNAs other than PEgRNA. For example, methods described herein are suitable for synthesizing guide RNA for other Cas9 or Cas12 gene editing systems. In some embodiments, the long guide RNAs of this disclosure have a length of over 100 nucleotides. In some embodiments, the long guide RNAs have a length of about 100 nucleotides, about 110 nucleotides, about 120 nucleotides, about 130 nucleotides, about 140 nucleotides, about 150 nucleotides, about 160 nucleotides, about 170 nucleotides, about 180 nucleotides, about 190 nucleotides, about 200 nucleotides, about 210 nucleotides, about 220 nucleotides, about 230 nucleotides, about 240 nucleotides, about 250 nucleotides, about 275 nucleotides, about 300 nucleotides, about 325 nucleotides, about 350 nucleotides, about 375 nucleotides, about 400 nucleotides, about 425 nucleotides, about 450 nucleotides, about 475

nucleotides, or about 500 nucleotides. In some embodiments, the long guide RNA has a length of about 140 nucleotides. In some embodiments, the long guide RNA has a length of about 150 nucleotides. In some embodiments, the long guide RNA has a length of about 170 nucleotides. In some embodiments, the long guide RNA has a length of about 190 nucleotides. In some embodiments, the long guide RNA has a length of about 200 nucleotides. In some embodiments, the long guide RNA has a length of about 210 nucleotides. In some embodiments, the long guide RNA has a length of about 220 nucleotides. In some embodiments, the long guide RNA has a length of about 230 nucleotides. In some embodiments, the long guide RNA has a length of about 240 nucleotides. In some embodiments, the long guide RNA has a length of about 250 nucleotides. In some embodiments, the long guide RNA has a length of about 300 nucleotides. In some embodiments, the long guide RNA has a length of about 400 nucleotides. In some embodiments, the long guide RNA has a length of about 500 nucleotides.

[141] In certain aspects, the disclosure provides methods for synthesizing long RNAs. In some aspects, the disclosure provides methods for synthesizing prime editing guide RNA (PEgRNA). In some embodiments, the disclosure provides methods for synthesizing prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core. In some embodiments, the disclosure provides methods for synthesizing prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core. In some embodiments, the disclosure provides methods for synthesizing prime editing guide RNA (PEgRNA) comprising a non-chemically-modified guide RNA (gRNA) core. In some embodiments, the disclosure provides methods for synthesizing full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core.

[142] In certain aspects, provided herein is a method comprising,

- contacting a first RNA sequence with a second RNA sequence,
 - wherein the first RNA sequence comprises a first chemical moiety at its 3' end
 - and the second RNA sequence comprises a second chemical moiety at its 5' end; and
- ligating the first RNA sequence and the second RNA sequence to form a synthesized RNA by reacting the first chemical moiety of the first RNA sequence with the second chemical moiety of the second RNA sequence;

wherein the ligation forms a linker in a stem loop structure, a tetraloop structure, and/or an extension arm of the synthesized RNA.

[143] Also provided herein is a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the method comprising,

contacting a first RNA sequence with a second RNA sequence,

wherein the first RNA sequence comprises a first chemical moiety at its 3' end and the second RNA sequence comprises a second chemical moiety at its 5' end; and

ligating the first RNA sequence and the second RNA sequence to obtain the synthesized full-length PEgRNA,

wherein the ligation forms a linker between the first chemical moiety of the first RNA sequence and the second chemical moiety of the second RNA sequence; and

wherein the linker is present in the gRNA core of the synthesized full-length PEgRNA.

[144] The term “prime editing guide RNA,” or “PEgRNA,” refers to a guide polynucleotide that comprises one or more intended nucleotide edits for incorporation into the target DNA. In some embodiments, the PEgRNA associates with and directs a prime editor to incorporate the one or more (e.g., two or more, three or more, four or more, or five or more) intended nucleotide edits into the target gene via prime editing.

[145] In some embodiments, the gRNA core is a modified gRNA core.

[146] In some embodiments, the synthesized full-length PEgRNA comprises, in 5' to 3' order, a spacer, a gRNA core, and an extension arm. In some embodiments, the synthesized full-length PEgRNA comprises, in 5' to 3' order, a spacer, a chemically-modified gRNA core, and an extension arm. In some embodiments, the extension arm comprises a reverse transcription template (RTT) and a primer binding site (PBS).

Spacers

[147] A spacer may guide a prime editing complex to a genomic locus with identical or substantially identical sequence during prime editing. In some embodiments, the PEgRNA comprises a spacer. In some embodiments, the length of the spacer varies from at least 10 nucleotides to 100 nucleotides. For examples, a spacer may be at least 10 nucleotides, at least 11 nucleotides, at least 12 nucleotides, at least 13 nucleotides, at least 14 nucleotides, at least 15 nucleotides, at least 16 nucleotides, at least 17 nucleotides, at least 18 nucleotides, at least 19 nucleotides, at least 20 nucleotides, at least 30 nucleotides, at least 40 nucleotides, at least

50 nucleotides, at least 60 nucleotides, at least 70 nucleotides, at least 80 nucleotides, at least 90 nucleotides, at least 100 nucleotides. In some embodiments, the spacer is 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23 nucleotides, 24 nucleotides, or 25 nucleotides in length. In some embodiments, the spacer is from 15 nucleotides to 30 nucleotides in length, 15 to 25 nucleotides in length, 18 to 22 nucleotides in length, 10 to 20 nucleotides in length, 20 to 30 nucleotides in length, 30 to 40 nucleotides in length, 40 to 50 nucleotides in length, 50 to 60 nucleotides in length, 60 to 70 nucleotides in length, 70 to 80 nucleotides in length, or 90 nucleotides to 100 nucleotides in length. In some embodiments, the spacer is 20 nucleotides in length. In some embodiments, the spacer is 17 to 18 nucleotides in length.

[148] In some embodiments, the length of the spacer varies from at least 10 nucleotides to 100 nucleotides. For examples, a spacer may be at least 10 nucleotides, at least 11 nucleotides, at least 12 nucleotides, at least 13 nucleotides, at least 14 nucleotides, at least 15 nucleotides, at least 16 nucleotides, at least 17 nucleotides, at least 18 nucleotides, at least 19 nucleotides, at least 20 nucleotides, at least 30 nucleotides, at least 40 nucleotides, at least 50 nucleotides, at least 60 nucleotides, at least 70 nucleotides, at least 80 nucleotides, at least 90 nucleotides, at least 100 nucleotides. In some embodiments, the spacer is 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23 nucleotides, 24 nucleotides, or 25 nucleotides in length. In some embodiments, the spacer is from 15 nucleotides to 30 nucleotides in length, 15 to 25 nucleotides in length, 18 to 22 nucleotides in length, 10 to 20 nucleotides in length, 20 to 30 nucleotides in length, 30 to 40 nucleotides in length, 40 to 50 nucleotides in length, 50 to 60 nucleotides in length, 60 to 70 nucleotides in length, 70 to 80 nucleotides in length, or 90 nucleotides to 100 nucleotides in length. In some embodiments, the spacer is 20 nucleotides in length. In some embodiments, the spacer is 17 to 18 nucleotides in length.

[149] As used herein in a PEGRNA, or components thereof such as a spacer, PBS, or RTT sequence, unless indicated otherwise, it should be appreciated that the letter “T” or “thymine” indicates a nucleobase in a DNA sequence that encodes the PEGRNA or guide RNA sequence, and is intended to refer to a uracil (U) nucleobase of the PEGRNA or guide RNA or any chemically modified uracil nucleobase known in the art, such as 5-methoxyuracil.

Primer binding site (PBS)

[150] A PEGRNA may comprise a primer binding site (PBS) and an editing template (*e.g.*, an RTT). The extension arm of a PEGRNA may comprise a PBS and an editing template.

[151] The length of the PBS sequence may vary depending on, *e.g.*, the prime editor components, the search target sequence and other components of the PEGRNA. In some embodiments, the length of the primer binding site (PBS) varies from at least 2 nucleotides to 50 nucleotides. For examples, a primer binding site (PBS) may be at least 2 nucleotides, at least 3 nucleotides, at least 4 nucleotides, at least 5 nucleotides, at least 6 nucleotides, at least 7 nucleotides, at least 8 nucleotides, at least 9 nucleotides, at least 10 nucleotides, at least 11 nucleotides, at least 12 nucleotides, at least 13 nucleotides, at least 14 nucleotides, at least 15 nucleotides, at least 16 nucleotides, at least 17 nucleotides, at least 18 nucleotides, at least 19 nucleotides, at least 20 nucleotides, at least 30 nucleotides, at least 40 nucleotides, or at least 50 nucleotides in length. In some embodiments, the PBS is at least 6 nucleotides in length. In some embodiments, the PBS is about 4 to 16 nucleotides, about 6 to 16 nucleotides, about 6 to 18 nucleotides, about 6 to 20 nucleotides, about 8 to 20 nucleotides, about 10 to 20 nucleotides, about 12 to 20 nucleotides, about 14 to 20 nucleotides, about 16 to 20 nucleotides, or about 18 to 20 nucleotides in length. In some embodiments, the PBS is about 7 to 15 nucleotides in length. In some embodiments, the PBS is 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In some embodiments, the PBS is 8, 9, 10, 11, 12, 13, or 14 nucleotides in length.

[152] An extension arm of a PEGRNA may comprise an editing template that serves as a DNA synthesis template for the DNA polymerase in a prime editor during prime editing.

[153] The length of an editing template may vary depending on, *e.g.*, the prime editor components, the search target sequence and other components of the PEGRNA. In some embodiments, the editing template serves as a DNA synthesis template for a reverse transcriptase, and the editing template is referred to as a reverse transcription editing template (RTT).

[154] In some embodiments, the length of the editing template varies from at least 2 nucleotides to 350 nucleotides. In some embodiments, the editing template is about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, or 350 nucleotides in length. In some embodiments, the editing template is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more nucleotides. In some embodiments, the editing template is about 5 to 10, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 5 to 40, 5 to 45, 5 to 50, 5 to 55, 5 to 60, 5 to 65, 5 to 70, 5 to 75, 5 to 80, 5 to 85, 5 to 90, 5 to 95, 5 to 100, 5 to 110, 5 to 120, 5 to 130, 5 to 140, 5 to 150, 15 to 20, 15 to 25, 15 to 30, 15 to 35, 15 to 40, 15 to 45, 15 to 50, 15 to 55, 15 to 60, 15 to 65, 15 to 70, 15 to 75, 15 to 80, 15 to 85, 15 to 90, 15 to 95, 15 to 100, 15 to 110, 15 to

120, 15 to 130, 15 to 140, 15 to 150, 25 to 30, 25 to 35, 25 to 40, 25 to 45, 25 to 50, 25 to 55, 25 to 60, 25 to 65, 25 to 70, 25 to 75, 25 to 80, 25 to 85, 25 to 90, 25 to 95, 25 to 100, 25 to 110, 25 to 120, 25 to 130, 25 to 140, 25 to 150, 35 to 40, 35 to 45, 35 to 50, 35 to 55, 35 to 60, 35 to 65, 35 to 70, 35 to 75, 35 to 80, 35 to 85, 35 to 90, 35 to 95, 35 to 100, 35 to 110, 35 to 120, 35 to 130, 35 to 140, 35 to 150, 45 to 50, 45 to 55, 45 to 60, 45 to 65, 45 to 70, 45 to 75, 45 to 80, 45 to 85, 45 to 90, 45 to 95, 45 to 100, 45 to 110, 45 to 120, 45 to 130, 45 to 140, 45 to 150, 55 to 60, 55 to 65, 55 to 70, 55 to 75, 55 to 80, 55 to 85, 55 to 90, 55 to 95, 55 to 100, 55 to 110, 55 to 120, 55 to 130, 55 to 140, 55 to 150, 65 to 70, 65 to 75, 65 to 80, 65 to 85, 65 to 90, 65 to 95, 65 to 100, 65 to 110, 65 to 120, 65 to 130, 65 to 140, 65 to 150, 75 to 80, 75 to 85, 75 to 90, 75 to 95, 75 to 100, 75 to 110, 75 to 120, 75 to 130, 75 to 140, 75 to 150, 85 to 90, 85 to 95, 85 to 100, 85 to 110, 85 to 120, 85 to 130, 85 to 140, 85 to 150, 95 to 100, 95 to 110, 95 to 120, 95 to 130, 95 to 140, 95 to 150, 105 to 110, 105 to 120, 105 to 130, 105 to 140, 105 to 150, 115 to 120, 115 to 130, 115 to 140, 115 to 150, 125 to 130, 125 to 140, 125 to 150, 135 to 140, 135 to 150, or 145 to 150 nucleotides in length.

[155] In some embodiments, the RTT is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In some embodiments, the RTT is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In some embodiments, the RTT is 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides in length.

[156] In some embodiments, a PEgRNA includes only RNA nucleotides and forms an RNA polynucleotide. In some embodiments, a PEgRNA is a chimeric polynucleotide that includes both RNA and DNA nucleotides. For example, a PEgRNA can include DNA in the spacer sequence, the gRNA core, or the extension arm. In some embodiments, a PEgRNA comprises DNA in the spacer sequence. In some embodiments, the entire spacer sequence of a PEgRNA is a DNA sequence. In some embodiments, the PEgRNA comprises DNA in the gRNA core, for example, in a stem region of the gRNA core. In some embodiments, the PEgRNA comprises DNA in the extension arm, for example, in the editing template. An editing template that comprises a DNA sequence may serve as a DNA synthesis template for a DNA polymerase in a prime editor, for example, a DNA-dependent DNA polymerase. Accordingly, the PEgRNA may be a chimeric polynucleotide that comprises RNA in the spacer, gRNA core, and/or the PBS sequences and DNA in the editing template.

[157] Components of a PEgRNA may be arranged in a modular fashion. In some embodiments, the spacer and the extension arm comprising a primer binding site sequence (PBS) and an editing template, *e.g.*, a reverse transcriptase template (RTT), can be

interchangeably located in the 5' portion of the PEgRNA, the 3' portion of the PEgRNA, or in the middle of the gRNA core. For example, in some embodiments, a PEgRNA comprises, from 5' to 3': a spacer, a gRNA core, an editing template, and a PBS. In some embodiments, a PEgRNA comprises, from 5' to 3': an editing template, a PBS, a spacer, and a gRNA core.

gRNA core

[158] In some embodiments, a gRNA core of a PEgRNA associates with a programmable DNA binding domain in a prime editor. In some embodiments, the gRNA core comprises a direct repeat, a first stem loop, and a second stem loop. In some embodiments, the gRNA core further comprises a third stem loop. A guide RNA core (also referred to herein as the gRNA core, gRNA scaffold, or gRNA backbone sequence) of a PEgRNA may contain a polynucleotide sequence that binds to a DNA binding domain (*e.g.*, Cas9) of a prime editor. The gRNA core may interact with a prime editor as described herein, for example, by association with a DNA binding domain, such as a DNA nickase of the prime editor.

[159] One of skill in the art will recognize that different prime editors having different DNA binding domains from different DNA binding proteins may require different gRNA core sequences specific to the DNA binding protein. In some embodiments, the gRNA core is capable of binding to a Cas9-based prime editor. In some embodiments, the gRNA core is capable of binding to a Cpf1-based prime editor. In some embodiments, the gRNA core is capable of binding to a Cas12b-based prime editor.

[160] In some embodiments, the gRNA core comprises regions and secondary structures involved in binding with specific CRISPR Cas proteins. For example, in a Cas9 based prime editing system, the gRNA core of a PEgRNA may comprise one or more regions of a base paired regions.

[161] In some embodiments, the gRNA core is a modified gRNA core. In some embodiments, the gRNA core is a chemically-modified gRNA core.

[162] In some embodiments, a gRNA core or a chemically-modified gRNA core comprises, from 5' to 3': a repeat sequence, a loop structure, an antirepeat sequence, a first stem loop, a second stem loop, and a third stem loop.

[163] In some embodiments, a gRNA core or a chemically-modified gRNA core comprises, from 5' to 3': a repeat sequence, a loop structure, an antirepeat sequence, a first stem loop, a second stem loop, and a third stem loop. As used herein, a repeat sequence and an antirepeat sequence refer to the nucleic acid secondary structure formed by the direct repeat region. The repeat sequence and the antirepeat sequence may be connected by a loop structure, and the

secondary structure formed by base pairing between the repeat and antirepeat sequence may be referred to as the direct repeat region. In some embodiments, the repeat, antirepeat, and the connecting loop structure may be referred to as a tetraloop.

[164] In some embodiments, a gRNA core or a chemically-modified gRNA core comprises, from 5' to 3': a tetraloop (TL), a first stem loop (SL1), a second stem loop (SL2), and a third stem loop (SL3). Exemplary structures of the gRNA core are shown in FIG. 1 and FIG. 2.

[165] In some embodiments, the tetraloop (TL) comprises a lower stem, a bulge, an upper stem, and a loop.

[166] In some embodiments, the tetraloop comprises one or more base paired regions: a base paired "lower stem" adjacent to the spacer sequence and a base paired "upper stem" following the lower stem, where the lower stem and upper stem may be connected by a "bulge" comprising unpaired RNAs.

[167] As used herein, a stem loop (or a hairpin loop) is base pairing pattern that can occur in single-stranded nucleic acids. In some embodiments, a stem loop may be formed when two regions of the same nucleic acid strand are at least partially complementary in nucleotide sequence when read in opposite directions, therefore, the base-pairs can form a double helix that comprises an unpaired loop. Stem loops within a gRNA core described herein may be numbered starting from the 5' to the 3' end of the gRNA core. For example, the "first stem loop" would be the first stem loop (not including any direct repeats) at the 5' end proximal to the direct repeat of the gRNA core sequence. A "second stem loop" would be the second stem loop (not including any direct repeats) following the first stem loop in a 5' to 3' direction, and the "third stem loop" would be the third stem loop following the second stem loop in a 5' to 3' direction, and so on.

[168] In some embodiments, the gRNA core comprises nucleotide alterations as compared to a wild type gRNA core. For example, in some embodiments, one or more nucleotides in the gRNA core is deleted, inserted, and/or substituted as compared to a wild type gRNA core. An exemplary wild type gRNA core sequence is
GUUUUAGAGCUAGAAAUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUU
GAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO.: 41).

[169] In some embodiments, the gRNA core comprises one or more nucleotide insertions, deletions, and/or substitutions in the lower stem or upper stem of the tetraloop. In some embodiments, the gRNA core comprises one or more nucleotide substitutions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises one or more nucleotide insertions in the upper stem of the tetraloop. In some embodiments, the gRNA

core comprises one or more nucleotide insertions, deletions, and/or substitutions in the second stem loop as compared to a wild type gRNA core. In some embodiments, the gRNA core comprises one or more nucleotide insertions in the second stem loop. In some embodiments, the gRNA core comprises one or more nucleotide insertions, deletions, and/or substitutions in the third stem loop as compared to a wild type gRNA core.

[170] In some embodiments, the gRNA core comprises one or more nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises one or more nucleotide insertions in the upper stem of the tetraloop. In some embodiments, the gRNA core comprises 1-30, 5-20, or 5-15 nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises 3-30, 3-20, 3-16 or 3-12 nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises 3-12 nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises 4-30, 4-20, 4-16 or 4-12 nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises 4-12 nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises 6-30, 6-20, 6-16 or 6-12 nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises 6-12 nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises 10, 11 or 12 nucleotide insertions in the lower stem of the tetraloop. In some embodiments, the gRNA core comprises 1-30, 5-20, or 5-15 nucleotide insertions in the upper stem of the tetraloop. In some embodiments, the gRNA core comprises 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotide insertions in the upper stem of the tetraloop.

[171] In some embodiments, the gRNA core has extensions in the TL or SL2 as shown in FIG. 5A and FIG. 5B. In some embodiments, the gRNA core has a 1 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 2 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 3 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 4 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 5 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 6 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 7 base pair extension in the upper stem of the

tetraloop. In some embodiments, the gRNA core has a 8 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 9 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 10 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 11 base pair extension in the upper stem of the tetraloop. In some embodiments, the gRNA core has a 12 base pair extension in the upper stem of the tetraloop.

[172] In some embodiments, the gRNA core comprises one or more nucleotide insertions in the SL2. In some embodiments, the gRNA core comprises 1-30, 5-20, or 5-15 nucleotide insertions in the SL2. In some embodiments, the gRNA core comprises 3-30, 3-20, 3-16 or 3-12 nucleotide insertions in the SL2. In some embodiments, the gRNA core comprises 3-12 nucleotide insertions in the SL2. In some embodiments, the gRNA core comprises 4-30, 4-20, 4-16 or 4-12 nucleotide insertions in the SL2. In some embodiments, the gRNA core comprises 4-12 nucleotide insertions in the SL2. In some embodiments, the gRNA core comprises 8-30, 8-20, 8-16 or 8-12 nucleotide insertions in the SL2. In some embodiments, the gRNA core comprises 8-12 nucleotide insertions in the SL2. In some embodiments, the gRNA core comprises 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotide insertions in the SL2. In some embodiments, the gRNA core comprises 10, 11 or 12 nucleotide insertions in the SL2.

[173] In some embodiments, the gRNA core has a 1 base pair extension in the the SL2. In some embodiments, the gRNA core has a 2 base pair extension in the the SL2. In some embodiments, the gRNA core has a 3 base pair extension in the the SL2. In some embodiments, the gRNA core has a 1 base pair extension in the the SL2. In some embodiments, the gRNA core has a 4 base pair extension in the the SL2. In some embodiments, the gRNA core has a 5 base pair extension in the the SL2. In some embodiments, the gRNA core has a 6 base pair extension in the the SL2. In some embodiments, the gRNA core has a 7 base pair extension in the the SL2. In some embodiments, the gRNA core has a 8 base pair extension in the the SL2. In some embodiments, the gRNA core has a 9 base pair extension in the the SL2. In some embodiments, the gRNA core has a 10 base pair extension in the the SL2. In some embodiments, the gRNA core has a 11 base pair extension in the the SL2. In some embodiments, the gRNA core has a 12 base pair extension in the the SL2.

Split Synthesis

[174] The split synthesis methods for preparing full-length PEgRNAs as described herein require a minimum of two RNA sequences (i.e., splitting the full-length PEgRNA into two or more parts), with each RNA sequence comprising at least one part of the full-length PEgRNA.

[175] An important factor to consider in the split synthesis methods is choosing the appropriate split site for chemical ligations. Due to the additional chemical structure formed after chemical ligation, it is important to avoid the chemical structure interfering with the Cas9 nuclease interaction and activity by disturbing the gRNA/Cas9 complex. Accordingly, and in some embodiments, the TL and SL2 are split sites for chemical ligations. By splitting the full-length PEgRNA at the TL or SL2, two RNA sequences are required, each sequence having at least one portion of the gRNA core. A “portion” of the gRNA core corresponds to a section of the gRNA core that is smaller than the full-length gRNA core. FIG. 3A and FIG. 3B shows exemplary schemes of the split sites at the TL (FIG. 3A) and SL2 (FIG. 3B) of the gRNA core.

[176] In some embodiments, the first RNA sequence comprises a spacer and a first portion of a chemically-modified gRNA core.

[177] In some embodiments, the second RNA sequence comprises a second portion of a chemically-modified gRNA core, an extension arm, a chemically-modified extension arm, an RTT, and/or a PBS. In some embodiments, the second RNA sequence comprises a second portion of a chemically-modified gRNA core and an extension arm. In some embodiments, the second RNA sequence comprises a second portion of a chemically-modified gRNA core, an RTT, and a PBS. In some embodiments, the second RNA sequence comprises a second portion of a chemically-modified gRNA core and a chemically modified extension arm.

[178] A “fragmented tetraloop” refer to a portion of a full-length tetraloop. In some embodiments, a fragmented tetraloop comprises 50% of the nucleotides of a full-length tetraloop. In some embodiments, a fragmented tetraloop comprises 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the nucleotides in length of a full-length tetraloop. In some embodiments, a fragmented tetraloop comprises 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, or 45% of the nucleotides in length of a full-length tetraloop.

[179] A “fragmented SL2” refer to a portion of a full-length SL2. In some embodiments, a fragmented SL2 comprises 50% of the nucleotides of a full-length SL2. In some embodiments, a fragmented SL2 comprises 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the nucleotides in length of a full-length SL2. In some embodiments, a fragmented

SL2 comprises 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, or 45% of the nucleotides in length of a full-length SL2.

[180] In some embodiments, the first portion of the chemically-modified gRNA core and the second portion of the chemically-modified gRNA core are different in length. In some embodiments, the first portion of the chemically-modified gRNA core and the second portion of the chemically-modified gRNA core are equal in length.

[181] In some embodiments, the first portion of the chemically-modified gRNA core is at least 5 nucleotides, at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 25 nucleotides, at least 30 nucleotides, at least 35 nucleotides, at least 40 nucleotides, at least 45 nucleotides, at least 50 nucleotides, at least 55 nucleotides, at least 60 nucleotides, at least 65 nucleotides, at least 70 nucleotides, at least 75 nucleotides, at least 80 nucleotides, at least 85 nucleotides, at least 90 nucleotides, at least 95 nucleotides, at least 100 nucleotides, at least 105 nucleotides, or at least 110 nucleotides in length. In some embodiments, the first portion of the chemically-modified gRNA core is 5-120 or 10-110 nucleotides in length. In some embodiments, the first portion of the chemically-modified gRNA core is 5 nucleotides, 10 nucleotides, 15 nucleotides, 20 nucleotides, 25 nucleotides, 30 nucleotides, 35 nucleotides, 40 nucleotides, 45 nucleotides, 50 nucleotides, 55 nucleotides, 60 nucleotides, 65 nucleotides, 70 nucleotides, 75 nucleotides, 80 nucleotides, 85 nucleotides, 90 nucleotides, 95 nucleotides, 100 nucleotides, 105 nucleotides, or 110 nucleotides in length.

[182] In some embodiments, the second portion of the chemically-modified gRNA core is at least 5 nucleotides, at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 25 nucleotides, at least 30 nucleotides, at least 35 nucleotides, at least 40 nucleotides, at least 45 nucleotides, at least 50 nucleotides, at least 55 nucleotides, at least 60 nucleotides, at least 65 nucleotides, at least 70 nucleotides, at least 75 nucleotides, at least 80 nucleotides, at least 85 nucleotides, at least 90 nucleotides, at least 95 nucleotides, at least 100 nucleotides, at least 105 nucleotides, or at least 110 nucleotides in length. In some embodiments, the second portion of the chemically-modified gRNA core is 5-120 or 10-110 nucleotides in length. In some embodiments, the second portion of the chemically-modified gRNA core is 5 nucleotides, 10 nucleotides, 15 nucleotides, 20 nucleotides, 25 nucleotides, 30 nucleotides, 35 nucleotides, 40 nucleotides, 45 nucleotides, 50 nucleotides, 55 nucleotides, 60 nucleotides, 65 nucleotides, 70 nucleotides, 75 nucleotides, 80 nucleotides, 85 nucleotides, 90 nucleotides, 95 nucleotides, 100 nucleotides, 105 nucleotides, or 110 nucleotides in length.

[183] In some embodiments, the fragmented tetraloop of the first portion of the chemically-modified gRNA core is 5 to 40, 10 to 30, or 10 to 20 nucleotides in length. In some

embodiments, the fragmented tetraloop of the first portion of the chemically-modified gRNA core is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides in length. In some embodiments, the fragmented tetraloop of the second portion of the chemically-modified gRNA core is 5 to 40, 10 to 30, or 10 to 20 nucleotides in length. In some embodiments, the fragmented tetraloop of the second portion of the chemically-modified gRNA core is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides in length.

[184] In some embodiments, the fragmented SL2 of the first portion of the chemically-modified gRNA core is 5 to 30, 10 to 20 nucleotides in length. In some embodiments, the fragmented SL2 of the first portion of the chemically-modified gRNA core is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In some embodiments, the fragmented SL2 of the second portion of the chemically-modified gRNA core is 5 to 30, 10 to 20 nucleotides in length. In some embodiments, the fragmented SL2 of the second portion of the chemically-modified gRNA core is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

[185] In some embodiments, the chemically-modified gRNA core comprises a tetraloop, an extended tetraloop, an SL2 and/or an extended SL2. In some embodiments, the chemically-modified gRNA core comprises a tetraloop. In some embodiments, the chemically-modified gRNA core comprises an extended tetraloop. In some embodiments, the extended tetraloop comprises an additional 1-20 nucleotides in length compared to a native tetraloop. In some embodiments the extended tetraloop comprises an additional 1-30, 5-20, or 5-15 nucleotides in length compared to a native tetraloop. In some embodiments the extended tetraloop comprises an additional 3-30, 3-20, 3-16 or 3-12 nucleotides in length compared to a native tetraloop. In some embodiments the extended tetraloop comprises an additional 3-12 nucleotides in length compared to a native tetraloop. In some embodiments the extended tetraloop comprises an additional 4-30, 4-20, 4-16 or 4-12 nucleotides in length compared to a native tetraloop. In some embodiments the extended tetraloop comprises an additional 4-12 nucleotides in length compared to a native tetraloop. In some embodiments the extended tetraloop comprises an additional 6-30, 6-20, 6-16 or 6-12 nucleotides in length compared to a native tetraloop. In some embodiments the extended tetraloop comprises an additional 6-12 nucleotides in length compared to a native tetraloop. In some embodiments, the extended tetraloop comprises an additional 1-20 nucleotides in the upper stem of the tetraloop

compared to the upper step of a native tetraloop. A “native tetraloop” is a tetraloop sequence that derives from a wild type gRNA core sequence. In some embodiments, a native tetraloop is a sequence of 5'-GUUUUAGAGCUAGAAAUAGCAAGUUAUAAA-3' (SEQ ID NO.: 22). In some embodiments, a native upper stem sequence of a tetraloop is the underlined nucleotides of the following sequence 5'-

GUUUUAGAGCUAGAAAUAGCAAGUUAUAAA-3' (SEQ ID NO.: 22). In some embodiments, the chemically-modified gRNA core comprises an SL2. In some embodiments, the chemically-modified gRNA core comprises an extended SL2. In some embodiments, the extended SL2 comprises an additional 1-20 nucleotides in length compared to a native SL2. In some embodiments the extended SL2 comprises an additional 1-30, 5-20, or 5-15 nucleotides in length compared to a native SL2. In some embodiments the extended SL2 comprises an additional 3-30, 3-20, 3-16 or 3-12 nucleotides in length compared to a native SL2. In some embodiments the extended SL2 comprises an additional 3-12 nucleotides in length compared to a native SL2. In some embodiments the extended SL2 comprises an additional 4-30, 4-20, 4-16 or 4-12 nucleotides in length compared to a native SL2. In some embodiments the extended SL2 comprises an additional 4-12 nucleotides in length compared to a native SL2. In some embodiments the extended SL2 comprises an additional 9-30, 9-20, 9-16 or 9-12 nucleotides in length compared to a native SL2. In some embodiments the extended SL2 comprises an additional 9-12 nucleotides in length compared to a native SL2. A “native SL2” is a SL2 sequence that derives from a wild type gRNA core sequence. In some embodiments, a native SL2 is a sequence of 5'-ACUUGAAAAAGU-3' (SEQ ID NO.: 23).

[186] Extension of the TL or SL2 where the chemical moiety or linker is located can avoid disturbance of the PEgRNA/Cas9 complex. By extending the TL or SL2, the linker is moved further away from the interactions between the PEgRNA and the Cas9 protein. In some embodiments the number of additional nucleotides of the extension is determined by the chemical moieties and the linker therebetween present in the gRNA core of the PEgRNA. For example, larger and/or bulkier chemical moieties and/or linkers may necessitate a longer extension to ensure they are located sufficiently far away from the interactions between the PEgRNA and the Cas9 protein. In some embodiments the extended TL or SL2 where the chemical moiety or linker is located comprises an additional 10, 11, 12, 13 or 14 nucleotides in length when the linker comprises a thioether linkage. In some embodiments the extended TL or SL2 where the chemical moiety or linker is located comprises an additional 10, 11, 12, 13 or 14 nucleotides in length when the linker comprises a bisamide linkage. In some

embodiments the extended TL or SL2 where the chemical moiety or linker is located comprises an additional 10, 11, 12, 13 or 14 nucleotides in length when the linker comprises a triazole linkage.

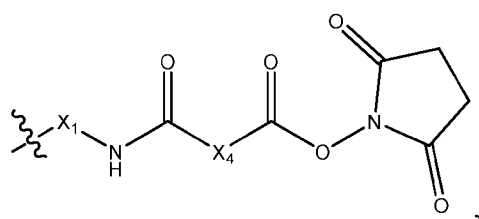
[187] For ligations of two RNA sequences in the split synthesis methods described herein, it is beneficial, and sometimes critical, to have strong interactions between the RNA sequences so as to bring the chemical moieties in proximity to increase the effective local concentration of the functional groups of the chemical moieties and facilitate the ligation. Having extensions in the TL and SL2 increases the number of base pairs between the RNA sequences comprising the chemical moieties, leading to efficient pre-annealing of the RNA sequences in solution.

Chemical Moieties

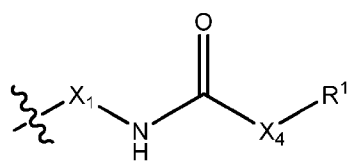
[188] The split synthesis methods described herein form a full-length PEgRNA by joining two chemical moieties present at the 3' end and/or 5' ends of the RNA sequences.

[189] In some embodiments, the first RNA sequence comprises a first chemical moiety at its 3' end. In some embodiments, the second RNA sequence comprises a second chemical moiety at its 5' end. The first RNA sequence and the second RNA sequence are ligated via a chemical ligation (chemical reaction). The first chemical moiety and the second chemical moiety react with each other or with a reagent thereby forming a linker between the two.

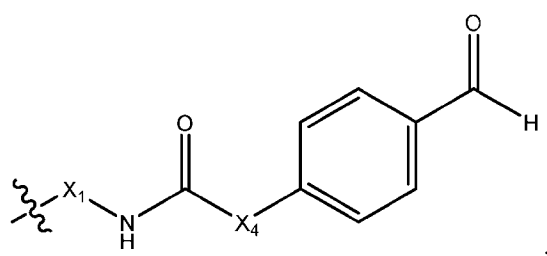
[190] In some embodiments, the first chemical moiety of the first RNA sequence comprises amino, $-X_1-NH_2$, $-X_1-OH$, $-X_1-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 1-6 or Formulas 9-11:



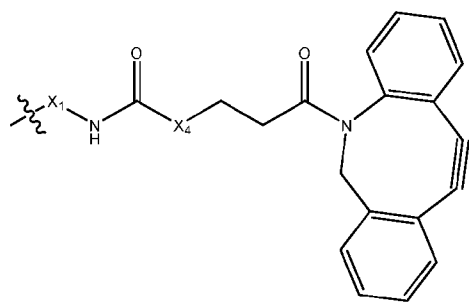
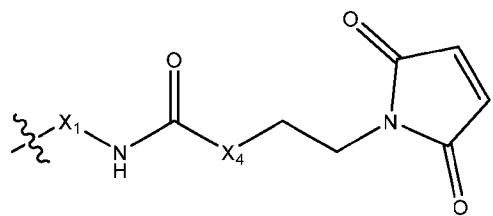
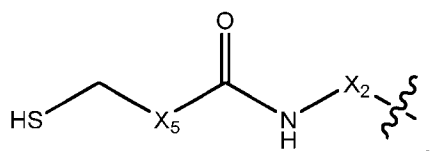
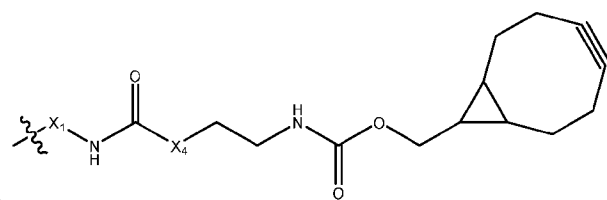
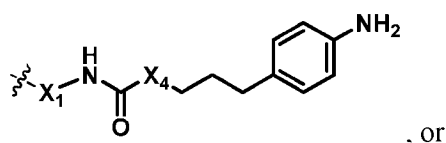
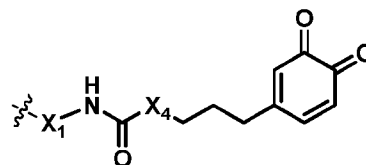
Formula 1



Formula 2



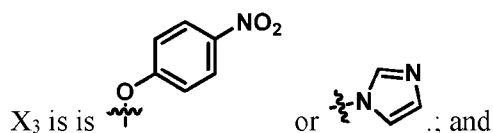
Formula 3

**Formula 4****Formula 5****Formula 6****Formula 9****Formula 10****Formula 11**

wherein:

each X_1 and X_4 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$,

“n” is an integer between 1 and 12;



R^1 is halogen.

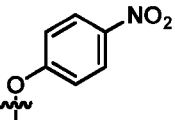
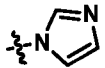
[191] In some embodiments, X_1 is absent. In some embodiments, X_1 is $(O-CH_2-CH_2)_n$. In some embodiments, X_1 is $(CH_2-CH_2-O)_n$. In some embodiments, X_1 is $((CH_2)_n-C(O)NH-CH_2)$. In some embodiments, X_1 is an alkylene. In some embodiments, X_1 is a C_1-C_{10} unsubstituted alkylene. In some embodiments, X_1 is a C_1-C_6 alkylene. In some embodiments, X_1 is $-CH_2-$. In some embodiments, X_1 is $-CH_2-CH_2-$. In some embodiments, X_1 is a C_6 unsubstituted alkylene. In some embodiments, X_1 is a C_1-C_{10} substituted alkylene with an $-OH$. In some embodiments, X_1 is a C_1-C_{10} substituted alkylene with an $-O-$. In some

embodiments, X_1 is a C_1 - C_{10} substituted alkylene with an $-OH$ and an $-O-$. In some embodiments, X_1 is $-O-$.

[192] In some embodiments, X_4 is absent. In some embodiments, X_4 is $(O-CH_2-CH_2)_n$. In some embodiments, X_4 is $(CH_2-CH_2-O)_n$. In some embodiments, X_4 is $((CH_2)_n-C(O)NH-CH_2)$. In some embodiments, X_4 is an alkylene. In some embodiments, X_4 is a C_1 - C_{10} unsubstituted alkylene. In some embodiments, X_4 is a C_1 - C_6 alkylene. In some embodiments, X_4 is $-CH_2-$. In some embodiments, X_4 is $-CH_2-CH_2-$. In some embodiments, X_4 is a C_1 - C_{10} substituted alkylene with an $-OH$. In some embodiments, X_4 is a C_1 - C_{10} substituted alkylene with an $-O-$. In some embodiments, X_4 is a C_1 - C_{10} substituted alkylene with an $-OH$ and an $-O-$. In some embodiments, X_4 is $-O-$.

[193] In some embodiments “n” is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12.

[194] In some embodiments, X_3 is 4-nitrophenoxy or imidazolyl. In some embodiments, X_3

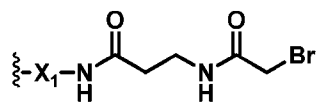
is  or . In some embodiments, X_3 is a 4-nitrophenyl derivative.

[195] In some embodiments, R^1 is Cl. In some embodiments, R^1 is Br. In some embodiments, R^1 is I.

[196] In some embodiments, the first chemical moiety of the first RNA sequence comprises an amino. In some embodiments, the first chemical moiety of the first RNA sequence comprises $-X_1-NH_2$. In some embodiments, the first chemical moiety of the first RNA sequence comprises $-X_1-OH$. In some embodiments, the first chemical moiety of the first RNA sequence comprises $-X_1-PO_4$. In some embodiments, the first chemical moiety of the first RNA sequence comprises $-O(CO)X_3$.

[197] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 1. In some embodiments, in the structure of Formula 1, X_4 is an alkylene. In some embodiments, in the structure of Formula 1, X_4 is $-CH_2-CH_2-$.

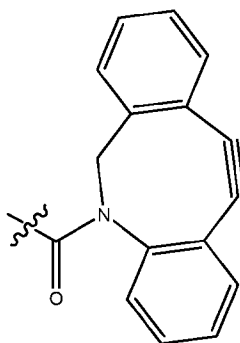
[198] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 2. In some embodiments, R^1 is Cl, Br, or I. In some embodiments, R^1 is Br. In some embodiments, R^1 is I. In some embodiments, the structure of Formula 2 is



[199] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 2. In some embodiments, in the structure of Formula 2, X_4 is an alkylene.

[200] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 3. In some embodiments, in the structure of Formula 3, X₄ is an alkylene. In some embodiments, in the structure of Formula 3, X₄ is absent.

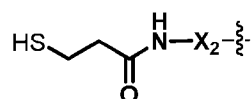
[201] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 4. In some embodiments, in the structure of Formula 4, X₄ is an alkylene. In some embodiments, in the structure of Formula 4, X₄ is absent. In some



embodiments, the structure of Formula 4 is

[202] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 5. In some embodiments, in the structure of Formula 5, X₄ is an alkylene. In some embodiments, in the structure of Formula 5, X₄ is absent.

[203] In some embodiments, the first chemical moiety of the second RNA sequence comprises a structure of Formula 6. In some embodiments, in the structure of Formula 6, X₅ is an alkylene. In some embodiments, in the structure of Formula 6, X₅ is $-\text{CH}_2-$. In some embodiments, the second chemical moiety of the second RNA sequence is

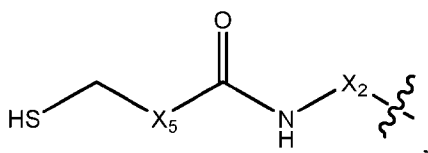


[204] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 9. In some embodiments, in the structure of Formula 9, X₄ is an alkylene. In some embodiments, in the structure of Formula 9, X₄ is absent.

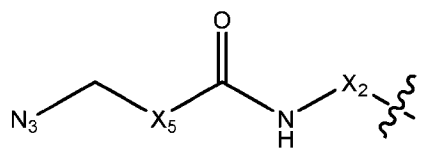
[205] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 10. In some embodiments, in the structure of Formula 10, X₄ is an alkylene. In some embodiments, in the structure of Formula 10, X₄ is absent.

[206] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 11. In some embodiments, in the structure of Formula 11, X₄ is an alkylene. In some embodiments, in the structure of Formula 11, X₄ is absent.

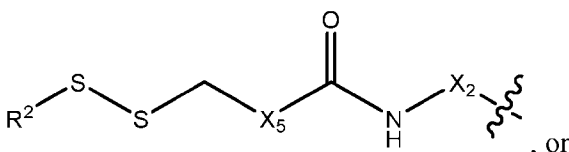
[207] In some embodiments, the second chemical moiety of the second RNA sequence comprises an amino, $-\text{X}_2-\text{NH}_2$, $-\text{X}_2-\text{OH}$, $-\text{X}_2-\text{PO}_4^-$, $-\text{O}(\text{CO})\text{X}_3$, or a structure of any one of Formulas 6-8 or Formula 12:



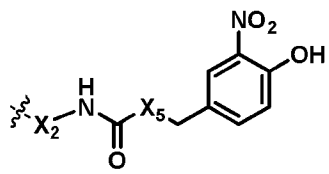
Formula 6



Formula 7



Formula 8

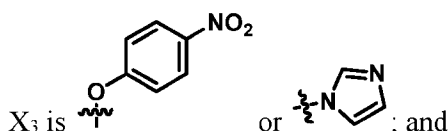


Formula 12

wherein:

each X_2 and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$,

“n” is an integer between 1 and 12;



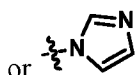
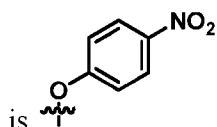
R^2 is a heteroaryl.

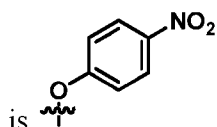
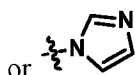
[208] In some embodiments, X_2 is absent. In some embodiments, X_2 is $(O-CH_2-CH_2)_n$. In some embodiments, X_2 is $(CH_2-CH_2-O)_n$. In some embodiments, X_2 is $((CH_2)_n-C(O)NH-CH_2)$. In some embodiments, X_2 is an alkylene. In some embodiments, X_2 is a C_1-C_{10} unsubstituted alkylene. In some embodiments, X_2 is a C_1-C_6 alkylene. In some embodiments, X_2 is $-CH_2-$. In some embodiments, X_2 is $-CH_2-CH_2-$. In some embodiments, X_2 is a C_1-C_{10} substituted alkylene with an $-OH$. In some embodiments, X_2 is a C_1-C_{10} substituted alkylene with an $-O-$. In some embodiments, X_2 is a C_1-C_{10} substituted alkylene with an $-OH$ and an $-O-$. In some embodiments, X_2 is $-O-$.

[209] In some embodiments, X_5 is absent. In some embodiments, X_5 is $(O-CH_2-CH_2)_n$. In some embodiments, X_5 is $(CH_2-CH_2-O)_n$. In some embodiments, X_5 is $((CH_2)_n-C(O)NH-CH_2)$. In some embodiments, X_5 is an alkylene. In some embodiments, X_5 is C_1-C_{10} unsubstituted alkylene. In some embodiments, X_5 is a C_1-C_6 alkylene. In some embodiments, X_5 is $-CH_2-$. In some embodiments, X_5 is $-CH_2-CH_2-$. In some embodiments, X_5 is a C_1-C_{10} substituted alkylene with an $-OH$ and an $-O-$. In some embodiments, X_5 is $-O-$.

[210] In some embodiments “n” is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12.

[211] In some embodiments, X_3 is 4-nitrophenoxy or imidazolyl. In some embodiments, X_3

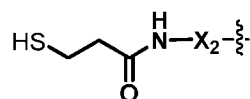


is  or . In some embodiments, X_3 is a 4-nitrophenyl derivative.

[212] In some embodiments, R^2 is pyridine. In some embodiments, R^2 is pyrimidine.

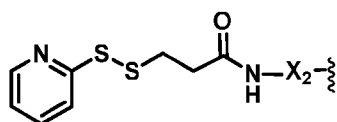
[213] In some embodiments, the second chemical moiety of the second RNA sequence comprises an amino.

[214] In some embodiments, the second chemical moiety of the second RNA sequence comprises a structure of Formula 6. In some embodiments, in the structure of Formula 6, X_5 is an alkylene. In some embodiments, in the structure of Formula 6, X_5 is $-\text{CH}_2-$. In some embodiments, the second chemical moiety of the second RNA sequence is



[215] In some embodiments, the second chemical moiety of the second RNA sequence comprises a structure of Formula 7. In some embodiments, in the structure of Formula 7, X_5 is an alkylene. In some embodiments, in the structure of Formula 7, X_5 is $-\text{CH}_2-$.

[216] In some embodiments, the second chemical moiety of the second RNA sequence comprises a structure of Formula 8. In some embodiments, in the structure of Formula 8, X_5 is an alkylene. In some embodiments, in the structure of Formula 8, X_5 is $-\text{CH}_2-$. In some embodiments, the second chemical moiety of the second RNA sequence is



[217] In some embodiments, the second chemical moiety of the second RNA sequence comprises a structure of Formula 12. In some embodiments, in the structure of Formula 12, X_5 is an alkylene. In some embodiments, in the structure of Formula 12, X_5 is $-\text{CH}_2-$.

[218] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 1, and the second chemical moiety of the second RNA sequence comprises an amino.

[219] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 2, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 6.

[220] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 3, and the second chemical moiety of the second RNA sequence comprises an amino.

[221] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 4, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 7.

[222] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 5, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 6.

[223] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 2, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 8.

[224] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 6, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 6.

[225] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 9, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 7.

[226] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 2, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 8.

[227] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 10, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 12.

[228] In some embodiments, the first chemical moiety of the first RNA sequence comprises a structure of Formula 10, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 6.

[229] In some embodiments, the first chemical moiety of the first RNA sequence comprises $-X_1-NH_2$, and the second chemical moiety of the second RNA sequence comprises $-X_2-NH_2$.

[230] In some embodiments, the first chemical moiety of the first RNA sequence comprises $-X_1-OH$, and the second chemical moiety of the second RNA sequence comprises $-X_2-PO_4^-$.

[231] In some embodiments, the first chemical moiety of the first RNA sequence comprises an amino, and the second chemical moiety of the second RNA sequence comprises $-O(CO)X_3$.

[232] In some embodiments, the first chemical moiety of the first RNA sequence comprises $-O(CO)X_3$, and the second chemical moiety of the second RNA sequence comprises an amino

[233] In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US4, US5, US6, US7, or US8 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US4 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US5 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US6 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US7 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US8 nucleotide of the tetraloop.

[234] In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the SL2-4, SL2-5, SL2-6, SL2-7, or SL2-8 nucleotide of the SL2. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the SL2-4 nucleotide of the SL2. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the SL2-5 nucleotide of the SL2. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the SL2-6 nucleotide of the SL2. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the SL2-7 nucleotide of the SL2. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the SL2-8 nucleotide of the SL2.

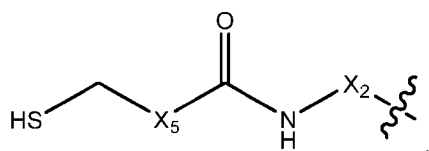
[235] In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the US5, US6, US7, US8, or US9 nucleotide of the tetraloop. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the US5 nucleotide of the tetraloop. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the US6 nucleotide of the tetraloop. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the US7 nucleotide of the tetraloop. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the US8 nucleotide of the tetraloop. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the US9 nucleotide of the tetraloop.

[236] In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-5, SL2-6, SL2-7, SL2-8, or SL2-9 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-5 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-6 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-7 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-8 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-9 nucleotide of the SL2.

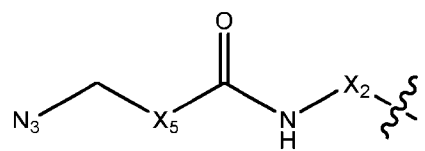
[237] In some embodiments, the method further comprises a third RNA sequence comprising a third chemical moiety at its 5' end and a fourth chemical moiety at its 3' end.

[238] In some embodiments, the third RNA sequence comprises a third portion of the chemically-modified gRNA core. In some embodiments, the third portion of the chemically-modified gRNA core comprises a fragmented tetraloop, a first stem loop, and a fragmented SL2.

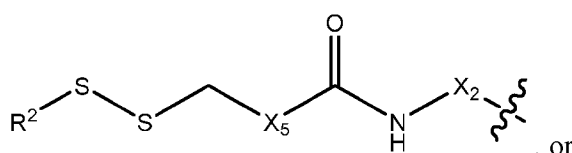
[239] In some embodiments, the third chemical moiety of the third RNA sequence comprises an amino, $-X_2-NH_2$, $-X_2-OH$, $-X_2-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 6-8 or Formula 12:



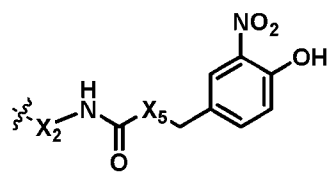
Formula 6



Formula 7



Formula 8

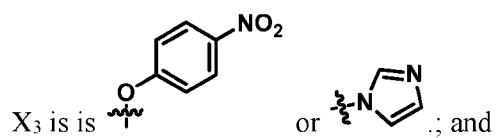


Formula 12

wherein:

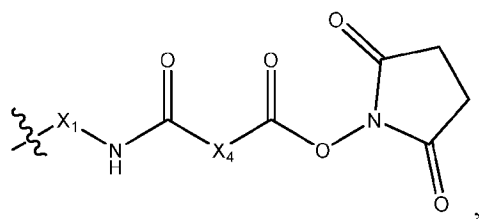
each X_2 and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$,

“n” is an integer between 1 and 12;

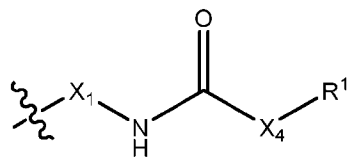


R² is a heteroaryl.

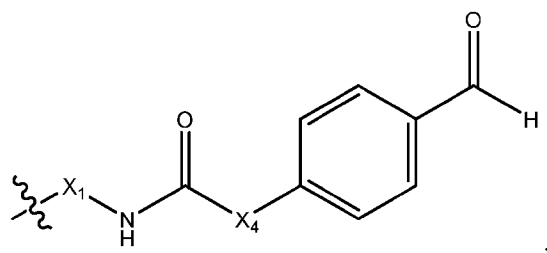
[240] In some embodiments, the fourth chemical moiety of the third RNA sequence comprises an amino, -X₁-NH₂, -X₁-OH, -X₁-PO₄⁻, -O(CO)X₃, or a structure of any one of Formulas 1-6 or Formulas 9-11:



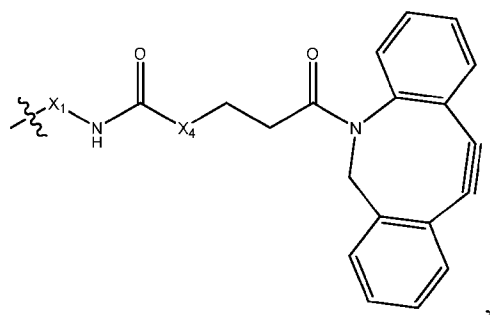
Formula 1



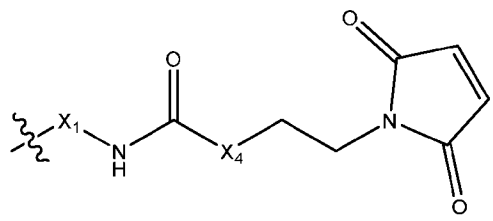
Formula 2



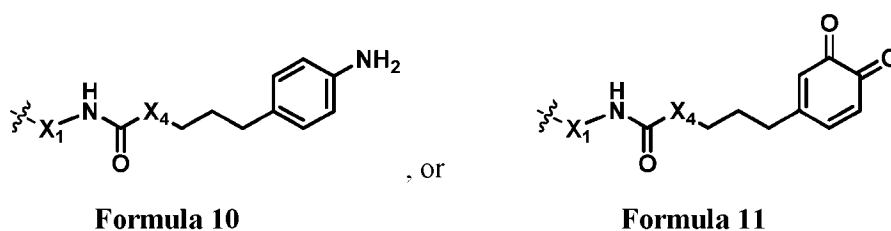
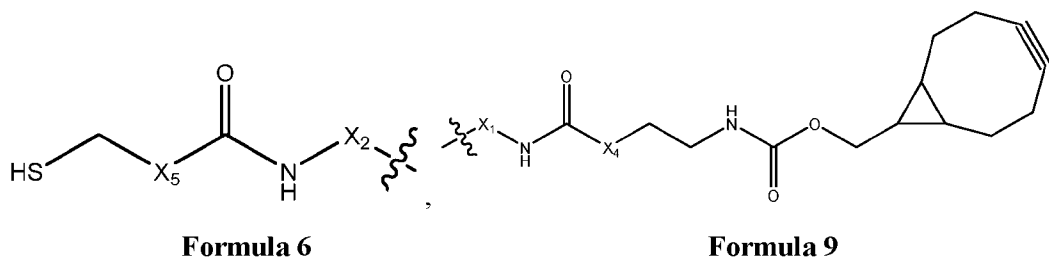
Formula 3



Formula 4



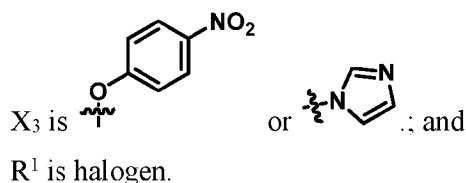
Formula 5



wherein:

each X_1 and X_4 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$,

“n” is an integer between 1 and 12;



Three-part Split Synthesis

[241] Another method to synthesize long RNAs involves a three-part split synthesis. Accordingly, one aspect of the disclosure described herein includes method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

the method comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end;

providing a second RNA sequence, wherein the second RNA sequence comprises a second chemical moiety at its 5' end;

providing a third RNA sequence, wherein the third RNA sequence comprises a third chemical moiety at its 5' end and a fourth chemical moiety at its 3' end;

ligating the first, second, and third RNA sequences to obtain the synthesized full-length PEgRNA,

wherein the ligation forms (i) a first linker between the first chemical moiety of the first RNA sequence and the third chemical moiety of the third RNA sequence, and (ii) a second linker between the second chemical moiety of the second RNA sequence and the fourth chemical moiety of the third RNA sequence;

wherein the first linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA, and

wherein the second linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA or in a junction of the synthesized full-length PEgRNA.

[242] In some embodiments, the second linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[243] In some embodiments, the second linker is present in the junction of the synthesized full-length PEgRNA.

[244] In some embodiments, the synthesized full-length PEgRNA comprises, in 5' to 3' order, a spacer, a chemically-modified gRNA core, a junction, and an extension arm.

[245] In some embodiments, the extension arm comprises a reverse transcription template (RTT) and a primer binding site (PBS). In some embodiments, the extension arm is a chemically-modified extension arm.

[246] In some embodiments, the chemically-modified gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2) and a third stem loop (SL3). Embodiments regarding gRNA core described above are embodiments of this method as well. In some embodiments, the tetraloop comprises a lower stem, a bulge, an upper stem, and a loop. In some embodiments, the junction is present, in 5' to 3' order, between the chemically-modified gRNA core and the extension arm.

[247] In some embodiments, the first RNA sequence comprises a spacer and a first portion of the chemically-modified gRNA core. In some embodiments, the first portion of the chemically-modified gRNA core comprises a fragmented tetraloop.

[248] In some embodiments, each chemical moiety is a functional group appended to the 3' end and/or the 5' end of a polynucleotide. In some embodiments, the chemical moiety is structurally different from a natural nucleotide, a natural sugar, and/or a natural internucleoside linkage.

[249] In some embodiments, the second RNA sequence comprises a second portion of the chemically-modified gRNA core, an extension arm, a chemically-modified extension arm, an RTT, and/or a PBS. In some embodiments, the second RNA sequence comprises a second portion of the chemically-modified gRNA core and an extension arm. In some embodiments, the second portion of the chemically-modified gRNA core comprises a fragmented SL2. In some embodiments, the second RNA sequence comprises a chemically-modified extension arm.

[250] In some embodiments, the third RNA sequence comprises a third portion of the chemically-modified gRNA core, a fourth portion of the chemically-modified gRNA core, and/or a junction. In some embodiments, the third RNA sequence comprises a third portion of the chemically-modified gRNA core and a fourth portion of the chemically-modified gRNA core. In some embodiments, the third portion of the chemically-modified gRNA core comprises a fragmented tetraloop and the fourth portion of the chemically-modified gRNA core comprises a fragmented SL2. In some embodiments, the third RNA sequence comprises a third portion of the chemically-modified gRNA core and a junction. In some embodiments, the third portion of the chemically-modified gRNA core comprises a fragmented tetraloop. In some embodiments, the junction comprises the fourth chemical moiety.

[251] In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US4, US5, US6, US7, or US8 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US4 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US5 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US6 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US7 nucleotide of the tetraloop. In some embodiments, the first chemical moiety of the first RNA sequence is located on the 3' end of the US8 nucleotide of the tetraloop.

[252] In some embodiments, the third chemical moiety of the third RNA sequence is located on the 5' end of the US5, US6, US7, US8, or US9 nucleotide of the tetraloop. In some embodiments, the third chemical moiety of the third RNA sequence is located on the 5' end of the US5 nucleotide of the tetraloop. In some embodiments, the third chemical moiety of the third RNA sequence is located on the 5' end of the US6 nucleotide of the tetraloop. In some embodiments, the third chemical moiety of the third RNA sequence is located on the 5' end of the US7 nucleotide of the tetraloop. In some embodiments, the v chemical moiety of

the third RNA sequence is located on the 5' end of the US8 nucleotide of the tetraloop. In some embodiments, the third chemical moiety of the third RNA sequence is located on the 5' end of the US9 nucleotide of the tetraloop.

[253] In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-5, SL2-6, SL2-7, SL2-8, or SL2-9 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-5 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-6 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-7 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-8 nucleotide of the SL2. In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-9 nucleotide of the SL2.

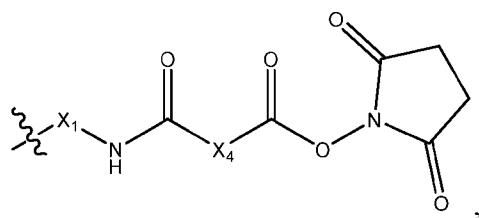
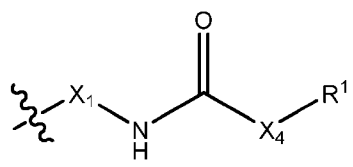
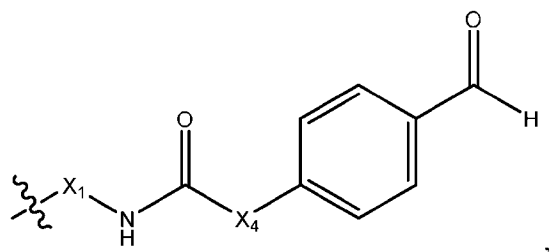
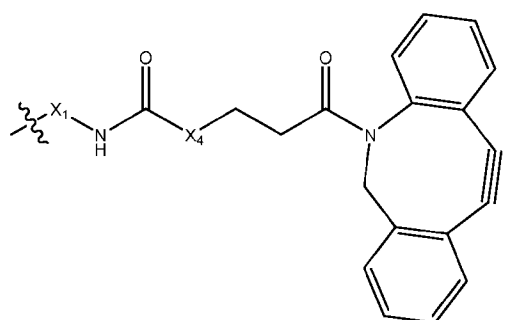
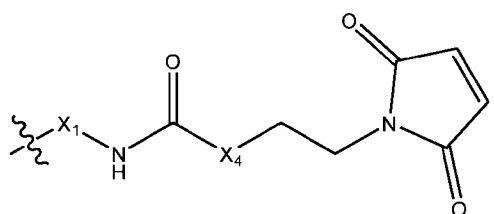
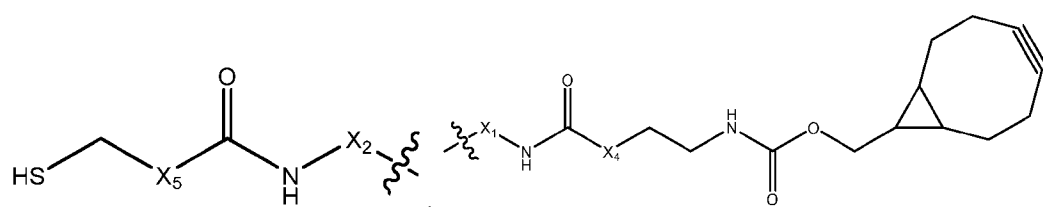
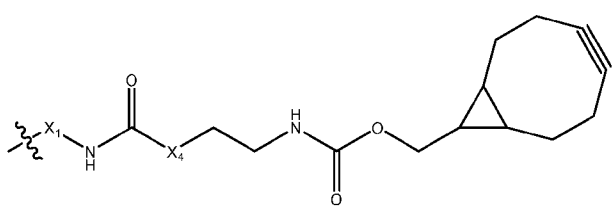
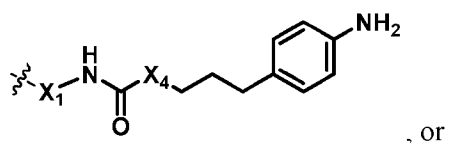
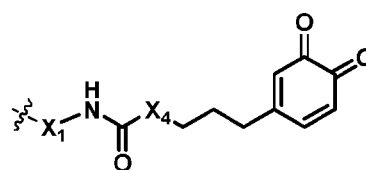
[254] In some embodiments, the fourth chemical moiety of the third RNA sequence is located on the 3' end of the SL2-4, SL2-5, SL2-6, SL2-7, or SL2-8 nucleotide of the SL2. In some embodiments, the fourth chemical moiety of the third RNA sequence is located on the 3' end of the SL2-4 nucleotide of the SL2. In some embodiments, the fourth chemical moiety of the third RNA sequence is located on the 3' end of the SL2-5 nucleotide of the SL2. In some embodiments, the fourth chemical moiety of the third RNA sequence is located on the 3' end of the SL2-6 nucleotide of the SL2. In some embodiments, the fourth chemical moiety of the third RNA sequence is located on the 3' end of the SL2-7 nucleotide of the SL2. In some embodiments, the fourth chemical moiety of the third RNA sequence is located on the 3' end of the SL2-8 nucleotide of the SL2.

[255] In some embodiments, the second chemical moiety of the second RNA sequence is located on the 5' end of the chemically-modified extension arm.

[256] In some embodiments, the fourth chemical moiety of the third RNA sequence is located on the 3' end of the junction.

[257] In some embodiments, the first chemical moiety of the first RNA sequence and the fourth chemical moiety of the third RNA sequence each comprise:

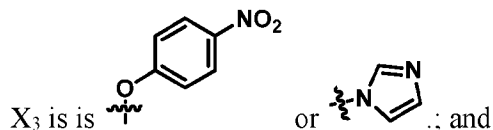
an amino, $-X_1-NH_2$, $-X_1-OH$, $-X_1-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 1-6 or Formulas 9-11:

**Formula 1****Formula 2****Formula 3****Formula 4****Formula 5****Formula 6****Formula 9****Formula 10****Formula 11**

wherein:

each X_1 and X_4 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$,

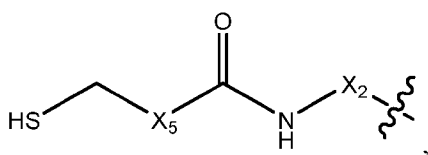
"n" is an integer between 1 and 12;



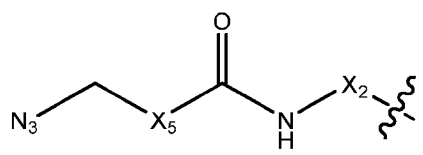
R^1 is halogen.

[258] In some embodiments, the second chemical moiety of the second RNA sequence and the third chemical moiety of the third RNA sequence each comprise:

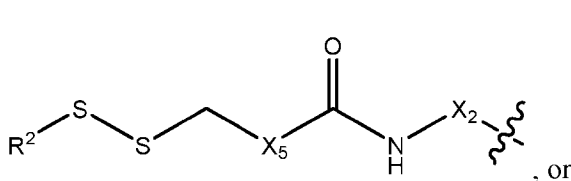
an amino, $-X_2-NH_2$, $-X_2-OH$, $-X_2-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 6-8 or Formula 12:



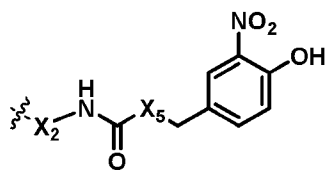
Formula 6



Formula 7



Formula 8

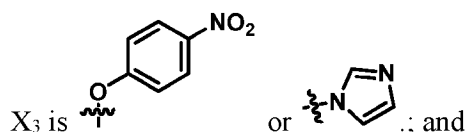


Formula 12

wherein:

each X_2 and X_5 is independently absent or $(-O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$,

"n" is an integer between 1 and 12;



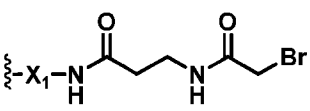
R^2 is a heteroaryl.

[259] In some embodiments, the first chemical moiety of the first RNA sequence and/or the fourth chemical moiety of the third RNA sequence comprises an amino. In some

embodiments, the first chemical moiety of the first RNA sequence and/or the fourth chemical moiety of the third RNA sequence comprises $-X_1-NH_2$. In some embodiments, the first chemical moiety of the first RNA sequence and/or the fourth chemical moiety of the third RNA sequence comprises $-X_1-OH$. In some embodiments, the first chemical moiety of the first RNA sequence and/or the fourth chemical moiety of the third RNA sequence comprises $-X_1-PO_4$. In some embodiments, the first chemical moiety of the first RNA sequence and/or the fourth chemical moiety of the third RNA sequence comprises $-O(CO)X_3$.

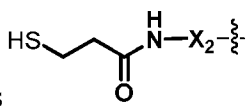
[260] In some embodiments, the first chemical moiety of the first RNA sequence and/or the fourth chemical moiety of the third RNA sequence comprises a structure of Formula 1. In some embodiments, in the structure of Formula 1, X_4 is an alkylene. In some embodiments, in the structure of Formula 1, X_4 is $-CH_2-CH_2-$.

[261] In some embodiments, the first chemical moiety of the first RNA sequence and/or the fourth chemical moiety of the third RNA sequence comprises a structure of Formula 2. In some embodiments, R^1 is Cl, Br, or I. In some embodiments, R^1 is Br. In some embodiments,

R^1 is I. In some embodiments, the structure of Formula 2 is 

[262] In some embodiments, the second chemical moiety of the second RNA sequence and/or the third chemical moiety of the third RNA sequence comprises an amino.

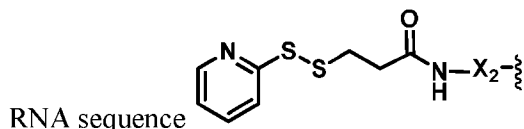
[263] In some embodiments, the second chemical moiety of the second RNA sequence and/or the third chemical moiety of the third RNA sequence comprises a structure of Formula 6. In some embodiments, in the structure of Formula 6, X_5 is an alkylene. In some embodiments, in the structure of Formula 6, X_5 is $-CH_2-$. In some embodiments, the second chemical moiety of the second RNA sequence and/or the third chemical moiety of the third

RNA sequence is 

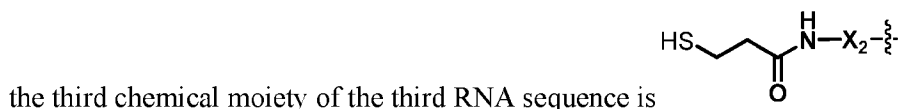
[264] In some embodiments, the second chemical moiety of the second RNA sequence and/or the third chemical moiety of the third RNA sequence comprises a structure of Formula 7. In some embodiments, in the structure of Formula 7, X_5 is an alkylene. In some embodiments, in the structure of Formula 7, X_5 is $-CH_2-$.

[265] In some embodiments, the second chemical moiety of the second RNA sequence and/or the third chemical moiety of the third RNA sequence comprises a structure of Formula 8. In some embodiments, in the structure of Formula 8, X_5 is an alkylene. In some embodiments, in the structure of Formula 8, X_5 is $-CH_2-$. In some embodiments, the second

chemical moiety of the second RNA sequence is and/or the third chemical moiety of the third



[266] In some embodiments, the first chemical moiety of the first RNA sequence and the fourth chemical moiety of the third RNA sequence comprises an amino. In some embodiments, the first chemical moiety of the first RNA sequence and the fourth chemical moiety of the third RNA sequence comprises $-X_1-NH_2$. In some embodiments, the second chemical moiety of the second RNA sequence and the third chemical moiety of the third RNA sequence comprises a structure of Formula 6. In some embodiments, in the structure of Formula 6, X_5 is an alkylene. In some embodiments, in the structure of Formula 6, X_5 is $-CH_2-$. In some embodiments, the second chemical moiety of the second RNA sequence and



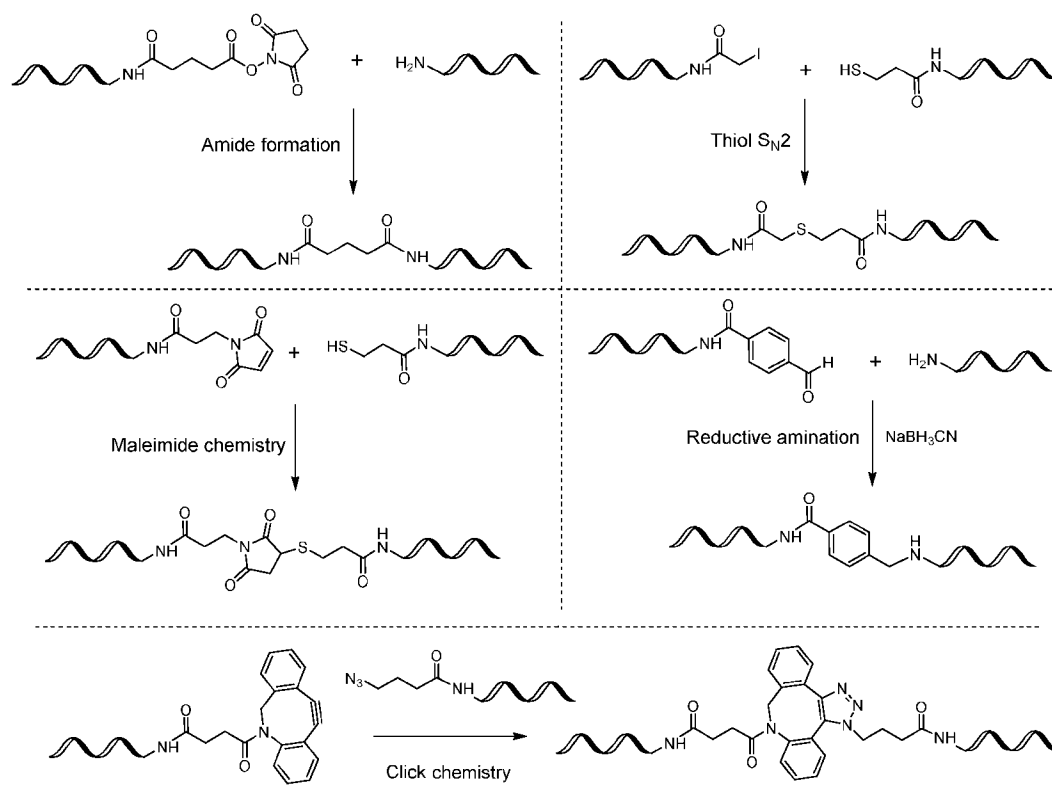
Ligation

[267] Enzymatic ligation or chemical ligation can be utilized to ligate the chemical moieties and produce the long RNAs described herein. In some embodiments, enzymatic ligation is used to ligate the chemical moieties. In some embodiments, chemical ligation is used to ligate the chemical moieties. In some embodiments, both enzymatic ligation and chemical ligation are used to ligate the chemical moieties.

[268] Chemical Ligation

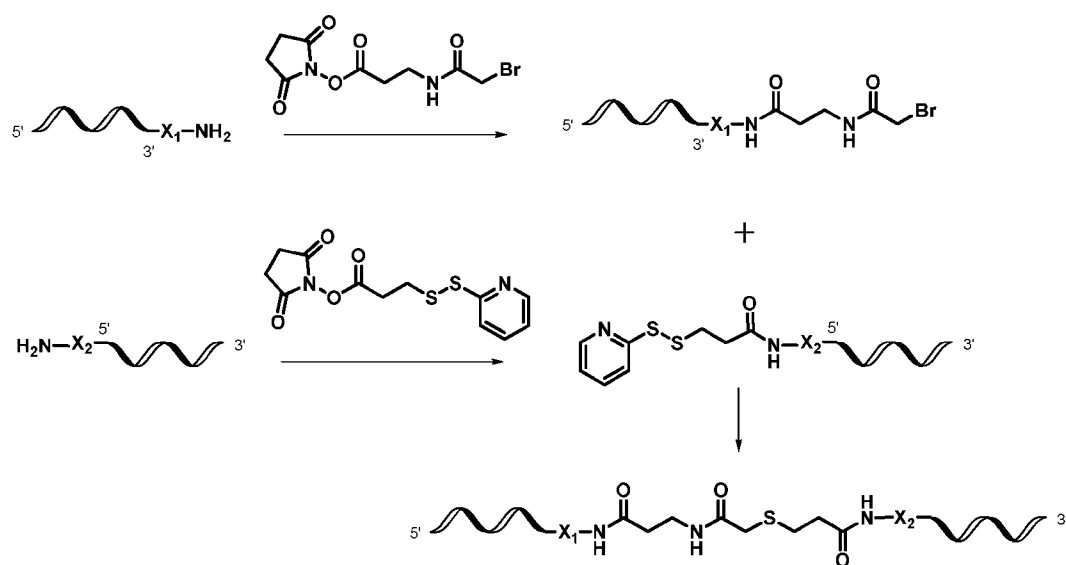
[269] In some embodiments, chemical ligation is used to ligate the chemical moieties. To ligate the chemical moieties using the chemical ligation, appropriate ligation chemistries can be designed. The chemical moieties described herein can incorporate functional groups that are specific to particular ligation chemistries.

[270] Some examples of chemistries suitable for chemical ligations in the split synthesis methods described herein are shown in Scheme 1 below:

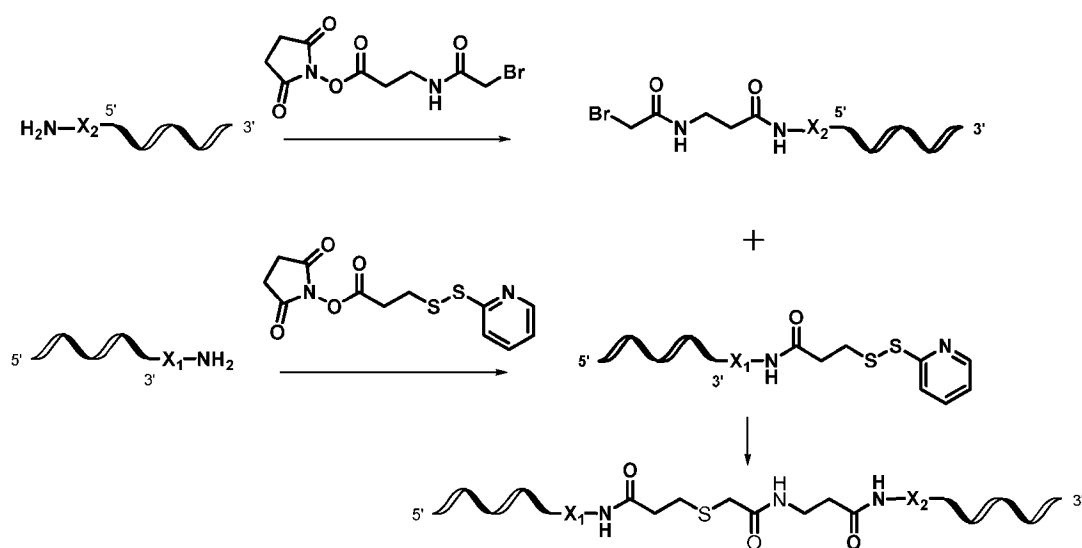


Scheme 1

[271] Another exemplary chemistry for use in the split synthesis methods described herein, includes the use of a thioether ligation as shown in Schemes 2 and 3 below. The thioether ligation strategy uses a thiol modified RNA sequence to react with the bromine containing RNA sequence to form a thioether linker. This strategy does not require the two RNA sequences to be pre-annealed. In some embodiments, the split site can be in a native TL or SL2 without any extension. In some embodiments, this strategy is referred to as a non-templated strategy.



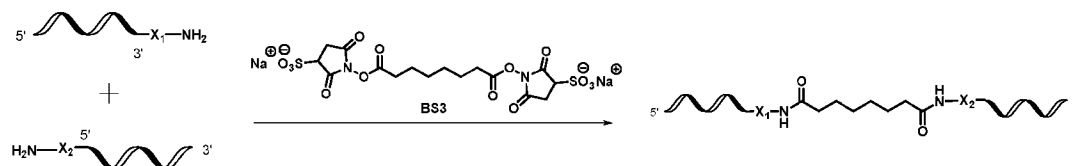
Scheme 2



Scheme 3

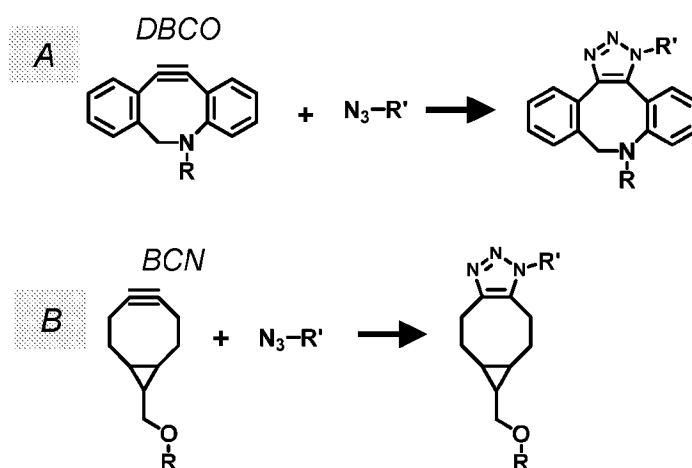
[272] Another exemplary chemistry for use in the split synthesis methods described herein, includes the use of a bisamide ligation as shown in Scheme 4 below. The bisamide strategy uses one molecule of BS3 (bis(sulfosuccinimidyl)suberate) with two NHS ester groups to link two amino modified RNA sequences through two amide bond formations on a single BS3 molecule (Scheme 4). This requires the two RNA sequences to be pre-annealed as the two amino groups need to be positioned in proximity before adding the BS3 reagent for the ligation. In some embodiments, the fragmented gRNA core comprises an extended TL or SL2. It is noteworthy to mention that in our this strategy the semi complementarity of the two

RNA sequences bring their respective reactive amines in close proximity for a conjugation to happen and thus requiring no traditional DNA/RNA template. In some embodiments, this strategy is referred to as a self-templated conjugation.



Scheme 4

[273] Another exemplary chemistry includes the cycloaddition reaction between two RNA sequences carrying an azide and alkyne functional groups at the respective ends. The reaction is driven by the ring strain of alkyne functional group in the cyclic ring instead of traditional alkyne activation by copper. In here, an azide is either partnered with A) DBCO-alkyne or B) BCN-alkyne (Scheme 4.1).

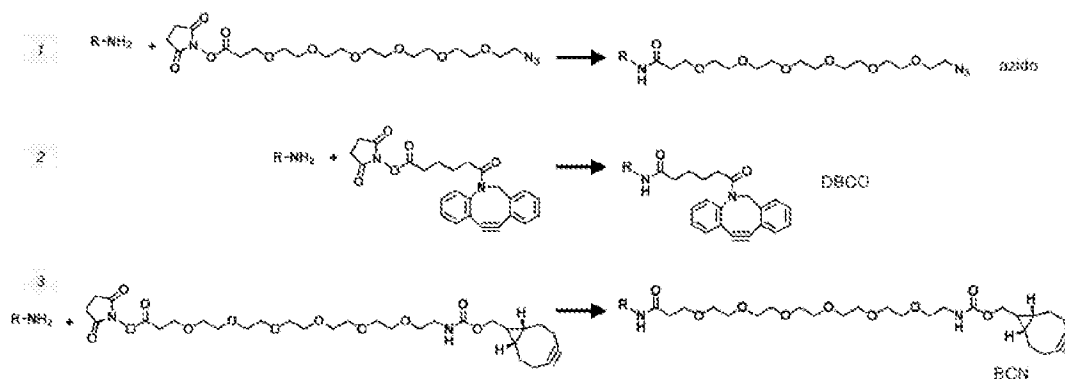


where R = RNA-1

R' = RNA-2

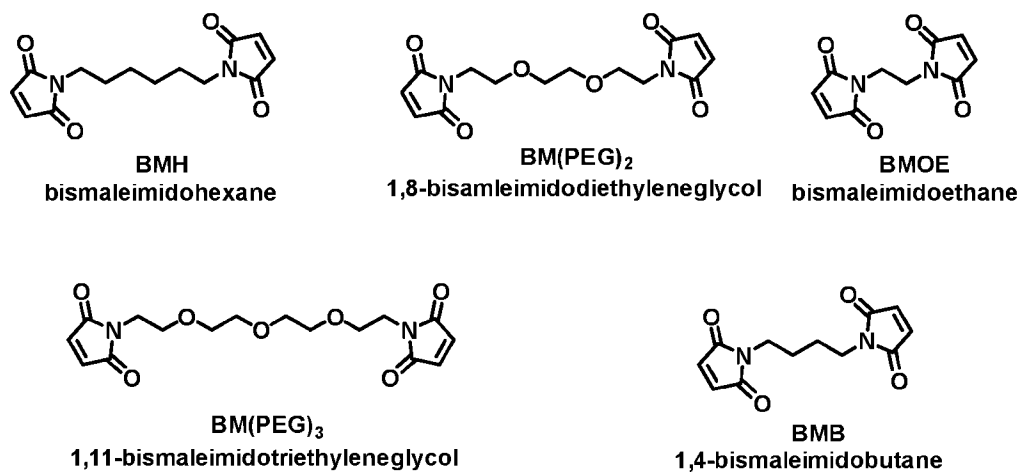
Scheme 4.1

[274] The amine functionalized RNAs can be treated with the activated NHS-esters of azide or alkyne partners (Scheme 4.1.1). Additionally, azide functional groups can be introduced on the RNAs on the nucleotides directly at the 5'-end of the last nucleotide in the position of 5'-OH or as a modification on the purine or pyrimidine bases without requiring using the amine handle and NHS-esters.



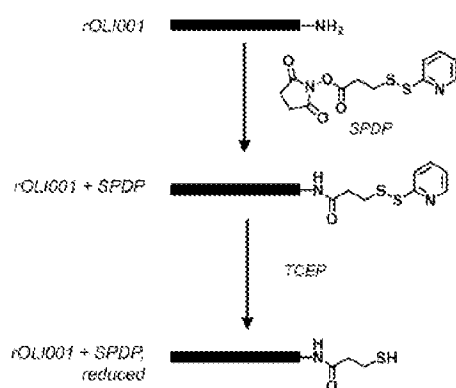
Scheme 4.1.1

[275] Another exemplary chemistry includes bismaleimide ligation with native stem loops using a splint. In here, two thiol containing RNAs are ligated together using BM $\underline{X}$ reagent, where $\underline{X}$ is a variable in length and nature of the linker itself as shown in Scheme 4.2.



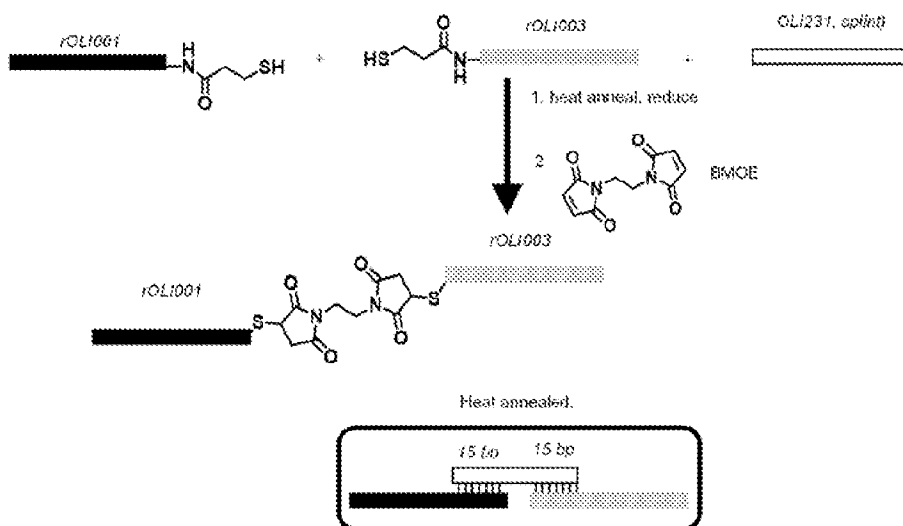
Scheme 4.2

[276] The amine functionalized RNAs can be treated with succinimidy 3-(2-pyridyldithio)propionate, SPDP, reagent followed by TCEP-mediated reduction to produce the thiolated RNA (Scheme 4.2.1).



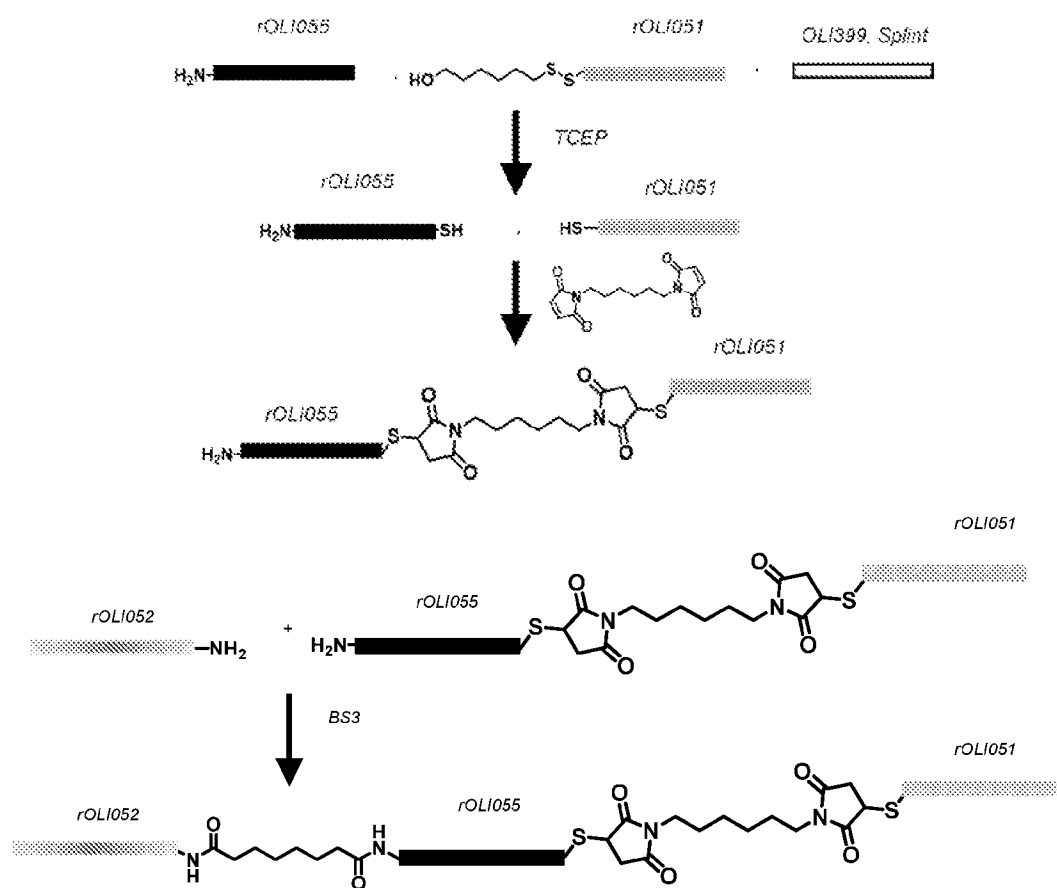
Scheme 4.2.1

[277] The desired RNAs with two thiol groups at respective ends can be ligated by heat annealing of the two RNA strands in the presence of a splint, TCEP reduction, followed by the addition of bismaleimide linker, as exemplified below with BMOE (Scheme 4.2.2).



Scheme 4.2.2

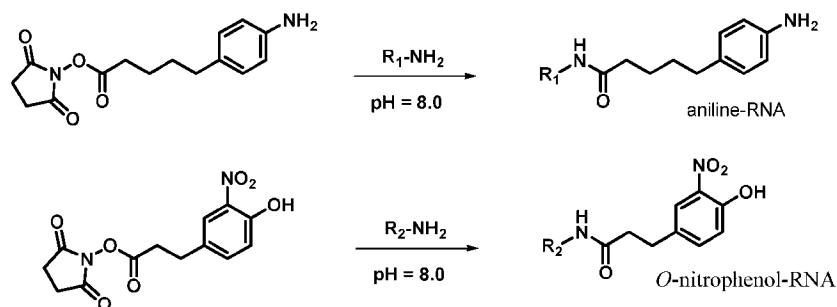
[278] The bismaleimide ligation and BS3 ligation chemistries can be combined in a 3-part ligation method, as shown in Scheme 4.3.



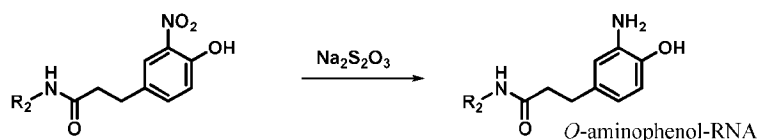
Scheme 4.3

[279] Another exemplary chemistry includes oxidative ligation of aniline-RNA and aminophenol-RNA. The selective reactivity of phenols with anilines can be used as a ligation chemistry for synthesizing longer RNAs. Short RNA sequences carrying functional groups (at the ends or internally through a side chain on the ribose sugar or nucleobase) such as aniline derivatives can be selectively coupled under oxidation conditions to phenol derivatives to produce a ligated RNA, Scheme 4.4.

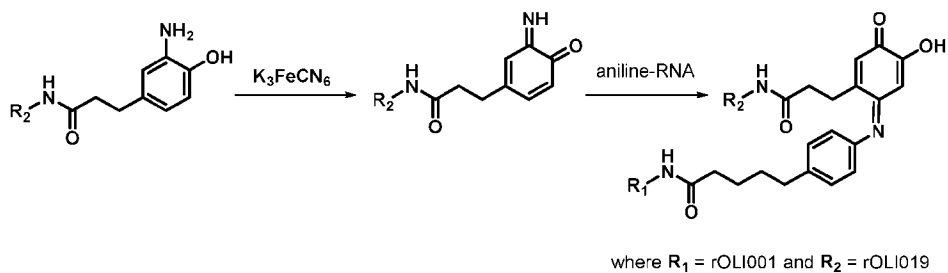
Step 1: Derivatization of RNAs with aniline and *O*-nitrophenol NHS esters



Step 2: Reduction of *O*-nitrophenol-RNA to *O*-aminophenol-RNA



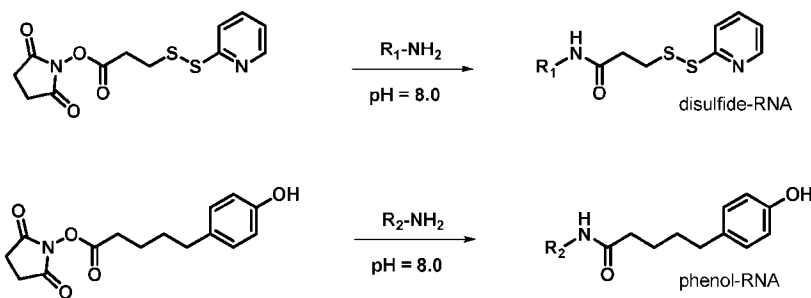
Step 3: Oxidative coupling of *O*-aminophenol-RNA to aniline-RNA



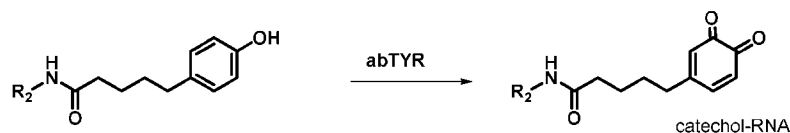
Scheme 4.4

[280] Another exemplary chemistry includes oxidative ligation of thiol-RNA and Catechol-RNA. The selective reactivity of thiols with catechols can be used as a ligation chemistry for synthesizing longer RNAs. Short RNA sequences carrying functional groups (at the ends or internally through a side chain on the ribose sugar or nucleobase) such as thiol derivatives or catechols derivatives can be selectively coupled under oxidation conditions to produce a ligated RNA, Scheme 4.5.

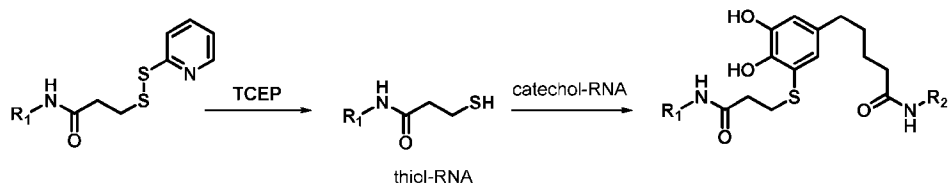
Step 1: Derivatization of RNAs with SPDP and phenol NHS esters



Step 2: Oxidation of phenol-RNA to catechol-RNA



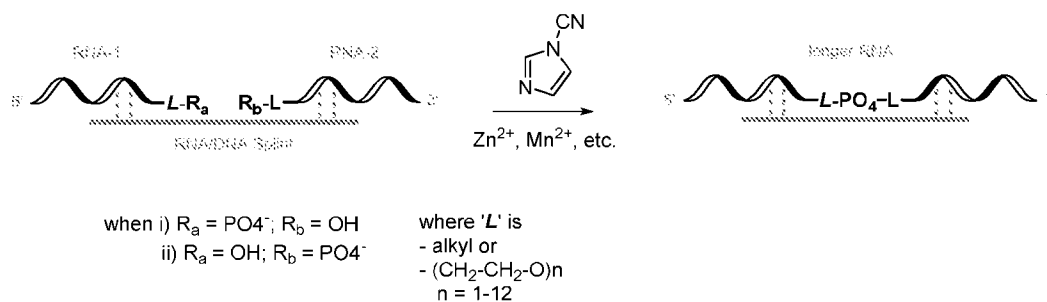
Step 3: Coupling of catechol-RNA to thiol-RNA



where abTYR = Tyrosinase from *Agaricus bisporus* R_1 = rOLI001 and R_2 = rOLI019

Scheme 4.5

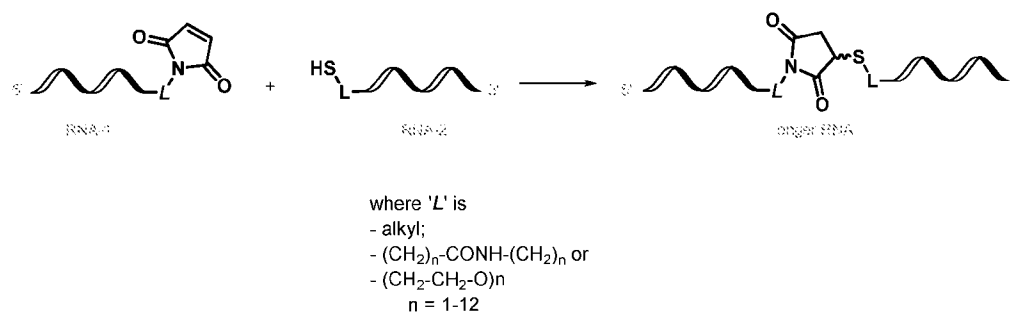
[281] Another exemplary chemistry includes the ligation of phosphorylated (5' or 3') RNA sequence with a free OH (5' or 3') RNA sequence in the presence of an N-cyanoimidazole activated phosphate of another RNA producing longer RNAs with native phosphodiester linkage as well as phosphodiester linkages with additional spacers, Scheme 5.



Scheme 5

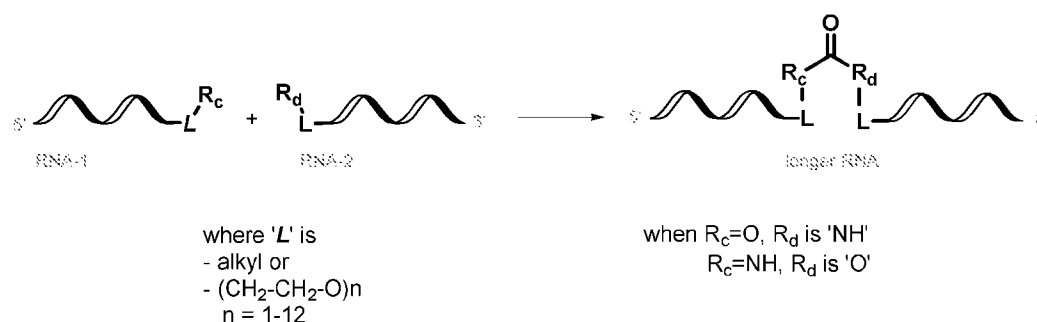
[282] Another exemplary chemistry includes chemical thiolation of 3'-maleimide functionalized RNA sequence with an appropriate 5'-thiol (SH) of a second RNA sequence, where the resultant succinimide derivative may undergo hydrolysis to produce an open-chain

linker. Thiolation of maleimide is highly chemo selective and may need no template or splints, Scheme 6.



Scheme 6

[283] Another exemplary chemistry includes producing a carbamate linkage, which is a mimic of phosphate that contains the same number of atoms as to native phosphate. This mimic allows maintaining similar internucleotide distance and exert nuclease resistance compared to phosphodiester linkage. As an example, as shown in Scheme 7 below, a 3'-C₆-NH₂ of a RNA sequence could react with an activated ester 5'-X(CO)O of a second RNA sequence resulting in a synthetic carbamate bridge.



Scheme 7

[284] Enzymatic Ligation

[285] In some embodiments, enzymatic ligation is used to ligate the chemical moieties. In here, a long RNA, such as a PEgRNA, is split into two or more short fragments. Then, the fragments are functionalized and ligated by enzymatic ligation to generate full-length PEgRNA.

[286] In certain aspects, the disclosure provides a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the method comprising,

contacting a first RNA sequence with a second RNA sequence,

wherein the first RNA sequence comprises a free 3' OH and the second RNA sequence comprises a 5'-monophosphate;
ligating the first RNA sequence and the second RNA sequence with a nucleic acid ligase to obtain the synthesized full-length PEGRNA,
wherein the ligation forms a linker between the 3' OH and the 5'-monophosphate; and
wherein the linker is present in the spacer, gRNA core, RTT, or PBS of the synthesized full-length PEGRNA.

[287] In certain aspects, the disclosure provides a method for synthesizing a full-length prime editing guide RNA (PEGRNA) comprising a guide RNA (gRNA) core, the method comprising,

contacting a first RNA sequence with a second RNA sequence and a splint sequence,
wherein the first RNA sequence comprises a free 3' OH, the second RNA sequence comprises a 5'-monophosphate, and the splint sequence comprises a sequence partially complementary to both the first and second RNA sequences;

ligating the first RNA sequence and the second RNA sequence with a nucleic acid ligase to obtain the synthesized full-length PEGRNA,

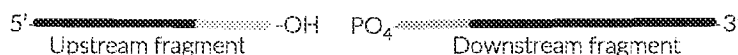
wherein the ligation forms a linker between the 3' OH and the 5'-monophosphate; and

wherein the linker is present in the spacer, gRNA core, RTT, or PBS of the synthesized full-length PEGRNA.

[288] In some embodiments, the chemical moieties described herein can incorporate functional groups that are specific for enzymatic ligation. In some embodiments, the two or more fragments may contain chemical modifications of the sugar, phosphodiester backbone, or nucleobase canonical structures. In some embodiments, the PEGRNA can be generated from one 5' fragment, and one or more 3' fragments which are 5' terminally modified with a free monophosphate to facilitate the ligation by way of a nucleotide ligase enzyme. Free 3' ends and phosphorylated 5' ends are brought into proximity by way of sequence complementarity resulting in base pairing hybridization either between the RNA fragments directly (self-templated) or via an additional complementary DNA splint (splinted). Subsequent enzymatic catalysis by a nucleic acid ligase can generate a natural phosphodiester linkage between fragments, yielding the full-length PEGRNA. In some

embodiments, a nucleic acid ligase is T4 RNA Ligase I, T4 RNA Ligase II, or T4 DNA Ligase.

[289] Scheme 8 below shows a potential fragment functionalization for enzymatic split synthesis, where “Upstream” or 5’ end RNA fragments have a free 3’ OH available for ligation, while “Downstream” or 3’ end RNA fragments are terminally functionalized with a 5’-monophosphate.



Scheme 8

Schematic of upstream and downstream fragments are shown. The light shaded segments are complementary and facilitate self-templated ligation.

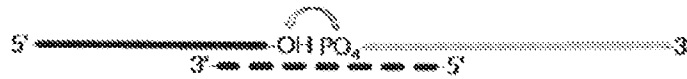
[290] **Self-templated Ligation:** In some embodiments, two functionalized, single-stranded RNA fragments with complementary base pairs are hybridized for enzymatic ligation (e.g., ligase-mediated conjugation). In some embodiments, the fragments take natural advantage of the sequence complementarity driven structure of any region within the PEgRNA to help facilitate a very specific ligation reaction between the fragments. Once these fragments are annealed under controlled buffered conditions, the RNA duplex forms a native secondary structure mimic to the full length PEgRNA, resulting in a net increase in the effective local concentration of the terminal 3’-OH and 5’-monophosphate functional ends required for ligase activity. Subsequent ligase catalysis forms a phosphodiester bond between the RNA fragments which covalently seals the nick and generates a full-length PEgRNA. Accordingly, and in some embodiments, the ligation occurs at any region within the PEgRNA. In some embodiments, the ligation occurs between and within the spacer of the PEgRNA. In some embodiments, the ligation occurs between and within the gRNA core of the PEgRNA. In some embodiments, the ligation occurs between and within the RTT of the PEgRNA. In some embodiments, the ligation occurs between and within the PBS of the PEgRNA. In some embodiments, the ligation occurs between and within the spacer and the gRNA core of the PEgRNA. In some embodiments, the ligation occurs between and within the TL, SL1, SL2, or SL3 of the gRNA core of the PEgRNA. In some embodiments, the ligation occurs between and within the TL of the gRNA core of the PEgRNA. In some embodiments, the ligation occurs between and within the SL1 of the gRNA core of the PEgRNA. In some embodiments, the ligation occurs between and within the SL2 of the gRNA core of the PEgRNA. In some embodiments, the ligation occurs between and within the SL3 of the

gRNA core of the PEGRNA. In some embodiments, the ligation occurs between and within the gRNA core and the RTT of the PEGRNA. In some embodiments, the ligation occurs between and within the RTT and the PBS of the PEGRNA.

[291] In some embodiments, self-templated ligation requires a minimum of two fragments (2-part ligation) and can be increased further to three fragments (3-part ligation), four fragments (4-part ligation), five fragments (5-part ligation), six fragments (6-part ligation), seven fragments (7-part ligation), eight fragments (8-part ligation), nine fragments (9-part ligation), or ten fragments (10-part ligation). The location of the fragments in the two-part to ten-part ligations is similar to that described in the sections above.

[292] In some embodiments, the efficiency of self-templated ligation reactions can be improved by modifying the conjugation sites. In some embodiments, stem-loop modifications in the form of stem extensions and sequence changes help facilitate secondary structure formation that strengthen favorable structures that promote efficient ligations to form. By increasing the number of complementary base pairs between the fragments and the melt temperature of the RNA duplex they form, the conjugation site is more stable, and the ultimate effect we hypothesize would be an increase in ligation efficiency. As indicated in sections above, designing modified stem-loops, it is critical to avoid disrupting the formation of the PEGRNA-Cas9 complex. In some embodiments, the Tetraloop and Stem-loop 2 are amenable to sequence modifications and secondary structure extensions ranging from 1 to 8 base pairs.

[293] **Template Ligation:** In some embodiments, a template ligation is used for enzymatic split synthesis of PEGRNA. In here, two functionalized, single-stranded RNA fragments are conjugated with ligase, similarly to self-templated ligation. In addition, a short, complementary nucleic acid splint facilitates a trimeric secondary structure formation during templated ligation. The nucleic acid splint is designed such that it bears proximal sequence complementarity of both the 3' end of the upstream RNA fragment and 5' end of the downstream RNA fragment to facilitate a splinted trimeric structure as shown in Scheme 9 below. Consequently, the RNA fragments may or may not require complementarity at the ligation site, and so the advantage of templated ligation for PEGRNA synthesis is that the splinted approach enables ligation at PEGRNA locations which lack secondary structure and anywhere in the sequence without extensions. In some embodiments, the splint is a DNA strand. In some embodiments, the splint is a modified nucleic acid strand.



Scheme 9

Schematic representation of a splint ligation. Black and light solid lines indicate upstream and downstream fragments, respectively. Dotted line indicates splint that is complementary to the 3'-end of upstream fragment and 5'-end of downstream fragment. Splint facilitates bringing the functional ends of the two fragments to proximity for ligation.

[294] In some embodiments, the ligations are possible at non-structured regions of the PEgRNA. In some embodiments, the regions are between and within SL1 and SL2. In some embodiments, the regions are within the 3' extension region to further elongate the RTT portion of PEgRNA. In here, we can obtain longer incorporated sequences that may correct larger mutations. Furthermore, and in some embodiments, because a splint can be designed across any region of the PEgRNA, ligations in SL1 and SL3 are also possible without the need to extend the SL3 sequence. In some embodiments, this ligation may require a stepwise annealing approach to ensure proper triplex hybridization to occur prior to enzymatic ligation. In some embodiments, to permit ligation, the splint base pairs with each RNA fragment, bridging them together to form a nicked, double-stranded splint-RNA hybrid. Subsequent ligase catalysis forms a phosphodiester bond between the RNA fragments of the splint-RNA hybrid, covalently sealing the nick and generating a full-length PEgRNA annealed to a splint. Unlike in self-templated ligations, the splint will be an extra oligonucleotide that must be removed during purification steps to yield the desired PEgRNA product, free from impurities.

[295] Accordingly, and in some embodiments, the splint ligation occurs at any region within the PEgRNA. In some embodiments, the splint ligation occurs between and within the spacer of the PEgRNA. In some embodiments, the splint ligation occurs between and within the gRNA core of the PEgRNA. In some embodiments, the splint ligation occurs between and within the RTT of the PEgRNA. In some embodiments, the splint ligation occurs between and within the PBS of the PEgRNA. In some embodiments, the splint ligation occurs between and within the spacer and the gRNA core of the PEgRNA. In some embodiments, the splint ligation occurs between and within the TL, SL1, SL2, or SL3 of the gRNA core of the PEgRNA. In some embodiments, the splint ligation occurs between and within the TL of the gRNA core of the PEgRNA. In some embodiments, the splint ligation occurs between and within the SL1 of the gRNA core of the PEgRNA. In some embodiments, the splint ligation occurs between and within the SL2 of the gRNA core of the PEgRNA. In some

embodiments, the splint ligation occurs between and within the SL3 of the gRNA core of the PEGRNA. In some embodiments, the splint ligation occurs between and within the gRNA core and the RTT of the PEGRNA. In some embodiments, the splint ligation occurs between and within the RTT and the PBS of the PEGRNA.

[296] In some embodiments, splint ligation requires a minimum of two fragments (2-part ligation) and can be increased further to three fragments (3-part ligation), four fragments (4-part ligation), five fragments (5-part ligation), six fragments (6-part ligation), seven fragments (7-part ligation), eight fragments (8-part ligation), nine fragments (9-part ligation), or ten fragments (10-part ligation). The location of the fragments in the two-part to ten-part splint ligations is similar to that described in the sections above.

[297] Another aspect of the disclosure provides a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core,

the method comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end;

providing a second RNA sequence, wherein the second RNA sequence comprises a second chemical moiety at its 5' end and a free 3' OH;

providing a third RNA sequence, wherein the third RNA sequence comprises a 5'-monophosphate;

ligating the first, second, and third RNA sequences to obtain the synthesized full-length PEGRNA,

wherein the ligation forms (i) a first linker between the first chemical moiety of the first RNA sequence and the second chemical moiety of the second RNA sequence, and (ii) a second linker between the 3' OH of the second RNA sequence and the 5'-monophosphate of the third RNA sequence;

wherein the first linker is present in the chemically-modified gRNA core of the synthesized full-length PEGRNA, and

wherein the second linker is present in the spacer, gRNA core, RTT, or PBS of the synthesized full-length PEGRNA.

[298] In some embodiments, the method further comprises providing a splint sequence, wherein the splint sequence comprises a sequence partially complementary to both the second and third RNA sequences.

[299] In some embodiments, the method further comprises providing at least one additional RNA sequence comprising a chemical moiety at its 3' end, and at least one additional RNA sequence comprising a chemical moiety at its 5' end.

[300] In some embodiments, the method further comprises providing at least one additional RNA sequence comprising a free 3' OH, and at least one additional RNA sequence comprising 5'-monophosphate.

[301] In some embodiments, ligating the additional RNA sequences results in the formation of a third linker.

[302] In some embodiments, ligating the additional RNA sequences results in the formation of a third and a fourth linker.

[303] In some embodiments, ligating the additional RNA sequences results in the formation of a third, fourth, and fifth linker.

[304] In some embodiments, ligating the additional RNA sequences results in the formation of a third, fourth, fifth, and sixth linker.

[305] Another aspect of the disclosure provides a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the method comprising chemically and enzymatically ligating RNA fragments.

[306] Another aspect of the disclosure provides a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core,

the method comprising,

providing at least two RNA sequences comprising chemical moieties at their 3' and/or 5' ends, at least two RNA sequences comprising a free 3' OH, and at least two RNA sequences comprising a 5'-monophosphate; and

chemically and enzymatically ligating the RNA sequences, wherein ligating the RNA sequences produce one to three linkers formed with the chemical ligation and one to three linkers formed with the enzymatic ligation.

Linker

[307] The split synthesis methods described herein form a full-length PEgRNA that comprises a linker. The linker derived from the joining of the first chemical moiety of the first RNA sequence and the second chemical moiety of the second RNA sequence.

[308] In some embodiments, the linker is present in the tetraloop or SL2 of the gRNA core. In some embodiments, the linker is present in the tetraloop or SL2 of the modified gRNA core. In some embodiments, the linker is present in the tetraloop or SL2 of the chemically-

modified gRNA core. In some embodiments, the linker is present in the tetraloop of the chemically-modified gRNA core. In some embodiments, the linker is present in the SL2 of the chemically-modified gRNA core. In some embodiments, the linker is a chemical linker.

[309] In some embodiments, the linker is present between the US4 and US9 nucleotides of the tetraloop. In some embodiments, the linker is present between the US4 and US5 nucleotides of the tetraloop. In some embodiments, the linker is present between the US5 and US6 nucleotides of the tetraloop. In some embodiments, the linker is present between the US6 and US7 nucleotides of the tetraloop. In some embodiments, the linker is present between the US7 and US8 nucleotides of the tetraloop. In some embodiments, the linker is present between the US8 and US9 nucleotides of the tetraloop.

[310] In some embodiments, the linker is present between the SL2-4 and SL2-9 nucleotides of the SL2. In some embodiments, the linker is present between the SL2-4 and SL2-5 nucleotides of the SL2. In some embodiments, the linker is present between the SL2-5 and SL2-6 nucleotides of the SL2. In some embodiments, the linker is present between the SL2-6 and SL2-7 nucleotides of the SL2. In some embodiments, the linker is present between the SL2-7 and SL2-8 nucleotides of the SL2. In some embodiments, the linker is present between the SL2-8 and SL2-9 nucleotides of the SL2.

[311] Another aspect of the disclosure described herein is a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

the method comprising,

providing a first RNA sequence,

wherein the first RNA sequence comprises an amino modified phosphodiester backbone at its 3' end;

functionalizing the 3' amino group of the first RNA sequence with a first chemical moiety;

providing a second RNA sequence;

wherein the second RNA sequence comprises an amino modified phosphodiester backbone at its 5' end;

functionalizing the 5' amino group of the second RNA sequence with a second chemical moiety;

reacting the first chemical moiety of the first RNA sequence with the second chemical moiety of the second RNA sequence to form a linker,

wherein forming the linker results in the formation of the synthesized full-length PEGRNA; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEGRNA.

[312] Another aspect of the disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

the method comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises an amino modified phosphodiester backbone at its 3' end;

providing a second RNA sequence; wherein the second RNA sequence comprises an amino modified phosphodiester backbone at its 5' end;

annealing the first and second RNA sequences to form a pre-annealed duplex;

providing a reagent;

contacting the pre-annealed duplex with the reagent to form a linker,

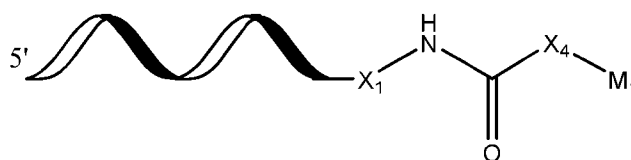
wherein forming the linker results in the formation of the synthesized full-length PEGRNA; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEGRNA.

[313] Another aspect of the disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core,

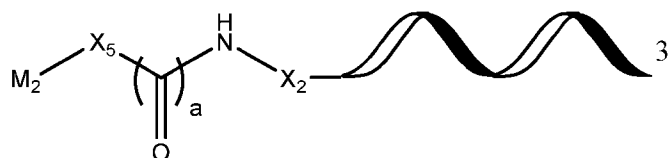
the method comprising:

providing a first RNA sequence of Formula A:



Formula A;

providing a second RNA sequence of Formula B:



Formula B;

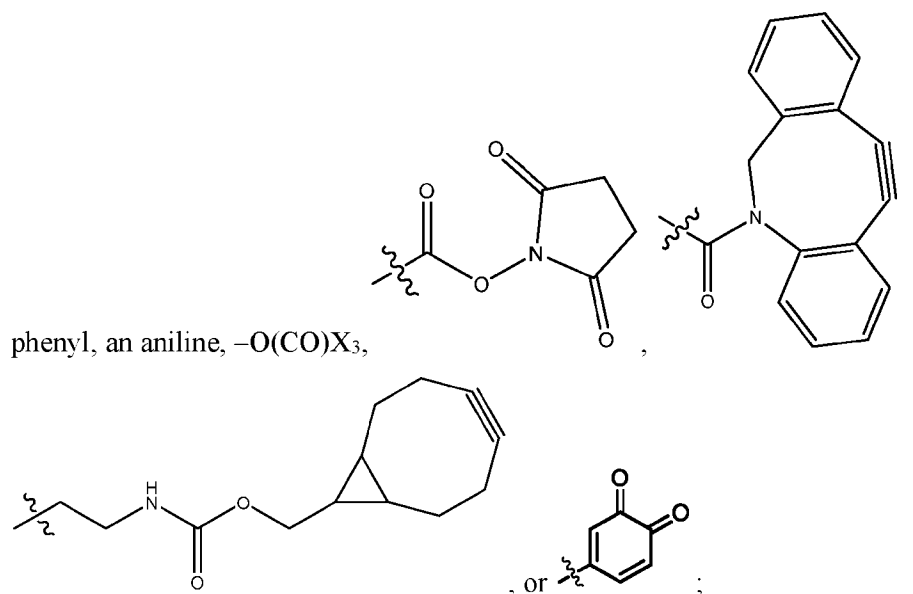
reacting M_1 with M_2 to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA;

wherein:

each X_1 , X_2 , X_4 , and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

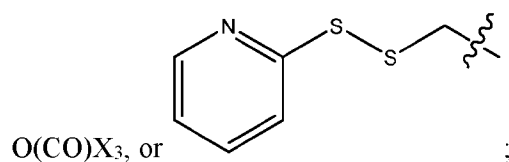
“n” is an integer between 1 and 12;

M_1 is an amino, a halogen, a hydroxyl, a phosphate ion, a maleimide, an aldehyde-substituted

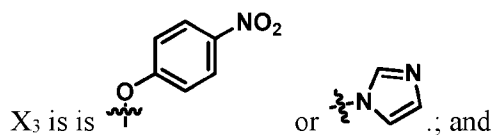


phenyl, an aniline, $-O(CO)X_3$,

M_2 is an amino, a hydroxyl, a phosphate ion, a thiol, an azido, an O-aminophenyl, –



$O(CO)X_3$, or



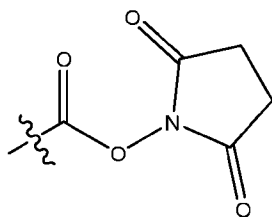
X_3 is is

or

“a” is an integer of 0 or 1;

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEGRNA.

[314] In some embodiments of this aspect in Formula A, X_4 is an alkylene and M_1 is

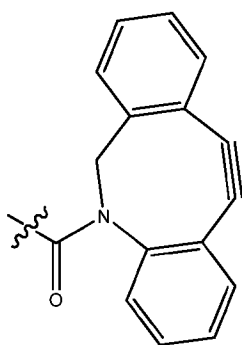


[315] In some embodiments, in Formula A, X_4 is an alkylene and M_1 is a halogen.

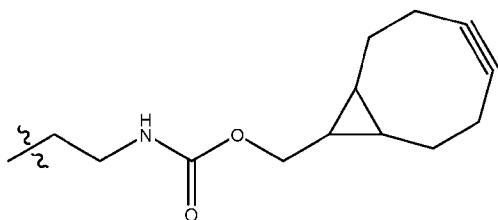
[316] In some embodiments, in Formula A, X_4 is $((CH_2)_n-C(O)NH-CH_2)$, and M_1 is a halogen.

[317] In some embodiments, in Formula A, X_4 is absent and M_1 is an aldehyde-substituted phenyl.

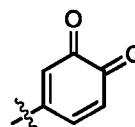
[318] In some embodiments, in Formula A, X_4 is an alkylene and M_1 is



[319] In some embodiments, in Formula A, X_4 is an alkylene and M_1 is



[320] In some embodiments, in Formula A, X_4 is an alkylene and M_1 is



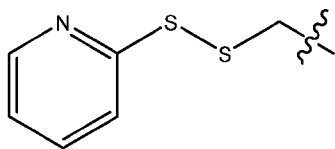
[321] In some embodiments, in Formula A, X_4 is an alkylene and M_1 is a maleimide.

[322] In some embodiments, in Formula B, “a” is 0, X_5 is absent, and M_2 is an amino.

[323] In some embodiments, in Formula B, “a” is 1, X_5 is an alkylene, and M_2 is a thiol.

[324] In some embodiments, in Formula B, “a” is 1, X_5 is an alkylene, and M_2 is an azide.

[325] In some embodiments, in Formula B, “a” is 1, X₅ is an alkylene, and M₂ is



[326] In some embodiments, in Formula B, “a” is 1, X₅ is an alkylene, and M₂ is an an O-aminophenyl.

[327] In some embodiments, each X₁ and X₂ is independently a C₁-C₁₀ unsubstituted alkylene.

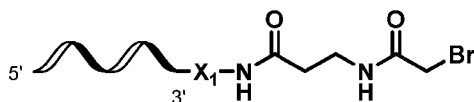
[328] In some embodiments, each X₁ and X₂ is independently a C₆ unsubstituted alkylene.

[329] In some embodiments, X₁ is a C₁-C₁₀ substituted alkylene with an -OH and an -O- group. In some embodiments, X₁ is a C₁-C₁₀ substituted alkylene with an -OH. In some embodiments, X₁ is a C₁-C₁₀ substituted alkylene with an -O- group. In some embodiments, X₁ is an -O- group.

[330] In some embodiments, X₂ is (O-CH₂-CH₂)_n.

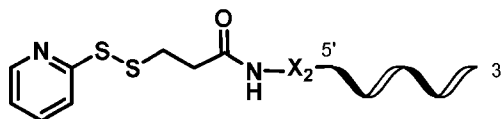
[331] Another aspect of the disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula C:



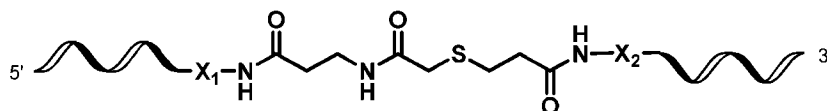
Formula C;

providing a second RNA sequence of Formula D:



Formula D;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula E:



Formula E;

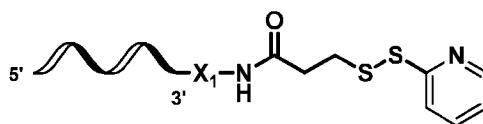
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

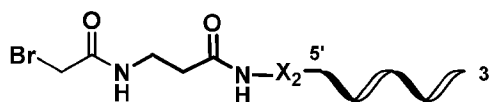
[332] Another aspect of the disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula C-1:



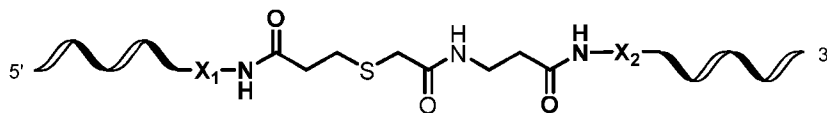
Formula C-1;

providing a second RNA sequence of Formula D-1:



Formula D-1;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula E-1:



Formula E-1;

wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[333] In some embodiments of this aspect, each X_1 and X_2 is independently a C_1 - C_{10} unsubstituted alkylene. In some embodiments, each X_1 and X_2 is independently a C_6 unsubstituted alkylene. In some embodiments, X_1 is a C_1 - C_{10} substituted alkylene with an –

OH and an $-O-$. In some embodiments, X_1 is an $-O-$. In some embodiments, X_2 is $(-O-CH_2-CH_2)_n$.

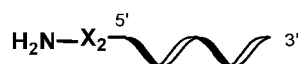
[334] Another aspect of the present disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula F:



Formula F;

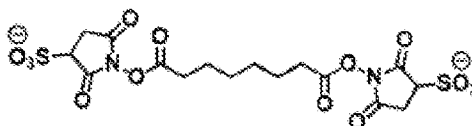
providing a second RNA sequence of Formula G:



Formula G;

annealing the first and second RNA sequences to form a pre-annealed duplex;

providing a BS3 reagent of the formula:



BS3;

contacting the pre-annealed duplex with the BS3 reagent to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula H:



Formula H;

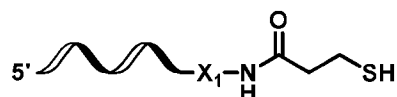
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

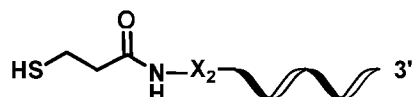
[335] Another aspect of the present disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula I:



Formula I;

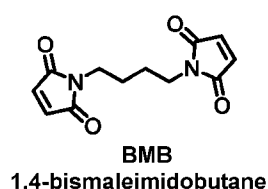
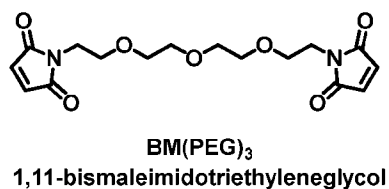
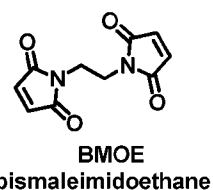
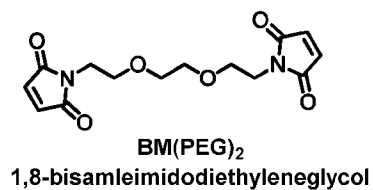
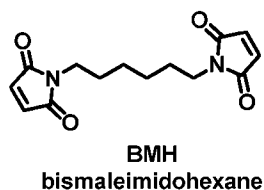
providing a second RNA sequence of Formula J:



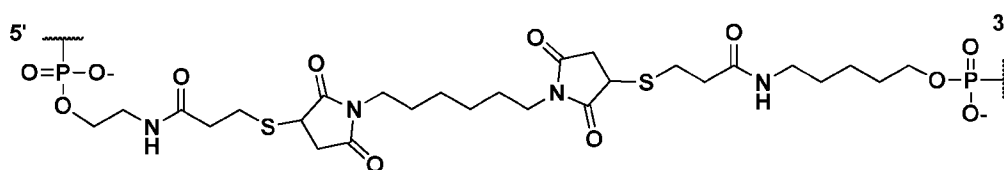
Formula J;

annealing the first and second RNA sequences to form a pre-annealed duplex;

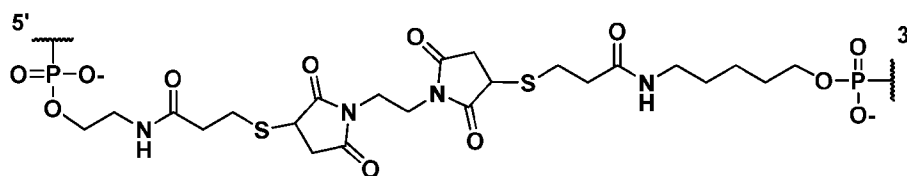
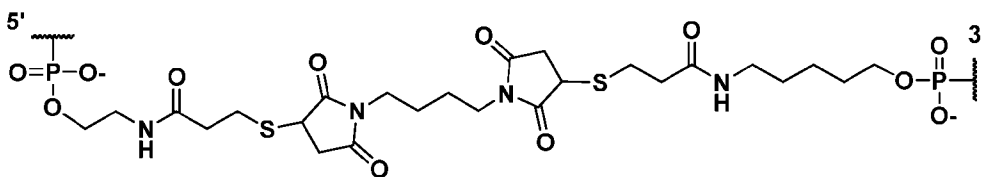
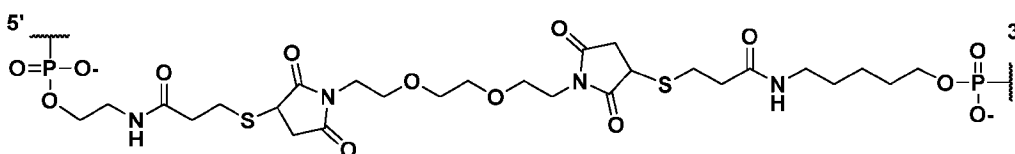
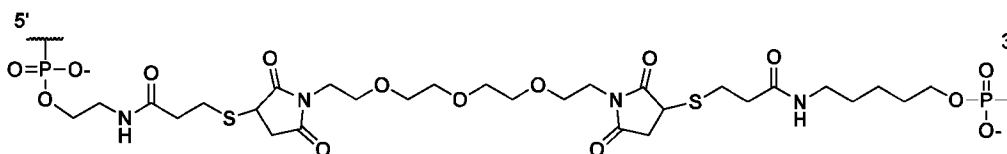
providing a BMH, BM(PEG)₂, BMOE, BM(PEG)₃, or BMB reagent of the formulas:



contacting the pre-annealed duplex with the BMH, BM(PEG)₂, BMOE, BM(PEG)₃, or BMB reagent to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formulas K-O:



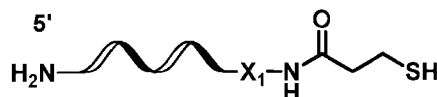
Formula K;

**Formula L;****Formula M;****Formula N; or****Formula O;**

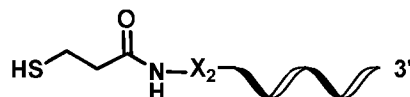
wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[336] Another aspect of the present disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula I-1:

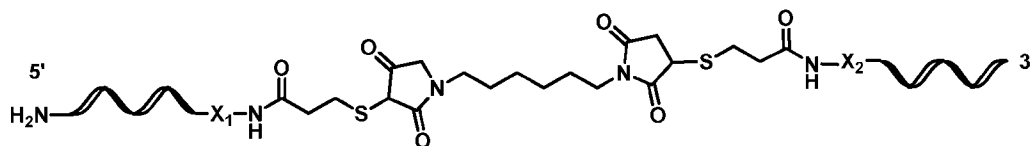
**Formula I;**

providing a second RNA sequence of Formula J:

**Formula J;**

annealing the first and second RNA sequences to form a pre-annealed duplex;
providing a BMH reagent;

contacting the pre-annealed duplex with the BMH reagent to form a linker, wherein forming the linker results in the formation of Formula P:



Formula P;

wherein Formula P comprises an amine at its 5' end;
providing a third RNA sequence of Formula F:

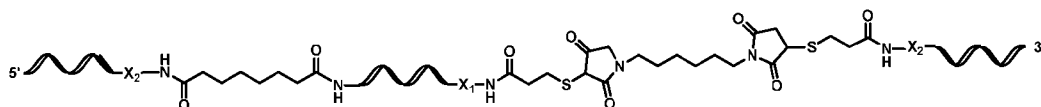


Formula F;

contacting Formula P with Formula F to form a second pre-annealed duplex;
providing a BS3 reagent;

contacting the second pre-annealed duplex with the BS3 reagent to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of

Formula Q:



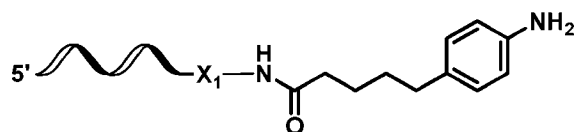
Formula Q;

wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“n” is an integer between 1 and 12.

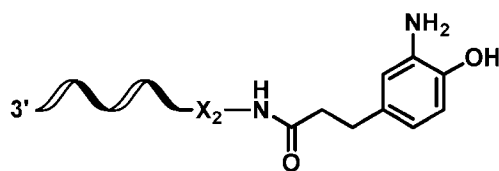
[337] Another aspect of the present disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula R:

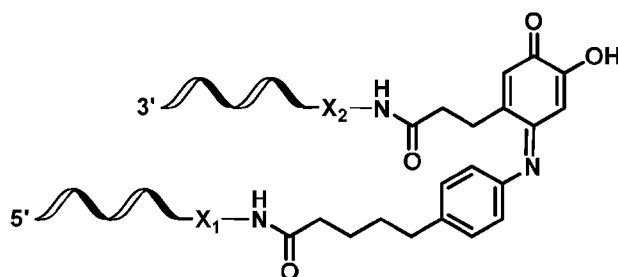


Formula R;

providing a second RNA sequence of Formula S:

**Formula S;**

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula T:

**Formula T;**

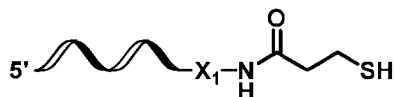
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

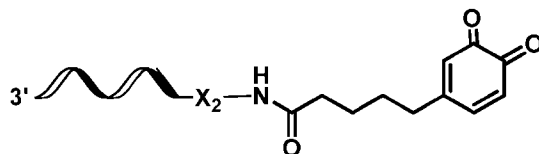
wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[338] Another aspect of the present disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula I:

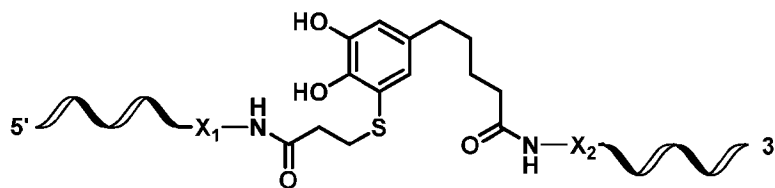
**Formula I;**

providing a second RNA sequence of Formula U:

**Formula U;**

annealing the first and second RNA sequences to form a pre-annealed duplex;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula W:



Formula W;

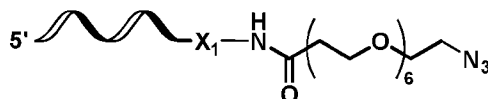
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

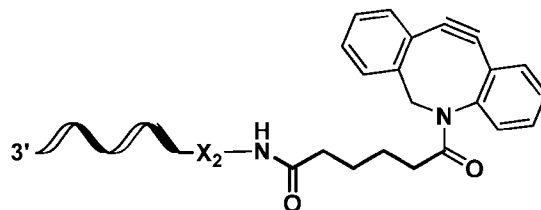
[339] Another aspect of the present disclosure includes a method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula X:



Formula X;

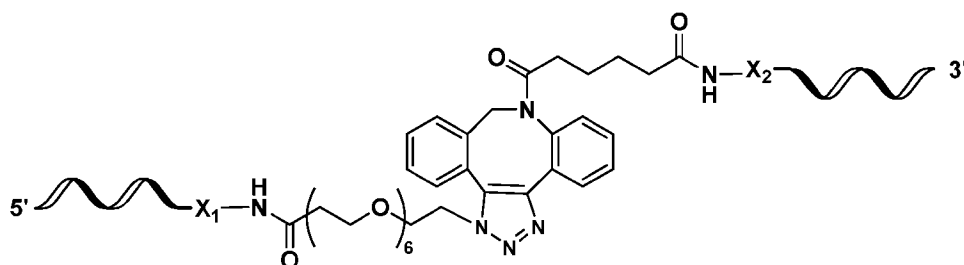
providing a second RNA sequence of Formula Y:



Formula Y;

annealing the first and second RNA sequences to form a pre-annealed duplex;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula Z:

**Formula Z;**

wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

[340] In some embodiments, each X_1 and X_2 is independently a C_1 - C_{10} unsubstituted alkylene. In some embodiments, each X_1 and X_2 is independently a C_6 unsubstituted alkylene. In some embodiments, X_1 is a C_1 - C_{10} substituted alkylene with an $-OH$ and an $-O-$. In some embodiments, X_1 is an $-O-$. In some embodiments, X_2 is $(-O-CH_2-CH_2)_n$.

[341] In embodiments of certain aspects, the linker is present in the tetraloop or SL2 of the chemically-modified gRNA core. In some embodiments, the linker is present in the tetraloop of the chemically-modified gRNA core. In some embodiments, the linker is present in the upper stem of the tetraloop. In some embodiments, the linker is present in the SL2 of the chemically-modified gRNA core.

[342] In some embodiments, the linker is located between the US4 and US5 nucleotides, between the US5 and US6 nucleotides, between the US6 and US7 nucleotides, between the US7 and US8 nucleotides, or between the US8 and US9 nucleotides of the tetraloop.

[343] In some embodiments, the linker is located between the SL2-4 and SL2-5 nucleotides, between the SL2-5 and SL2-6 nucleotides, between the SL2-6 and SL2-7 nucleotides, between the SL2-7 and SL2-8 nucleotides, or between the SL2-8 and SL2-9 nucleotides of the SL2.

[344] Another aspect of the present disclosure includes a full-length PEgRNA comprising a chemically-modified gRNA core, wherein the synthetic full-length PEgRNA is produced by: contacting a first RNA sequence with a second RNA sequence,

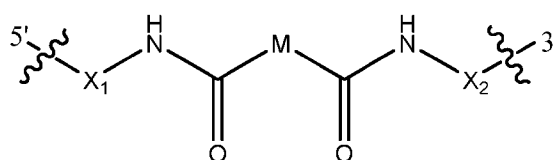
wherein the first RNA sequence comprises a first chemical moiety at its 3' end and the second RNA sequence comprises a second chemical moiety at its 5' end; and;

ligating the first RNA sequence and the second RNA sequence to obtain the synthetic full-length PEgRNA,

wherein the ligation forms a linker between the first chemical moiety of the first RNA sequence and the second chemical moiety of the second RNA sequence; and

wherein the linker is present in the chemically-modified gRNA core of the synthetic full-length PEgRNA.

[345] Another aspect of the present disclosure includes a full-length PEgRNA comprising a chemically-modified gRNA core, wherein the chemically-modified gRNA core comprises one or more linkers of Formula AA:



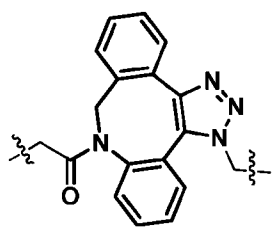
Formula AA

wherein:

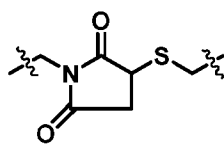
each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12;

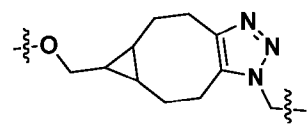
M is a linker, wherein the linker comprises an alkylene optionally substituted with one or more of $-OH$, $-O-$, $-S-$, or $(O-CH_2-CH_2)_n$; or a structure of any one of Formulas AA-1 to AA-10:



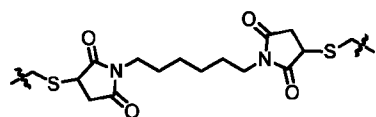
Formula AA-1



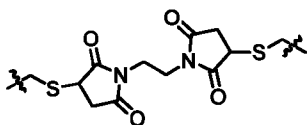
Formula AA-2



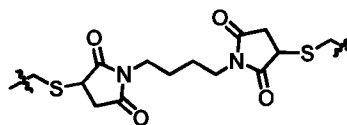
Formula AA-3



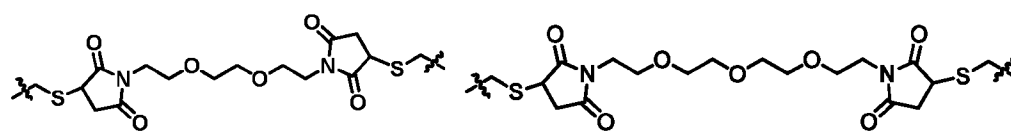
Formula AA-4



Formula AA-5

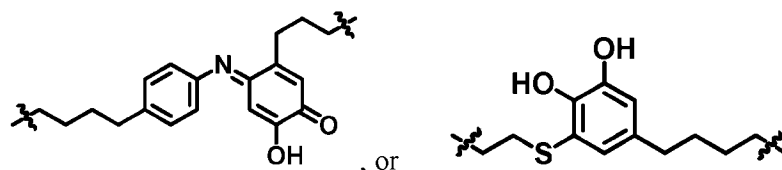


Formula AA-6



Formula AA-7

Formula AA-8



Formula AA-9

Formula AA-10.

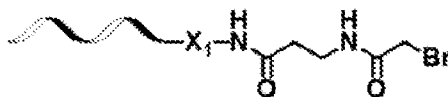
[346] In embodiments of this aspect, the linker is a C₆ unsubstituted alkylene. In some embodiments, the linker is a C₁-C₁₀ substituted alkylene with a -S-. In some embodiments, the linker is a structure of any one of of Formulas AA-1 to AA-10. In some embodiments, the linker is a structure of Formula AA-1. In some embodiments, the linker is a structure of Formula AA-2. In some embodiments, the linker is a structure of Formula AA-3. In some embodiments, the linker is a structure of Formula AA-4. In some embodiments, the linker is a structure of Formula AA-5. In some embodiments, the linker is a structure of Formula AA-6. In some embodiments, the linker is a structure of Formula AA-7. In some embodiments, the linker is a structure of Formula AA-8. In some embodiments, the linker is a structure of Formula AA-9. In some embodiments, the linker is a structure of Formula AA-10.

[347] In some embodiments, X₁ is a C₁-C₁₀ unsubstituted alkylene. In some embodiments, X₁ is a C₆ unsubstituted alkylene. In some embodiments, X₁ is a C₁-C₁₀ substituted alkylene with an -OH and an -O-. In some embodiments, X₁ is a C₁-C₁₀ substituted alkylene with an -OH. In some embodiments, X₁ is a C₁-C₁₀ substituted alkylene with an -O-. In some embodiments, X₁ is an -O-. In some embodiments, X₁ is (-O-CH₂-CH₂)_n.

[348] In some embodiments, the linker is present in the tetraloop or SL2 of the chemically-modified gRNA core. In some embodiments, the linker is present in the tetraloop of the chemically-modified gRNA core. In some embodiments, the linker is present in the upper stem of the tetraloop. In some embodiments, the linker is present in the SL2 of the chemically-modified gRNA core.

[349] In some embodiments, the linker is located between the US4 and US5 nucleotides, between the US5 and US6 nucleotides, between the US6 and US7 nucleotides, between the US7 and US8 nucleotides, or between the US8 and US9 nucleotides of the tetraloop. In some embodiments, the linker is located between the SL2-4 and SL2-5 nucleotides, between the SL2-5 and SL2-6 nucleotides, between the SL2-6 and SL2-7 nucleotides, between the SL2-7 and SL2-8 nucleotides, or between the SL2-8 and SL2-9 nucleotides of the SL2.

[350] Another aspect of the disclosure includes an RNA sequence of Formula C:

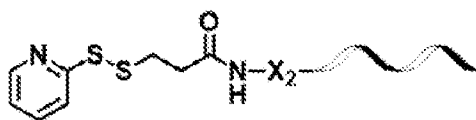


Formula C;

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

[351] Another aspect of the disclosure includes an RNA sequence of Formula D:

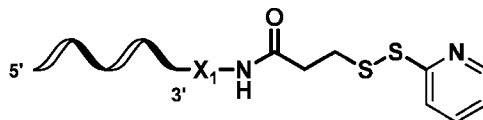


Formula D;

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

[352] Another aspect of the disclosure includes an RNA sequence of Formula C-1:

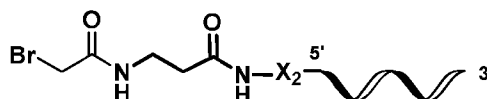


Formula C-1;

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

[353] Another aspect of the disclosure includes an RNA sequence of Formula D-1:



Formula D-1;

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

[354] Another aspect of the disclosure includes an RNA sequence of Formula F:

**Formula F;**

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

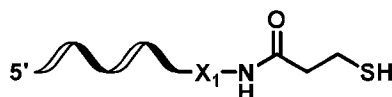
[355] Another aspect of the disclosure includes an RNA sequence of Formula G:

**Formula G;**

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

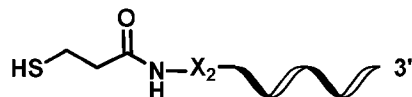
[356] Another aspect of the disclosure includes an RNA sequence of Formula I:

**Formula I;**

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

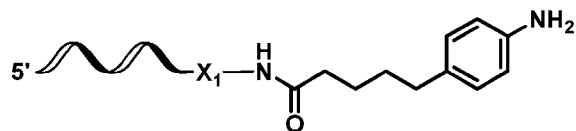
[357] Another aspect of the disclosure includes an RNA sequence of Formula J:

**Formula J;**

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“ n ” is an integer between 1 and 12.

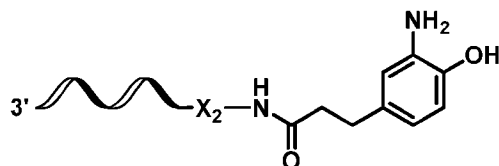
[358] Another aspect of the disclosure includes an RNA sequence of Formula R:

**Formula R;**

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“n” is an integer between 1 and 12.

[359] Another aspect of the disclosure includes an RNA sequence of Formula S:

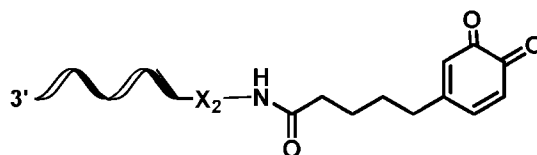


Formula S:

wherein X₂ is absent or (O-CH₂-CH₂)_n, (CH₂-CH₂-O)_n, ((CH₂)_n-C(O)NH-CH₂), or an alkylene optionally substituted with one or more -OH or -O-, or -O-; and

“n” is an integer between 1 and 12.

[360] Another aspect of the disclosure includes an RNA sequence of Formula U:

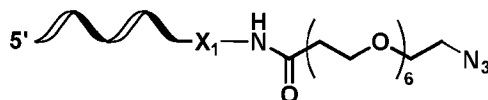


Formula U:

wherein X₂ is absent or (O-CH₂-CH₂)_n, (CH₂-CH₂-O)_n, ((CH₂)_n-C(O)NH-CH₂), or an alkylene optionally substituted with one or more -OH or -O-, or -O-; and

“n” is an integer between 1 and 12.

[361] Another aspect of the disclosure includes an RNA sequence of Formula X:

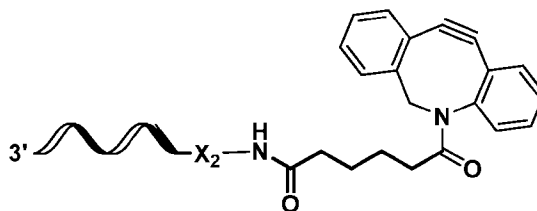


Formula X.

wherein X₁ is absent or (O-CH₂-CH₂)_n, (CH₂-CH₂-O)_n, ((CH₂)_n-C(O)NH-CH₂), or an alkylene optionally substituted with one or more -OH or -O-, or -O-; and

“n” is an integer between 1 and 12.

[362] Another aspect of the disclosure includes an RNA sequence of Formula Y:



Formula Y:

wherein X₂ is absent or (O-CH₂-CH₂)_n, (CH₂-CH₂-O)_n, ((CH₂)_n-C(O)NH-CH₂), or an alkylene optionally substituted with one or more -OH or -O-, or -O-; and

“n” is an integer between 1 and 12.

Full-length PEgRNAs

[363] In some embodiments, the synthetic RNA or synthesized full-length PEgRNA is at least 85 nucleotides, at least 90 nucleotides, at least 95 nucleotides, at least 100 nucleotides, at least 110 nucleotides, at least 120 nucleotides, at least 130 nucleotides, at least 140 nucleotides, at least 150 nucleotides, at least 160 nucleotides, at least 170 nucleotides, at least 180 nucleotides, at least 190 nucleotides, at least 200 nucleotides, at least 210 nucleotides, at least 220 nucleotides, at least 230 nucleotides, at least 240 nucleotides, at least 250 nucleotides, at least 275 nucleotides, at least 300 nucleotides, at least 325 nucleotides, at least 350 nucleotides, at least 375 nucleotides, at least 400 nucleotides, at least 425 nucleotides, at least 450 nucleotides, at least 475 nucleotides, or at least 500 nucleotides in length.

[364] In some embodiments, the full-length PEgRNA is at least 85 nucleotides, at least 90 nucleotides, at least 95 nucleotides, at least 100 nucleotides, at least 110 nucleotides, at least 120 nucleotides, at least 130 nucleotides, at least 140 nucleotides, at least 150 nucleotides, at least 160 nucleotides, at least 170 nucleotides, at least 180 nucleotides, or at least 190 nucleotides in length at least 200 nucleotides, at least 210 nucleotides, at least 220 nucleotides, at least 230 nucleotides, at least 240 nucleotides, at least 250 nucleotides in length, at least 275 nucleotides, at least 300 nucleotides, at least 325 nucleotides, at least 350 nucleotides, at least 375 nucleotides, at least 400 nucleotides, at least 425 nucleotides, at least 450 nucleotides, at least 475 nucleotides, or at least 500 nucleotides.

[365] In some embodiments, the first RNA sequence is SEQ ID NO.: 1, 3, 5, 7, 9, 19, 24, 30, 60, 62, 66, 69, 74, 89, 91, 93, or 112.

[366] In some embodiments, the second RNA sequence is SEQ ID NO.: 2, 4, 6, 8, 10, 20, 25, 31, 61, 63, 67, 70, 73, 75, 90, 108, or 121.

[367] In some embodiments, the synthesized full-length PEgRNA is SEQ ID NO.: 17, 18, 21, 26, 32, 64, 65, 68, 72, 77-88, 97-102, 104-106, 109-111, 114-116, 118-120, or 122-123.

[368] In some embodiments, when using chemical ligation, the first RNA sequence is SEQ ID NO.: 1, 3, 5, 7, 9, 19, 60, 62, 66, 69, 74, 89, 91, 93, or 112.

[369] In some embodiments, when using chemical ligation, the second RNA sequence is SEQ ID NO.: 2, 4, 6, 8, 10, 20, 61, 63, 67, 70, 73, 75, 90, 108, or 121.

[370] In some embodiments, when using chemical ligation, the synthesized full-length PEgRNA is SEQ ID NO.: 17, 18, 21, 64, 65, 68, 72, 77-88, 97-102, 104-106, 109-111, 114-116, 118-120, or 122-123.

[371] In some embodiments, such as those utilizing a three-part split synthesis, the first RNA sequence is SEQ ID NO.: 1, 3, 5, 7, 9, 69, 75, 89, 91, 93, or 112; the second RNA sequence is SEQ ID NO.: 2, 4, 6, 8, 10, 75, 90, or 108, and the third RNA sequence is SEQ ID NO.: 12, 13, 14, 15, 92, 94, 103, 107, 113, or 117.

[372] In some embodiments, such as those utilizing a three-part split synthesis, the synthesized full-length PEGRNA is SEQ ID NO.: 97-102, 104-106, 109-111, 114-116, or 118-120.

[373] In some embodiments, such as those utilizing a three-part split synthesis, the first RNA sequence is SEQ ID NO.: 89, 91, or 93, the second RNA sequence is SEQ ID NO.: 90, and the third RNA sequence is SEQ ID NO.: 92 or 94.

[374] In some embodiments, such as those utilizing a three-part split synthesis, the first RNA sequence is SEQ ID NO.: 69, the second RNA sequence is SEQ ID NO.: 75, and the third RNA sequence is SEQ ID NO.: 103.

[375] In some embodiments, such as those utilizing a three-part split synthesis, the first RNA sequence is SEQ ID NO.: 69, the second RNA sequence is SEQ ID NO.: 107, and the third RNA sequence is SEQ ID NO.: 108.

[376] In some embodiments, such as those utilizing a three-part split synthesis, the first RNA sequence is SEQ ID NO.: 112, the second RNA sequence is SEQ ID NO.: 75, and the third RNA sequence is SEQ ID NO.: 113.

[377] In some embodiments, such as those utilizing a three-part split synthesis, the first RNA sequence is SEQ ID NO.: 112, the second RNA sequence is SEQ ID NO.: 108, and the third RNA sequence is SEQ ID NO.: 117.

[378] In some embodiments, when using enzymatic ligation, the first RNA sequence is SEQ ID NO.: 24 or 30.

[379] In some embodiments, when using enzymatic ligation, the second RNA sequence is SEQ ID NO.: 25 or 31.

[380] In some embodiments, when using enzymatic ligation, the synthesized full-length PEGRNA is SEQ ID NO.: 26 or 32.

[381] In some embodiments, the linker of the full-length PEGRNA does not affect CRISPR associated (Cas) protein binding and/or prime editor binding. In some embodiments, the linker of the chemically-modified PEGRNA does not affect CRISPR associated (Cas) protein binding and/or prime editor.

[382] In some embodiments, long RNAs, such as a full-length PEGRNA, are prepared using the chemical ligation methods described herein. In some embodiments, long RNAs, such as a

full-length PEgRNA, are prepared using the enzymatic ligation methods described herein. In some embodiments, long RNAs, such as a full-length PEgRNA, are prepared using a combination of the chemical ligation and enzymatic ligation methods described herein.

[383] In one example, when preparing a full-length PEgRNA, the gRNA core can be prepared with any of the chemical ligation methods described herein. The rest of the PEgRNA elements (e.g., spacer, RTT, and PBS) can be prepared with the enzymatic ligation methods described herein. The chemically-ligated gRNA core can then be incorporated to the other elements via the enzymatic ligation methods described herein. The chemically-ligated gRNA core can also be incorporated to the other elements via other methods known to a person of ordinary skill in the art.

[384] In another example, when preparing a full-length PEgRNA, the gRNA core can be prepared with a 3-part chemical ligation, as shown in FIG. 4A and FIG. 4B, and as described above. The rest of the PEgRNA elements (e.g., spacer, RTT, and PBS) can be prepared with the enzymatic ligation methods described herein. The chemically-ligated gRNA core can then be incorporated to the other elements via the enzymatic ligation methods described herein. The chemically-ligated gRNA core can also be incorporated to the other elements via other methods known to a person of ordinary skill in the art.

[385] It is to be understood that any combination of chemical and enzymatic ligation can be utilized to prepare the full-length PEgRNA and it is not limited to the examples provided above. In some embodiments, a combination of one chemical ligation and one enzymatic ligation are utilized to prepare the full-length PEgRNA. In some embodiments, a combination of two chemical ligations and two enzymatic ligations are utilized to prepare the full-length PEgRNA. In some embodiments, a combination of three chemical ligations and three enzymatic ligations are utilized to prepare the full-length PEgRNA. In some embodiments, a combination of one chemical ligation and two enzymatic ligations are utilized to prepare the full-length PEgRNA. In some embodiments, a combination of one chemical ligation and three enzymatic ligations are utilized to prepare the full-length PEgRNA. In some embodiments, a combination of two chemical ligations and one enzymatic ligation are utilized to prepare the full-length PEgRNA. In some embodiments, a combination of three chemical ligations and one enzymatic ligation are utilized to prepare the full-length PEgRNA. In some embodiments, a combination of three chemical ligations and two enzymatic ligations are utilized to prepare the full-length PEgRNA.

[386] Additional PEGRNA elements

[387] The full-length PEGRNA synthesized with the methods described herein may comprise, or further comprise, additional polynucleotide components such as a structural motif at the 3' end of the extension arm, tag sequences, and/or flap endonucleases as described in PCT publication No.: WO 2023/096977 and WO 2023/086558, the contents of each are herein incorporated by reference in their entireties.

[388] **Tag Sequence**

[389] In some embodiments, the PEGRNA synthesized by the methods described herein comprises a tag sequence in addition to the other elements, e.g., spacer, gRNA core (e.g., chemically-modified gRNA core), primer binding site, and editing template. In some embodiments, the tag sequence comprises a region of complementarity to the editing template. In some embodiments, the tag sequence comprises a region of complementarity to the PBS. In some embodiments, the tag sequence comprises a region of complementarity to the editing template and/or the PBS. In some embodiments, the tag sequence comprises a region of complementarity to the editing template and does not have substantial complementarity to the PBS. In some embodiments, the tag sequence comprises a region of complementarity to the editing template and does not have complementarity to the PBS. In some embodiments, the tag sequence does not have perfect complementarity with the PBS. In some embodiments, the tag sequence does not have perfect complementarity with the gRNA core. In some embodiments, the tag sequence and the editing template each comprises a region of complementarity to each other, wherein the 3' end of the region of complementarity in the editing template is at a position 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or more bases 5' of the 3' half of the editing template. In some embodiments, the region of complementarity in the tag sequence is at a 5' portion of the tag sequence. In some embodiments, the tag sequence does not have substantial complementarity to the spacer. In some embodiments, the tag does not have complementarity to the spacer. In some embodiments, the tag sequence does not have perfect complementarity to the spacer.

[390] In some embodiments, the tag sequence is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22 nucleotides in length. In some embodiments, the tag sequence is at least 4, at least 6, at least 8 nucleotides in length. In some embodiments, the tag sequence is from 4 to 22, 4 to 10, or 6 to 8 nucleotides in length. In some embodiments, the tag sequence comprises a nucleic acid sequence selected from any nucleic acid sequence disclosed in SEQ ID NOs 62-1960 of PCT Publication No.: WO 2023/096977.

[391] **Additional Motifs**

[392] In some embodiments, the PEGRNA synthesized by the methods described herein comprises a motif at the 3' end of the PBS in addition to the other elements, e.g., spacer, gRNA core (e.g., chemically-modified gRNA core), primer binding site, and editing template.

[393] In some embodiments, the one or more nucleic acid moieties comprise a hairpin (e.g., hairpin comprising a region of self-complementarity, optionally wherein the region of self-complementary comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or more contiguous complementary basepairs), a quadruplex (e.g., a G-quadruplex or a C-quadruplex, optionally wherein the G-quadruplex or the C-quadruplex is derived from a VEGF gene promoter), a tRNA sequence (e.g., a tRNA sequence, optionally wherein the tRNA sequence is a tRNA (Proline) sequence), an aptamer (e.g., an aptamer derived from a viral protein-binding sequence, optionally wherein the aptamer comprises a viral reverse transcriptase recruitment sequence, optionally wherein the aptamer comprises a MS2 protein binding sequence or a Moloney Murine leukemia (MMLV) reverse transcriptase recruitment sequence), and/or a pseudoknot (e.g. pseudoknot is derived from a potato roll leaf virus (PLRV)), or any combination thereof.

[394] In some embodiments, the one or more nucleic acid moieties comprises a hairpin. In some embodiments, the hairpin comprises a sequence of any one of SEQ ID NOs: 42-44 or 46-48.

[395] In some embodiments, the one or more nucleic acid moieties comprises a pseudoknot. In some embodiments, the pseudoknot is derived from potato roll-leaf virus. In some embodiments, the pseudoknot comprises the sequence of SEQ ID NO.: 45. In some embodiments, the one or more nucleic acid moieties comprises a MS2 hairpin. In some embodiments, the nucleotide sequence of the MS2 hairpin (or also referred to as the "MS2 aptamer") is: GCCAACATGAGGATCACCCATGTCTGCAGGGCC (SEQ ID NO.: 58). In some embodiments, the nucleotide sequence of the MS2 aptamer comprises the sequence of SEQ ID NO.: 50. In some embodiments, a MS2 coat protein (MCP) recognizes the MS2 hairpin. In some embodiments, the amino acid sequence of the MCP is:

GSASNFTQFVLVDNGGTGDVTVAPSNFANGVAEWISSNSRSQAYKVTCSVRQSSAQ
NR

KYTIKVEVPKVATQTVGGEELPVAGWRSYLNMEITIPFATNSDCELVKAMQGLLK
DG NPIPSAIA ANSGIY (SEQ ID NO.: 59).

[396] In some embodiments, the one or more nucleic acid moieties comprises a G-quadruplex or a C-quadruplex. In some embodiments, the one or more nucleic acid moieties

comprises a quadruplex from a VEGF gene promoter. In some embodiments, the quadruplex comprises the sequence of SEQ ID NO.: 51 or 52.

[397] In some embodiments, the one or more nucleic acid moieties comprise a structure derived from a replication recognition sequence of a retrovirus. In some embodiments, the nucleic acid moiety comprises a sequence derived from a replication recognition sequence of a Moloney Murine leukemia virus (MMLV). In some embodiments, the one or more nucleic acid moieties comprise a nucleic acid sequence selected from SEQ ID Nos: 53-56.

[398] **Table A. Exemplary PEGRNA secondary structure motifs**

SEQ ID NO.	Name	Name description	Motif Sequence	Motif length
42	hp_1	hairpin 1	CGGGTCTCTACGTGGGGGCCCCG	22
43	hp_1	hairpin 1	CGCGTCTCTACGTGGGG GCGCG	22
44	hp_3	hairpin 3	GGCGCGAAAGCGCC	14
45	PLRV_22	potato roll leaf virus pseudoknot	GCGGCACCGTCCGCCCCAAACGG	22
46	hp_5	hairpin 5	GCCCGGCGAAAGCCGGGC	18
47	hp_4	hairpin 4	GCCCGGCTTCGGCCGGGC	18
48	hp_2	hairpin 2	GGCGCTTCGGCGCC	14
49	MMLV-RT aptamer	MMLV aptamer sequence that can recruit MMLV RT	TTACCACGCGCTCTTAAGTCTA GCGCCATGGC	33
50	MS2	MS2 protein binding sequence.	ACATGAGGATCACCCATGT	19
51	G quad/ G4 VEGF	G-quadruplex in VEGF promoter	GGGCGGGCCGGGGGCGGG	18
52	C quad/ iM VEGF	C-quadruplex in VEGF promoter	CCCCGCCCCGGCCGCCCC	18
53	tRNA_PBS _long	MMLV endogenous binding for replication	GCTCCTCTGATTGACTACCCGTC AGCGGGGTCTTTTGGGGGCTC GTCCGGGATCGGGAGT	61
54	tRNA_PBS _long_RC	MMLV endogenous binding for replication (reverse complement)	ACTCCCGATCCCGGACG AGCCCCAAAAGACCCC CGCTGACGGGTAGTCAA TCAGAGGAGC	61
55	tRNA_PBS _short	MMLV endogenous binding for replication	TGGGGGCTCGTCCGGGAT	18
56	tRNA_PBS short RC	MMLV endogenous binding for replication (reverse complement)	ATCCCGGACGAGCCCCCA	18
57	evopreQ1	Prequeosine1-1 riboswitch aptamer	CGCGGTTCTATCTAGTTACGCGT TAAACCAACTAGAA	37

[399] Prime Editors with Flap Endonucleases

[400] In some embodiments, the prime editor comprises additional polypeptides or polypeptide domains involved in prime editing, for example, a polypeptide domain having 5' endonuclease activity, e.g., a 5' endogenous DNA flap endonucleases (e.g., FEN1), for helping to drive the prime editing process towards the edited product formation. In some embodiments, the prime editor further comprises an RNA-protein recruitment polypeptide, for example, a MS2 coat protein.

[401] A prime editor may be engineered. In some embodiments, the polypeptide components of a prime editor do not naturally occur in the same organism or cellular environment. In some embodiments, the polypeptide components of a prime editor may be of different origins or from different organisms. In some embodiments, a prime editor comprises a DNA binding domain and a DNA polymerase domain that are derived from different species. In some embodiments, a prime editor comprises a Cas polypeptide and a reverse transcriptase polypeptide that are derived from different species. For example, a prime editor may comprise a *S. pyogenes* Cas9 polypeptide and a Moloney murine leukemia virus (M-MLV) reverse transcriptase polypeptide.

[402] In some embodiments, polypeptide domains of a prime editor may be fused or linked by a peptide linker to form a fusion protein. In other embodiments, a prime editor comprises one or more polypeptide domains provided in *trans* as separate proteins, which are capable of being associated to each other through non-peptide linkages or through aptamers or recruitment sequences. For example, a prime editor may comprise a DNA binding domain and a reverse transcriptase domain associated with each other by an RNA-protein recruitment aptamer, e.g., an MS2 aptamer, which may be linked to a PEGRNA. Prime editor polypeptide components may be encoded by one or more polynucleotides in whole or in part. In some embodiments, a single polynucleotide, construct, or vector encodes the prime editor fusion protein. In some embodiments, multiple polynucleotides, constructs, or vectors each encode a polypeptide domain or portion of a domain of a prime editor, or a portion of a prime editor fusion protein. For example, a prime editor fusion protein may comprise an N-terminal portion fused to an intein-N and a C-terminal portion fused to an intein-C, each of which is individually encoded by an AAV vector.

[403] Flap Endonuclease

[404] In some embodiments, a prime editor further comprises additional polypeptide components, for example, a flap endonuclease (FEN, e.g., FEN1). In some embodiments, the flap endonuclease excises the 5' single stranded DNA of the edit strand of the target gene and

assists incorporation of the intended nucleotide edit into the target gene. In some embodiments, the FEN is linked or fused to another component. In some embodiments, the FEN is provided in trans, for example, as a separate polypeptide or polynucleotide encoding the FEN.

[405] In some embodiments, a prime editor or prime editing composition comprises a flap nuclease. In some embodiments, the flap nuclease is a FEN1, or any FEN1 functional variant, functional mutant, or functional fragment thereof. In some embodiments, the flap nuclease is a TREX2, EXO1, or any other flap nuclease known in the art, or any functional variant, functional mutant, or functional fragment thereof. In some embodiments, the flap nuclease has an amino acid sequence that is at least about 70% identical, at least about 80% identical, at least about 90% identical, at least about 95% identical, at least about 96% identical, at least about 97% identical, at least about 98% identical, at least about 99% identical, at least about 99.5% identical, or at least about 99.9% identical to any of the flap nucleases described herein or known in the art.

[406] *PEgRNA modular arrangement*

[407] Components of a PEgRNA may be arranged in a modular fashion. In some embodiments, the spacer, the primer binding site sequence (PBS) and the editing template, *e.g.*, a reverse transcriptase template (RTT), can be interchangeably located in the 5' portion of the PEgRNA, the 3' portion of the PEgRNA, or in the middle of the gRNA core. In some embodiments, a PEgRNA comprises a PBS and an editing template sequence in 5' to 3' order. In some embodiments, the gRNA core of a PEgRNA of this disclosure may be located in between a spacer and an extension arm (*i.e.*, the PBS and editing template) of the PEgRNA. In some embodiments, the gRNA core of a PEgRNA may be located at the 3' end of a spacer. In some embodiments, the gRNA core of a PEgRNA may be located at the 5' end of a spacer. In some embodiments, the gRNA core of a PEgRNA may be located at the 3' end of an extension arm. In some embodiments, the gRNA core of a PEgRNA may be located at the 5' end of an extension arm. In some embodiments, a PEgRNA comprises, from 5' to 3': a spacer, a gRNA core, and an extension arm. In some embodiments, a PEgRNA comprises, from 5' to 3': a spacer, a gRNA core, an editing template, and a PBS. In some embodiments, a PEgRNA comprises, from 5' to 3': an extension arm, a spacer, and a gRNA core. In some embodiments, the PEgRNA comprises, from 5' to 3': an editing template a PBS, a spacer, and a gRNA core. In some embodiments, a first PEgRNA comprises a structure: 5' -[first spacer]-[first gRNA core]-[first editing template]-[first primer binding site sequence]-3'. In some embodiments, a first PEgRNA comprises a structure: 5' - [first editing template]-[first

primer binding site sequence]- [first spacer]-[first gRNA core]-3'. In some embodiments, a second PEGRNA comprises a structure: 5'-[second spacer]-[second gRNA core]-[second editing template]-[second primer binding site sequence]-3'. In some embodiments, a second PEGRNA comprises a structure: 5'- [second editing template]-[second primer binding site sequence]- [second spacer]-[second gRNA core]-3'.

[408] In some embodiments, a spacer sequence may further comprise additional nucleotides beside a region of complementarity to genomic search target sequence. For example, in some embodiments, a spacer sequence (as well as the full PEGRNA sequence) may comprise an additional G at the 5' end, for example, wherein the 5' most nucleotide of the spacer (or the PEGRNA) is not a G.

[409] The extension arm of a first PEGRNA may be partially complementary to the spacer of the first PEGRNA. In some embodiments, the editing template (*e.g.*, RTT) of a first PEGRNA is partially complementary to the spacer of the first PEGRNA. In some embodiments, the editing template (*e.g.*, RTT) and the primer binding site (PBS) of the first PEGRNA are each partially complementary to the spacer of the first PEGRNA. The extension arm of a PEGRNA may comprise a primer binding site (PBS) and an editing template (*e.g.*, an RTT). The extension arm of a second PEGRNA may be partially complementary to the spacer of the second PEGRNA. In some embodiments, the editing template (*e.g.*, RTT) of a second PEGRNA is partially complementary to the spacer of the second PEGRNA. In some embodiments, the editing template (*e.g.*, RTT) and the primer binding site (PBS) of the second PEGRNA are each partially complementary to the spacer of the second PEGRNA.

[410] An extension arm of a PEGRNA may comprise a primer binding site sequence (also referred to as a primer binding site, PBS, or PBS sequence) that hybridizes with a free 3' end of a single stranded DNA in the target gene generated by nicking with a prime editor. The length of the PBS sequence may vary depending on, *e.g.*, the prime editor components, the search target sequence and other components of the PEGRNA. In some embodiments, the length of the primer binding site (PBS) varies from at least 2 nucleotides to 50 nucleotides. For examples, a primer binding site (PBS) may be at least 2 nucleotides, at least 3 nucleotides, at least 4 nucleotides, at least 5 nucleotides, at least 6 nucleotides, at least 7 nucleotides, at least 8 nucleotides, at least 9 nucleotides, at least 10 nucleotides, at least 11 nucleotides, at least 12 nucleotides, at least 13 nucleotides, at least 14 nucleotides, at least 15 nucleotides, at least 16 nucleotides, at least 17 nucleotides, at least 18 nucleotides, at least 19 nucleotides, at least 20 nucleotides, at least 30 nucleotides, at least 40 nucleotides, or at least 50 nucleotides in length. In some embodiments, the PBS is at least 4 nucleotides in length. In

some embodiments, the PBS is at least 6 nucleotides in length. In some embodiments, the PBS is about 4 to 12 nucleotides, about 6 to 12 nucleotides, about 8 to 12 nucleotides, about 10 to 12 nucleotides, 4 to 14 nucleotides, about 6 to 14 nucleotides, about 8 to 14 nucleotides, about 10 to 14 nucleotides, about 12 to 14 nucleotides, 4 to 16 nucleotides, about 6 to 16 nucleotides, about 8 to 16 nucleotides, about 10 to 16 nucleotides, about 6 to 18 nucleotides, about 6 to 20 nucleotides, about 8 to 20 nucleotides, about 10 to 20 nucleotides, about 12 to 20 nucleotides, about 14 to 20 nucleotides, about 16 to 20 nucleotides, or about 18 to 20 nucleotides in length. In some embodiments, the PBS is about 7 to 15 nucleotides in length. In some embodiments, the PBS is 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In some embodiments, the PBS is 8, 9, 10, 11, 12, 13, or 14 nucleotides in length.

[411] The PBS of a first PEgRNA may be complementary or substantially complementary to a DNA sequence in the second strand of the target gene. The PBS of a second PEgRNA may be complementary or substantially complementary to a DNA sequence in the first strand of the target gene. By annealing with the edit strand at a free hydroxy group, *e.g.*, a free 3' end generated by prime editor nicking, a PBS may initiate synthesis of a new single stranded DNA encoded by the editing template at the nick site. In some embodiments, a PBS is at least about 70%, 75%, 80%, 85%, 90%, 95%, or 100% complementary to a region of the edit strand of the target gene. In some embodiments, a PBS is perfectly complementary, or 100% complementary, to a region of the edit strand of the target gene.

Prime Editing

[412] Another aspect of the present disclosure includes a prime editing complex comprising: (i) the full-length PEgRNA synthesized by the methods described herein; and (ii) a prime editor comprising a DNA binding domain and a DNA polymerase domain. In some embodiments, the DNA binding domain is a CRISPR associated (Cas) protein domain.

[413] The term “prime editing” refers to programmable editing of a target DNA using a prime editor complexed with a PEgRNA to incorporate an intended nucleotide edit into the target DNA through target-primed DNA synthesis. A target polynucleotide, *e.g.*, a target gene of prime editing may comprise a double stranded DNA molecule having two complementary strands: a first strand that may be referred to as a “target strand” or a “non-edit strand”, and a second strand that may be referred to as a “non-target strand,” or an “edit strand.” In some embodiments, in a prime editing guide RNA (PEgRNA), a spacer sequence is complementary or substantially complementary to a specific sequence on the target strand, which may be

referred to as a “search target sequence”. In some embodiments, the spacer sequence anneals with the target strand at the search target sequence. The target strand may also be referred to as the “non-Protospacer Adjacent Motif (non-PAM strand).” In some embodiments, the non-target strand may also be referred to as the “PAM strand”. In some embodiments, the PAM strand comprises a protospacer sequence and optionally a protospacer adjacent motif (PAM) sequence. In prime editing using a Cas-protein-based prime editor, a PAM sequence refers to a short DNA sequence immediately adjacent to the protospacer sequence on the PAM strand of the target gene. A PAM sequence may be specifically recognized by a programmable DNA binding protein, e.g., a Cas nickase or a Cas nuclease. In some embodiments, a specific PAM is characteristic of a specific programmable DNA binding protein, e.g., a Cas nickase or a Cas nuclease. A protospacer sequence refers to a specific sequence in the PAM strand of the target gene that is complementary to the search target sequence. In a PEGRNA, a spacer sequence may have a substantially identical sequence as the protospacer sequence on the edit strand of a target gene, except that the spacer sequence may comprise Uracil (U) and the protospacer sequence may comprise Thymine (T).

[414] In some embodiments, the double stranded target DNA comprises a nick site on the PAM strand (or non-target strand). As used herein, a “nick site” refers to a specific position in between two nucleotides or two base pairs of the double stranded target DNA. In some embodiments, the position of a nick site is determined relative to the position of a specific PAM sequence. In some embodiments, the nick site is the particular position where a nick will occur when the double stranded target DNA is contacted with a nickase, for example, a Cas nickase, that recognizes a specific PAM sequence. In some embodiments, the nick site is upstream of a specific PAM sequence on the PAM strand of the double stranded target DNA. In some embodiments, the nick site is downstream of a specific PAM sequence on the PAM strand of the double stranded target DNA. In some embodiments, the nick site is 3 base pairs upstream of the PAM sequence, and the PAM sequence is recognized by a *Streptococcus pyogenes* Cas9 nickase, a *P. lavamentivorans* Cas9 nickase, a *C. diphtheriae* Cas9 nickase, a *N. cinerea* Cas9, a *S. aureus* Cas9, or a *N. lari* Cas9 nickase. In some embodiments, the nick site is 3 base pairs upstream of the PAM sequence, and the PAM sequence is recognized by a Cas9 nickase, wherein the Cas9 nickase comprises a nuclease active HNH domain and a nuclease inactive RuvC domain. In some embodiments, the nick site is 2 base pairs upstream of the PAM sequence, and the PAM sequence is recognized by a *S. thermophilus* Cas9 nickase.

[415] In some embodiments, a PEgRNA complexes with and directs a prime editor to bind to the search target sequence of the target gene. In some embodiments, the bound prime editor generates a nick on the edit strand (PAM strand) of the target gene at the nick site. In some embodiments, a primer binding site (PBS) of the PEgRNA anneals with a free 3' end formed at the nick site, and the prime editor initiates DNA synthesis from the nick site, using the free 3' end as a primer. Subsequently, a single-stranded DNA encoded by the editing template of the PEgRNA is synthesized. In some embodiments, the newly synthesized single-stranded DNA comprises one or more intended nucleotide edits compared to the endogenous target gene sequence. In some embodiments, the editing template of a PEgRNA is complementary to a sequence in the edit strand except for one or more mismatches at the intended nucleotide edit positions in the editing template partially complementary to the editing template may be referred to as an "editing target sequence". Accordingly, in some embodiments, the newly synthesized single stranded DNA has identity or substantial identity to a sequence in the editing target sequence, except for one or more insertions, deletions, or substitutions at the intended nucleotide edit positions.

[416] In some embodiments, the newly synthesized single-stranded DNA equilibrates with the editing target on the edit strand of the target gene for pairing with the target strand of the target gene. In some embodiments, the editing target sequence of the target gene is excised by a flap endonuclease (FEN), for example, FEN1. In some embodiments, the FEN is an endogenous FEN, for example, in a cell comprising the target gene. In some embodiments, the FEN is provided as part of the prime editor, either linked to other components of the prime editor or provided in *trans*. In some embodiments, the newly synthesized single stranded DNA, which comprises the intended nucleotide edit, replaces the endogenous single stranded editing target sequence on the edit strand of the target gene. In some embodiments, the newly synthesized single stranded DNA and the endogenous DNA on the target strand form a heteroduplex DNA structure at the region corresponding to the editing target sequence of the target gene. In some embodiments, the newly synthesized single-stranded DNA comprising the nucleotide edit is paired in the heteroduplex with the target strand of the target DNA that does not comprise the nucleotide edit, thereby creating a mismatch between the two otherwise complementary strands. In some embodiments, the mismatch is recognized by DNA repair machinery, *e.g.*, an endogenous DNA repair machinery. In some embodiments, through DNA repair, the intended nucleotide edit is incorporated into the target gene.

[417] In some embodiments, the Cas protein domain has nickase activity. In some embodiments, the Cas protein domain is a Cas9. In some embodiments, the Cas9 comprises a

mutation in an HNH domain. In some embodiments, the Cas9 comprises a H840A mutation in the HNH domain. In some embodiments, the Cas protein domain is a Cas12b. In some embodiments, the Cas protein domain is a Cas12a, Cas12b, Cas12c, Cas12d, Cas12e, Cas14a, Cas14b, Cas14c, Cas14d, Cas14e, Cas14f, Cas14g, Cas14h, Cas14u, or a Cas ϕ .

[418] In some embodiments, the DNA polymerase domain is a reverse transcriptase. In some embodiments, the reverse transcriptase is a retrovirus reverse transcriptase. In some embodiments, the reverse transcriptase is a Moloney murine leukemia virus (M-MLV) reverse transcriptase. In some embodiments, the DNA polymerase and the DNA binding domain are fused or linked to form a fusion protein.

Delivery

[419] Prime editing compositions described herein can be delivered to a cellular environment with any approach known in the art. Components of a prime editing composition can be delivered to a cell by the same mode or different modes. For example, in some embodiments, a prime editor can be delivered as a polypeptide or a polynucleotide (DNA or RNA) encoding the polypeptide. In some embodiments, a PEGRNA can be delivered directly as an RNA or as a DNA encoding the PEGRNA.

[420] In some embodiments, the polynucleotide encoding one or more prime editing composition components is a part of, or is encoded by, a vector. In some embodiments, the vector is a viral vector. In some embodiments, the vector is a non-viral vector. Exemplary delivery methods are shown in Table 1 below.

[421] Table 1: Exemplary delivery methods

Delivery	Vector/Mode	Delivery into Non-Dividing Cells	Duration of Expression	Genome Integration	Type of Molecule Delivered
Physical	(e.g., electroporation, particle gun, Calcium phosphate transfection)	YES	Transient	NO	Nucleic Acids and Proteins
Viral	Retrovirus	NO	Stable	YES	RNA
	Lentivirus	YES	Stable	YES/NO with modification	RNA
	Adenovirus	YES	Transient	NO	DNA
	Adeno-Associated Virus (AAV)	YES	Stable	NO	DNA
	Vaccinia Virus	YES	Very Transient	NO	DNA
	Herpes Simplex Virus	YES	Stable	NO	DNA

Delivery	Vector/Mode	Delivery into Non-Dividing Cells	Duration of Expression	Genome Integration	Type of Molecule Delivered
Non-Viral	Cationic	YES	Transient	Depends on what is delivered	Nucleic acids and Proteins
	Polymeric Nanoparticles	YES	Transient	NO	Nucleic Acids
Biological	Attenuated Bacteria	YES	Transient	NO	Nucleic Acids
Non-Viral Delivery Vehicles	Engineered Bacteriophages	YES	Transient	NO	Nucleic Acids
	Mammalian Virus-like Particles	YES	Transient	NO	Nucleic Acids
	Biological liposomes: Erythrocyte Ghosts and Exosomes	YES	Transient	NO	Nucleic Acids

[422] In some embodiments, a prime editing composition, for example, prime editor polypeptide components and PEgRNA/ngRNA are introduced to a target cell by nanoparticles. In some embodiments, the prime editor polypeptide components and the PEgRNA and/or ngRNA form a complex in the nanoparticle. Any suitable nanoparticle design can be used to deliver genome editing system components or nucleic acids encoding such components. In some embodiments, the nanoparticle is inorganic. In some embodiments, the nanoparticle is organic. In some embodiments, a prime editing composition is delivered to a target cell, *e.g.*, a hepatocyte, in an organic nanoparticle, *e.g.*, a lipid nanoparticle (LNP) or polymer nanoparticle.

[423] In some embodiments, LNPs are formulated from cationic, anionic, neutral lipids, or combinations thereof. In some embodiments, neutral lipids, such as the fusogenic phospholipid DOPE or the membrane component cholesterol, are included to enhance transfection activity and nanoparticle stability. In some embodiments, LNPs are formulated with hydrophobic lipids, hydrophilic lipids, or combinations thereof. Lipids may be formulated in a wide range of molar ratios to produce an LNP. Any lipid or combination of lipids that are known in the art can be used to produce an LNP. Exemplary lipids used to produce LNPs are provided in Table 2 below.

[424] In some embodiments, components of a prime editing composition form a complex prior to delivery to a target cell. For example, a PEgRNA can form a complex prior to delivery to the target cell. In some embodiments, a prime editing polypeptide (*e.g.*, a prime editor fusion protein) and a guide polynucleotide (*e.g.*, a PEgRNA or ngRNA) form a ribonucleoprotein (RNP) for delivery to a target cell. In some embodiments, the RNP

comprises a prime editor fusion protein in complex with a PEGRNA. RNPs may be delivered to cells using known methods, such as electroporation, nucleofection, or cationic lipid-mediated methods, or any other approaches known in the art. In some embodiments, delivery of a prime editing composition or complex to the target cell does not require the delivery of foreign DNA into the cell. In some embodiments, the RNP comprising the prime editing complex is degraded over time in the target cell. Exemplary lipids for use in nanoparticle formulations and/or gene transfer are shown in Table 2 below.

[425] Table 2: Exemplary lipids for nanoparticle formulation or gene transfer

Lipid	Abbreviation	Feature
1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine	DOPC	Helper
1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine	DOPE	Helper
Cholesterol		Helper
N41-(2,3-Dioleoyloxy)propyl-N,N,N-trimethylammonium chloride	DOTMA	Cationic
1,2-Dioleoyloxy-3-trimethylammonium-propane Diocetadecylamidoglycylspermine	DOGS	Cationic
N-(3-Aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide	GAP-DLRIE	Cationic
Cetyltrimethylammonium bromide	CTAB	Cationic
6-Lauroxyhexyl ornithinate	LHON	Cationic
1-(2,3-Dioleoyloxypropyl)-2,4,6-trimethylpyridinium	2Oc	Cationic
2,3-Dioleoyloxy-N-P(spenninecarboxamido-ethyl)-N,Ndimethyl-1-propanaminium trifluoroacetate	DOSPA	Cationic
1,2-Dioleoyl-3-trimethylammonium-propane	DOPA	Cationic
N-(2-Hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide	MDRIE	Cationic
Dimyristoxypropyl dimethyl hydroxyethyl ammonium bromide	DMRI	Cationic
3β-[N-(N', N'-Dimethylaminoethane)-carbamoyl]cholesterol	DC-Chol	Cationic
Bis-guanidium-tren-cholesterol	BGTC	Cationic
1,3-Dioleoyl-2-(6-carboxy-spermyl)-propylamide	DOSPER	Cationic
Dimethyloctadecylammonium bromide	DDAB	Cationic
Diocetadecylamidoglycylspermidin	DSL	Cationic
rac-[(2,3-Dioctadecyloxypropyl)(2-hydroxyethyl)]-dimethylammonium chloride	CLIP-1	Cationic
rac-[2(2,3-Dihexadecyloxypropyloxymethyloxy)ethyl]trimethylammonium bromide	CLIP-6	Cationic
Ethyl dimyristoylphosphatidylcholine	EDMPC	Cationic
1,2-Dioleoyloxy-N,N-dimethyl-3-aminopropane	DSDMA	Cationic
1,2-Dimyristoyl-trimethylammonium propane	DMTAP	Cationic
O,O'-Dimyristyl-N-lysyl aspartate	DMKE	Cationic
1,2-Dioleoyl-sn-glycero-3-ethylphosphocholine	DSEPC	Cationic
N-Palmitoyl D-erythro-sphingosyl carbamoyl-spermine	CCS	Cationic
N-t-Butyl-N0-tetradecyl-3-tetradecylaminopropionamidine	diC14-amidine	Cationic
Octadecenolyoxy[ethyl-2-heptadecenyl-3 hydroxyethyl]	DOTIM	Cationic

Lipid	Abbreviation	Feature
imidazolium chloride		
N1 -Cholesteryl oxycarbonyl-3,7-diazanone-1,9-diamine	CDAN	Cationic
2-(3-Bis(3-amino-propyl)-amino)propylamino)-N-ditetradecylcarbamoylme-ethyl-acetamide	RPR209120	Cationic
1,2-dilinoleyloxy-3-dimethylaminopropane	DLinDMA	Cationic
2,2-dilinoleyloxy-4-dimethylaminoethyl-[1,3]-dioxolane	DLin-KC2-DMA	Cationic
dilinoleyloxy-methyl-4-dimethylaminobutylate	DLin-MC3-DMA	Cationic

[426] Exemplary polymers for use in nanoparticle formulations and/or gene transfer are shown in Table 3 below.

[427] **Table 3: Exemplary lipids for nanoparticle formulation or gene transfer**

Polymer	Abbreviation
Poly(ethylene)glycol	PEG
Polyethylenimine	PEI
Dithiobis (succinimidyl)propionate	DSP
Dimethyl-3,3'-dithiobispropionimidate	DTBP
Poly(ethylene imine)biscarbamate	PEIC
Poly(L-lysine)	PLL
Histidine modified PLL	
Poly(N-vinylpyrrolidone)	PVP
Poly(propylenimine)	PPI
Poly(amidoamine)	PAMAM
Poly(amidoethylenimine)	SS PAEI
Triethylenetetramine	TETA
Poly(β -aminoester)	
Poly(4-hydroxy-L-proline ester)	PHP
Poly(allylamine)	
Poly(α -[4-aminobutyl]-L-glycolic acid)	PAGA
Poly(D,L-lactic-co-glycolic acid)	PLGA
Poly(N-ethyl-4-vinylpyridinium bromide)	
Poly(phosphazene)s	PPZ
Poly(phosphoester)s	PPE
Poly(phosphoramidate)s	PPA
Poly(N-2-hydroxypropylmethacrylamide)	pHPMA
Poly (2-(dimethylamino)ethyl methacrylate)	pDMAEMA
Poly(2-aminoethyl propylene phosphate)	PPE-EA
Chitosan	
Galactosylated chitosan	
N-dodacylated chitosan	
Histone	
Collagen	
Dextran-spermine	D-SPM

Pharmaceutical compositions

[428] Disclosed herein are pharmaceutical compositions comprising the full-length PEgRNA, the prime editing complex, or the LNP or RNP described herein.

[429] The term “pharmaceutical composition”, as used herein, refers to a composition formulated for pharmaceutical use. In some embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition comprises additional agents, *e.g.*, for specific delivery, increasing half-life, or other therapeutic compounds.

[430] In some embodiments, a pharmaceutically-acceptable carrier comprises any vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (*e.g.*, lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the compound from one site (*e.g.*, the delivery site) of the body, to another site (*e.g.*, organ, tissue or portion of the body). A pharmaceutically acceptable carrier is “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the tissue of the subject (*e.g.*, physiologically compatible, sterile, physiologic pH, etc.)

[431] Formulations of the pharmaceutical compositions described herein can be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient(s) into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, shaping and/or packaging the product into a desired single- or multi-dose unit. Pharmaceutical formulations can additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired.

Methods of Editing

[432] The methods and compositions disclosed herein can be used to edit a target gene of interest by prime editing.

[433] In some embodiments, the prime editing method comprises contacting a target gene, with a PEGRNA prepared by the methods described herein and a prime editor (PE) comprising a DNA binding domain and a DNA polymerase domain, wherein the PEGRNA directs the prime editor to incorporate the intended nucleotide edit in the gene, thereby editing the gene.

[434] In some embodiments, a method for editing a gene comprises contacting the gene with the prime editing complex described herein, wherein the PEGRNA directs the prime editor to incorporate the intended nucleotide edit in the gene, thereby editing the gene.

[435] In some embodiments, the editing efficiency is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.9% equivalent as compared to editing with an unmodified control PEgRNA.

[436] In some embodiments, the prime editor synthesizes a single stranded DNA encoded by the editing template, wherein the single stranded DNA replaces the editing target sequence and results in incorporation of the intended nucleotide edit into a region corresponding to the editing target sequence.

[437] In some embodiments, the subject is a human.

[438] In some embodiments, the method further comprising administering the cell to the subject after incorporation of the intended nucleotide edit.

EXAMPLES

[439] EXAMPLE 1 – Split Synthesis of Long RNAs

[440] The CRISPR-Cas9 gene editing technology is a powerful and simple tool that has been widely used in fundamental biological studies and therapeutics development. The therapeutic potential of gene editing technology is significant, and the second-generation base editing and prime editing technological advancements triggered further interest in the field. However, there are still some technical challenges that limit the rapid growth of gene editing technology in the therapeutic areas. One of the major challenges is the production of high-quality guide RNAs ($\geq 90\%$ purity).

[441] In general, the synthesis of RNA/DNA oligonucleotides is achieved by solid phase synthesis on automated synthesizers. However, the solid phase synthesis of long RNA oligonucleotides (>100 nt length) suffers from low yield and purity of the final product. The coupling efficiency is not quantitative, thus limiting the synthesis yield for long-RNA oligonucleotides. These non-quantitative couplings generate shorter impurities, N-X (X nucleotides shorter than the full-length product (FLP; N), X= 1, 2, etc.) species. Impurities with longer length called N+X (X nucleotides longer than the FLP, X= 1, 2, etc.) species can also be generated during the coupling step. It is difficult to remove shorter and longer impurities that have close lengths compared to the FLP due to the similarity of the chemical/physical properties of the impurities and the FLP. This is particularly problematic for long RNAs > 100 -nt, because just several nucleotides shorter or longer becomes a trivial difference for long oligonucleotides (> 100 -nt length) compared to much shorter

oligonucleotides (20-nt length). Hence, alternate synthesis, and purification methods are required for long-RNA synthesis.

[442] Here we present an approach to synthesizing long RNAs, such as PEGRNAs, via split synthesis using functionalized fragments. The functionalized fragments can be joined together via enzymatic ligation or chemical conjugation.

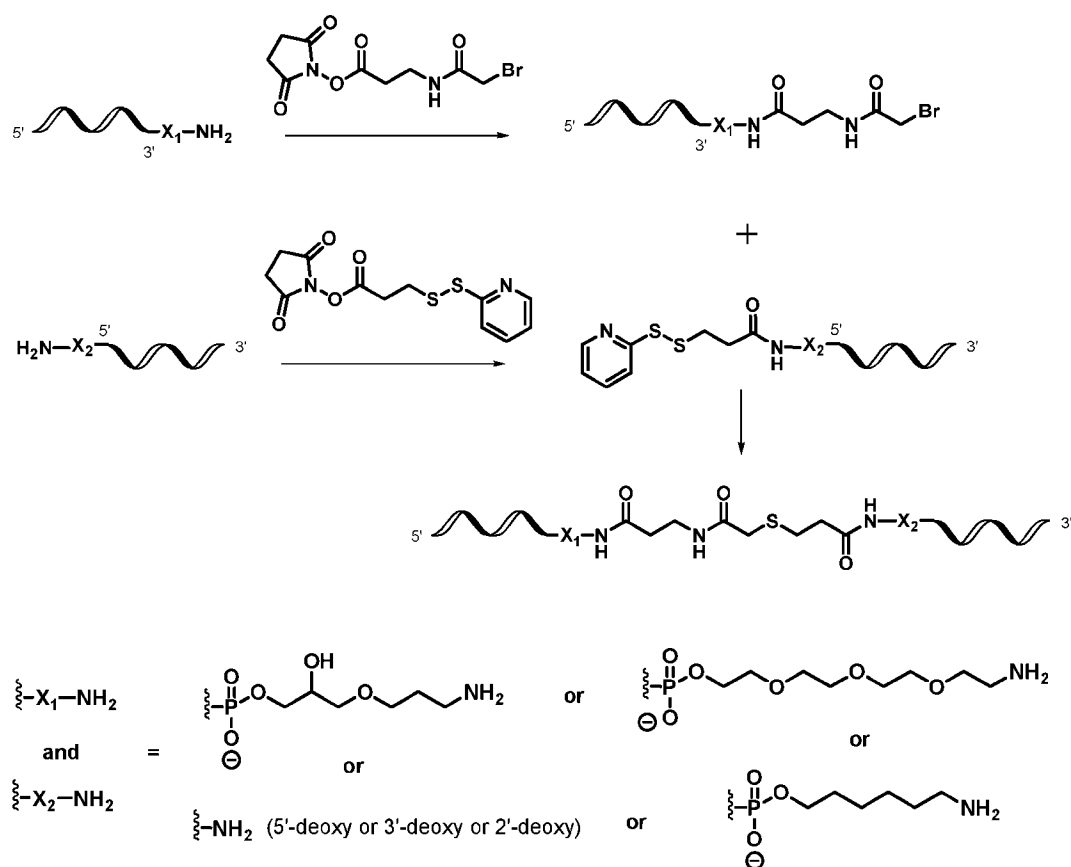
[443] *Split Site of PEGRNAs*

[444] PEGRNAs have four major components: a spacer, a gRNA core, a Reverse Transcription Template (RTT), and a Primer Binding Site (PBS), in 5' to 3' order. The gRNA core has four major components: a tetraloop (TL), a Stem Loop 1 (SL1), a Stem Loop 2 (SL2), and a Stem Loop 3 (SL3). An illustration of these major components is shown in FIGs. 1 and 2.

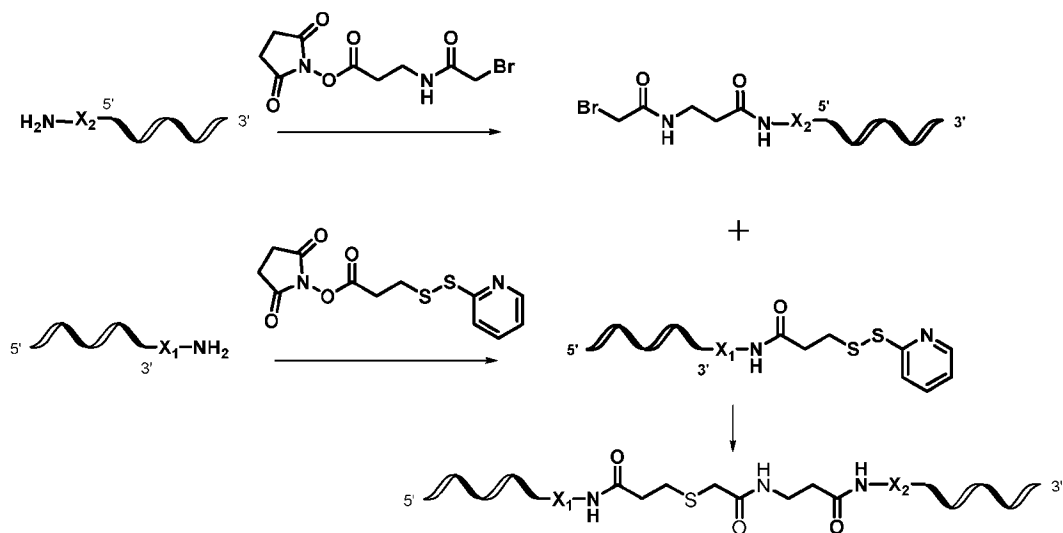
[445] An important factor to consider in long RNA split synthesis methods is choosing the appropriate split site for chemical ligations. In PEGRNAs, due to the additional chemical structure formed after chemical ligation, it is important the chemical structure avoids interfering with the (a) Cas9 nuclease interaction and (b) its activity, by disturbing the gRNA/Cas9 complex. A crystal structure analysis of gRNA/Cas9 complex has showed that the TL and SL2 in the gRNA core have minimal interactions with the Cas9 protein. Therefore, TL and SL2 are considered as the potential split sites for chemical ligations. Other potential split sites include the junction between the gRNA core and the RTT and the end of the PBS and extensions of the 3' end.

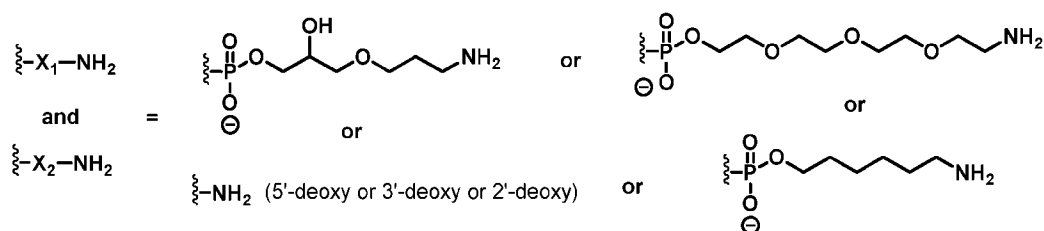
[446] EXAMPLE 2 – Split Synthesis of Long RNAs via Thioether Ligation

[447] The thioether ligation uses a thiol modified fragment to react with the bromine containing fragment to form a thioether linkage (Schemes 10 and 11). One amino-modified fragment is functionalized by bromine containing NHS ester SBAP (succinimidyl 3-(bromoacetamido)propionate), and the other amino-modified fragment is functionalized by pyridyl disulfide NHS ester SPDP (succinimidyl 3-(2-pyridyldithio)propionate). The SPDP functionalized fragment is then reduced with reducing agent TCEP (tris(2-carboxyethyl)phosphine) to generate free thiol group and react with the SBAP functionalized fragment to form the thioether linkage.



Scheme 10





Scheme 11

[448] Methods

[449] Succinimidyl 3-(bromoacetamido)propionate (SBAP) functionalization of 5' or 3' amine ends of RNA is optimized. Amino-functionalized RNA-001, Table 4 (60 nmol, 1 eq., 28.4 μL of 2.115 mM) in water is treated with freshly prepared solution of SBAP in DMF (12000 nmol) in phosphate buffer at pH 8.0 under denaturing conditions. After 2 h at room temperature, the reaction mixture is quenched with 1 M Tris.HCl and the mixture is desalted using a SephadexTM G25 DNA grade Nap-25 column as per the manufacture's recommendations. The resulting desalted bromo product is then concentrated. The product without purification is then either used immediately in a next step or stored at -20 °C until the next use.

[450] Step 2: SPDP modification

[451] Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) functionalization of 5' or 3' amine ends of RNA is optimized. Amino-functionalized RNA-002, Table 4 (60 nmol in water is treated with freshly prepared solution of SPDP in DMF/phosphate buffer at pH 8.0 under denaturing conditions. After 2 h at room temperature, the reaction mixture is quenched with 1 M Tris.HCl and the mixture is desalted using a SephadexTM G25 DNA grade Nap-25 column as per the manufacture's recommendations. The desalted RNA product is then concentrated. The concentrated disulfide product is analyzed via RP-HPLC and observed a quantitative conversion in general. The product without purification is then either used immediately in a next step or stored at -20 °C until the next use.

[452] Step 3: Thioether conjugation of SBAP and SPDP modified RNA fragments

[453] In final conjugation, disulfide-RNA-002 product is reduced to reveal the reactive thiol-RNA-002 product using tris-(2-carboxyethyl)phosphine solution (TCEP, neutral pH) and conjugated to bromo-RNA-001 product under denaturing conditions. In brief, disulfide-RNA-002 product is reduced with 50 eq. of TCEP solution at room temperature for 30 minutes. The reaction mixture is desalted using a SephadexTM G25 DNA grade Nap-5 column as per the manufacture's recommendations. After the quantification, the resultant thiol-RNA-

002 product (28.4 nmol) is mixed with bromo-RNA-001 product (31.24 nmol) and concentrated. To a solution of 100 mM phosphate buffer (pH 7), the above concentrate of bromo-RNA-001 product and thiol-RNA-002 product mixture (26.75 nmol) is added followed by the addition of DMF and allowed to react at room temperature for 2 h. The mixture is desalted using a Sephadex™ G25 DNA grade Nap-25 column as per the manufacture's recommendations. The crude products are analyzed on analytical RP-HPLC and denaturing TBE-urea PAGE analysis.

[454] Results

[455] The thioether ligation was tested using model fragment sequences RNA010 and RNA011 (Table 4). The RNA010 was treated with SBAP and the RNA011 was treated with SPDP to functionalize amino groups on each fragment. The ligation conditions were optimized for parameters like pH (6.0 – 8.0), DMF solvent volume percentage (0-50%), without pre-annealing.

[456] The ligation reaction products were analyzed by denaturing PAGE (Polyacrylamide Gel Electrophoresis), FIG. 6. The ligated (FLP) yield is quantified as 65% based on the band intensity on the gel image. The ligation reaction was also verified by RP-HPLC (FIG. 7) and LC-MS (FIG. 8).

[457] After the thioether ligation conditions were optimized and verified by the model fragment sequences, this conjugation was used for split synthesis of a full-length PEGRNAL1 (Table 5), which provided two products PEGRNAL1001 and PEGRNAL1005 (Table 5).

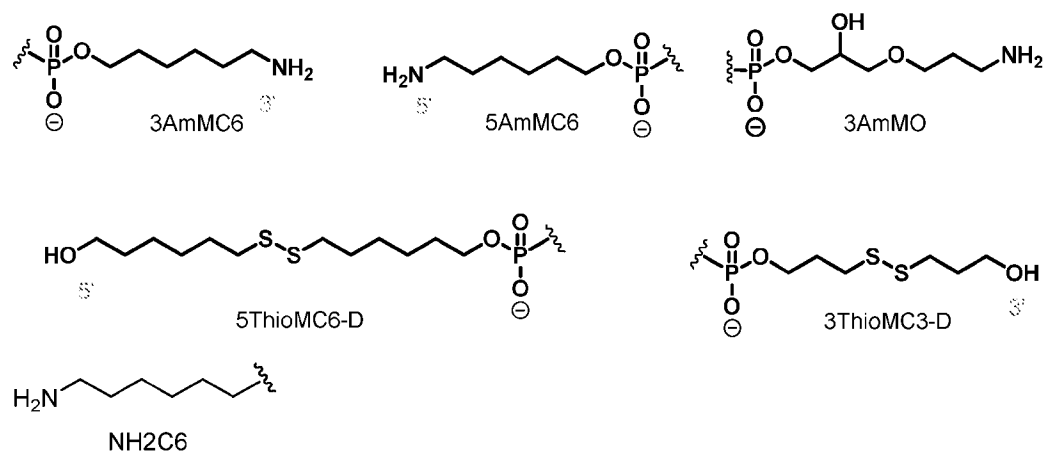
[458] PEGRNAL1001 was synthesized with a thioether linkage in the TL using SBAP functionalized RNA001 and SPDP functionalized RNA002. PEGRNAL1005 was synthesized with a thioether linkage in the SL2 using SPDP functionalized RNA003 and SBAP functionalized RNA004. The ligated products, PEGRNAL1001 and PEGRNAL1005, were purified by reverse phase HPLC and analyzed by PAGE (FIG. 9A and FIG. 10A, respectively) and RP-HPLC (FIG. 9B and FIG. 10B, respectively). PEGRNAL1005 purified product was also verified by LC-MS (FIG. 11).

[459] Table 4. RNA fragments

SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>Sequences for 2-part split synthesis</i>			
1	RNA001	34	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC UAGA/3AmMO/
2	RNA002	92	/5AmMC6/AAUAGCAAGUUAAAAUAAGGCUAGUCCG UUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU UGCCCUUGGAAGGUGGCUGGAACACmU*mU*mU*U
3	RNA003	74	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC UAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUA UCAACUUGA/3AmMO/
4	RNA004	52	/5AmMC6/AAAAGUGGCACCGAGUCGGUGCUUGCCCU GGGAAGGUGGCUGGAACACmU*mU*mU*U
5	RNA005	45	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC UAGCAGCAAGCACGA/3AmMO/
6	RNA006	108	/5AmMC6/AGUGCUUGCUGCUAGCAAGUUAAAAUA AGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACC GAGUCGGUGCAUUGCCCUGGGGCGGUGGCUGGAAC ACUUGGmU*mU*mU*U
7	RNA007	85	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC UAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUA UCAACUUGCAGCAAGCACGA/3AmMO/
8	RNA008	68	/5AmMC6/AGUGCUUGCUGCAAGUGGCACCGAGUCG GUGCAUUGCCCUGGGGCGGUGGCUGGAACACUUGG mU*mU*mU*U
9	RNA009	87	mA*mG*mA*AAGACAAUAUAGUUCUUGUUUAAGAG CGGGGAAUCCGCAAGUUUAAAUAAAGGCUAGUCCG UUAUCAGCGUGCAGCAAGCACGA/3AmMO/
10	RNA012	106	/5AmMC6/AGUGCUUGCUGCACGCGGCACCGAGUCG GUGCUUCCACCUUCUCCAAGAACUAUUAUUGUCCAU UCGCGGUUCUAUCUAGUUACGCGUUAACCAACUA GAAmU*mU*mU*mU
<i>Sequences for 3-part split synthesis</i>			
1	RNA001	34	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC UAGA/3AmMO/
4	RNA004	52	/5AmMC6/AAAAGUGGCACCGAGUCGGUGCUUGCCCU GGGAAGGUGGCUGGAACACmU*mU*mU*U
11	RNA013	45	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC UAGCAGCAAGCACGA/3AmMC6/
12	RNA014	51	/NH2C6/AAGUGCUUGCUGCUAGCAAGUUAAAAUAAG GCUAGUCCGUUAUCAACUUGA/3ThioMC3-D
13	RNA015	51	/5ThioMC6-D/ AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAA CUUGCAGCAAGCACGA/3AmMC6/
14	RNA016	63	/5AmMC6/AGUGCUUGCUGCAAGUGGCACCGAGUCG GUGCUUGCCCUGGGAAGGUGGCUGGAACACmU*mU* mU*U
15	RNA017	62	/5AmMC6/AGUGCUUGCUGCUAGCAAGUUAAAAUA AGGCUAGUCCGUUAUCAACUUGCAGCAAGCACGA/3 AmMC6/

mU = 2'-O-Me uridine; mG = 2'-O-Me guanosine; * = Phosphorothioate internucleotide

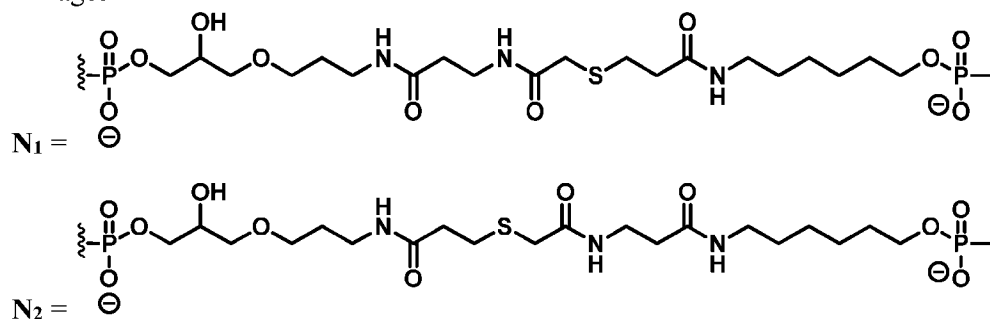
linkages



[460] Table 5. PEGRNA sequences synthesized

SEQ ID NO.:	Name	RNA Sequence (5'-3')
16	PEGRNAL1	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC UAGAAAUAGCAAGUUAAAAUAAAGGCUAGUCCGUUA UCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUGC CCUGGGAAGGUGGCUGGAACACmU*mU*mU*U
17	PEGRNAL1001	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC UAGAN ₁ AAUAGCAAGUUAAAAUAAAGGCUAGUCCGU UAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUU GCCCUGGGAAGGUGGCUGGAACACmU*mU*mU*U
18	PEGRNAL1005	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC UAGAAAUAGCAAGUUAAAAUAAAGGCUAGUCCGUUA UCAACUUGAN ₂ AAAAGUGGCACCGAGUCGGUGCUU GCCCUGGGAAGGUGGCUGGAACACmU*mU*mU*U

mU = 2'-O-Me uridine; mG = 2'-O-Me guanosine; * = Phosphorothioate internucleotide linkages



[461] EXAMPLE 3 – In-vitro Gene Editing

[462] The split-synthesized PEGRNAL1001 and PEGRNAL1005 were tested in-vitro to evaluate the editing efficiency. As shown in Table 6 and FIG. 12, the split-synthesized

PEGRNAL1001 and PEGRNAL1005 had comparable editing efficiencies to a non-split-synthesized PEGRNA (PEGRNAL1).

[463] Method

[464] WT Primary Human Hepatocytes Hu8391 were obtained from Thermo Scientific and were cultured according to the manufacturer protocols. 40 K cells were plated in 96-well plate and twenty-four later cells were transfected with Messenger Max according to the manufacturer's directions with PE2 mRNA (228 ngs), pegRNA (129.6 ngs) and ngRNA (43.2 ngs). The total Nucleic acid was combined and diluted 1.2-fold following 2.5-fold dilution respectively. Three days after transfection, gDNA was harvested in quick DNA extract for high throughput sequencing and sequenced using miseq.

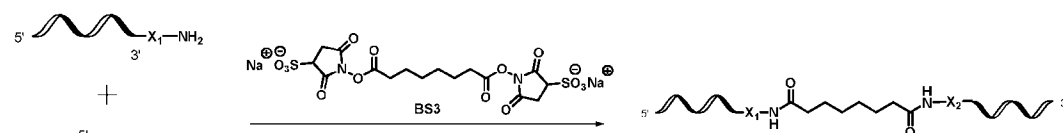
[465] Table 6. *In vitro* prime editing in primary human hepatocytes

EC50 (ngs)	PE3	PE3b	Max Edit (PE3 & PE3b) %	Max Indel (PE3 & PE3b)
PEGRNAL1	11.45	14.5	34; 32	1.7%; 1.07%
PEGRNAL1001	14.12	15.97	26; 20	1.2%; 0.8%
PEGRNAL1005	13.34	19.85	25; 31	1.7%; 0.7%

Prime editing systems, including PE3 and PE3b, are described in Anzalone, A.V., Randolph, P.B., Davis, J.R. *et al.*, "Search-and-replace genome editing without double-strand breaks or donor DNA," Nature 576, 149–157 (2019) which is incorporated herein by reference.

[466] EXAMPLE 4 – Split Synthesis of Long RNAs via Bisamide Conjugation

[467] The bisamide conjugation uses one molecule of BS3 (bis(sulfosuccinimidyl)suberate) with two NHS ester groups to link two amino modified fragments through two amide bond formations on a single BS3 molecule (Scheme 12). This conjugation requires the two fragments to be pre-annealed and the two amino groups need to be positioned in proximity before adding the BS3 reagent. In this case, the split site has an extended stem loop so that a stable duplex forms, which in turn facilitates the reaction between the amino groups of the two fragments and the BS3 reagent. It is noteworthy that in this method the semi complementarity of the two RNA fragments bring their respective reactive amines in the close proximity for a conjugation to happen and thus requiring no traditional DNA/RNA template.



Scheme 12

[468] Methods

[469] Step 1: Pre-annealing

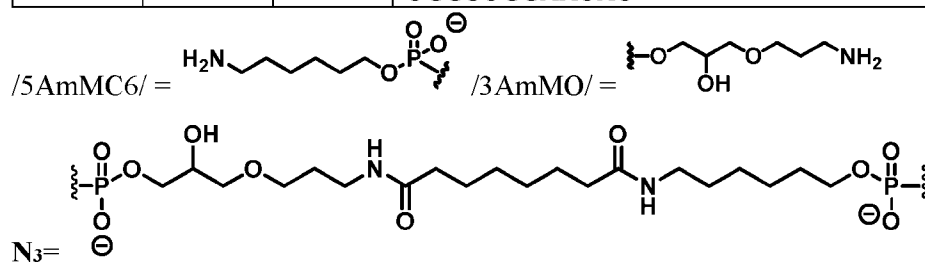
[470] Equimolar ratio of RNA011 (1.3 nmol, 3.03 μ L) and RNA010 (1.3 nmol, 3.03 μ L) each carrying a reactive amine at 5'/3'-ends in ultrapure water (40.34 μ L) with NaCl (5.2 μ L, 1.5 M) was annealed in a thermal cycler. The annealing involved a rapid heating of the above mixture to 95 $^{\circ}$ C followed by a controlled cooling (6 $^{\circ}$ C/min) to 4 $^{\circ}$ C and maintained at 4 $^{\circ}$ C for at least 5 minutes prior to the bis-amide conjugation with BS3.

[471] Step 2: BS3-mediated conjugation

[472] A Freshly prepared 10X stock solution of BS3 reagent (13 μ L, variable equivalents from 500 to 3000) was added to the above pre-annealed RNA mixture (52 μ L) after diluting with CHES (N-Cyclohexyl-2-aminoethanesulfonic acid) buffer (65 μ L), pH 9. The reaction is quenched after 10 min with 1M tris buffer (pH 7.5) to a final concentration of 50 mM. The above mixture is desalted using Nap-5 column followed by concentration with Amicon centrifugal device 0.5-mL (10 KDa cutoff). The products are analyzed by RP-HPLC and denaturing PAGE analysis.

[473] **Table 7. Model RNA fragments and the conjugation**

SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
19	RNA010	51	AAUAGCAAGUAAAAUAAGGCUAGUCCGUUAUC AACUUGCAGCAAGCACGA/3AmMO/
20	RNA011	59	/5AmMC6/AAGUGCUUGCUGCAAGUGGCACCGAGU CGGUGCUUGCCCUGGGAAGGUGGCUGGAACAC
21	RNAL1	100	AAUAGCAAGUAAAAUAAGGCUAGUCCGUUAUC AACUUGCAGCAAGCACGAN ₃ AAGUGCUUGCUGCA AGUGGCACCGAGUCGGUGCUUGCCCUGGGAAGG UGGCUGGAACAC



[474] Results

[475] The conjugation was analyzed by PAGE (FIG. 13). The yield of the conjugated product (RNAL1) was up to 70% based on the band intensity on the gel image. The ligation reaction was also verified by RP-HPLC (FIG. 14) and LC-MS (FIG. 15).

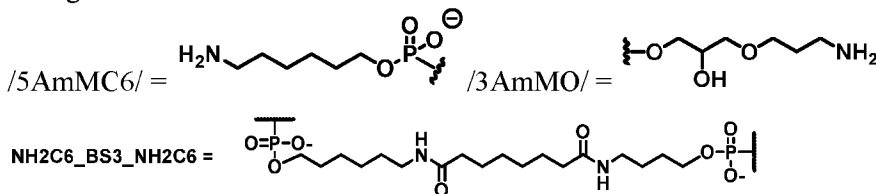
[476] **EXAMPLE 4.1 – Split Synthesis of Long RNAs via Bisamide Conjugation**

[477] Using similar conjugation to the one described in Example 4, the following 153-nt long PEgRNAs were synthesized (Table 7.1). The ligation was performed at either the tetraloop (TL) or SL2, where each had an 11-bp extension at their respective stem structures.

[478] **Table 7.1. RNA fragments and the conjugation**

SEQ ID NO.	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments</i>			
60	RNA018	45	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUU AGAGCUAGCAGCAAGCACGA/3AmMC6/
61	RNA019	108	/5AmMC6/AAGUGCUUGCUGCUAGCAAGUUA AAUAAGGCUAGUCCGUUAUCAACUUGAAAA AGUGGCACCGAGUCGGUGCNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
62	RNA020	85	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUU AGAGCUAGAAAUAGCAAGUUAUAAUAAAGGC UAGUCCGUUAUCAACUUGCAGCAAGCACGA/3 AmMC6/
63	RNA021	68	/5AmMC6/AAGUGCUUGCUGCAAGUGGCACCG AGUCGGUGCNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNmU*mU*mU*U
<i>Ligated PEgRNAs</i>			
64	RNA022 (RNA018+ RNA019)	153	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUU AGAGCUAGCAGCAAGCACGA(NH2C6)_BS3_(N H2C6)AAGUGCUUGCUGCUAGCAAGUUAUAA UAAGGCUAGUCCGUUAUCAACUUGAAAAAG UGGCACCGAGUCGGUGCNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
65	RNA023 (RNA020 + RNA021)	153	mU*mU*mG*CCAAGUGUCCAGCCACGUUUU AGAGCUAGAAAUAGCAAGUUAUAAUAAAGGC UAGUCCGUUAUCAACUUGCAGCAAGCACGA(NH2C6)_BS3_(NH2C6)AAGUGCUUGCUGCAAG UGGCACCGAGUCGGUGCNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNmU*mU*mU*U

mU = 2'-O-Me uridine; mG = 2'-O-Me guanosine; * = Phosphorothioate internucleotide linkages



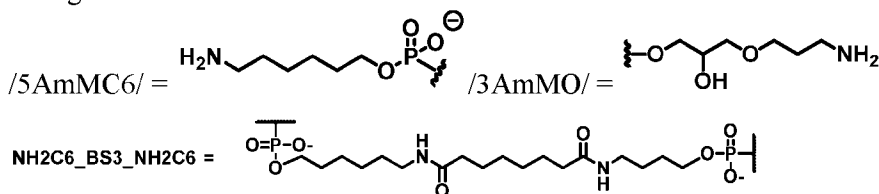
[479] EXAMPLE 4.2 – Split Synthesis of Long RNAs via Bisamide Conjugation

[480] To further demonstrate the value of ligation chemistries for building long RNAs, we synthesized a longer 193-nt long PEGRNA. SL2 ligation was appropriate to synthesize the target with almost similar length 5'- and 3'-split fragments. The SL2 had an 11-bp extension at the stem structure. The bisamide ligated PEGRNA and the corresponding fragments for SL2 ligation are shown in Table 7.2.

[481] Table 7.2. RNA fragments and the conjugation

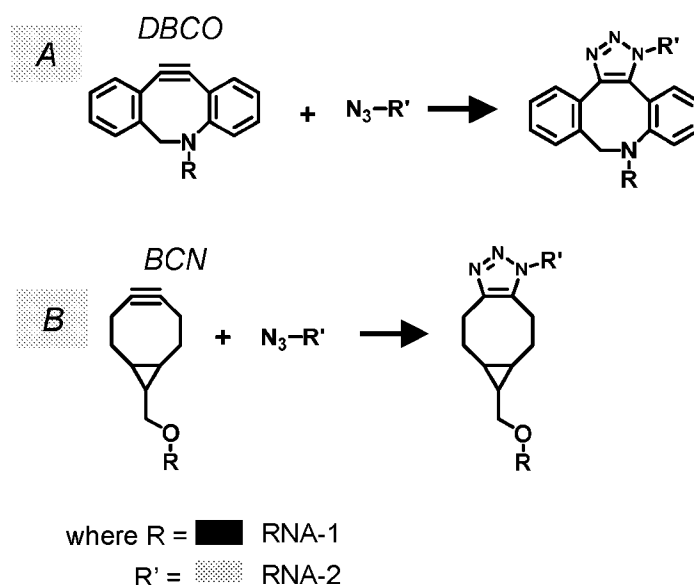
[illegible]

mU = 2'-O-Me uridine; mG = 2'-O-Me guanosine; * = Phosphorothioate internucleotide linkages



[482] EXAMPLE 5 – Strain-promoted copper-free azide and alkyne cycloaddition reaction (SPAAC)

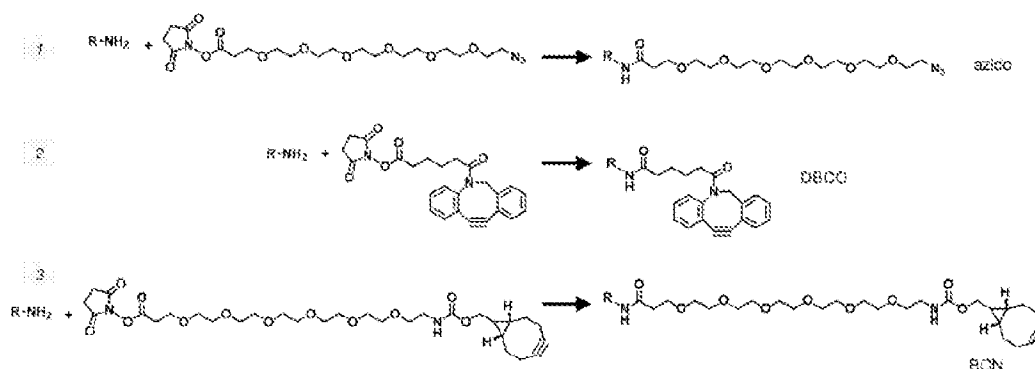
[483] This strategy involves the cycloaddition reaction between two RNA sequences carrying an azide and alkyne functional groups at the respective ends. The reaction is driven by the ring strain of alkyne functional group in the cyclic ring instead of traditional alkyne activation by copper. We developed two chemical ligation strategies, wherein azide is either partnered with A) DBCO-alkyne or B) BCN-alkyne (Scheme 13). A splint was used to bring the both the reactive functional groups in the proximity for a successful ligation reaction.



Scheme 13

[484] Functionalization of Amine-RNAs with azide and alkynes

[485] The amine functionalized RNAs were treated with the activated NHS-esters of azide or alkyne partners (Scheme 14). Additionally, azide functional groups can be introduced on the RNAs on the nucleotides directly at the 5'-end of the last nucleotide in the position of 5'-OH or as a modification on the purine or pyrimidine bases without requiring using the amine handle and NHS-esters.



Scheme 14

[486] Methods**[487]** *Azido modification of amino-functionalized RNA oligonucleotides*

[488] The input for this process is azide-functionalized rOLI001 and alkyne-functionalized rOLI019 in nuclease-free water. rOLI001 with a 3' amino-end (typically at a concentration of

10 μ M – 50 μ M, 1 equiv.) was modified with azido-PEG6-NHS ester (200 equiv.) for 2 h at room temperature at pH 8.0 under denaturing conditions (40/60 v/v% DMF and 50 mM phosphate buffer pH 8.0), as shown in FIG. 23. After 2 h, the reaction was quenched with tris (~1,000 equiv., as a solution of 1 M Tris HCl pH 7.5). This reaction was then desalted using Cytiva NAPTM columns to remove residual azido-PEG6-NHS ester from rOLI001. Following desalting, the azido-modified rOLI001 was analyzed by RP-HPLC to confirm modification, and then used in the cycloaddition reaction with DBCO-modified rOLI019 in presence of OLI231 under heat annealing conditions.

[489] *DBCO modification of amino-functionalized RNA oligonucleotides*

[490] The input for this process is azide-functionalized rOLI001 and alkyne-functionalized rOLI019 in UltraPureTM water. rOLI019 with a 5' amino-end (typically at a concentration of 10 μ M – 50 μ M, 1 equiv.) was modified with DBCO-C6-NHS ester (200 equiv.) for 2 h at room temperature at pH 8.0 under denaturing conditions (40/60 v/v% DMF and 50 mM phosphate buffer pH 8.0), as shown in FIG. 23. After 2 h, the reaction was quenched with tris (~1,000 equiv., as a solution of 1 M Tris HCl pH 7.5). This reaction was then desalted using Cytiva NAPTM columns to remove residual DBCO-C6-NHS ester from rOLI019. Following desalting, the DBCO-modified rOLI019 was analyzed by RP-HPLC to confirm modification. This DBCO-modified rOLI019 was annealed to azido-modified rOLI001 and OLI231 and then ligated to RNA-X in the reaction buffer as shown in FIG. 23.

[491] *2-part ligation of RNA oligonucleotides at the native TL site using SPAAC*

[492] The input for this process is azido-rOLI001 (containing a 3' azido), alkyne-rOLI019 (containing 5' DBCO), and OLI231 (splint oligonucleotide with complementarity to TL ligation site). azido-rOLI001 and alkyne-rOLI019 were annealed (at a concentration of 20-50 μ M per RNA, 1 equiv. azido-rOLI001 and 1 equiv. alkyne-rOLI019), with sodium chloride (150 mM) and a bridging oligonucleotide, OLI231, (1:1:1 equiv. for azido-rOLI001: alkyne-rOLI019:OLI231), as shown in Table 8. azido-rOLI001 and alkyne-rOLI019 were then combined (at a concentration of 10 μ M per RNA, 1 equiv.) and the reaction proceeded for 2 h at room temperature at pH 7.5 under aqueous conditions (20 mM HEPES buffer pH 7.5, 5 mM EDTA, 158 mM NaCl). This reaction was then desalted using Cytiva NAPTM columns to remove residual small molecule from RNA. Following desalting, the RNA was analyzed by TBE-Urea and RP-HPLC to confirm ligation and purified the ligated RNA. The fragment and ligated sequences are shown in Table 9.

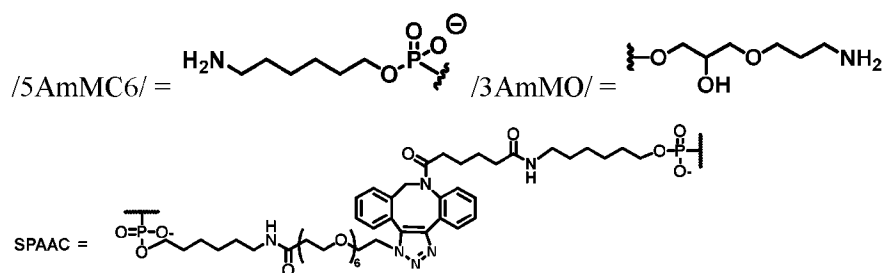
[493] Table 8

Ligation Name	RNA 1	RNA 2	Splint DNA	Conversion
SPAAC-1	rOLI020-DBCO	azido-rOLI007		3%
SPAAC-2	rOLI020-DBCO	azido-rOLI007	OLI232	40%
SPAAC-3	rOLI020-azido	DBCO-rOLI007		2%
SPAAC-4	rOLI020-azido	DBCO-rOLI007	OLI232	30%

[494] Table 9

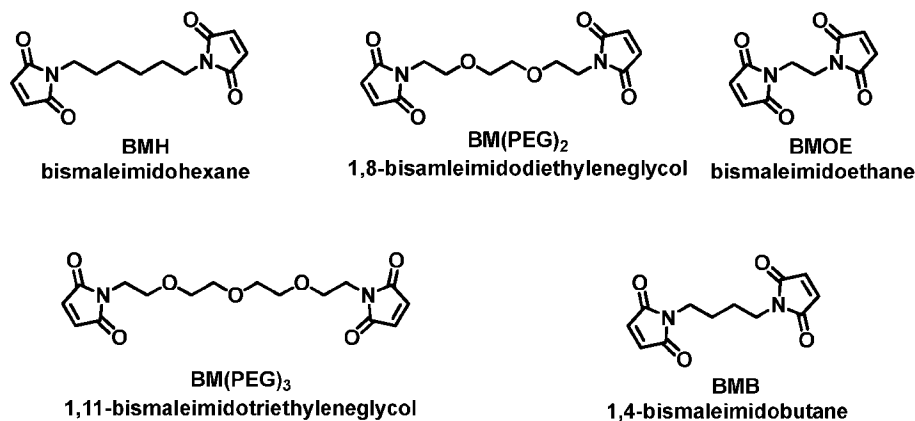
SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments and splints</i>			
69	rOLI001	34	mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUA GAGCUAGA/3AmMO/
70	rOLI019		/5AmMC6/AAUAGCAAGUUA AAAUAAGGCUAGU CCGUUAUCAACUUGA
71	OLI231	30	mUmUmUmU-d(AACTTGCTATTTCTAGCTCT)- mAmAmAmAmCmG
<i>Ligated PEGRNAs</i>			
72	rOLI001 _SPAAC_ rOLI019		mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUA GAGCUAGA SPAAC AAUAGCAAGUUA AAAUA GGCUAGUCCGUUAUCAACUUGA

mN= 2'-O-Me ribonucleotide; * = Phosphorothioate internucleotide linkages



[495] EXAMPLE 6 – Bismaleimide ligation with native stem loops using a splint

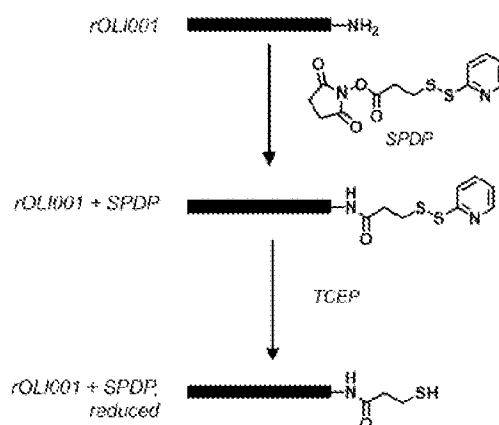
[496] Two thiol containing RNAs are ligated together using BM_X reagent, where _X is a variable in length and nature of the linker itself as shown in Scheme 15.



Scheme 15

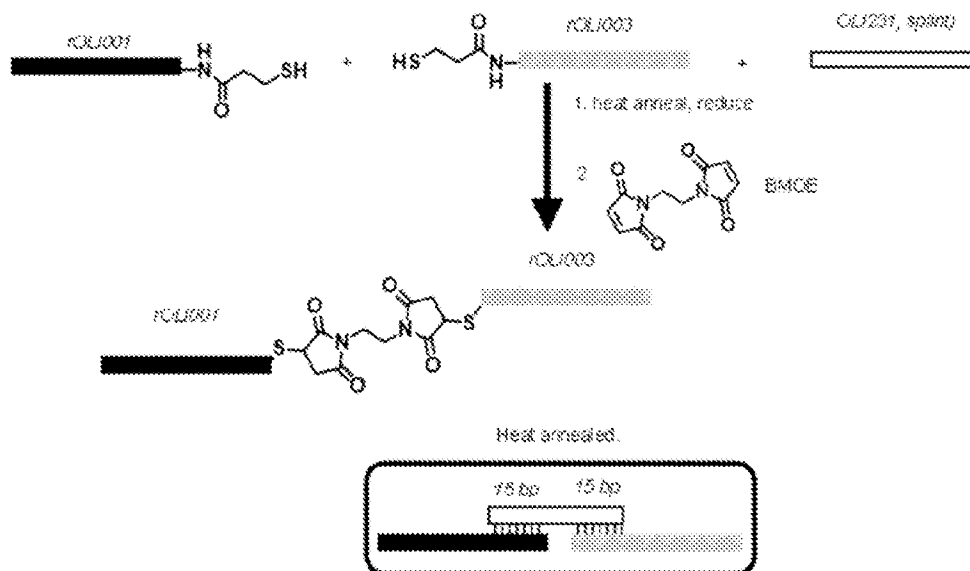
[497] Thiolation of amine-RNAs:

[498] Amine functionalized RNAs are treated with succinimidyl 3-(2-pyridyldithio)propionate, SPDP, reagent followed by TCEP-mediated reduction to produce the thiolated RNA (Scheme 16).



Scheme 16

[499] The desired RNAs with two thiol groups at respective ends are ligated by heat annealing of the two RNA strands in the presence of a splint, TCEP reduction, followed by the addition of bismaleimide linker, as exemplified below with BMOE (Scheme 17).



Scheme 17

[500] Methods

[501] *SPDP modification of amino-functionalized RNA oligonucleotides and BMX ligations*

[502] Amino-functionalized RNAs (rOLI001/rOLI003/rOLI005/rOLI007, at a concentration of 10 μ M – 50 μ M, 1 equiv.) were modified with SPDP (160 equiv.) for 2 h at room temperature at pH 8.0 under denaturing conditions (40/60 v/v% DMF and 50 mM phosphate buffer pH 8.0). After 2 h, the reaction was quenched with tris (~1,000 equiv., as a solution of 1 M Tris HCl pH 7.5). This reaction was then desalted using Cytiva NAPTM columns to remove residual SPDP from RNAs. Following desalting, the crude was analyzed by RP-HPLC to confirm modification followed by concentration. This SPDP-modified RNA was annealed to another complimentary SPDP-modified RNA in presence of a splint oligonucleotide (OLI231 for TL ligation and OLI232 for SL2 ligation), reduced, and then ligated to form a longer RNAs using BMX reagents.

[503] *Exemplary ligation between rOLI001 and rOLI003 using BMH (BMX) reagent*

[504] The input for this process is rOLI001 (containing a 3' protected thiol), rOLI003 (containing 5' protected thiol), and OLI231 (complementarity to TL ligation site). rOLI001 and rOLI003 were annealed (at a concentration of 20-50 μ M per RNA, 1 equiv. rOLI001 and 1 equiv. rOLI003) in presence of sodium chloride (150 mM) and a bridging oligonucleotide, OLI231, (1:1:1 equiv. for rOLI001:rOLI003:OLI231). rOLI001 and rOLI003 were then reduced using TCEP to generate thiol moieties. BMH (250 equiv.) was then added to the desalted, reduced rOLI001 and rOLI003 (typically at a concentration of 10 μ M per RNA, 1 equiv.) and the reaction proceeded for 2 h at room temperature at pH 6.5 under aqueous conditions (50 mM phosphate buffer pH 6.5, 2.5% DMF). This reaction was then desalted using Cytiva NAPTM columns to remove residual small molecule from RNA. The crudes were purified by a RP-HPLC followed by the concentration of the appropriately pooled fractions and sodium ion-exchange yielding the pure ligated RNAs.

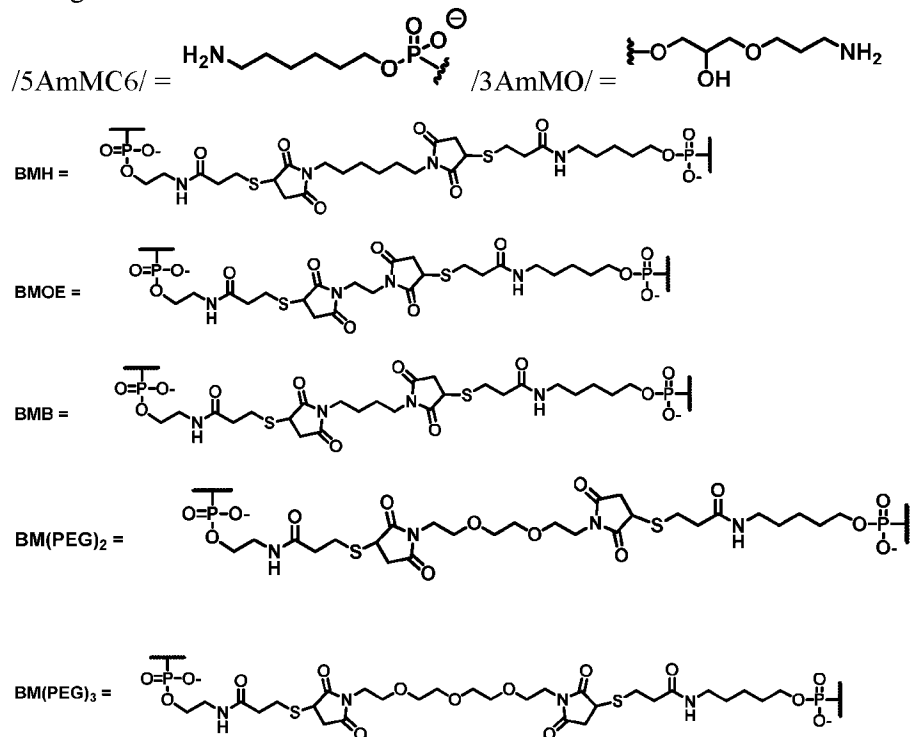
[505] Table 10 shows the sequences utilized for these ligations and the resulting PEGRNAs.

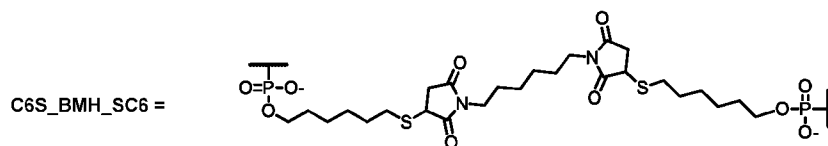
[506] Table 10.

SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments and splints</i>			
69	rOLI001	34	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUA GAGCUAGA/3AmMO/
73	rOLI003	92	/5AmMC6/AAUAGCAAGUUA AAAUAAGGCUAGU CCGUUAUCAACUUGAAAAAGUGGCACCGAGUC GGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNN mU*mU*mU*U
74	rOLI005	74	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUA GAGCUAGAAAUAGCAAGUUA AAAUAAGGCUA GUCCGUUAUCAACUUGA/3AmMO/
75	rOLI007	52	/5AmMC6/AAAAGUGGCACCGAGUCGGUGCNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU* U
71	OLI231 (Splint)	30	mUmUmUmUm-d(AACTTGCTATTTCTAGCTCT)- AmAmAmAmCmG
76	OLI232 (Splint)	30	mCmUmCmGmG-d(TGCCACTTTTCAAGTTGAT)- mAmAmCmGmG
<i>Ligated PEgRNAs</i>			
77	rOLI001_B MH_rOLI0 03		mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUA GAGCUAGA_ BMH _AAUAGCAAGUUA AAAUA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGC ACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNN NNNNNNNNmU*mU*mU*U
78	rOLI001_B MOE_rOL I003		mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUA GAGCUAGA_ BMOE _AAUAGCAAGUUA AAAUA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGC ACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNN NNNNNNNNmU*mU*mU*U
79	rOLI001_B MB_rOLI0 03		mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUA GAGCUAGA_ BMB _AAUAGCAAGUUA AAAUAAG GCUAGUCCGUUAUCAACUUGAAAAAGUGGCAC CGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNN NNNNNNNmU*mU*mU*U
80	rOLI001_B M(PEG)2_ rOLI003		mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUA GAGCUAGA_ BM(PEG)2 _AAUAGCAAGUUA AAAA UAAGGCUAGUCCGUUAUCAACUUGAAAAAGU GGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNN NNNNNNNNNNNmU*mU*mU*U
81	rOLI001_B M(PEG)3_ rOLI003		mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUA GAGCUAGA_ BM(PEG)3 _AAUAGCAAGUUA AAAA UAAGGCUAGUCCGUUAUCAACUUGAAAAAGU GGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNN NNNNNNNNNNNmU*mU*mU*U
82	rOLI005_B MH_rOLI0 07		mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUA GAGCUAGAAAUAGCAAGUUA AAAUAAGGCUA GUCCGUUAUCAACUUGA_ BMH _AAAAGUGGCA CCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNN NNNNNNNmU*mU*mU*U
83	rOLI005_B MOE_rOL I007		mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUA GAGCUAGAAAUAGCAAGUUA AAAUAAGGCUA GUCCGUUAUCAACUUGA_ BMOE _AAAAGUGGC

[illegible]

mN = 2'-O-Me ribonucleotide; mU = 2'-O-Me uridine; * = Phosphorothioate internucleotide linkages





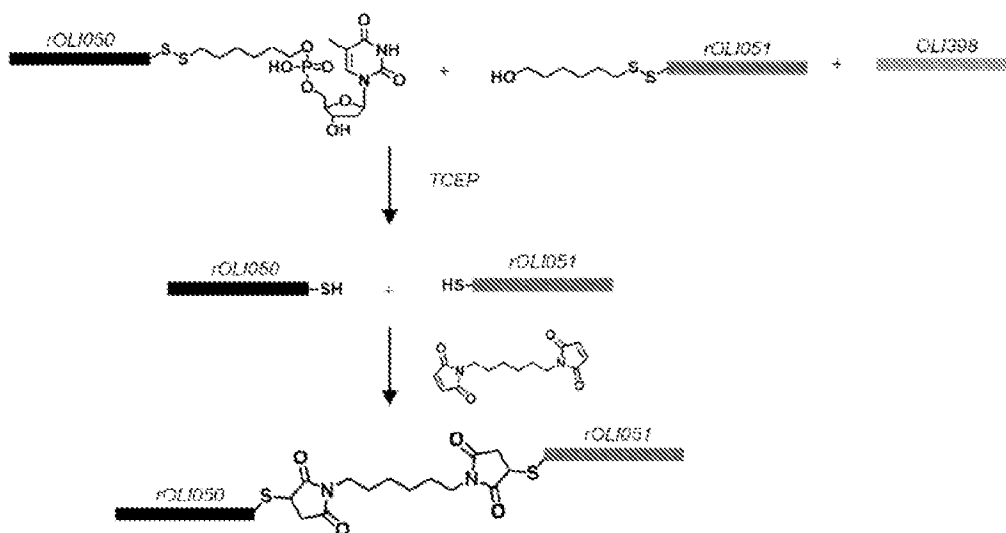
[507] EXAMPLE 7 – BMH ligation between SL3 and RTT

[508] EXAMPLE 7.1 - Two-part ligations

[509] In here, PEgRNAs were ligated between SL3 and RTT, which in turn can help achieve a longer sequence of about ≥ 200 -nt via two-part ligation. The ligation chemistry is similar to the ligation chemistry described in Example 6 herein, where both RNAs are functionalized to have reactive thiol functional groups followed by the reaction with bismaleimide reagent, as exemplified in Scheme 18 with BMH reagent. The splint OLI398 brings the reactive thiols of both RNAs in the reacting distance with BMH reagent. We also tested the BMH ligation efficiency with and without a 4-nt extension at the end-of-SL3, as part of 2-part and 3-part ligation (Example 7.2 below) approaches as shown in Table 11.

[510] Table 11.

Ligation	SL3 nt extension	Split at	5'-fragment	Middle fragment	3'-fragment	Chemistry	Splint
2-part	0	end of SL3 and between RTT	rOLI050	---	rOLI051	BMH	OLI398
3-part	0	TL and end of SL3 and between RTT	rOLI052	rOLI053	rOLI051	BS3 & BMH	OLI398
2-part	4	end of SL3 and between RTT	rOLI054	---	rOLI051	BMH	OLI399
3-part	4	TL and end of SL3 and between RTT	rOLI052	rOLI055	rOLI051	BS3 & BMH	OLI399



Scheme 18

[511] Methods

[512] The sequences used for this ligation are shown in Table 12.

[513] *Two-part ligation of guide RNA oligonucleotides at the end of SL3*

[514] The input for this process is rOLI050 (containing a 3' protected thiol), rOLI051 (containing 5' protected thiol), and OLI230 (complementarity to SL3 ligation site). rOLI050 and rOLI051 were annealed (typically at a concentration of 20-50 μM per RNA, 1 equiv. rOLI050 and 2 equiv. rOLI051) in presence of sodium chloride (150 mM) and a bridging oligonucleotide, OLI230, (1:2:1 equiv. for rOLI050:rOLI051:OLI230). rOLI050 and rOLI051 were then reduced using TCEP to generate thiol moieties. BMH (250 equiv.) was then added to the desalted, reduced rOLI050 and rOLI051 (at a concentration of 10 μM per RNA, 1 equiv.) and the reaction proceeded for 2 h at room temperature at pH 6.5 under aqueous conditions (50 mM phosphate buffer pH 6.5, 2.5% DMF). This reaction was then desalted using Cytiva NAPTM columns to remove residual small molecule from RNA. The crudes were purified by a RP-HPLC followed by the concentration of the appropriately pooled fractions and sodium ion-exchange yielding the pure ligated RNAs.

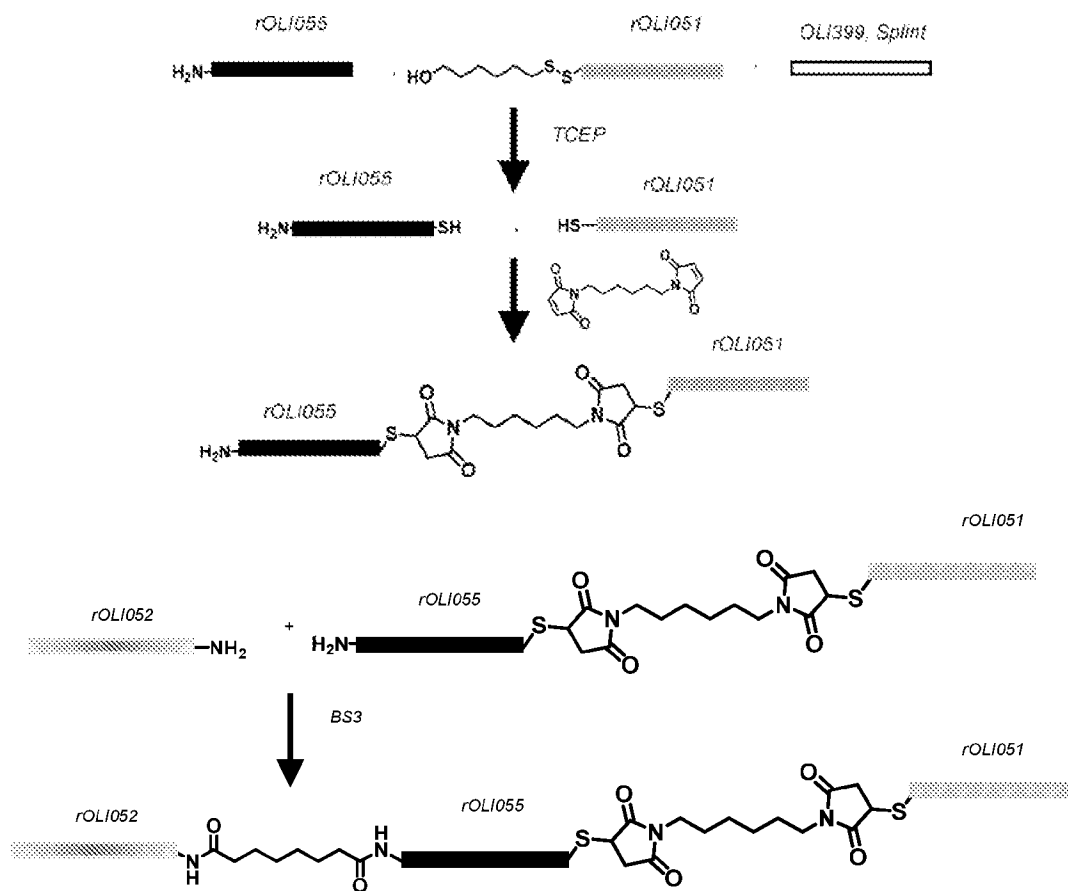
[515] *Two-part ligation of guide RNA oligonucleotides at the end of SL3 with four nucleotide extension*

[516] The input for this process is rOLI054 (containing a 3' protected thiol and 4 nt extension), rOLI051 (containing 5' protected thiol), and a bridging oligonucleotide at the ligation site, OLI399. rOLI054 and rOLI051 were annealed (at a concentration of 20-50 μM per RNA, 1 equiv. rOLI054 and 2 equiv. rOLI051) with sodium chloride (150 mM) and a

bridging oligonucleotide, OLI399, (1:2:1 equiv. for rOLI054:rOLI051:OLI399). rOLI054 and rOLI051 were then reduced using TCEP to generate thiol moieties. BMH (250 equiv.) was then added to the desalted, reduced rOLI054 and rOLI051 (at a concentration of 10 μ M per RNA, 1 equiv.) and the reaction proceeded for 2 h at room temperature at pH 6.5 under aqueous conditions (50 mM phosphate buffer pH 6.5, 2.5% DMF). This reaction was then desalted using Cytiva NAPTM columns to remove residual small molecule from RNA. The crudes were purified by a RP-HPLC followed by the concentration of the appropriately pooled fractions and sodium ion-exchange yielding the pure ligated RNAs.

[517] EXAMPLE 7.1 - Three-part ligations

[518] We also tested the BMH ligation efficiency with a 3-part ligation. An exemplary scheme of this ligation is shown in Scheme 19. The sequences used for this ligation are shown in Table 12.



Scheme 19

[519] Methods

[520] *3-part ligation of guide RNA oligonucleotides at TL with extension and at the end of SL3*

[521] The input for this process is rOLI052 with a 3'-amino-end, rOLI053 with 5'-amino-end and 3' protected thiol, and rOLI0051 with a 5' protected thiol). rOLI052, rOLI053 and rOLI051 were annealed (at a concentration of 20-50 μ M per RNA, 1 equiv.) with sodium chloride (150 mM) and a bridging oligonucleotide, OLI231, (1 equiv. for rOLI052:rOLI053:rOLI051:OLI231). rOLI053 and rOLI051 were then reduced using TCEP to generate thiol moieties. BMH (250 equiv.) was then added to the desalted, reduced rOLI053 and rOLI051 (at a concentration of 10 μ M per RNA, 1 equiv.) and the reaction proceeded for 2 h at room temperature at pH 6.5 under aqueous conditions (50 mM phosphate buffer pH 6.5, 2.5% DMF). This reaction was then desalted to remove excess reagents, concentrated, and buffer exchanged into 50 mM CHES pH 9.0. The annealed RNA complex (at a concentration of 10 μ M per RNA, 1 equiv.) was reacted with BS3 (500 equiv.) for 1 h at room temperature. After 1 h, the reaction was quenched with tris (as a solution of 1 M Tris HCl pH 7.5, ~1,000 equiv.). This reaction was then desalted using Cytiva NAPTM columns to remove residual small molecule from RNA. The crudes were purified by a RP-HPLC followed by the concentration of the appropriately pooled fractions and sodium ion-exchange yielding the pure ligated RNAs.

[522] *3-part ligation of guide RNA oligonucleotides at TL extension and at the end of SL3 with four nucleotide extension*

[523] The input for this process is rOLI052 (containing a 3' amino and 3' 11-mer extension at the TL site), rOLI055 (containing 5' amino and 3' protected thiol, a 5' 11-mer extension at the TL site, and a 3' 4-mer extension at the end of the SL3), and rOLI051 (containing 5' protected thiol). rOLI052, rOLI055 and rOLI051 were annealed (at a concentration of 20-50 μ M per RNA, 1 equiv.) with sodium chloride (150 mM) and a bridging oligonucleotide, OLI399, (1 equiv. for rOLI052:rOLI055:rOLI051:OLI399). rOLI055 and rOLI051 were then reduced using TCEP to generate thiol moieties. BMH (250 equiv.) was then added to the desalted, reduced rOLI055 and rOLI051 (at a concentration of 10 μ M per RNA, 1 equiv.) and the reaction proceeds for 2 h at room temperature at pH 6.5 under aqueous conditions (50 mM phosphate buffer pH 6.5, 2.5% DMF). This reaction was then desalted using Cytiva NAPTM columns to remove residual small molecule from RNA. The solution was then concentrated, and buffer exchanged into 50 mM CHES pH 9.0. The annealed RNA (at a concentration of 10 μ M per RNA, 1 equiv.) were reacted with BS3 (500 equiv.) for 1 h at

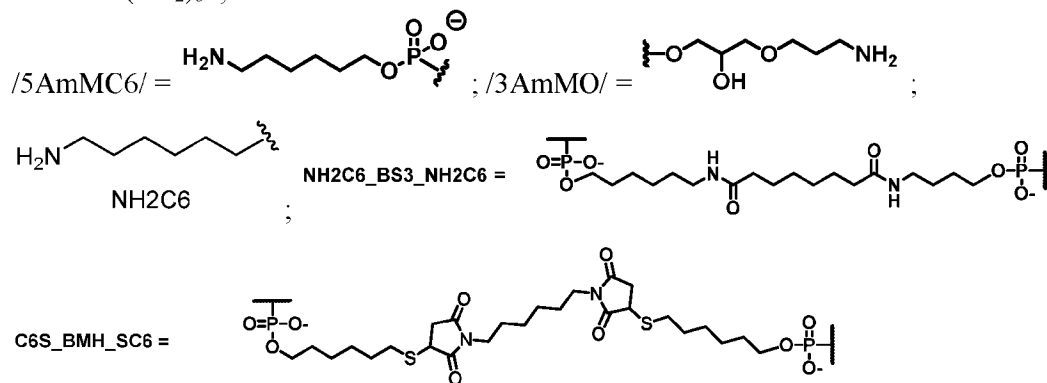
room temperature. After 1 h, the reaction was quenched with tris (as a solution of 1 M Tris HCl pH 7.5, ~1,000 equiv.). This reaction was then desalted using Cytiva NAPTM columns to remove residual small molecule from RNA. The crudes were purified by a RP-HPLC (FIG. 24) followed by the concentration of the appropriately pooled fractions and sodium ion-exchange yielding the pure ligated RNAs. The polyacrylamide gel analysis is shown in FIG. 25.

[524] Table 12.

SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments and splints</i>			
89	rOLI050	96	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC(C6SSC6)dT
90	rOLI051	30	(C6SSC6)NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
91	rOLI052	45	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUAGAGCUAGCAGCAAGCACGA(NH2C6)
92	rOLI053	73	(NH2C6)AAGUGCUUGCUGCUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC(C6SSC6)dT
93	rOLI054	100	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUCUC(C6SSC6)dT
94	rOLI055	77	(NH2C6)AAGUGCUUGCUGCUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUCUC(C6SSC6)dT
95	OLI398 (Splint)	35	d(CAGCCACCTTCCAGGGCAAGCACCGACTCGGTGC)
96	OLI399 (Splint)	39	d(CAG CCA CCT TCC CAG GGC AAG AGA GCA CCG ACT CGG TGC)
<i>Ligated PEgRNAs</i>			
97	rOLI050_BMH_rOLI051	126	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC(C6S)_BMH_(SC6)NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
98	rOLI054_BMH_rOLI051	130	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUCUC(C6S)_BMH_(SC6)NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
99	rOLI053_BMH_rOLI051	Precursor of 3-part synthesis	(NH2C6)AAGUGCUUGCUGCUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC(C6S)_BMH_(SC6)NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
100	rOLI052_BS3_rOLI05	148	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUAGAGCUAGCAGCAAGCACGA(NH2C6)_BS3_(NH2C6)AAGU

	3_BMH_r OLI051		GCUUGCUGCUAGCAAGUAAAAUAAGGCUAGUCCG UUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC(C6S)_BMH_(SC6)NNNNNNNNNNNNNNNNNNNNNN NNNmU*mU*mU*U
101	rOLI055_B MH_rOLI0 51	Precursor of 3- part synthesis	(NH2C6)AAGUGCUUGCUGCUAGCAAGUAAAAUA GGCUGAGUCCGUUAUCAACUUGAAAAAGUGGCACCG AGUCGGUGCUCUC(C6S)_BMH_(SC6)NNNNNNNNNN NNNNNNNNNNNNNNNNNmU*mU*mU*U
102	rOLI052_B S3_rOLI05 5_BMH_r OLI051	152	mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUAGAG CUAGCAGCAAGCACGA(NH2C6)_BS3_(NH2C6)AAGU GCUUGCUGCUAGCAAGUAAAAUAAGGCUAGUCCG UUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU CUC(C6S)_BMH_(SC6)NNNNNNNNNNNNNNNNNNNNNN NNNNNNmU*mU*mU*U

mN = 2'-O-Me ribonucleotide; mU = 2'-O-Me uridine; mG = 2'-O-Me guanosine; * = Phosphorothioate internucleotide linkages; C6SSC6 = -S-S(CH₂)₆OH; C6S = -(CH₂)₆S-; SC6 = -S(CH₂)₆-;



[525] EXAMPLE 8. 3-part ligations in TL and SL2

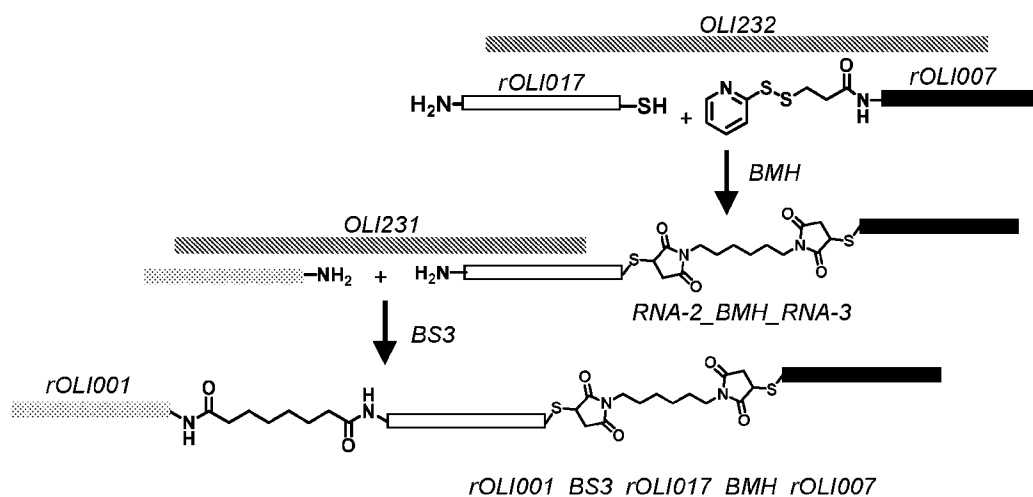
[526] We tested ligation efficiency with a 3-part ligation, where the ligation occurred at the TL and SL2. Various ligation strategies were tested (Strategies A through D) as shown in Table 13. Each ligation strategy is described in Examples 8.1 to 8.4 (Strategies A to D, respectively).

[527] Table 13. Ligation Strategies

Example	Strategy	TL	SL2	Final Product
8.1	A	native	native	rOLI001 BS3 rOLI017 BMH rOLI007
8.2	B	native	extension	rOLI001 BMH rOLI027 BS3 rOLI028
8.3	C	extension	native	rOLI009 BS3 rOLI026 BMH rOLI007
8.4	D	extension	extension	rOLI009 BS3 rOLI029 BS3 rOLI028

[528] Example 8.1 – 3-part Ligation, Strategy A

[529] Scheme 20 shows a schematic of this ligation strategy.



Scheme 20

[530] Methods

[531] 3-part ligation of TL native and SL2 native RNA oligonucleotides, Strategy A (Scheme 20)

[532] The input for this process is rOLI001 with an amine functional group at 3'-end, rOLI017 with an amine functional group at 5'-end and a thiol group 3'-end, and rOLI007 containing a 5' thiol group. rOLI017 and rOLI007 were annealed (at a concentration of 20-50 μ M per RNA, 1 equiv.) with sodium chloride (150 mM) in the presence of a bridging splint oligo, OLI232 (1 equiv. for rOLI017:rOLI007:OLI232). rOLI017 and rOLI007 were reduced using TCEP to generate thiol moieties. BMH (250 equiv.) was then added to the desalted, reduced rOLI017 and rOLI007 (at a concentration of 10 μ M, 1 equiv.) and the reaction proceeded for 2 h at room temperature at pH 6.5 under aqueous conditions (50 mM phosphate buffer pH 6.5, 2.5% DMF) to generate the BMH ligated product, rOLI017_BMH_rOLI007. This reaction was then desalted using Cytiva NAPTM followed by concentration, crude analysis and RP-HPLC purification.

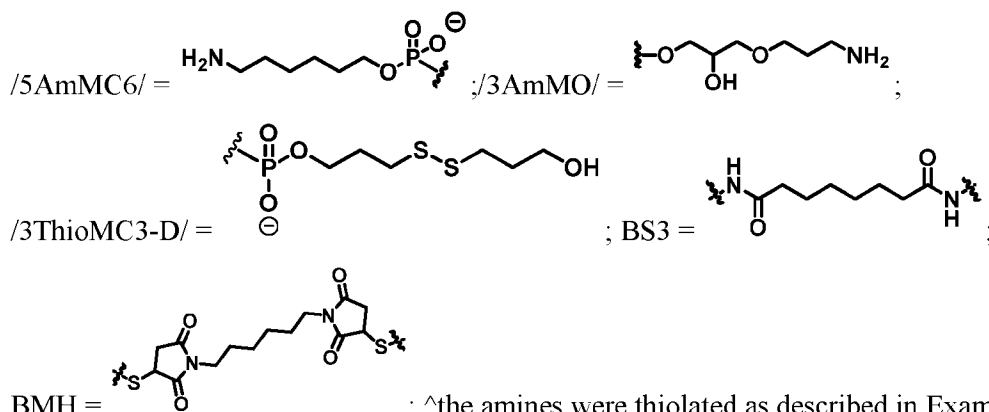
[533] Following purification, rOLI017_BMH_rOLI007 was next annealed with rOLI001 (at a concentration of 20-50 μ M, 1 equiv.) in presence of sodium chloride (150 mM) and a bridging splint, OLI231 (1 equiv. for rOLI001: rOLI017_BMH_rOLI007:OLI231). The annealed RNA mixture (at a concentration of 10 μ M per RNA, 1 equiv.) was reacted with BS3 (2000 equiv.) for 1 h at room temperature at pH 9.0 under aqueous conditions (50 mM CHES pH 9.0). After 1 h, the reaction was quenched with tris (as a solution of 1 M Tris HCl pH 7.5, ~1,000 equiv.). This reaction was then desalted using Cytiva NAPTM columns, followed by the crude analysis on RP-HPLC. The crudes were finally purified by a RP-HPLC

followed by the concentration of the appropriately pooled fractions and sodium ion-exchange yielding the pure ligated RNAs. Table 14 shows the sequences used in this example.

[534] Table 14.

SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments and splints</i>			
69	rOLI001^	34	mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGA GCUAGA/3AmMO/
75	rOLI007^	52	/5AmMC6/AAAAGUGGCACCGAGUCGGUGCNNNNNN NNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
103	rOLI017	40	/5AmMC6/AAUAGCAAGUUAAAAUAAGGCUAGUCCG UUAUCAACUUGA/3ThioMC3-D/
71	OLI231 (Splint)	30	mUmUmUmUm-d(AACTTGCTATTTCTAGCTCT)- AmAmAmAmCmG
76	OLI232 (Splint)	30	mCmUmCmGmG-d(TGCCACTTTTCAAGTTGAT)- mAmAmCmGmG
<i>Ligated PEgRNAs</i>			
104	rOLI001_B S3_rOLI01 7	Precursor for 3-part synthesis	mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGA GCUAGA_BS3_AAUAGCAAGUUAAAAUAAGGCUAG UCCGUUAUCAACUUGA/3ThioMC3-D/
105	rOLI017_B MH_rOLI0 07	Precursor for 3-part synthesis	/5AmMC6/AAUAGCAAGUUAAAAUAAGGCUAGUCCG UUAUCAACUUGA_BMH_AAAAGUGGCACCGAGUC GGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU* mU*mU*U
106	rOLI001_B S3_rOLI01 7_BMH_r OLI007		mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGA GCUAGA_BS3_AAUAGCAAGUUAAAAUAAGGCUAG UCCGUUAUCAACUUGA_BMH_AAAAGUGGCACCG AGUCGGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NmU*mU*mU*U

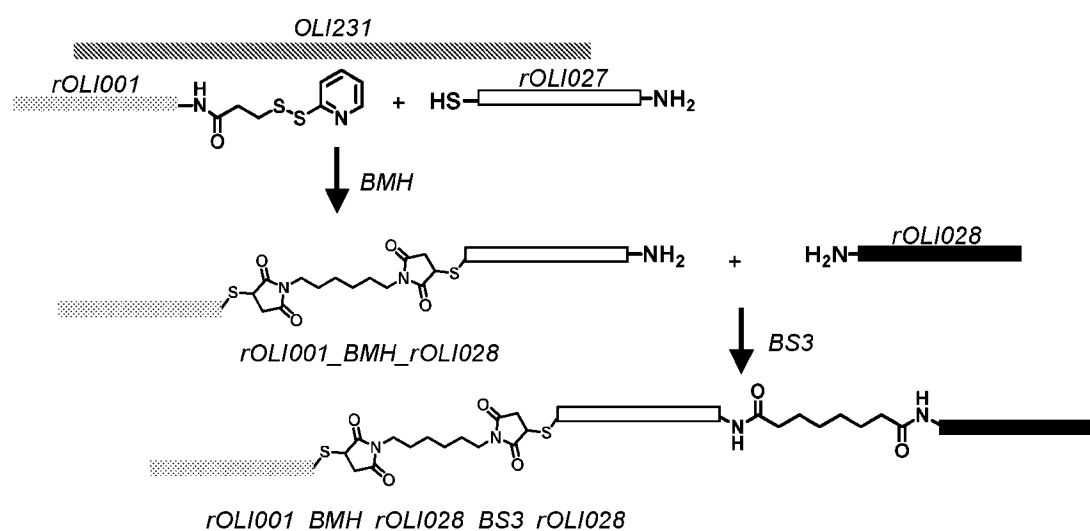
mN= 2'-O-Me ribonucleotide; mU = 2'-O-Me uridine; * = Phosphorothioate internucleotide linkages;



; ^the amines were thiolated as described in Example 6 herein.

[535] Example 8.2 – 3-part Ligation, Strategy B

[536] Scheme 21 shows a schematic of this ligation strategy.



Scheme 21

[537] Methods

[538] 3-part ligation of TL native and SL2 extension RNA oligonucleotides, Strategy B (Scheme 21)

[539] The input for this process is rOLI001 with an amine functional group at 3'-end, rOLI027 with an amine functional group at 5'-end and a thiol group 3'-end, and rOLI028 containing a 5' thiol group. rOLI001 and rOLI027 were annealed (at a concentration of 20-50 μ M per RNA, 1 equiv.) with sodium chloride (150 mM) and a bridging splint, OLI231, (1 equiv. for rOLI001:rOLI027:OLI231). rOLI001 and rOLI027 were then reduced using TCEP to generate thiol moieties. BMH (250 equiv.) was then added to the desalted, reduced rOLI001 and rOLI027 (at a concentration of 10 μ M per RNA, 1 equiv.) and the reaction proceeds for 2 h at room temperature at pH 6.5 under aqueous conditions (50 mM phosphate buffer pH 6.5, 2.5% DMF). This reaction was then desalted using Cytiva NAPTM columns to remove residual small molecule from RNA. The solution was then concentrated, and buffer exchanged into UltraPureTM water using centrifugal filtration devices. Following desalting, the RNA was analyzed by RP-HPLC to confirm ligation, and then frozen until purification.

[540] In the next step, rOLI001_BMH_rOLI027 and rOLI028 were annealed (at a concentration of 20-50 μ M per RNA, 1 equiv.) with sodium chloride (150 mM). The annealed RNA mixture (at a concentration of 10 μ M per RNA, 1 equiv.) was reacted with BS3 (500 equiv.) for 1 h at room temperature at pH 9.0 under aqueous conditions (50 mM CHES pH 9.0). After 1 h, the reaction was quenched with tris (as a solution of 1 M Tris HCl pH 7.5, ~1,000 equiv.). This reaction was then desalted using Cytiva NAPTM columns and

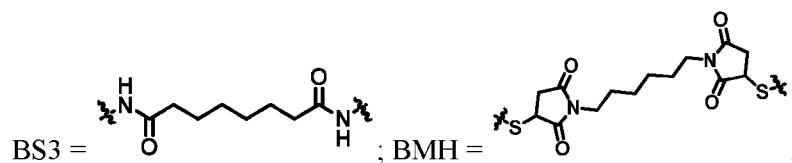
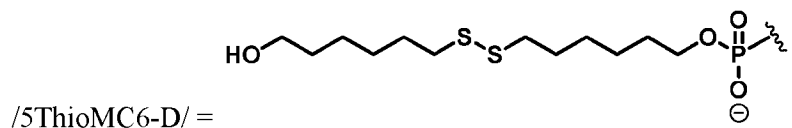
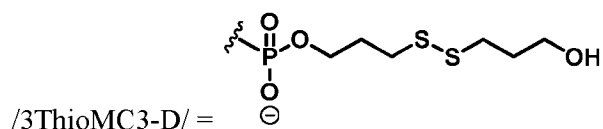
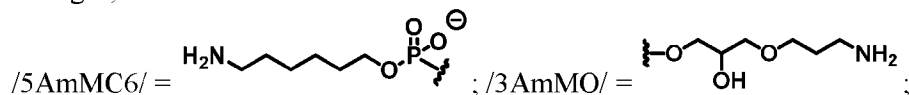
concentrated. The crudes were purified by a RP-HPLC followed by the concentration of the appropriately pooled fractions and sodium ion-exchange yielding the pure ligated RNAs.

Table 15 shows the sequences used in this example.

[541] Table 15.

SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments and splints</i>			
69	rOLI001 [^]	34	mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGA/3AmMO/
107	rOLI027	51	/5ThioMC6-D/AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGCAGCAAGCACGA/3AmMO/
108	rOLI028	63	/5AmMC6/AAGUGCUUGCUGCAAGUGGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
71	OLI231 (Splint)	30	mUmUmUmUm-d(AACTTGCTATTCTAGCTCT)-AmAmAmAmCmG
<i>Ligated PEgRNAs</i>			
109	rOLI001_B MH_rOLI027	Precursor for 3-part synthesis	mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGA_ BMH _AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGCAGCAAGCACGA/3AmMO/
110	rOLI027_B S3_rOLI028	Precursor for 3-part synthesis	/5ThioMC6-D/AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGCAGCAAGCACGA_ BS3 _AAGUGCUUGCUGCAAGUGGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
111	rOLI001_B MH_rOLI027_BS3_rOLI028		mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGA_ BMH _AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGCAGCAAGCACGA_ BS3 _AAGUGCUUGCUGCAAGUGGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U

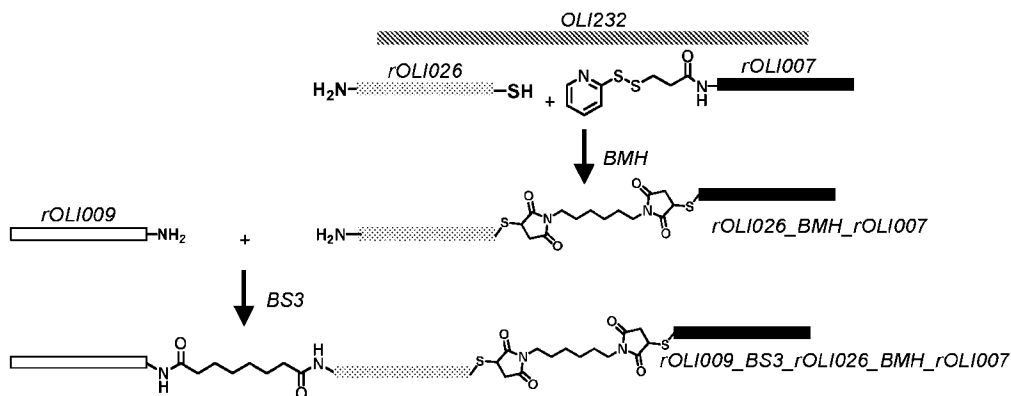
mN = 2'-O-Me ribonucleotide; mU = 2'-O-Me uridine; * = Phosphorothioate internucleotide linkages;



the amines were thiolated as described in Example 6 herein

[542] Example 8.3 – 3-part Ligation, Strategy C

[543] Scheme 22 shows a schematic of this ligation strategy.



Scheme 22

[544] Methods

[545] 3-part ligation of TL extension and SL2 native RNA oligonucleotides, Strategy C (Scheme 22)

[546] The input for this process is rOLI009 with an amine functional group at 3'-end, rOLI026 with an amine functional group at 5'-end and a thiol group 3'-end, and rOLI007 containing a 5' thiol group. rOLI026 and rOLI007 were annealed (at a concentration of 20-50 μ M per RNA, 1 equiv.) with sodium chloride (150 mM) and a bridging splint OLI232, (1 equiv. for rOLI026:rOLI007:OLI232). RNA-2 and RNA-3 were then reduced using TCEP to generate thiol moieties. BMH (250 equiv.) was then added to the desalted, reduced rOLI026 and rOLI007 (at a concentration of 10 μ M per RNA, 1 equiv.) and the reaction proceeded for 2 h at room temperature at pH 6.5 under aqueous conditions (50 mM phosphate buffer pH 6.5, 2.5% DMF). This reaction was then desalted using Cytiva NAPTM followed by concentration, crude analysis and RP-HPLC purification.

[547] Following purification, rOLI026_BMH_rOLI007 and rOLI009 were annealed (at a concentration of 20-50 μ M per RNA, 1 equiv.) with sodium chloride (150 mM). The annealed RNA (at a concentration of 10 μ M per RNA, 1 equiv.) were reacted with BS3 (500 equiv.) for 1 h at room temperature at pH 9.0 under aqueous conditions (50 mM CHES pH 9.0). After 1 h, the reaction was quenched with tris (as a solution of 1 M Tris HCl pH 7.5, ~1,000 equiv.). This reaction was then desalted using Cytiva NAPTM columns and concentrated. The crudes were purified by a RP-HPLC followed by the concentration of the

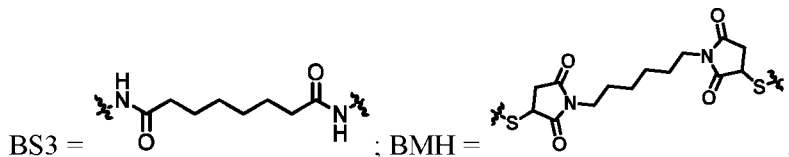
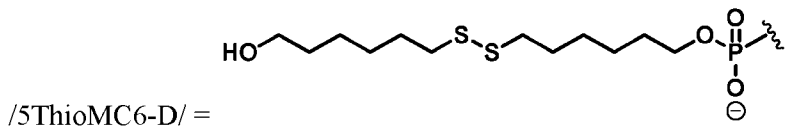
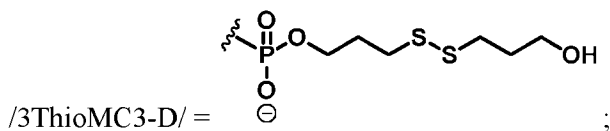
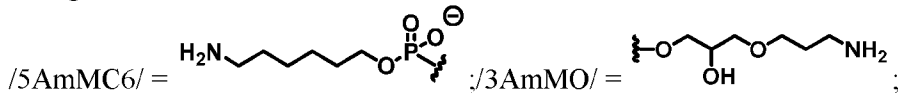
appropriately pooled fractions and sodium ion-exchange yielding the pure ligated RNAs.

Table 16 shows the sequences used in this example.

[548] Table 16.

SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments and splints</i>			
75	rOLI007	52	/5AmMC6/AAAAGUGGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*rU
112	rOLI009		mN*mN*mN*NNNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGCAGCAAGCACGA/3AmMO/
113	rOLI026		/5AmMC6/AAGUGCUUGCUGCUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGA/3ThioMC3-D/
76	OLI232 (Splint)	30	mCmUmCmGmG-d(TGCCACTTTTCAAGTTGAT)-mAmAmCmGmG
<i>Ligated PEgRNAs</i>			
114	rOLI009_BS3_rOLI026	Precursor of 3-part synthesis	mN*mN*mN*NNNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGCAGCAAGCACGA_BS3_AAGUGCUUGCUGCUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGA/3ThioMC3-D/
115	rOLI026_BMH_rOLI007	Precursor of 3-part synthesis	/5AmMC6/AAGUGCUUGCUGCUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGA_BMH_AAAAGUGGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
116	rOLI009_BS3_rOLI026_BMH_rOLI007		mN*mN*mN*NNNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGCAGCAAGCACGA_BS3_AAGUGCUUGCUGCUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGA_BMH_AAAAGUGGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U

mN= 2'-O-Me ribonucleotide; mU = 2'-O-Me uridine; * = Phosphorothioate internucleotide linkages;

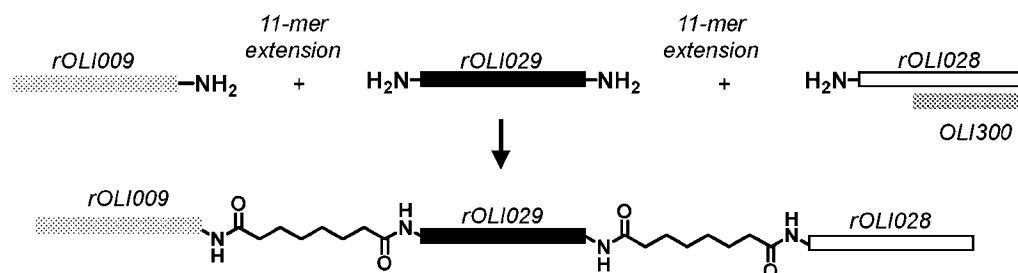


^the amines were thiolated as described in Example 6 herein

[549] Example 8.4 – 3-part Ligation, Strategy D

[550] Scheme 23 shows a schematic of this ligation strategy.

[551] As this strategy involves both TL and SL2 extensions with 11-bp, both these 11-bp sequence extensions at each loop can be exactly same or different or even different lengths. If its same extension at both loop structures, blocking the third fragment RNA, rOLI028 with a splint led to improved conversions.



Scheme 23

[552] Methods

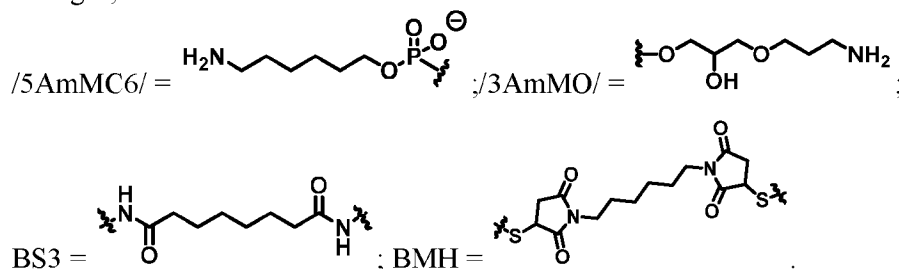
[553] *3-part ligation of TL extension and SL2 extension RNA oligonucleotides, Strategy D (Scheme 23)*

[554] The input for this process is rOLI009 with an amine functional group at 3'-end, rOLI029 with amine functional groups at both 5'- and 3'-ends, and rOLI028 with an amine functional group at 5'-end, as well as a short splint sequence, OLI300, that is complementary to the 3' portion of RNA-3. rOLI009, rOLI029, and rOLI028 were annealed (at a concentration of 20-50 μ M per RNA, 1:1:1 equiv.) with sodium chloride (150 mM) and splint sequence (2 equiv.) to promote the maximum desired product formation. A solution of BS3 (500 equiv.) was then added to annealed rOLI009, rOLI029, and rOLI028, and splint OLI300 in the reaction buffer (50 mM CHES pH 9.0). The reaction proceeded for 1 h at room temperature. After 1 h, the reaction is quenched with tris (~1000 equiv., as a solution of 1 M Tris pH 7.5). This reaction was then desalted using Cytiva NAPTM columns, followed by the crude analysis on RP-HPLC. The crudes were eventually purified by a RP-HPLC, concentration of the appropriately pooled fractions, sodium ion-exchange and the ligated material was ready for in vitro or in vivo analysis. Table 17 shows the sequences used in this example.

[555] Table 17.

SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments and splints</i>			
112	rOLI009		mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUAGAGCUAGCAGCAAGCACGA/3AmMO/
108	rOLI028		/5AmMC6/AAGUGCUUGCUGCAAGUGGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
117	rOLI029		/5AmMC6/AAGUGCUUGCUGCUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGCAGCAAGCACGA/3AmMO/
<i>Ligated PEgRNAs</i>			
118	rOLI009_BS3_rOLI029	Precursor of 3-part synthesis	mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUAGAGCUAGCAGCAAGCACGA_BS3_AAGUGCUUGCUGCUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGCAGCAAGCACGA/3AmMO/
119	rOLI029_BS3_rOLI028	Precursor of 3-part synthesis	/5AmMC6/AAGUGCUUGCUGCUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGCAGCAAGCACGA_BS3_AAGUGCUUGCUGCAAGUGGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U
120	rOLI009_BS3_rOLI029_BS3_rOLI028		mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUAGAGCUAGCAGCAAGCACGA_BS3_AAGUGCUUGCUGCUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGCAGCAAGCACGA_BS3_AAGUGCUUGCUGCAAGUGGCACCGAGUCGGUGCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNmU*mU*mU*U

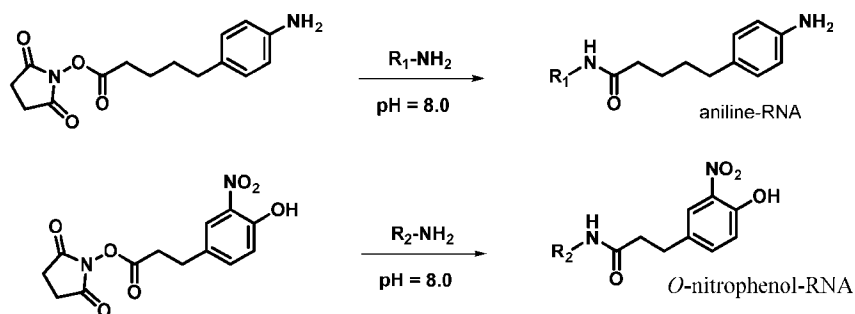
mN= 2'-O-Me ribonucleotide; mU = 2'-O-Me uridine; * = Phosphorothioate internucleotide linkages;



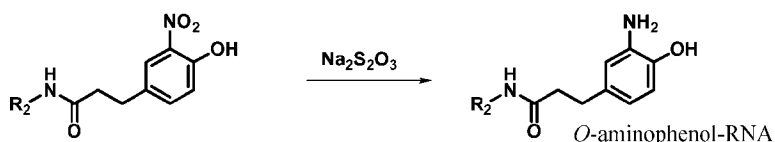
[556] EXAMPLE 9 – Oxidative ligation of aniline-RNA and aminophenol-RNA

[557] The selective reactivity of phenols with anilines can be used as a ligation chemistry for synthesizing longer RNAs. Short RNA sequences carrying functional groups (at the ends or internally through a side chain on the ribose sugar or nucleobase) such as aniline derivatives can be selectively coupled under oxidation conditions to phenol derivatives to produce a ligated RNA as shown in Scheme 24.

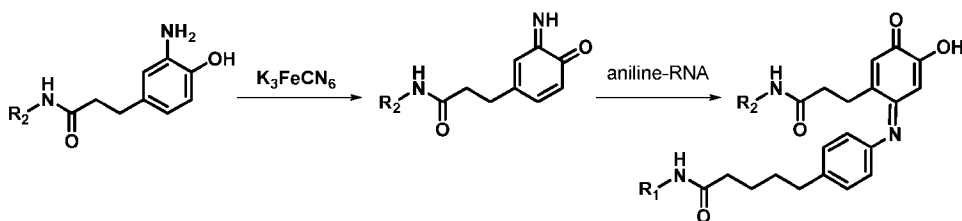
Step 1: Derivatization of RNAs with aniline and *O*-nitrophenol NHS esters



Step 2: Reduction of *O*-nitrophenol-RNA to *O*-aminophenol-RNA



Step 3: Oxidative coupling of *O*-aminophenol-RNA to aniline-RNA



where R_1 = rOLI001 and R_2 = rOLI019

Scheme 24

[558] Methods

[559] *Oxidative coupling of aniline-RNA with o-aminophenol-RNA*

[560] **Step-1:** Amino-functionalized RNAs (rOLI001/rOLI019, at a concentration of 10 μM - 50 μM , 1 equiv.) were modified with corresponding NHS-esters (160 equiv.) for 2 h at room temperature at pH 8.0 under denaturing conditions (40/60 v/v% DMF and 50 mM phosphate buffer pH 8.0), Scheme 24. After 2 h, the reaction was quenched with tris (~1,000 equiv., as a solution of 1 M Tris HCl pH 7.5). This reaction was then desalted using Cytiva NAPTM columns to remove the unreacted small molecule reagents from RNAs. Following desalting, the crude was analyzed by RP-HPLC to confirm modification followed by concentration and used it in the step-3 coupling reaction, Scheme 24.

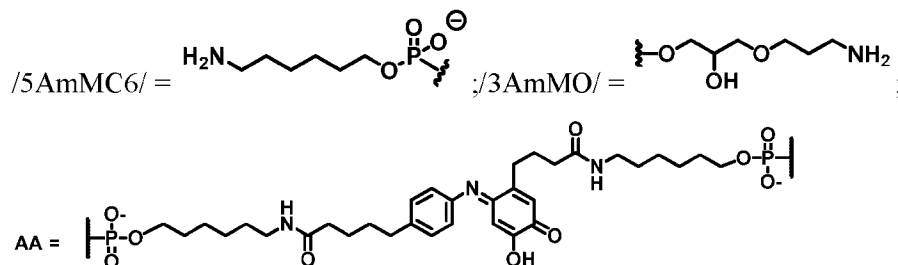
[561] **Step-2:** Reduction of *O*-nitrophenol-RNA to *O*-aminophenol-RNA was performed at 10-100 μ M of RNA with 100 mM sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_3$) at pH 6.5 in 100 mM phosphate buffer for 20 minutes at room temperature. This reaction was then desalted using Cytiva NAPTM columns to remove the unreacted small molecule reagents from RNAs and used in the next coupling reaction, step-3.

[562] **Step-3:** Aniline-RNA, rOLI001 (1 eq.) was coupled with *O*-aminophenol-RNA, rOLI019 (5-10 eq.) at pH 6.0 under oxidative conditions of 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ (100 eq.) at room temperature for 30 minutes. The reaction was quenched with TCEP followed by the desalting using Cytiva NAPTM columns resulted in a ligated RNA which is analyzed by the PAGE-urea (FIG. 26) and LCMS. Table 18 shows the sequences used in this example.

[563] **Table 18.**

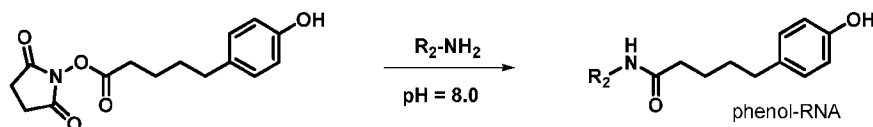
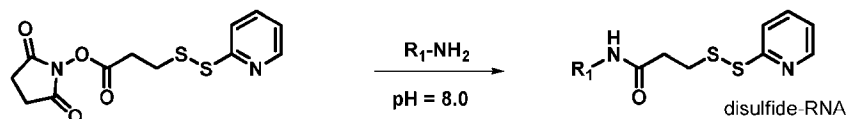
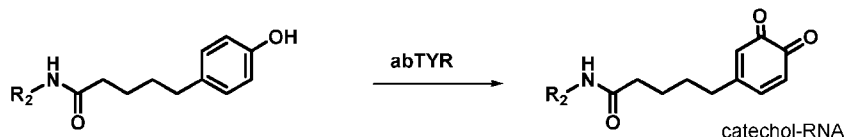
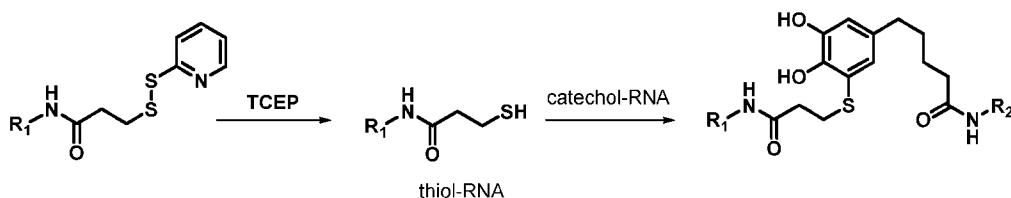
SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments and splints</i>			
69	rOLI001	34	mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGA/3AmMO/
121	rOLI019		/5AmMC6/AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGA
<i>Ligated PEgRNAs</i>			
122	rOLI001-AA-rOLI019		mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGA-AA-AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGA

mN= 2'-O-Me ribonucleotide; * = Phosphorothioate internucleotide linkages;



[564] **EXAMPLE 10 – Oxidative ligation of thiol-RNA and Catechol-RNA**

[565] The selective reactivity of thiols with catechols can be used as a ligation chemistry for synthesizing longer RNAs. Short RNA sequences carrying functional groups (at the ends or internally through a side chain on the ribose sugar or nucleobase) such as thiol derivatives or catechols derivatives can be selectively coupled under oxidation conditions to produce a ligated RNA, Scheme 25.

Step 1: Derivatization of RNAs with SPDP and phenol NHS esters**Step 2: Oxidation of phenol-RNA to catechol-RNA****Step 3: Coupling of catechol-RNA to thiol-RNA**

where abTYR = Tyrosinase from *Agaricus bisporus* R_1 = rOLI001 and R_2 = rOLI019

Scheme 25

[566] Methods

[567] Step-1: Amino-functionalized RNAs (rOLI001/rOLI019, at a concentration of 10 μ M - 50 μ M, 1 equiv.) were modified with corresponding NHS-esters (160 equiv.) for 2 h at room temperature at pH 8.0 under denaturing conditions (40/60 v/v% DMF and 50 mM phosphate buffer pH 8.0), Scheme 25. After 2 h, the reaction was quenched with tris (~1,000 equiv., as a solution of 1 M Tris HCl pH 7.5). This reaction was then desalted using Cytiva NAPTM columns to remove the unreacted small molecule reagents from RNAs. Following desalting, the crude was analyzed by RP-HPLC to confirm modification followed by concentration and used it in the next steps.

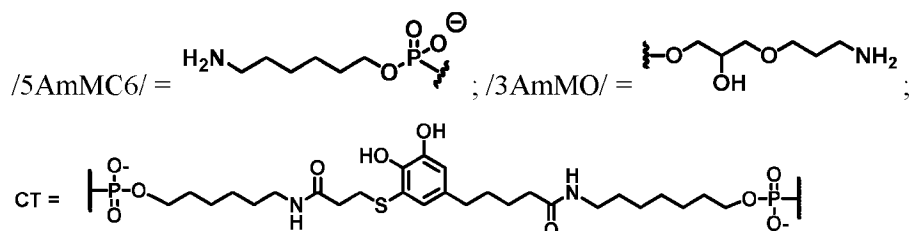
[568] Step-2 and step-3: Oxidation of phenol-RNA obtained in the above step to catechol-RNA was performed using abTYR (*Tyrosinase* isolated from *Agaricus bisporus*). The oxidation was typically performed at 10-50 μ M of RNA concentration (10 eq.) with abTYR (14 U/L, 0.2 eq.) in 20 mM phosphate buffer pH 6.5 for 20 min at room temperature. To the above mixture was added the freshly TCEP-reduced thiol-RNA (1 eq.) and allowed the

ligation for 30 minutes. The reaction was quenched with tropolone followed by the desalting using Cytiva NAPTM columns resulted in a ligated RNA which is analyzed by the PAGE-urea (FIG. 27) and LCMS. Table 19 shows the sequences used in this example.

[569] **Table 19.**

SEQ ID NO.:	Name	Length (nt)	RNA Sequence (5'-3')
<i>RNA fragments</i>			
69	rOLI001	34	mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUAGAGCUAGA/3AmMO/
121	rOLI019		/5AmMC6/AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGA
<i>Ligated PEgRNAs</i>			
123	rOLI001-CT-rOLI019		mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUUAGAGCUAGA-CT-AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGA

mN= 2'-O-Me ribonucleotide; * = Phosphorothioate internucleotide linkages;

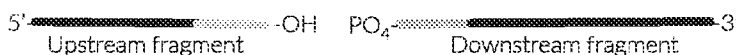


[570] **EXAMPLE 11 –Enzymatic Ligation**

[571] Gene editing is a new technological advancement in which genetic material is inserted, deleted, modified, or replaced in the genome of a living organism. Gene editing inserts genetic material into a host genome at site-specific locations to replace defective genes. The nucleic acid fragments used are called sgRNA and are about 100-nt long. In contrast, prime editing technology requires >100-nt long RNAs. Synthesis of oligonucleotides (RNA or DNA) is commonly achieved on automated DNA/RNA synthesizers using a solid support. Synthetic methods have been well established for producing several therapeutic oligonucleotides used for antisense (20-30-nt), siRNA (19-21-nt), aptamers (30-90-nt), and sgRNAs up to 100-nt. However, making >120-nt long nucleic acids faces technical challenges of synthesis and their purification. These challenges demand finding alternate methods to build >120-nt long prime editors at the quantities and purities required. To overcome this challenge, we aim to enzymatically ligate two or more oligoribonucleotides synthesized by solid phase synthesis which may or may not contain chemically modifications of the sugar, phosphodiester backbone, or nucleobase canonical structures. In here, the PEgRNA can be generated from one 5' fragment, and one or more 3'

fragments which are 5' terminally modified with a free monophosphate to facilitate the ligation by way of a nucleotide ligase enzyme. Free 3' ends and phosphorylated 5' ends are brought into proximity by way of sequence complementarity resulting in base pairing hybridization either between the RNA fragments directly (self-templated) or via an additional complementary DNA splint (splinted). Subsequent enzymatic catalysis by a nucleic acid ligase, such as, but not limited to, T4 RNA Ligase I, T4 RNA Ligase II, or T4 DNA Ligase can generate a natural phosphodiester linkage between fragments, yielding the full-length PEgRNA. Other ligase variants can also be used to ligate the fragments.

[572] Scheme 26 below shows a potential fragment functionalization for enzymatic split synthesis, where “Upstream” or 5' end RNA fragments have a free 3' OH available for ligation, while “Downstream” or 3' end RNA fragments are terminally functionalized with a 5'-monophosphate.



Scheme 26

Schematic of upstream and downstream fragments are shown. The light shaded segments are complementary and facilitate self-templated ligation.

[573] EXAMPLE 11.1 – Self-templated Enzymatic Ligation

[574] In this approach, two functionalized, single-stranded RNA fragments with complementary base pairs are hybridized for ligase-mediated conjugation (Scheme 26). In here, the full-length PEgRNA is split into two or more fragments that take natural advantage of the sequence complementarity driven structure of either the Tetraloop or Stem-loop 2 features within the scaffold of a PEgRNA to help facilitate a very specific ligation reaction between the fragments. Once these fragments are annealed under controlled buffered conditions, the RNA duplex can form a native secondary structure mimic to the full length PEgRNA, resulting in a net increase in the effective local concentration of the terminal 3'-OH and 5'-monophosphate functional ends required for ligase activity. Subsequent ligase catalysis forms a phosphodiester bond between the RNA fragments which covalently seals the nick and generates a full-length PEgRNA. Accordingly, during self-templated ligation two RNA fragments with significant complementarity are enzymatically conjugated. The first step is to anneal the fragments, bringing the reactive functional groups together and creating a nicked double-stranded substrate for ligase catalysis. In the presence of ligase, a phosphodiester bond is formed between the 5'-monophosphate and native 3'-hydroxyl of the fragments, yielding a full-length PEgRNA.

[575] Methods**[576]** Step 1: Hybridization

[577] Equimolar ratio of RNA A and 5'-phosphorylated RNA B (0.01nmol, 1μL each) were combined in molecular biology grade water (5μL) and annealed in a thermocycler. The thermocycling program included heating the RNA mixture to 65°C for 3 minutes, followed by snap cooling on ice for 2 minutes. The RNA was maintained on ice for no longer than 5 minutes before enzyme addition.

[578] Step 2: Enzymatic conjugation

[579] Freshly prepared enzyme master mix (13μL) was added to the annealed RNA mixture (7μL). The master mix contained molecular biology grade water (5.5μL), T4 RNA Ligase 2 buffer (10X, 2μL), PEG8000 (50% w/v, 4μL), RNase inhibitor (40U/μL, 0.5μL), and T4 RNA Ligase 2 (10U/μL, 1μL). The reaction proceeded in a thermocycler at 25°C for 120 minutes. The synthesis products were then analyzed by 10% TBE-Urea denaturing PAGE and fluorescence gel imaging.

[580] Discussion

[581] Self-templated ligation was tested using fragments RNA A and RNA B (Table 8) to split synthesize PEgRNA C (Table 8). RNA's A and B were designed to anneal and form SL2, and thus, the nicked, double-stranded substrate necessary for ligase activity.

Furthermore, RNA B was functionalized with a 5'-monophosphate to enable ligation to the native 3'-hydroxyl of RNA A. RNA's A and B were first pre-annealed and then treated with ligase to catalyze the formation of a phosphodiester bond. The ligation reaction was analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) and compared to a full-length PEgRNA C control (FIG. 16). Gel image density measurements revealed a synthesis efficiency of 75% for PEgRNA C by self-templated ligation.

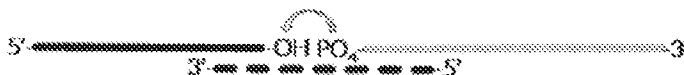
[582] **Table 20. RNA fragments and the conjugation product**

SEQ ID NO.:	Name	RNA Sequence (5'-3')
24	RNA A	mU*mC*mA*CCAGGAACAUGUACCUGGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAGCGUUC GCC
25	RNA B	/5Phos/AAGGAAAUUUGGCGGACGCGGCACCGAGUCGGUGCU UUCCCCCAGGUGUACAUGUCCUGGmU*mU*mU*U
26	PEgRNA C	mU*mC*mA*CCAGGAACAUGUACCUGGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAGCGUUC GCCAAGGAAAUUUGGCGGACGCGGCACCGAGUCGGUGCUU UUCCCCCAGGUGUACAUGUCCUGGmU*mU*mU*U

mN = 2'-O-Me ribonucleotide; N = Ribonucleotide; * = Phosphorothioate internucleotide linkage

[583] EXAMPLE 11.2 – Templated Enzymatic Ligation

[584] Templated ligation is another strategy used for enzymatic split synthesis of PEgRNAs. In here, two functionalized, single-stranded RNA fragments are conjugated with ligase, similarly to self-templated ligation. In addition, a short, complementary nucleic acid splint facilitates a trimeric secondary structure formation during templated ligation. The nucleic acid splint is designed such that it bears proximal sequence complementarity of both the 3' end of the upstream RNA fragment and 5' end of the downstream RNA fragment to facilitate a splinted trimeric structure as shown in Scheme 27 below. Consequently, the RNA fragments do not require complementarity at the ligation site, and so the advantage of templated ligation for PEgRNA synthesis is that the splinted approach enables ligation at PEgRNA locations which lack secondary structure and anywhere in the sequence without extensions.



Scheme 27

Schematic representation of templated ligation. Black and light solid lines indicate upstream and downstream fragments, respectively. Dotted line indicates splint that is complementary to the 3'-end of upstream fragment and 5'-end of downstream fragment. Splint facilitates bringing the functional ends of the two fragments to proximity for ligation.

[585] In templated ligation, the first step is to anneal the DNA-RNA hybrid complex, bringing the RNA fragments and their functional groups together. The DNA splint is designed such that DNA-RNA hybridization forms a double-stranded structure with a nick between the RNA fragments. Upon ligase addition, a phosphodiester bond is formed between the 5' monophosphate and native 3'-hydroxyl of the RNA fragments, yielding a full-length pegRNA.

[586] Methods**[587] Step 1: Hybridization**

[588] Equimolar ratio of RNA X, 5'-phosphorylated RNA Y, and DNA splint (0.01nmol, 1μL each) were combined in molecular biology grade water (4μL) and annealed in a thermocycler. The thermocycling program included heating the DNA-RNA mixture to 65°C for 3 minutes, followed by snap cooling on ice for 2 minutes. The DNA-RNA hybrid was maintained on ice for no longer than 5 minutes before enzyme addition.

[589] Step 2: Enzymatic conjugation

[590] Freshly prepared enzyme master mix (13 μ L) was added to the annealed DNA-RNA mixture (7 μ L). The master mix contained molecular biology grade water (5.5 μ L), T4 RNA Ligase 2 buffer (10X, 2 μ L), PEG8000 (50% w/v, 4 μ L), RNase inhibitor (40U/ μ L, 0.5 μ L), and T4 RNA Ligase 2 (10U/ μ L, 1 μ L). The reaction proceeded in a thermocycler at 25°C for 120 minutes. The synthesis products were then analyzed by 10% TBE-Urea denaturing PAGE and fluorescence gel imaging.

[591] Discussion

[592] The templated ligation approach was tested using DNA splints L, M, and N (Table 21) to bridge fragments RNA X and RNA Y (Table 22) and split synthesized pegRNA Z (Table 22). DNA splints L, M, and N were designed to anneal to RNA's X and Y, facilitating the formation of a nicked, double-stranded substrate for ligase activity. Furthermore, RNA Y was functionalized with a 5'-monophosphate to enable ligation to the native 3'-hydroxyl of RNA X. During the reaction, RNA's X and Y were first annealed to a single DNA splint. Subsequently, the DNA-RNA hybrid was treated with ligase to catalyze the formation of a phosphodiester bond between the splinted RNA fragments. Ligation reactions were analyzed by denaturing PAGE and compared to both a self-templated split synthesis ligation reaction (i.e. splint-less) and a full-length PEGRNA Z control (FIG. 17). Gel image density measurements revealed an increase in synthesis efficiency of PEGRNA Z from 37% by self-templated ligation to 47, 50, and 51% by templated ligation with DNA splints L, M, and N, respectively.

[593] **Table 21. DNA Splints**

SEQ ID NO.:	Name	DNA Sequence (5'-3')
27	DNA splint L	CGGATTTCCCCGCTC
28	DNA splint M	CCCCGCTCTAAAACG
29	DNA splint N	GATTTCCCCGCTCTAAAACG

[594] Table 22. RNA fragments and the conjugation product

SEQ ID NO.:	Name	RNA Sequence (5'-3')
30	RNA X	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGC
31	RNA Y	/5Phos/GGGGAAAUCCGCAAGUUAUUAAAGGCUAGUCCGU UAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUGCCCU GGGACGGUGGCUGGAACACmU*mU*mU*U
32	PEgRNA Z	mU*mU*mG*CCAAGUGUCCAGCCACGUUUUAGAGCGGGG AAAUCCGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUU GAAAAAGUGGCACCGAGUCGGUGCUUGCCCUUGGGACGGUG GCUGGAACACmU*mU*mU*U

mN = 2'-O-Me ribonucleotide; N = Ribonucleotide; * = Phosphorothioate internucleotide linkage

[595] EXAMPLE 11.3 –Enzymatic Ligation**[596] Methods**

[597] All control/comparative RNA sequences prepared through linear solid-phase synthesis are synthesized and purified by Axo Labs.

[598] Enzymatic ligation protocol

[599] A self-splinted guide RNA ligation approach was utilized for enzymatic ligation. Both the acceptor and donor split fragments (A1+D1 or A2+D2) were used in 1:1 molar ratio. The solutions of both oligos were mixed along with 2X annealing solution (0.2 mM EDTA + 200 mM KCl). The resulting solution was heated to 75 °C and cooled slowly to 25 °C at 0.1 °C/sec in a thermocycler. The annealed oligo mixture was used for the ligation reaction. The ligation reaction was setup by mixing the annealing mixture, RNase-free T4 RNA Ligase II (NEB, Cat. No. M0239L), T4 Rnl2 Reaction Buffer, SUPERase•In™ RNase Inhibitor (Invitrogen, Cat. No. AM2694), 50% aq. PEG8000, and RNase-free water to a final oligo concentration of 0.5 mM. The reaction mixture was incubated at 37 °C for 1 h. The ligated RNA product was isolated by using Monarch® RNA Cleanup Kit (NEB, Cat. No. T2030S/T2040S/T2050S) using manufacturer's protocols. The resultant full-length RNA was obtained in good purity and could be directly used for downstream applications without further LC-based purification.

[600] Analytical UPLC method

[601] The purity of the ligation products was analyzed by Agilent 1290 Infinity II UPLC system. A buffer system consisting of mobile phase A: 0.1M Hexylamine acetate (HAA) buffer pH 6.9 – 7.0 and mobile phase B: 0.1M HAA in H₂O/ACN/MeOH (5/16/4 v/v/v) was used at a flow rate of 0.2 ml/min. An ACQUITY, BEH C18, 130 Å, 1.7 µm, 2.1 x 150 mm

column at 80 °C was used using 0.00-40.00 min – 54-72% B, 40-42.60 min – 72-90% B, 42.60-45.60 min – 90% B, 45.20-45.30 min, 90-54% B, 45.30-52.00 min – 54% B gradient. Product was detected with a UV detector at 260 nm.

[602] Primary Human Hepatocytes (PHH) cell culture and transfection

[603] WT Primary Human Hepatocytes Hu8391 were obtained from Thermo Scientific and were cultured according to the manufacturer's protocols. 40 K cells/well were plated in 96-well plate and twenty-four hours later cells were transfected with Messenger Max according to the manufacturer's directions with PE2 mRNA (120 ngs), PEgRNA (54 ngs) and ngRNA (18 ngs). The total nucleic acid 192 ngs was combined and diluted 2-fold to 7 doses. Three days after transfection, gDNA was harvested in quick DNA extract for high throughput sequencing and sequenced using miseq.

[604] Results

[605] Acceptor and donor fragments are listed in Table 23. Acceptor A1 and donor D1 fragments were synthesized with a split in the tetraloop (TL) and acceptor A2 and donor D2 were synthesized with a split in the stem-loop 2 (SL2).

[606] The ligation reactions were conducted as described above. At the end of the ligation reactions, all samples were analyzed by UPLC utilizing the buffer gradient described in the analytical UPLC method. FIG. 18 shows the UPLC profiles of A1, D1 fragments and A1-D1 ligated product (FLP1-T4). The UPLC profile of A1-D1 product (FLP1-T4) shown in the bottom panel of FIG. 18 suggests that the ligated product is clean with <1% of unreacted A1 and D1 fragments remaining.

[607] Similarly, ligation reactions were conducted between A2 and D2. At the end of the ligation reaction, samples were analyzed by UPLC utilizing the buffer gradient described in the analytical UPLC method. FIG. 19 shows the UPLC profiles of A1, D1 fragments and A1-D1 ligated product (FLP2-T4). The UPLC profile of A1-D1 product (FLP2-T4) shown in the bottom panel of FIG. 19 suggests that the ligated product is clean with <3% of unreacted A2 and D2 fragments remaining.

[608] Table 23. Sequence information for acceptor and donor oligos along with full-length products and corresponding molecular weights.

Oligo Name*	SEQ ID NO.	Length (nt)	RNA Sequences (5'-3')**	Molecular Weight, free acid (Da)***
A1	33	31	mU*mU*mG* CCAAGUGUCCAGCCACGUUUUAGAGCG	9962.2
D1	34	99	/5Phos/GUCGAAAGGCCGCAAGUUAAAAUA AGGCUAGUCCGUUAUCAACUUGAAAAAGU GGCACCAGUCGUGUCUUGCCCUGGGACG GUGGCUUGGAACACmU* mU*mU*U	32129.4
FLP1-T4	35	130	mU*mU*mG*CCAAGUGUCCAGCCACGUU UUAGAGCGGUCGAAAGGCCGCAAGUUAAA AUAAGGCUAGUCCGUUAUCAACUUGAAAA AGUGGCACCAGUCGUGUCUUGCCCUGGG ACGGUGGCUUGGAACACmU*mU*mU*U	42097.4
A2	36	75	mU*mU*mG*CCAAGUGUCCAGCCACGUU UUAGAGCUAGAAAUAGCAAGUUAAAAUA AGGCUAGUCCGUUAUCAGCGUUAC	24130.6
D2	37	63	/5Phos/ACCGAAAGGUGUGACGCGGCACCGA GUCGGUGCUUGCCCUGGGACGGUGGCUUG AACACmU* mU*mU*U	20556.4
FLP2-T4	38	138	mU*mU*mG*CCAAGUGUCCAGCCACGUU UUAGAGCUAGAAAUAGCAAGUUAAAAUA AGGCUAGUCCGUUAUCAGCGUUACACCGA AAGGUGUGACGCGGCACCGAGUCGGUGCU UGCCCUGGGACGGUGGCUUGGAACACmU* mU*mU*U	44668.9

* A1, A2: Acceptor oligos with a free 3'-OH on ribose sugar; D1, D2: Donor oligos with a 5'-phosphate on ribose sugar; FLP1, FLP2: Full-length products for corresponding A1+D1 and A2+D2 RNA ligation respectively.

** All sequences are represented from the 5'→3'. All are ribonucleotides, wherein mN and * represent 2'-O-methylribonucleotides and phosphorothioate linkages, respectively.

*** Molecular Weight, free acid (Da) calculated by IDT OligoAnalyzer™ Tool.

[609] Separately, RNAs equivalent to A1, D1 and A2, D2 ligation products were synthesized on an automated DNA/RNA synthesizer. Both the enzymatically ligated and synthetic full-length products were analyzed on UPLC for comparison. FIG. 20 shows the comparison of enzymatically ligated A1-D1, and synthetic products and FIG. 21 shows the comparison of enzymatically ligated A2-D2 and synthetic products. Analysis of the results suggest that in both cases, the enzymatically ligated products show higher purity than the fully synthesized products (Table 24).

[610] Table 24. Comparison of enzymatically ligated RNA with linearly synthesized RNA through solid-phase synthesis

Oligo Name (Length ; nt)	SEQ ID NO	Synthesis	RNA Sequences*	Molecular Weight, free acid (Da)**	Observed Mass (Da)***	Calculated Purity (%)****
FLP1-T4 (130 nt)	35	Enzymatic ligation	mU*mU*mG*CCAAGUGUCC AGCCACGUUUUAGAGCGGU CGAAAGGCCGCAAGUAAA AUAAGGCUAGUCCGUUAUC AACUUGAAAAAGUGGCACC GAGUCGGUGCUUGCCCUGG GACGGUGGCUGGAACACmU *mU*mU*U	42097.4	42100.5	76.0
FLP1-LS (130 nt)	39	Linear Synthesis	mU*mU*mG*CCAAGUGUCC AGCCACGUUUUAGAGCGGU CGAAAGGCCGCAAGUAAA AUAAGGCUAGUCCGUUAUC AACUUGAAAAAGUGGCACC GAGUCGGUGCUUGCCCUGG GACGGUGGCUGGAACACmU *mU*mU*U	42097.4	42100.0	50.1
FLP2-T4 (138 nt)	38	Enzymatic ligation	mU*mU*mG*CCAAGUGUCC AGCCACGUUUUAGAGCUAG AAAUAGCAAGUAAAAUAA GGCUAGUCCGUUAUCAGCG UUACACCGAAAGGUGUGAC GCGGCACCGAGUCGGUGCU UGCCCUGGGACGGUGGCUG GAACACmU* mU*mU*U	44668.9	44672.0	82.8
FLP2-LS (138 nt)	40	Linear Synthesis	mU*mU*mG*CCAAGUGUCC AGCCACGUUUUAGAGCUAG AAAUAGCAAGUAAAAUAA GGCUAGUCCGUUAUCAGCG UUACACCGAAAGGUGUGAC GCGGCACCGAGUCGGUGCU UGCCCUGGGACGGUGGCUG GAACACmU* mU*mU*U	44668.9	44672.0	44.8

* All sequences are represented from the 5'→3'. All are ribonucleotides, wherein mN and * represent 2'-O-methylribonucleotides and phosphorothioate linkages, respectively.

** Molecular Weight, free acid (Da) calculated by IDT OligoAnalyzer™ Tool.

*** Observed Mass (Da) obtained by Novatia Oligo HTCS system.

**** Calculated purity (%) is determined by calculating area under the curve (AUC) from analytical UPLC traces.

[611] In-vitro gene editing

[612] The enzymatically ligated PEgRNAs FLP1-T4 and FLP2-T4, fully synthetic linear products FLP1-LS, and FLP1-LS and control were tested in-vitro in primary hepatocytes for their editing efficiency. As shown in Table 25 and FIG. 22, the enzymatically ligated PEgRNAs FLP1-T4 and FLP2-T4 show higher max edit as compared to control (8.87%). Between enzymatically ligated FLP1-T4 and linear synthetic product FLP1-LS, FLP1-LS showed slightly higher max editing (14.49%) compared to FLP1-T4 (12.44%). Between

enzymatically ligated FLP2-T4 and linear synthesized FLP2-LS, FLP2-LS showed higher max editing (16.42%) compared with FLP2-T4 (12.80%). The calculated ED50 and ED90 for each pegRNA are shown in Table 25.

[613] Table 25. *In vitro* prime editing of split synthesized PEgRNAs in primary human hepatocytes

PEgRNA/ngRNA	Synthesis	ED50 [nM]	ED90 [nM]	Max Edit %
FLP1-T4/ ngRNA001	Enzymatically	10.38	26.87	12.44
FLP1-LS/ ngRNA001	Linear	13.05	41.99	14.49
FLP2-T4/ ngRNA001	Enzymatically	23.19	87.82	12.80
FLP2-LS/ ngRNA001	Linear	20.2	74.13	16.42
Control PEgRNA/ ngRNA001	Linear	5.79	15.51	8.87

EQUIVALENTS

[614] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the methods and compositions provided herein. Such equivalents are intended to be encompassed by the following claims.

OTHER EMBODIMENTS

[615] The above examples are to be understood as illustrative examples. Further examples are envisaged, which include combinations of features as indicated in the following list, which contains various envisaged claim dependencies for the claims originally filed with this application. Hence, in addition to the description above, this list provides basis for examples having a combination of features of claims filed herewith. Hence, the following list also discloses embodiments of the invention.

WHAT IS CLAIMED IS:

1. A method comprising,
contacting a first RNA sequence with a second RNA sequence,
wherein the first RNA sequence comprises a first chemical moiety at its 3' end
and the second RNA sequence comprises a second chemical moiety at its 5'
end; and
ligating the first RNA sequence and the second RNA sequence to form a synthesized
RNA by reacting the first chemical moiety of the first RNA sequence with the second
chemical moiety of the second RNA sequence;
wherein the ligation forms a linker in a stem loop structure, a tetraloop
structure, and/or an extension arm of the synthesized RNA.
2. A method for synthesizing a full-length prime editing guide RNA (PEgRNA)
comprising a guide RNA (gRNA) core,
the method comprising,
contacting a first RNA sequence with a second RNA sequence,
wherein the first RNA sequence comprises a first chemical moiety at its 3' end
and the second RNA sequence comprises a second chemical moiety at its 5'
end; and
ligating the first RNA sequence and the second RNA sequence to obtain the
synthesized full-length PEgRNA,
wherein the ligation forms a linker between the first chemical moiety
of the first RNA sequence and the second chemical moiety of the second RNA
sequence; and
wherein the linker is present in the gRNA core of the synthesized full-
length PEgRNA.
3. The method of claim 1 or 2, wherein the ligation occurs via a chemical ligation or an
enzymatic ligation.
4. The method of claim 2, wherein the gRNA core is a modified gRNA core, optionally,
wherein the gRNA core is a chemically-modified gRNA core.

5. The method of claim 2, wherein the synthesized full-length PEgRNA comprises, in 5' to 3' order, a spacer, a gRNA core, and an extension arm.
6. The method of claim 2, wherein the synthesized full-length PEgRNA comprises, in 5' to 3' order, a spacer, a chemically-modified gRNA core, and an extension arm.
7. The method of claim 5 or 6, wherein the extension arm comprises a reverse transcription template (RTT) and a primer binding site (PBS).
8. The method of any one of claims 2-7, wherein the first RNA sequence comprises a spacer and a first portion of a chemically-modified gRNA core.
9. The method of any one of claims 2-7, wherein the second RNA sequence comprises a second portion of a chemically-modified gRNA core, an extension arm, a chemically-modified extension arm, an RTT, and/or a PBS.
10. The method of any one of claims 4-9, wherein the chemically modified gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2) and a third stem loop (SL3).
11. The method of claim 10, wherein the tetraloop comprises a lower stem, a bulge, an upper stem, and a loop.
12. The method of any one of claims 8-11, wherein the first portion and the second portion of the chemically-modified gRNA core each comprise a fragmented tetraloop or a fragmented SL2.
13. The method of any one of claims 8-12, wherein the first portion and the second portion of the chemically-modified gRNA core each comprise a fragmented tetraloop.
14. The method of any one of claims 8-12, wherein the first portion and the second portion of the chemically-modified gRNA core each comprise a fragmented SL2.

15. The method of any one of claims 5-14, wherein the spacer is 10 nucleotides to 30 nucleotides in length.
16. The method of any one of claims 7-15, wherein the PBS is 20 nucleotides to 50 nucleotides in length.
17. The method of any one of claims 7-15, wherein the RTT is 5 nucleotides to 25 nucleotides in length.
18. The method of any one of claims 8-17, wherein the first portion of the chemically-modified gRNA core and the second portion of the chemically-modified gRNA core are different in length.
19. The method of any one of claims 8-17, wherein the first portion of the chemically-modified gRNA core and the second portion of the chemically-modified gRNA core are equal in length.
20. The method of any one of claims 8-19, wherein the first portion of the chemically-modified gRNA core is at least 10 nucleotides in length.
21. The method of any one of claims 8-19, wherein the second portion of the chemically-modified gRNA core is at least 10 nucleotides in length.
22. The method of any one of claims 12-21, wherein the fragmented tetraloop of the first portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
23. The method of any one of claims 12-22, wherein the fragmented SL2 of the first portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
24. The method of any one of claims 12-23, wherein the fragmented tetraloop of the second portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.

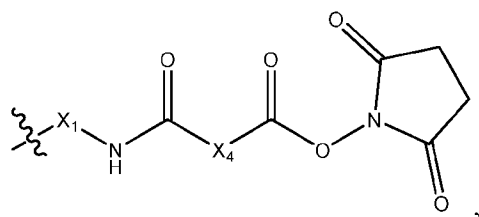
25. The method of any one of claims 12-24, wherein the fragmented SL2 of the second portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
26. The method of claim 4, wherein the chemically-modified gRNA core comprises a tetraloop, an extended tetraloop, an SL2 and/or an extended SL2.
27. The method of claim 26, wherein the extended tetraloop comprises an additional 1-20 nucleotides compared to the tetraloop of SEQ ID NO.: 22.
28. The method of claim 26, wherein the extended tetraloop comprises an additional 1-20 nucleotides in the upper stem compared to the upper stem of the tetraloop of SEQ ID NO.: 22.
29. The method of claim 23, wherein the extended SL2 comprises an additional 1-20 nucleotides compared the SL2 of SEQ ID NO.: 23.
30. The method of any one of claims 2-29, wherein the linker is present in the tetraloop or SL2 of the chemically-modified gRNA core.
31. The method of claim 30, wherein the linker is present in the tetraloop of the chemically-modified gRNA core.
32. The method of claim 31, wherein the linker is present in the upper stem of the tetraloop.
33. The method of claim 30, wherein the linker is present in the SL2 of the chemically-modified gRNA core.
34. The method of any one of claims 2-33, wherein the first chemical moiety of the first RNA sequence is located on the 3' end of the US4, US5, US6, US7, or US8 nucleotide of the tetraloop.

35. The method of any one of claims 2-33, wherein the first chemical moiety of the first RNA sequence is located on the 3' end of the SL2-4, SL2-5, SL2-6, SL2-7, or SL2-8 nucleotide of the SL2.

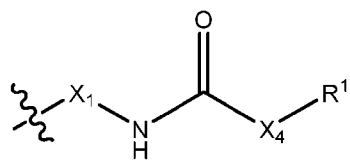
36. The method of any one of claims 2-35, wherein the second chemical moiety of the second RNA sequence is located on the 5' end of the US5, US6, US7, US8, or US9 nucleotide of the tetraloop.

37. The method of any one of claims 2-35, wherein the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-5, SL2-6, SL2-7, SL2-8, or SL2-9 nucleotide of the SL2.

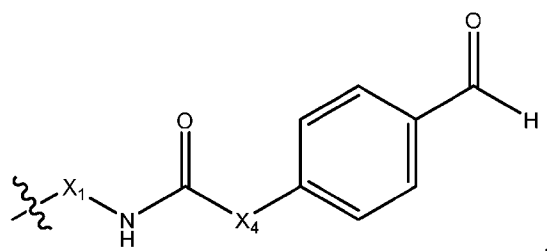
38. The method of any one of claims 1-37, wherein the first chemical moiety of the first RNA sequence comprises amino, $-X_1-NH_2$, $-X_1-OH$, $-X_1-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 1-6 or Formulas 9-11:



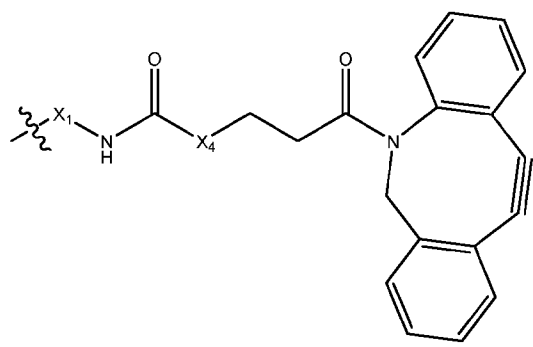
Formula 1



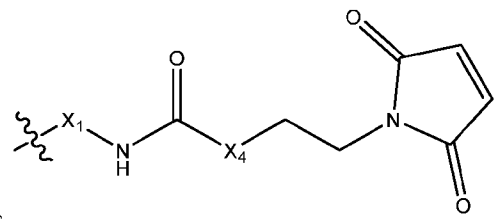
Formula 2



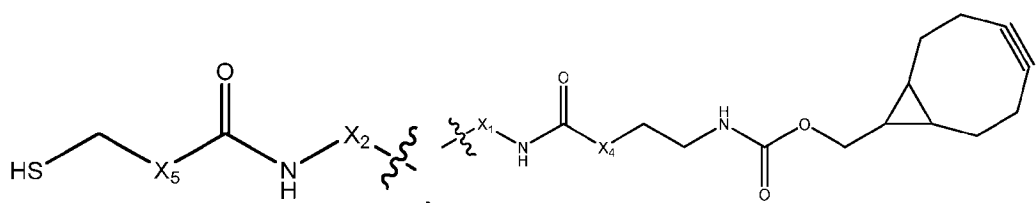
Formula 3



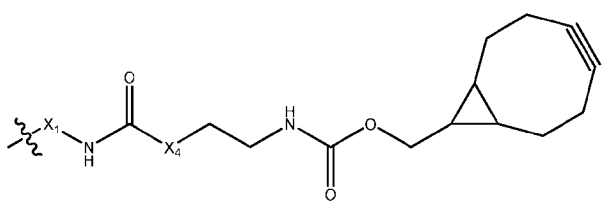
Formula 4



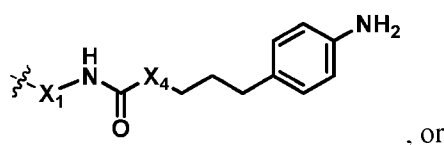
Formula 5



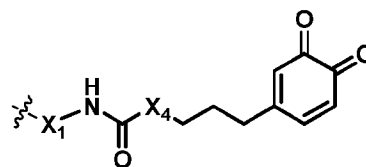
Formula 6



Formula 9



Formula 10

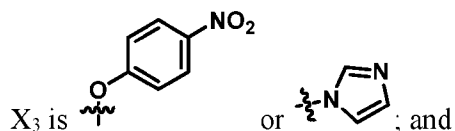


Formula 11

wherein:

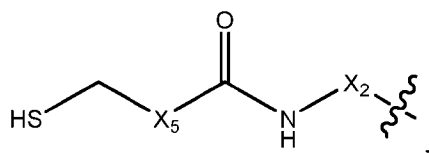
each X_1 , X_4 , and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12;

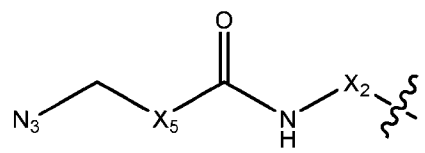


R^1 is halogen.

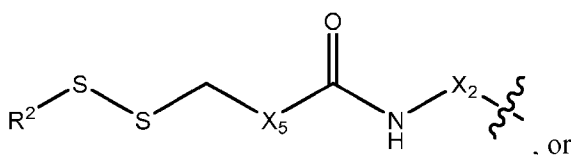
39. The method of any one of claims 1-38, wherein the second chemical moiety of the second RNA sequence comprises an amino, $-X_2-NH_2$, $-X_2-OH$, $-X_2-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 6-8 or Formula 12:



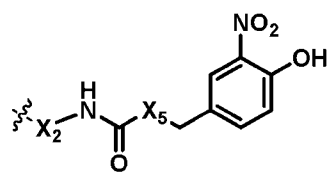
Formula 6



Formula 7



Formula 8

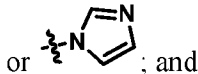
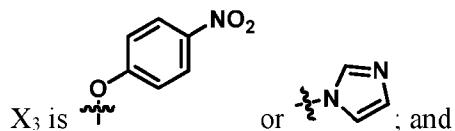


Formula 12

wherein:

each X_2 and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

"n" is an integer between 1 and 12;



R^2 is a heteroaryl.

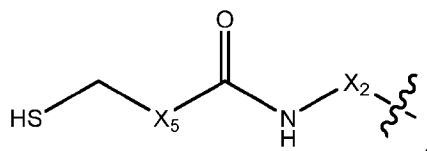
40. The method of claims 38 or 39, wherein the first chemical moiety of the first RNA sequence comprises a structure of Formula 1, and the second chemical moiety of the second RNA sequence comprises an amino.

41. The method of claims 38 or 39, wherein the first chemical moiety of the first RNA sequence comprises a structure of Formula 2, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 6.

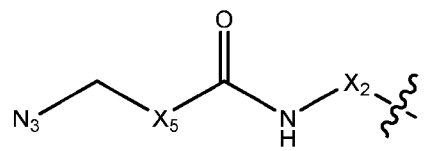
42. The method of claims 38 or 39, wherein the first chemical moiety of the first RNA sequence comprises a structure of Formula 3, and the second chemical moiety of the second RNA sequence comprises an amino.

43. The method of claims 38 or 39, wherein the first chemical moiety of the first RNA sequence comprises a structure of Formula 4, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 7.
44. The method of claims 38 or 39, wherein the first chemical moiety of the first RNA sequence comprises a structure of Formula 5, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 6.
45. The method of claims 38 or 39, wherein the first chemical moiety of the first RNA sequence comprises a structure of Formula 2, and the second chemical moiety of the second RNA sequence comprises a structure of Formula 8.
46. The method of claims 38 or 39, wherein the first chemical moiety of the first RNA sequence comprises $-X_1-NH_2$, and the second chemical moiety of the second RNA sequence comprises $-X_2-NH_2$.
47. The method of claims 38 or 39, wherein the first chemical moiety of the first RNA sequence comprises an amino, and the second chemical moiety of the second RNA sequence comprises $-O(CO)X_3$.
48. The method of claims 38 or 39, wherein the first chemical moiety of the first RNA sequence comprises $-O(CO)X_3$, and the second chemical moiety of the second RNA sequence comprises an amino.
49. The method of any one of claims 1-48, wherein the method further comprises a third RNA sequence comprising a third chemical moiety at its 5' end and a fourth chemical moiety at its 3' end.
50. The method of claim 49, wherein the third RNA sequence comprises a third portion of the chemically-modified gRNA core.
51. The method of claim 50, wherein the third portion of the chemically-modified gRNA core comprises a fragmented tetraloop, a first stem loop, and a fragmented SL2.

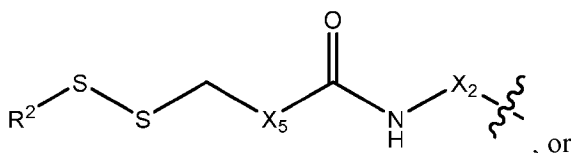
52. The method of any one of claims 49-51, wherein the third chemical moiety of the third RNA sequence comprises an amino, $-X_2-NH_2$, $-X_2-OH$, $-X_2-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 6-8 or Formula 12:



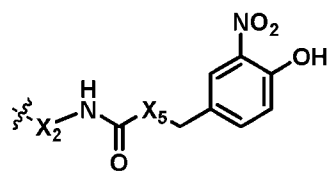
Formula 6



Formula 7



Formula 8

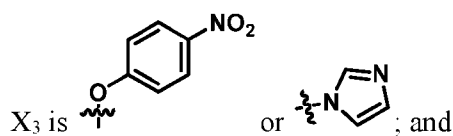


Formula 12

wherein:

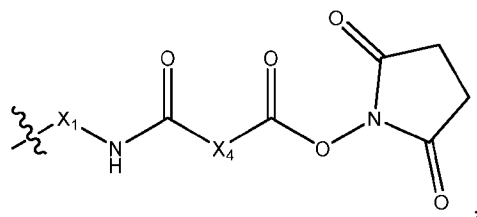
each X_2 and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

"n" is an integer between 1 and 12;

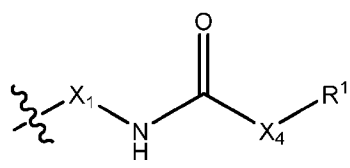


R^2 is a heteroaryl.

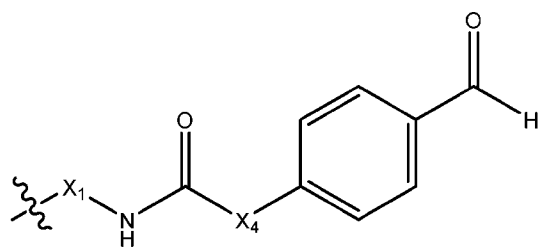
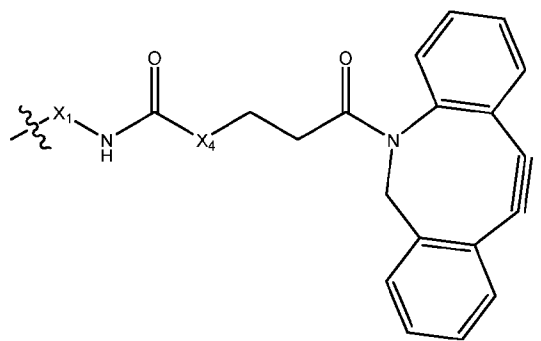
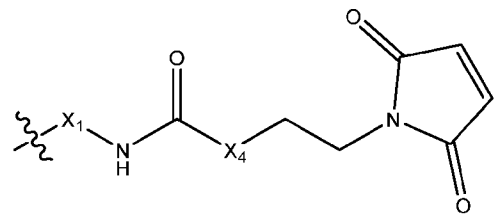
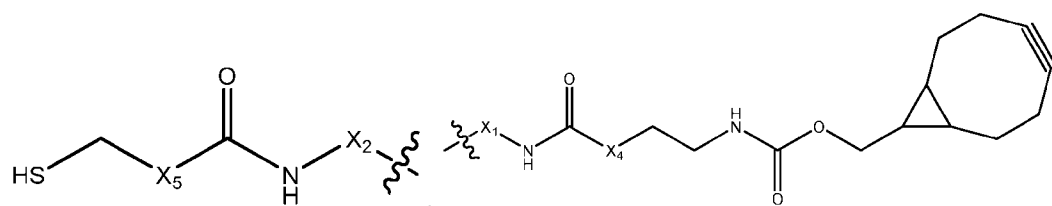
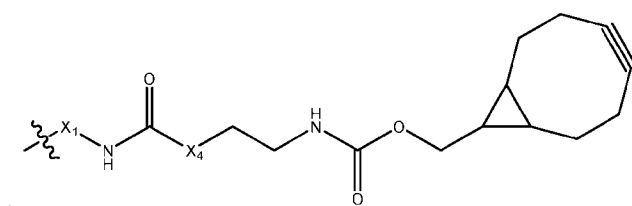
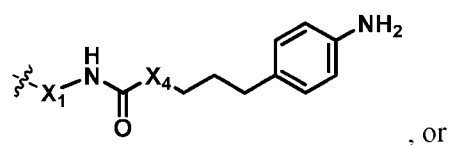
53. The method of any one of claims 49-51, wherein the fourth chemical moiety of the third RNA sequence comprises amino $-X_1-NH_2$, $-X_1-OH$, $-X_1-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 1-6 or Formulas 9-11:



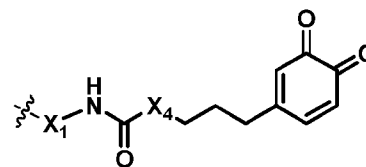
Formula 1



Formula 2

**Formula 3****Formula 4****Formula 5****Formula 6****Formula 9****Formula 10**

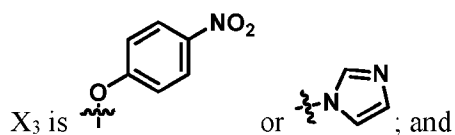
, or

**Formula 11**

wherein:

each X_1 , X_4 , and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“ n ” is an integer between 1 and 12;



R^1 is halogen.

54. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising,
- providing a first RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end
 - providing a second RNA sequence, wherein the second RNA sequence comprises a second chemical moiety at its 5' end;
 - providing a third RNA sequence, wherein the third RNA sequence comprises a third chemical moiety at its 5' end and a fourth chemical moiety at its 3' end;
 - ligating the first, second, and third RNA sequences to obtain the synthesized full-length PEgRNA,
 - wherein the ligation forms (i) a first linker between the first chemical moiety of the first RNA sequence and the third chemical moiety of the third RNA sequence, and (ii) a second linker between the second chemical moiety of the second RNA sequence and the fourth chemical moiety of the third RNA sequence;
 - wherein the first linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA, and
 - wherein the second linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA or in a junction of the synthesized full-length PEgRNA.
55. The method of claim 54, wherein the second linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.
56. The method of claim 54, wherein the second linker is present in the junction of the synthesized full-length PEgRNA.
57. The method of claim 54, wherein the synthesized full-length PEgRNA comprises, in 5' to 3' order, a spacer, a chemically-modified gRNA core, a junction, and an extension arm.
58. The method of claim 57, wherein the extension arm comprises a reverse transcription template (RTT) and a primer binding site (PBS).

59. The method of claim 57, wherein the extension arm is a chemically-modified extension arm.
60. The method of any one of claims 54-59, wherein the chemically-modified gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2) and a third stem loop (SL3).
61. The method of claim 60, wherein the tetraloop comprises a lower stem, a bulge, an upper stem, and a loop.
62. The method of any one of claims 54-61, wherein the junction is present, in 5' to 3' order, between the chemically-modified gRNA core and the extension arm.
63. The method of any one of claims 54-62, wherein the first RNA sequence comprises a spacer and a first portion of the chemically-modified gRNA core.
64. The method of claim 63, wherein the first portion of the chemically-modified gRNA core comprises a fragmented tetraloop.
65. The method of any one of claims 54-64, wherein the second RNA sequence comprises a second portion of the chemically-modified gRNA core, an extension arm, a chemically-modified extension arm, an RTT, and/or a PBS.
66. The method of any one of claims 54-64, wherein the second RNA sequence comprises a second portion of the chemically-modified gRNA core and an extension arm.
67. The method of claim 65 or 66, wherein the second portion of the chemically-modified gRNA core comprises a fragmented SL2.
68. The method of any one of claims 54-65, wherein the second RNA sequence comprises a chemically-modified extension arm.

69. The method of any one of claims 54-68, wherein the third RNA sequence comprises a third portion of the chemically-modified gRNA core, a fourth portion of the chemically-modified gRNA core, and/or a junction.

70. The method of any one of claims 54-68, wherein the third RNA sequence comprises a third portion of the chemically-modified gRNA core and a fourth portion of the chemically-modified gRNA core.

71. The method of claim 70, wherein the third portion of the chemically-modified gRNA core comprises a fragmented tetraloop and the fourth portion of the chemically-modified gRNA core comprises a fragmented SL2.

72. The method of any one of claims 54-69, wherein the third RNA sequence comprises a third portion of the chemically-modified gRNA core and a junction.

73. The method of claim 72, wherein the third portion of the chemically-modified gRNA core comprises a fragmented tetraloop.

74. The method of claim 72, wherein the junction comprises the fourth chemical moiety.

75. The method of any one of claims 57-74, wherein the spacer is 10 nucleotides to 30 nucleotides in length.

76. The method of any one of claims 58-75, wherein the PBS is 20 nucleotides to 50 nucleotides in length.

77. The method of any one of claims 58-75, wherein the RTT is 5 nucleotides to 25 nucleotides in length.

78. The method of any one of claims 63-77, wherein the first portion of the chemically-modified gRNA core is at least 10 nucleotides in length.

79. The method of any one of claims 65-78, wherein the second portion of the chemically-modified gRNA core is at least 10 nucleotides in length.

80. The method of any one of claims 69-79, wherein the third portion of the chemically-modified gRNA core is at least 10 nucleotides in length.
81. The method of any one of claims 69-80, wherein the fourth portion of the chemically-modified gRNA core is at least 10 nucleotides in length.
82. The method of claim 64, wherein the fragmented tetraloop of the first portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
83. The method of claim 73, wherein the fragmented tetraloop of the third portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
84. The method of claim 67, wherein the fragmented SL2 of the second portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
85. The method of claim 71, wherein the fragmented SL2 of the fourth portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
86. The method of any one of claims 54-86, wherein the chemically-modified gRNA core comprises a tetraloop, an extended tetraloop, an SL2 or an extended SL2.
87. The method of claim 86, wherein the extended tetraloop comprises an additional 1-20 nucleotides compared to the tetraloop of SEQ ID NO.: 22.
88. The method of claim 86, wherein the extended tetraloop comprises an additional 1-20 nucleotides in the upper stem compared to the upper stem of the tetraloop of SEQ ID NO.: 22.
89. The method of claim 86, wherein the extended SL2 comprises an additional 1-20 nucleotides compared the SL2 of SEQ ID NO.: 23.

90. The method of any one of claims 54-89, wherein the first chemical moiety of the first RNA sequence is located on the 3' end of the US4, US5, US6, US7, or US8 nucleotide of the tetraloop.

91. The method of any one of claims 54-89, wherein the third chemical moiety of the third RNA sequence is located on the 5' end of the US5, US6, US7, US8, or US9 nucleotide of the tetraloop.

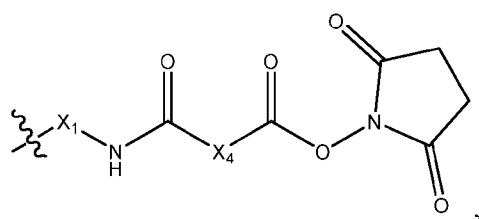
92. The method of any one of claims 54-89, wherein the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-5, SL2-6, SL2-7, SL2-8, or SL2-9 nucleotide of the SL2.

93. The method of any one of claims 54-89, wherein the fourth chemical moiety of the third RNA sequence is located on the 3' end of the SL2-4, SL2-5, SL2-6, SL2-7, or SL2-8 nucleotide of the SL2.

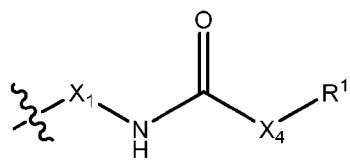
94. The method of any one of claims 54-89, wherein the second chemical moiety of the second RNA sequence is located on the 5' end of the chemically-modified extension arm.

95. The method of any one of claims 54-89, wherein the fourth chemical moiety of the third RNA sequence is located on the 3' end of the junction.

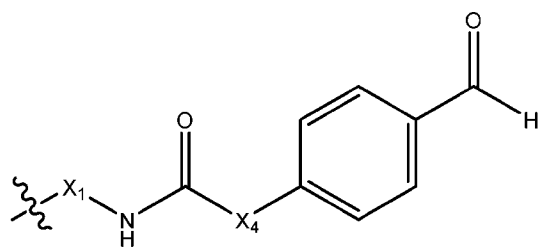
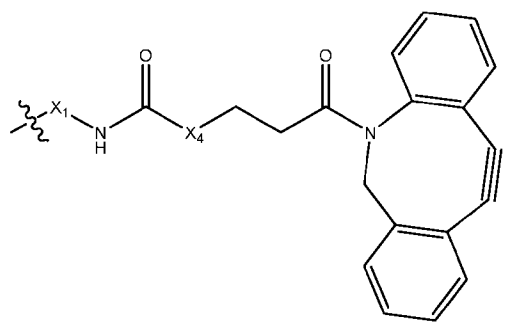
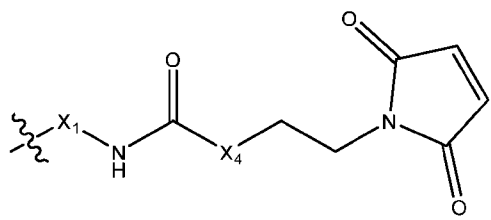
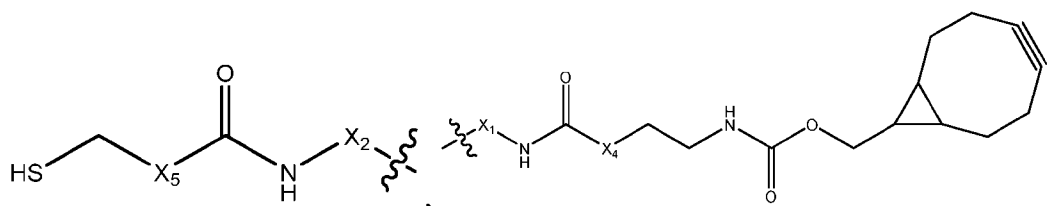
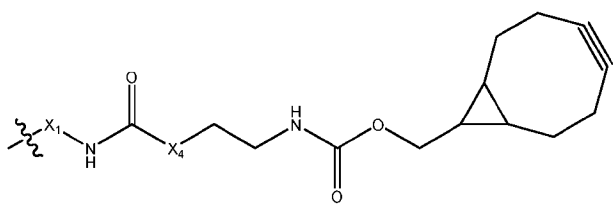
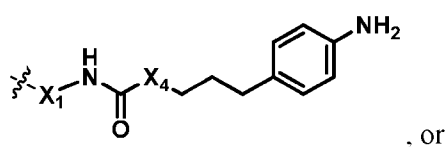
96. The method of any one of claims 54-95, wherein the first chemical moiety of the first RNA sequence and the fourth chemical moiety of the third RNA sequence each comprise: an amino, $-X_1-NH_2$, $-X_1-OH$, $-X_1-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 1-6 or Formulas 9-11:



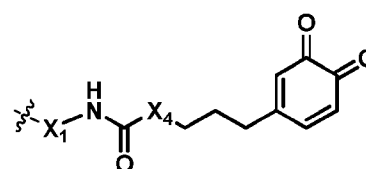
Formula 1



Formula 2

**Formula 3****Formula 4****Formula 5****Formula 6****Formula 9****Formula 10**

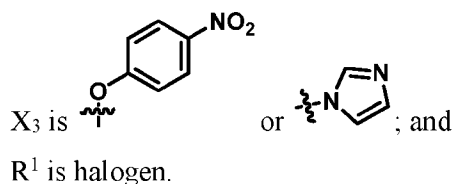
, or

**Formula 11**

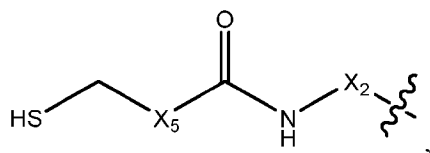
wherein:

each X_1 , X_4 , and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

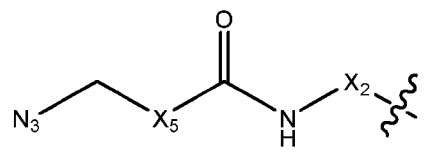
“n” is an integer between 1 and 12;



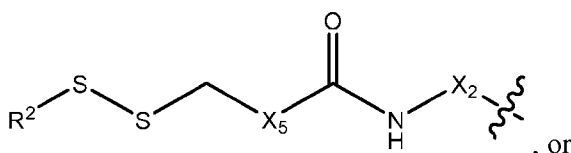
97. The method of any one of claims 54-96, wherein the second chemical moiety of the second RNA sequence and the third chemical moiety of the third RNA sequence each comprise: an amino, $-X_2-NH_2$, $-X_2-OH$, $-X_2-PO_4^-$, $-O(CO)X_3$, or a structure of any one of Formulas 6-8 or Formula 12:



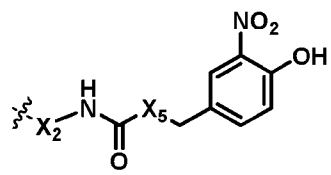
Formula 6



Formula 7



Formula 8

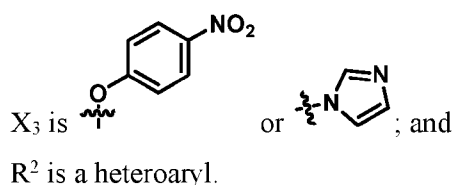


Formula 12

wherein:

each X₂ and X₅ is independently absent or (O-CH₂-CH₂)_n, (CH₂-CH₂-O)_n, ((CH₂)_n-C(O)NH-CH₂), or an alkylene optionally substituted with one or more -OH or -O-, or -O-;

“n” is an integer between 1 and 12;



98. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising,
 providing a first RNA sequence,
 wherein the first RNA sequence comprises an amino modified phosphodiester backbone at its 3' end;

functionalizing the 3' amino group of the first RNA sequence with a first chemical moiety;

providing a second RNA sequence;

wherein the second RNA sequence comprises an amino modified phosphodiester backbone at its 5' end;

functionalizing the 5' amino group of the second RNA sequence with a second chemical moiety;

reacting the first chemical moiety of the first RNA sequence with the second chemical moiety of the second RNA sequence to form a linker,

wherein forming the linker results in the formation of the synthesized full-length PEgRNA; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

99. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises an amino modified phosphodiester backbone at its 3' end;

providing a second RNA sequence; wherein the second RNA sequence comprises an amino modified phosphodiester backbone at its 5' end;

annealing the first and second RNA sequences to form a pre-annealed duplex;

providing a reagent;

contacting the pre-annealed duplex with the reagent to form a linker,

wherein forming the linker results in the formation of the synthesized full-length PEgRNA; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

100. The method of claim 98 or 99, wherein the synthesized full-length PEgRNA comprises, in 5' to 3' order, a spacer, a chemically-modified guide RNA (gRNA) core, and an extension arm.

101. The method of claim 100, wherein the extension arm comprises a reverse transcription template (RTT) and a primer binding site (PBS).
102. The method of any one of claims 98-101, wherein the first RNA sequence comprises a spacer and a first portion of the chemically-modified gRNA core.
103. The method of any one of claims 98-102, wherein the second RNA sequence comprises a second portion of the chemically-modified gRNA core, an extension arm, a chemically-modified extension arm, an RTT, and/or a PBS.
104. The method of any one of claims 98-103, wherein the chemically-modified gRNA core comprises, in 5' to 3' order, a tetraloop, a first stem loop (SL1), a second stem loop (SL2) and a third stem loop (SL3).
105. The method of claim 104, wherein the tetraloop comprises a lower stem, a bulge, an upper stem, and a loop.
106. The method of any one of claims 102-105, wherein the first portion and the second portion of the chemically-modified gRNA core each comprise a fragmented tetraloop or a fragmented SL2.
107. The method of any one of claims 102-105, wherein the first portion and the second portion of the chemically-modified gRNA core each comprise a fragmented tetraloop.
108. The method of any one of claims 102-105, wherein the first portion and the second portion of the chemically-modified gRNA core each comprise a fragmented SL2.
109. The method of any one of claims 100-108, wherein the spacer is 10 nucleotides to 30 nucleotides in length.
110. The method of any one of claims 101-109, wherein the PBS is 20 nucleotides to 50 nucleotides in length.

111. The method of any one of claims 101-110, wherein the RTT is 5 nucleotides to 25 nucleotides in length.
112. The method of any one of claims 102-111, wherein the first portion of the chemically-modified gRNA core and the second portion of the chemically-modified gRNA core are different in length.
113. The method of any one of claims 102-111, wherein the first portion of the chemically-modified gRNA core and the second portion of the chemically-modified gRNA core are equal in length.
114. The method of any one of claims 102-113, wherein the first portion of the chemically-modified gRNA core is at least 10 nucleotides in length.
115. The method of any one of claims 102-114, wherein the second portion of the chemically-modified gRNA core is at least 10 nucleotides in length.
116. The method of claim 106 or 107, wherein the fragmented tetraloop of the first portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
117. The method of claim 106 or 108, wherein the fragmented SL2 of the first portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
118. The method of claim 106 or 107, wherein the fragmented tetraloop of the second portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
119. The method of claim 106 or 108, wherein the fragmented SL2 of the second portion of the chemically-modified gRNA core is 5 nucleotides to 30 nucleotides in length.
120. The method of any one of claims 98-119, wherein the chemically-modified gRNA core comprises a tetraloop, an extended tetraloop, an SL2 or an extended SL2.
121. The method of claim 120, wherein the extended tetraloop comprises an additional 1-20 nucleotides compared to the tetraloop of SEQ ID NO.: 22.

122. The method of claim 120, wherein the extended tetraloop comprises an additional 1-20 nucleotides in the upper stem compared to the upper stem of the tetraloop of SEQ ID NO.: 22.

123. The method of claim 120, wherein the extended SL2 comprises an additional 1-20 nucleotides compared the SL2 of SEQ ID NO.: 23.

124. The method of any one of claims 98-123, wherein the linker is present in the tetraloop or SL2 of the chemically-modified gRNA core.

125. The method of claim 124, wherein the linker is present in the tetraloop of the chemically-modified gRNA core.

126. The method of claim 125, wherein the linker is present in the upper stem of the tetraloop.

127. The method of claim 124, wherein the linker is present in the SL2 of the chemically-modified gRNA core.

128. The method of any one of claims 98-127, wherein the first chemical moiety of the first RNA sequence is located on the 3' end of the US4, US5, US6, US7, or US8 nucleotide of the tetraloop.

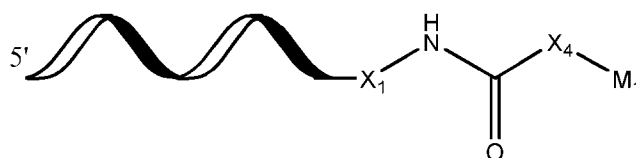
129. The method of any one of claims 98-127, wherein the first chemical moiety of the first RNA sequence is located on the 3' end of the SL2-4, SL2-5, SL2-6, SL2-7, or SL2-8 nucleotide of the SL2.

130. The method of any one of claims 98-129, wherein the second chemical moiety of the second RNA sequence is located on the 5' end of the US5, US6, US7, US8, or US9 nucleotide of the tetraloop.

131. The method of any one of claims 98-129, wherein the second chemical moiety of the second RNA sequence is located on the 5' end of the SL2-5, SL2-6, SL2-7, SL2-8, or SL2-9 nucleotide of the SL2.

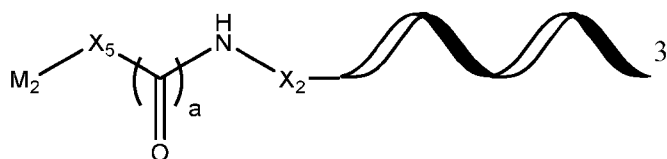
132. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula A:



Formula A;

providing a second RNA sequence of Formula B:



Formula B;

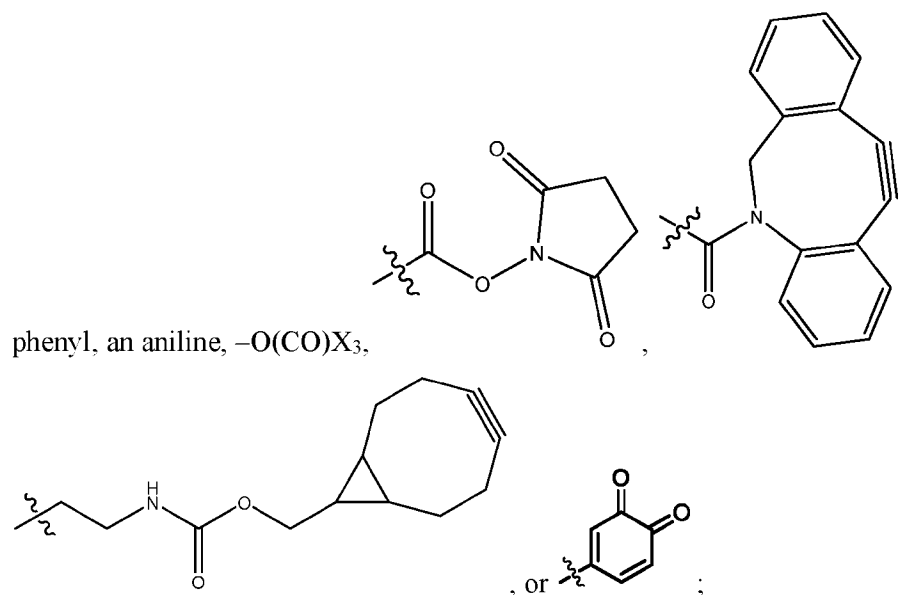
reacting M_1 with M_2 to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA;

wherein:

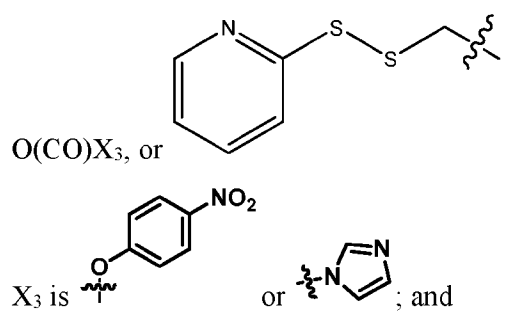
each X_1 , X_2 , X_4 , and X_5 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12;

M₁ is an amino, a halogen, a hydroxyl, a phosphate ion, a maleimide, an aldehyde-substituted



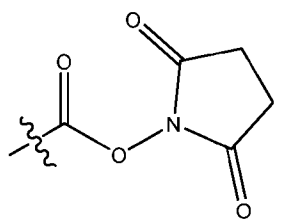
M₂ is an amino, a hydroxyl, a phosphate ion, a thiol, an azido, an O-aminophenyl, –



“a” is an integer of 0 or 1;

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

133. The method of claim 132, wherein in Formula A, X₄ is an alkylene and M₁ is

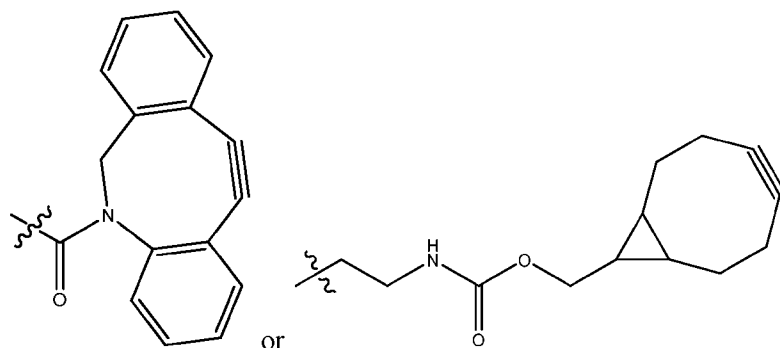


134. The method of claim 132, wherein in Formula A, X₄ is an alkylene and M₁ is a halogen.

135. The method of claim 132, wherein in Formula A, X_4 is $((CH_2)_n-C(O)NH-CH_2)$, and M_1 is a halogen.

136. The method of claim 132, wherein in Formula A, X_4 is absent and M_1 is an aldehyde-substituted phenyl.

137. The method of claim 132, wherein in Formula A, X_4 is an alkylene and M_1 is



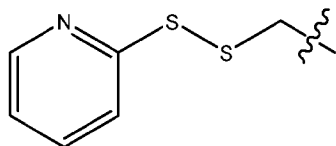
138. The method of claim 132, wherein in Formula A, X_4 is an alkylene and M_1 is a maleimide.

139. The method of claim 132, wherein in Formula B, "a" is 0, X_5 is absent, and M_2 is an amino.

140. The method of claim 132, wherein in Formula B, "a" is 1, X_5 is an alkylene, and M_2 is a thiol.

141. The method of claim 132, wherein in Formula B, "a" is 1, X_5 is an alkylene, and M_2 is an azide.

142. The method of claim 132, wherein in Formula B, "a" is 1, X_5 is an alkylene, and M_2 is



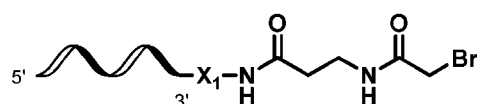
143. The method of any one of claims 132-142, wherein each X_1 and X_2 is independently a C_1 - C_{10} unsubstituted alkylene.

144. The method of claim 143, wherein each X_1 and X_2 is independently a C_6 unsubstituted alkylene.

145. The method of any one of claims 132-144, wherein X_1 is a C_1 - C_{10} substituted alkylene with an $-OH$ and/or an $-O-$ group.

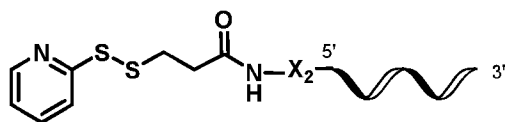
146. The method of any one of claims 132-145, wherein X_2 is $(O-CH_2-CH_2)_n$.

147. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:
providing a first RNA sequence of Formula C:



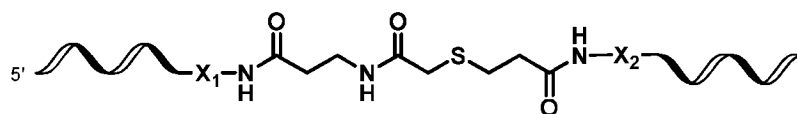
Formula C;

providing a second RNA sequence of Formula D:



Formula D;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula E:



Formula E;

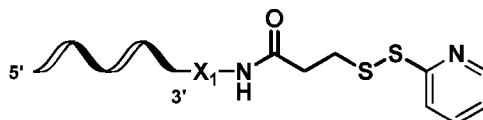
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

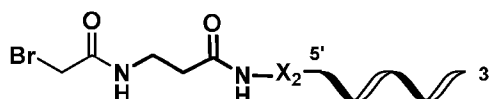
148. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula C-1:



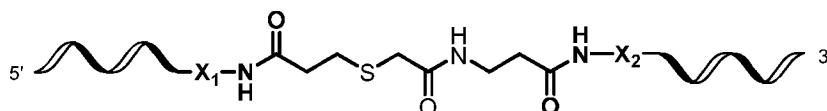
Formula C-1;

providing a second RNA sequence of Formula D-1:



Formula D-1;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula E-1:



Formula E-1;

wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

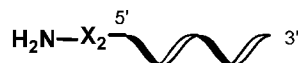
149. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula F:



Formula F;

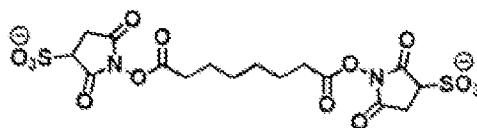
providing a second RNA sequence of Formula G:



Formula G;

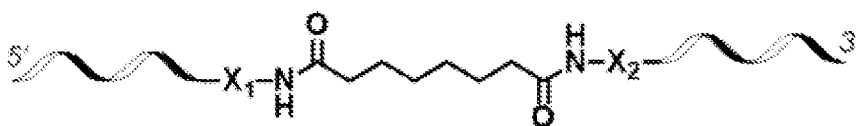
annealing the first and second RNA sequences to form a pre-annealed duplex;

providing a BS3 reagent of the formula:



BS3;

contacting the pre-annealed duplex with the BS3 reagent to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula H:



Formula H;

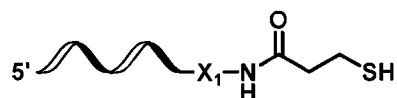
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

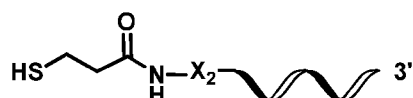
150. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula I:



Formula I;

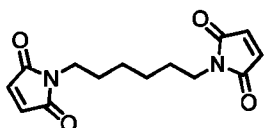
providing a second RNA sequence of Formula J:



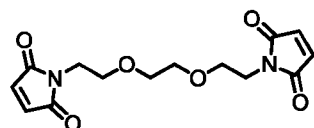
Formula J;

annealing the first and second RNA sequences to form a pre-annealed duplex;

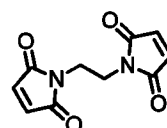
providing a BMH, BM(PEG)₂, BMOE, BM(PEG)₃, or BMB reagent of the formulas:



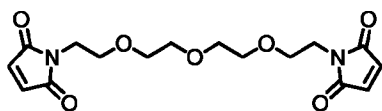
BMH
bismaleimidoethane



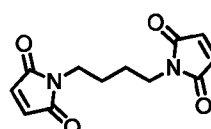
BM(PEG)₂
1,8-bis(maleimido)diethyleneglycol



BMOE
bismaleimidoethane

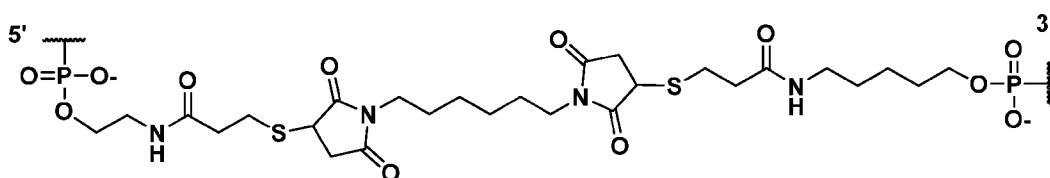


BM(PEG)₃
1,11-bis(maleimido)triethyleneglycol

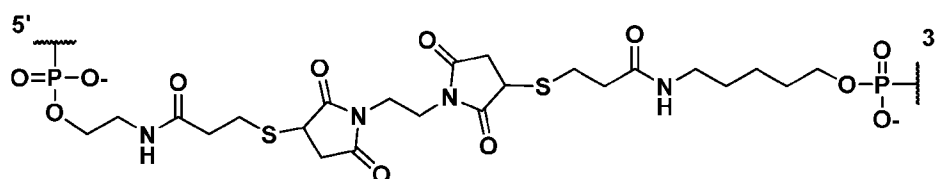


BMB
1,4-bis(maleimido)butane

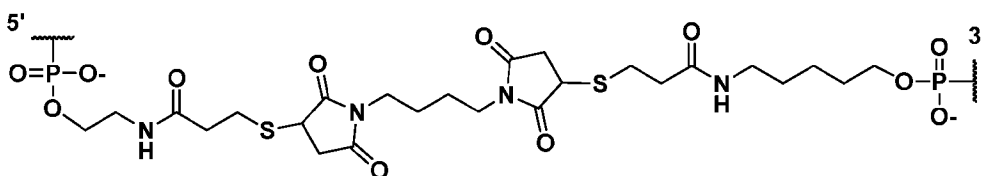
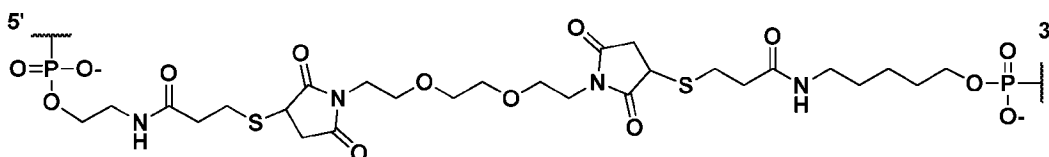
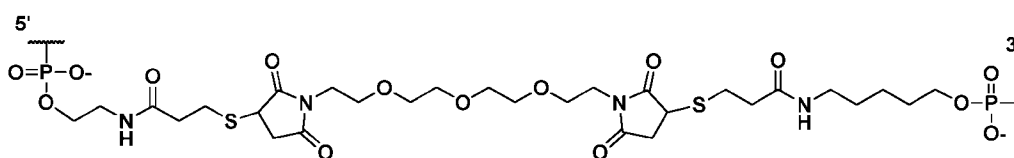
contacting the pre-annealed duplex with the BMH, BM(PEG)₂, BMOE, BM(PEG)₃, or BMB reagent to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formulas K-O:



Formula K;



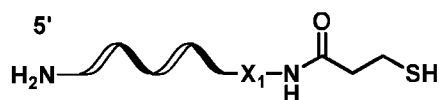
Formula L;

**Formula M;****Formula N; or****Formula O;**

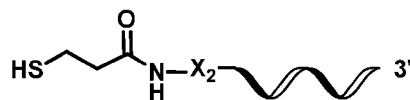
wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

151. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula I-1:

**Formula I-1;**

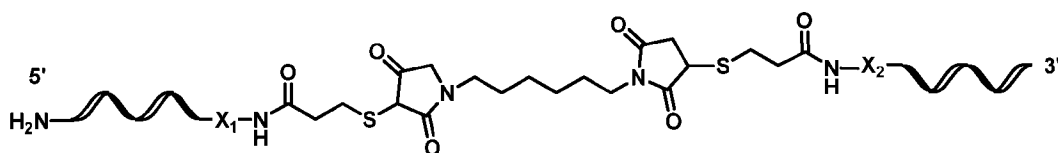
providing a second RNA sequence of Formula J:

**Formula J;**

annealing the first and second RNA sequences to form a pre-annealed duplex;

providing a BMH reagent;

contacting the pre-annealed duplex with the BMH reagent to form a linker, wherein forming the linker results in the formation of Formula P:

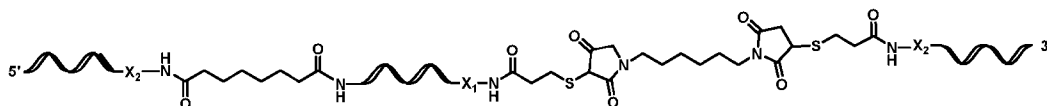
**Formula P;**

wherein Formula P comprises an amine at its 5' end;
providing a third RNA sequence of Formula F:

**Formula F;**

contacting Formula P with Formula F to form a second pre-annealed duplex;
providing a BS3 reagent;

contacting the second pre-annealed duplex with the BS3 reagent to form a linker,
wherein forming the linker results in the formation of the synthesized full-length PEgRNA of

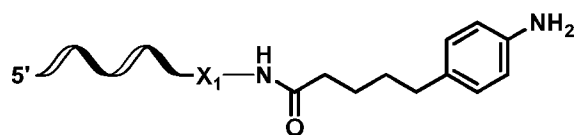
Formula Q:**Formula Q;**

wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

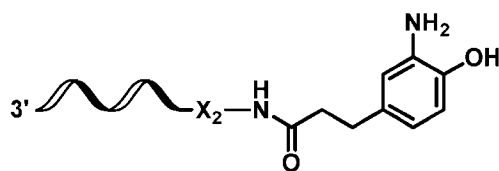
"n" is an integer between 1 and 12.

152. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

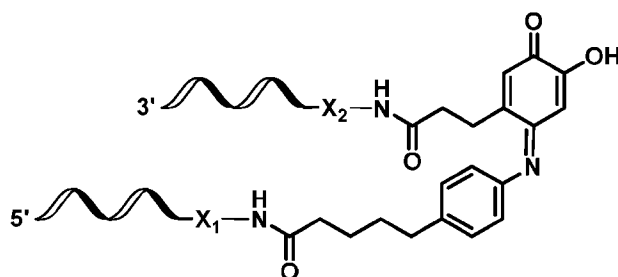
providing a first RNA sequence of Formula R:

**Formula R;**

providing a second RNA sequence of Formula S:

**Formula S;**

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula T:

**Formula T;**

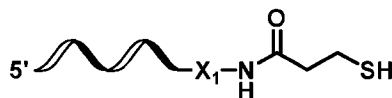
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

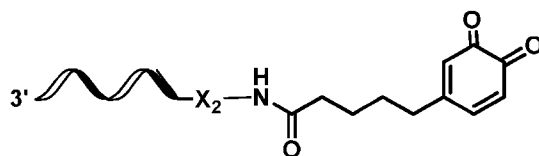
wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

153. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula I:

**Formula I;**

providing a second RNA sequence of Formula U:

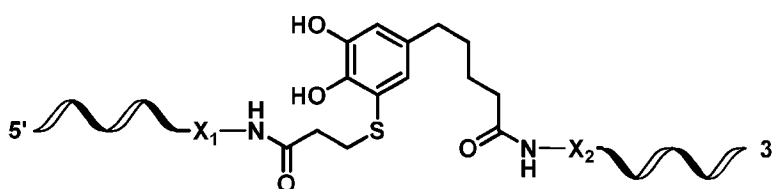


Formula U;

annealing the first and second RNA sequences to form a pre-annealed duplex;

contacting the first RNA sequence with the second RNA sequence to form a linker,

wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula W:



Formula W;

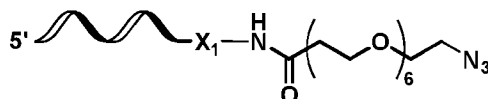
wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

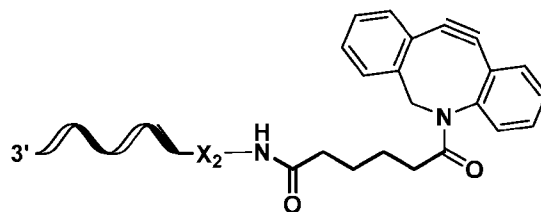
154. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a chemically-modified guide RNA (gRNA) core, the method comprising:

providing a first RNA sequence of Formula X:



Formula X;

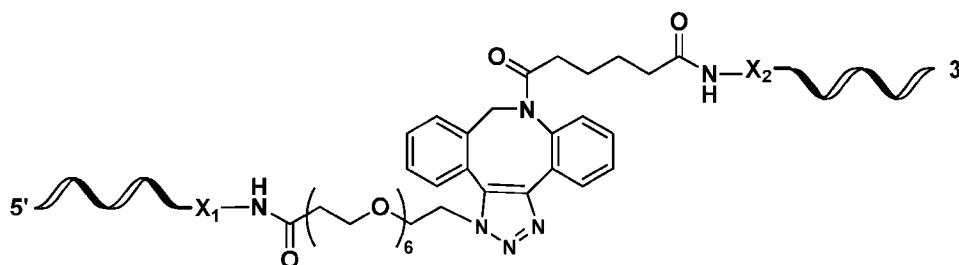
providing a second RNA sequence of Formula Y:



Formula Y;

annealing the first and second RNA sequences to form a pre-annealed duplex;

contacting the first RNA sequence with the second RNA sequence to form a linker, wherein forming the linker results in the formation of the synthesized full-length PEgRNA of Formula Z:



Formula Z;

wherein each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$;

“n” is an integer between 1 and 12; and

wherein the linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA.

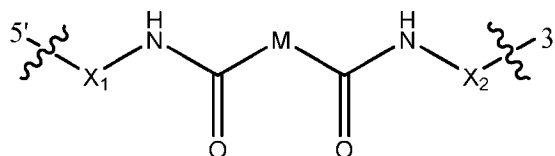
155. The method of any one of claims 147-154, wherein each X_1 and X_2 is independently a C_1 - C_{10} unsubstituted alkylene.

156. The method of any one of claims 147-154, wherein X_1 is a C_1 - C_{10} substituted alkylene with an $-OH$ and/or an $-O-$ group.

157. The method of any one of claims 132-156, wherein the linker is present in the tetraloop or SL2 of the chemically-modified gRNA core.

158. The method of claim 157, wherein the linker is present in the tetraloop of the chemically-modified gRNA core.
159. The method of claim 158, wherein the linker is present in the upper stem of the tetraloop.
160. The method of claim 157, wherein the linker is present in the SL2 of the chemically-modified gRNA core.
161. The method of any one of claims 132-156, wherein the linker is present in the SL3 of the chemically-modified gRNA core.
162. The method of any one of claims 132-161, wherein the linker is located between the US4 and US5 nucleotides, between the US5 and US6 nucleotides, between the US6 and US7 nucleotides, between the US7 and US8 nucleotides, or between the US8 and US9 nucleotides of the tetraloop.
163. The method of any one of claims 132-161, wherein the linker is located between the SL2-4 and SL2-5 nucleotides, between the SL2-5 and SL2-6 nucleotides, between the SL2-6 and SL2-7 nucleotides, between the SL2-7 and SL2-8 nucleotides, or between the SL2-8 and SL2-9 nucleotides of the SL2.
164. A full-length PEgRNA comprising a chemically-modified gRNA core, wherein the full-length PEgRNA is produced by:
- contacting a first RNA sequence with a second RNA sequence,
 - wherein the first RNA sequence comprises a first chemical moiety at its 3' end
 - and the second RNA sequence comprises a second chemical moiety at its 5' end; and;
 - ligating the first RNA sequence and the second RNA sequence to obtain the full-length PEgRNA,
 - wherein the ligation forms a linker between the first chemical moiety of the first RNA sequence and the second chemical moiety of the second RNA sequence; and
 - wherein the linker is present in the chemically-modified gRNA core of the full-length PEgRNA.

165. A full-length PEGRNA comprising a chemically-modified gRNA core, wherein the chemically-modified gRNA core comprises one or more linkers of Formula AA:

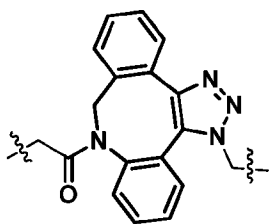


Formula AA

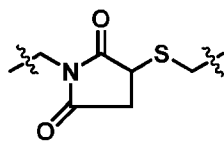
wherein:

each X_1 and X_2 is independently absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; “n” is an integer between 1 and 12;

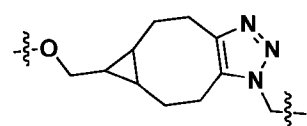
M is a linker, wherein the linker comprises an alkylene optionally substituted with one or more of $-OH$, $-O-$, $-S-$, or $(O-CH_2-CH_2)_n$; or a structure of any one of Formulas AA-1 to AA-10:



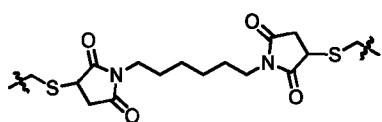
Formula AA-1



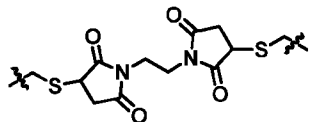
Formula AA-2



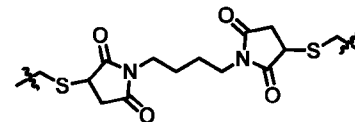
Formula AA-3



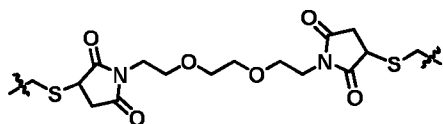
Formula AA-4



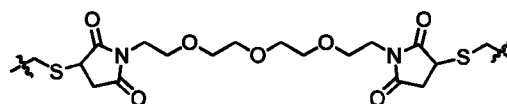
Formula AA-5



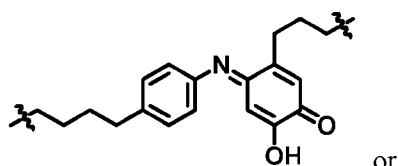
Formula AA-6



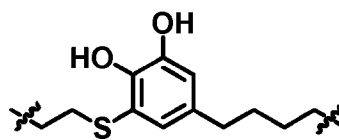
Formula AA-7



Formula AA-8



Formula AA-9



Formula AA-10

166. The full-length PEgRNA of claim 165, wherein the linker is a C₆ unsubstituted alkylene.
167. The full-length PEgRNA of claim 165, wherein the linker is a C₁-C₁₀ substituted alkylene with a -S-.
168. The full-length PEgRNA of claim 165, wherein the linker is a structure of any one of Formulas AA-1 to AA-10.
169. The full-length PEgRNA of any one of claims 165-168, wherein X₁ is a C₁-C₁₀ unsubstituted alkylene.
170. The full-length PEgRNA of claim 169, wherein X₁ is a C₆ unsubstituted alkylene.
171. The full-length PEgRNA of any one of claims 165-168, wherein X₁ is a C₁-C₁₀ substituted alkylene with an -OH and/or an -O-.
172. The full-length PEgRNA of any one of claims 165-168, wherein X₁ is (O-CH₂-CH₂)_n.
173. The full-length PEgRNA of any one of claims 165-172, wherein the linker is present in the tetraloop or SL2 of the chemically-modified gRNA core.
174. The full-length PEgRNA of claim 173, wherein the linker is present in the tetraloop of the chemically-modified gRNA core.
175. The full-length PEgRNA of claim 173, wherein the linker is present in the upper stem of the tetraloop.
176. The full-length PEgRNA of claim 173, wherein the linker is present in the SL2 of the chemically-modified gRNA core.
177. The full-length PEgRNA of any one of claims 165-176, wherein the linker is located between the US4 and US5 nucleotides, between the US5 and US6 nucleotides, between the

US6 and US7 nucleotides, between the US7 and US8 nucleotides, or between the US8 and US9 nucleotides of the tetraloop.

178. The full-length PEgRNA of any one of claims 165-176, wherein the linker is located between the SL2-4 and SL2-5 nucleotides, between the SL2-5 and SL2-6 nucleotides, between the SL2-6 and SL2-7 nucleotides, between the SL2-7 and SL2-8 nucleotides, or between the SL2-8 and SL2-9 nucleotides of the SL2.

179. The method of any one of claims 1-163, wherein the synthetic RNA or synthesized full-length PEgRNA is at least 85 nucleotides, at least 90 nucleotides, at least 95 nucleotides, at least 100 nucleotides, at least 110 nucleotides, at least 120 nucleotides, at least 130 nucleotides, at least 140 nucleotides, at least 150 nucleotides, at least 160 nucleotides, at least 170 nucleotides, at least 180 nucleotides, at least 190 nucleotides, at least 200 nucleotides, at least 210 nucleotides, at least 220 nucleotides, at least 230 nucleotides, at least 240 nucleotides, or at least 250 nucleotides in length.

180. The full-length PEgRNA of any one of claims 164-178, wherein the full-length PEgRNA is at least 85 nucleotides, at least 90 nucleotides, at least 95 nucleotides, at least 100 nucleotides, at least 110 nucleotides, at least 120 nucleotides, at least 130 nucleotides, at least 140 nucleotides, at least 150 nucleotides, at least 160 nucleotides, at least 170 nucleotides, at least 180 nucleotides, or at least 190 nucleotides in length at least 200 nucleotides, at least 210 nucleotides, at least 220 nucleotides, at least 230 nucleotides, at least 240 nucleotides, or at least 250 nucleotides in length.

181. The method of any one of claims 1-163, wherein the first RNA sequence is SEQ ID NO.: 1, 3, 5, 7, 9, 19, 24, 30, 60, 62, 66, 69, 74, 89, 91, 93, or 112.

182. The method of any one of claims 1-163, wherein the second RNA sequence is SEQ ID NO.: 2, 4, 6, 8, 10, 20, 25, 31, 61, 63, 67, 70, 73, 75, 90, 108, or 121.

183. The method of any one of claims 2-163, wherein the synthesized full-length PEgRNA is SEQ ID NO.: 17, 18, 21, 26, 30, 64, 65, 68, 72, 77-88, 97-102, 104-106, 109-111, 114-116, 118-120, or 122-123.

184. The full-length PEgRNA of any one of claims 164-178, wherein the first RNA sequence is SEQ ID NO.: 1, 3, 5, 7, 9, 19, 60, 62, 66, 69, 74, 89, 91, 93, or 112.
185. The full-length PEgRNA of any one of claims 164-178, wherein the first RNA sequence is SEQ ID NO.: 2, 4, 6, 8, 10, 20, 61, 63, 67, 70, 73, 75, 90, 108, or 121.
186. The full-length PEgRNA of any one of claims 164-178, wherein the full-length PEgRNA is SEQ ID NO.: 17, 18, 21, 64, 65, 68, 72, 77-88, 97-102, 104-106, 109-111, 114-116, 118-120, or 122-123.
187. The method of any one of claims 2-164, wherein the linker of the full-length PEgRNA does not affect CRISPR associated (Cas) protein binding and/or prime editor binding.
188. The full-length PEgRNA of any one of claims 164-178, wherein the linker of the chemically-modified PEgRNA does not affect CRISPR associated (Cas) protein binding and/or prime editor.
189. A prime editing complex comprising: (i) the full-length PEgRNA prepared by the method of any one of claims 2-163 or the full-length PEgRNA of any one of claims 164-178; and (ii) a prime editor comprising a DNA binding domain and a DNA polymerase domain.
190. The prime editing complex of claim 189, wherein the DNA binding domain is a CRISPR associated (Cas) protein domain.
191. The prime editing complex of claim 190, wherein the Cas protein domain has nickase activity.
192. The prime editing complex of claim 191, wherein the Cas protein domain is a Cas9.
193. The prime editing complex of claim 192, wherein the Cas9 comprises a mutation in an HNH domain.

194. The prime editing complex of claim 193, wherein the Cas9 comprises a H840A mutation in the HNH domain.
195. The prime editing complex of claim 191, wherein the Cas protein domain is a Cas12b.
196. The prime editing complex of claim 191, wherein the Cas protein domain is a Cas12a, Cas12b, Cas12c, Cas12d, Cas12e, Cas14a, Cas14b, Cas14c, Cas14d, Cas14e, Cas14f, Cas14g, Cas14h, Cas14u, or a Cas ϕ .
197. The prime editing complex of any one of claims 189-196, wherein the DNA polymerase domain is a reverse transcriptase.
198. The prime editing complex of claim 197, wherein the reverse transcriptase is a retrovirus reverse transcriptase.
199. The prime editing complex of claim 198, wherein the reverse transcriptase is a Moloney murine leukemia virus (M-MLV) reverse transcriptase.
200. The prime editing complex of any one of claims 189-199, wherein the DNA polymerase and the DNA binding domain are fused or linked to form a fusion protein.
201. A lipid nanoparticle (LNP) or ribonucleoprotein (RNP) comprising the prime editing complex of any one of claims 189-200, or a component thereof.
202. A cell comprising the full-length PEgRNA prepared by the method of any one of claims 1 to 163, the full-length PEgRNA of any one of claims 164-178, the prime editing complex of any one of claims 189-200, or the LNP or RNP of claim 201.
203. The cell of claim 202, wherein the cell is a human cell.
204. A pharmaceutical composition comprising (i) the full-length PEgRNA prepared by the method of any one of claims 1 to 163, the full-length PEgRNA of any one of claims 164-178, the prime editing complex of any one of claims 189-200, or the LNP or RNP of claim 201; and (ii) a pharmaceutically acceptable carrier.

205. A method for editing a gene, the method comprising contacting the gene with (i) the full-length PEgRNA prepared by the method of any one of claims 1 to 163 or the full-length PEgRNA of any one of claims 164-178, and (ii) a prime editor comprising a DNA binding domain and a DNA polymerase domain, wherein the PEgRNA directs the prime editor to incorporate the intended nucleotide edit in the gene, thereby editing the gene.

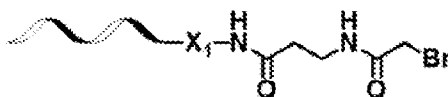
206. A method for editing a gene, the method comprising contacting the gene with the prime editing complex of any one of claims 189-200, wherein the PEgRNA directs the prime editor to incorporate the intended nucleotide edit in the gene, thereby editing the gene.

207. The method of claim 205 or 206, wherein the editing efficiency is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.9% equivalent as compared to editing with an unmodified control PEgRNA.

208. The method of any one of claims 205-207, wherein the prime editor synthesizes a single stranded DNA encoded by the editing template, wherein the single stranded DNA replaces the editing target sequence and results in incorporation of the intended nucleotide edit into a region corresponding to the editing target sequence.

209. The method of any one of claims 205-208, wherein the gene is in a human subject.

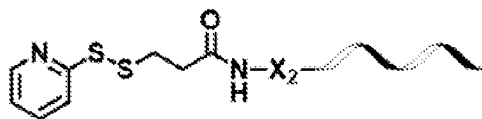
210. An RNA sequence of Formula C:



Formula C;

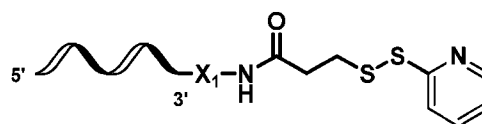
wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and
 “n” is an integer between 1 and 12.

211. An RNA sequence of Formula D:

**Formula D;**

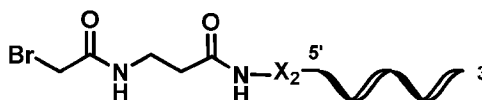
wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and
 “n” is an integer between 1 and 12.

212. An RNA sequence of Formula C-1:

**Formula C-1;**

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and
 “n” is an integer between 1 and 12.

213. An RNA sequence of Formula D-1:

**Formula D-1;**

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and
 “n” is an integer between 1 and 12.

214. An RNA sequence of Formula F:

**Formula F,**

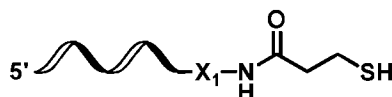
wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and
 “n” is an integer between 1 and 12.

215. An RNA sequence of Formula G:

**Formula G;**

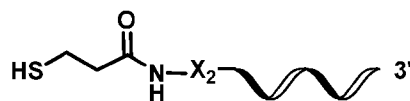
wherein X₂ is absent or (O-CH₂-CH₂)_n, (CH₂-CH₂-O)_n, ((CH₂)_n-C(O)NH-CH₂), or an alkylene optionally substituted with one or more -OH or -O-, or -O-; and
 “n” is an integer between 1 and 12.

216. An RNA sequence of Formula I:

**Formula I,**

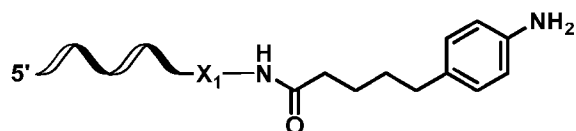
wherein X₁ is absent or (O-CH₂-CH₂)_n, (CH₂-CH₂-O)_n, ((CH₂)_n-C(O)NH-CH₂), or an alkylene optionally substituted with one or more -OH or -O-, or -O-; and
 “n” is an integer between 1 and 12.

217. An RNA sequence of Formula J:

**Formula J;**

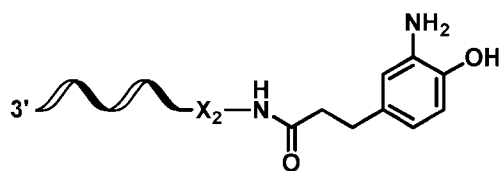
wherein X₂ is absent or (O-CH₂-CH₂)_n, (CH₂-CH₂-O)_n, ((CH₂)_n-C(O)NH-CH₂), or an alkylene optionally substituted with one or more -OH or -O-, or -O-; and
 “n” is an integer between 1 and 12.

218. An RNA sequence of Formula R:

**Formula R,**

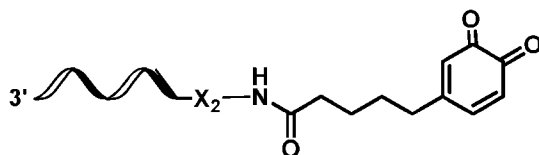
wherein X₁ is absent or (O-CH₂-CH₂)_n, (CH₂-CH₂-O)_n, ((CH₂)_n-C(O)NH-CH₂), or an alkylene optionally substituted with one or more -OH or -O-, or -O-; and
 “n” is an integer between 1 and 12.

219. An RNA sequence of Formula S:

**Formula S;**

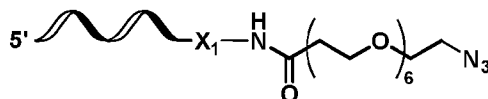
wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and “ n ” is an integer between 1 and 12.

220. An RNA sequence of Formula U:

**Formula U;**

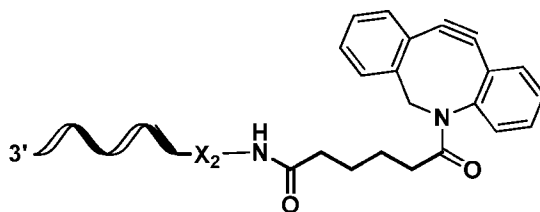
wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and “ n ” is an integer between 1 and 12.

221. An RNA sequence of Formula X:

**Formula X,**

wherein X_1 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and “ n ” is an integer between 1 and 12.

222. An RNA sequence of Formula Y:

**Formula Y;**

wherein X_2 is absent or $(O-CH_2-CH_2)_n$, $(CH_2-CH_2-O)_n$, $((CH_2)_n-C(O)NH-CH_2)$, or an alkylene optionally substituted with one or more $-OH$ or $-O-$, or $-O-$; and

“n” is an integer between 1 and 12.

223. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the method comprising,

- contacting a first RNA sequence with a second RNA sequence,
 - wherein the first RNA sequence comprises a free 3' OH and the second RNA sequence comprises a 5'-monophosphate;
- ligating the first RNA sequence and the second RNA sequence with a nucleic acid ligase to obtain the synthesized full-length PEgRNA,
 - wherein the ligation forms a linker between the 3' OH and the 5'-monophosphate; and
 - wherein the linker is present in the spacer, gRNA core, RTT, or PBS of the synthesized full-length PEgRNA.

224. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the method comprising,

- contacting a first RNA sequence with a second RNA sequence and a splint sequence,
 - wherein the first RNA sequence comprises a free 3' OH, the second RNA sequence comprises a 5'-monophosphate, and the splint sequence comprises a sequence partially complementary to both the first and second RNA sequences;
- ligating the first RNA sequence and the second RNA sequence with a nucleic acid ligase to obtain the synthesized full-length PEgRNA,
 - wherein the ligation forms a linker between the 3' OH and the 5'-monophosphate; and
 - wherein the linker is present in the spacer, gRNA core, RTT, or PBS of the synthesized full-length PEgRNA.

225. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the method comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end;

providing a second RNA sequence, wherein the second RNA sequence comprises a second chemical moiety at its 5' end;

providing a third RNA sequence, wherein the third RNA sequence comprises a third chemical moiety at its 5' end and a fourth chemical moiety at its 3' end;

ligating the first, second, and third RNA sequences to obtain the synthesized full-length PEgRNA,

wherein the ligation forms (i) a first linker between the first chemical moiety of the first RNA sequence and the third chemical moiety of the third RNA sequence, and (ii) a second linker between the second chemical moiety of the second RNA sequence and the fourth chemical moiety of the third RNA sequence;

wherein the first linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA, and

wherein the second linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA or in a junction of the synthesized full-length PEgRNA.

226. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core,
the method comprising,

providing a first RNA sequence, wherein the first RNA sequence comprises a first chemical moiety at its 3' end;

providing a second RNA sequence, wherein the second RNA sequence comprises a second chemical moiety at its 5' end and a free 3' OH;

providing a third RNA sequence, wherein the third RNA sequence comprises a 5'-monophosphate;

ligating the first, second, and third RNA sequences to obtain the synthesized full-length PEgRNA,

wherein the ligation forms (i) a first linker between the first chemical moiety of the first RNA sequence and the second chemical moiety of the second RNA sequence, and (ii) a second linker between the 3' OH of the second RNA sequence and the 5'-monophosphate of the third RNA sequence;

wherein the first linker is present in the chemically-modified gRNA core of the synthesized full-length PEgRNA, and

wherein the second linker is present in the spacer, gRNA core, RTT, or PBS of the synthesized full-length PEgRNA.

227. The method of claim 226, wherein the method further comprises providing a splint sequence, wherein the splint sequence comprises a sequence partially complementary to both the second and third RNA sequences.

228. The method of claim 226 or 227, wherein the method further comprises providing at least one additional RNA sequence comprising a chemical moiety at its 3' end, and at least one additional RNA sequence comprising a chemical moiety at its 5' end.

229. The method of any one of claims 226-228, wherein the method further comprises providing at least one additional RNA sequence comprising a free 3' OH, and at least one additional RNA sequence comprising 5'-monophosphate.

230. The method of claims 228 or 229, wherein ligating the additional RNA sequences results in the formation of a third linker.

231. The method of claims 228 or 229, wherein ligating the additional RNA sequences results in the formation of a third and a fourth linker.

232. The method of claims 228 or 229, wherein ligating the additional RNA sequences results in the formation of a third, fourth, and fifth linker.

233. The method of claims 228 or 229, wherein ligating the additional RNA sequences results in the formation of a third, fourth, fifth, and sixth linker.

234. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core,
the method comprising

providing at least two RNA sequences comprising chemical moieties at their 3' and/or 5' ends, at least two RNA sequences comprising a free 3' OH, and at least two RNA sequences comprising a 5'-monophosphate; and

chemically and enzymatically ligating the RNA sequences, wherein ligating the RNA sequences produce one to three linkers formed with the chemical ligation and one to three linkers formed with the enzymatic ligation.

235. A method for synthesizing a full-length prime editing guide RNA (PEgRNA) comprising a guide RNA (gRNA) core, the method comprising chemically and enzymatically ligating the RNA fragments to form the full-length PEgRNA.

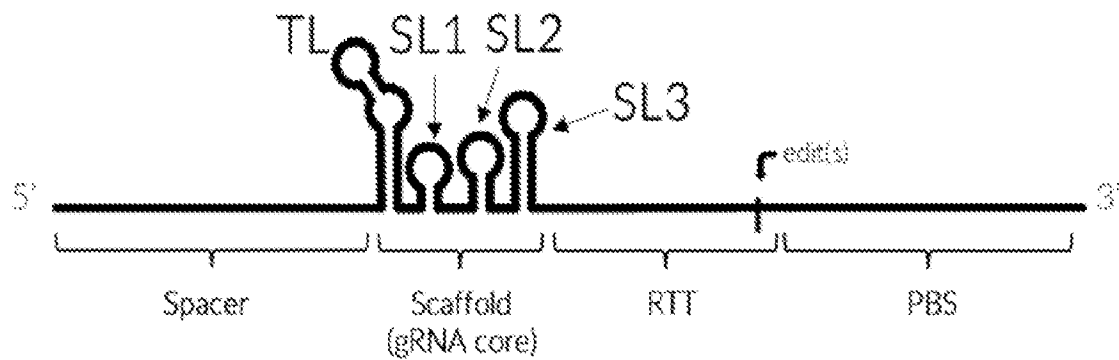


FIG. 1

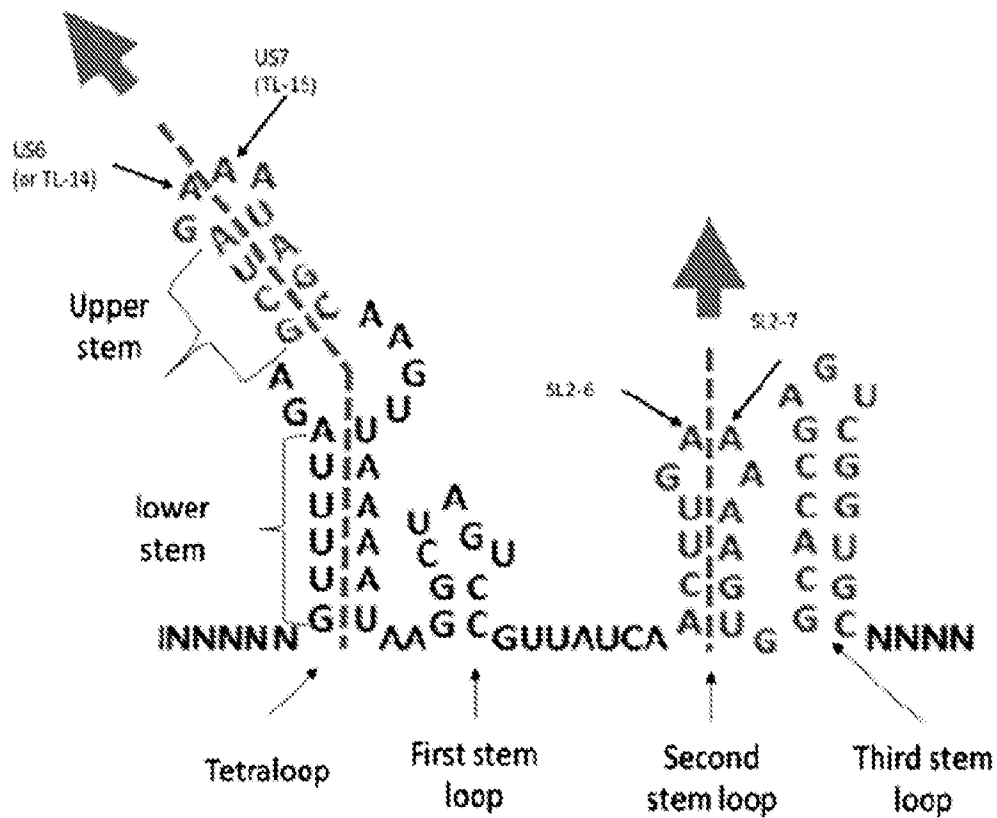


FIG. 2

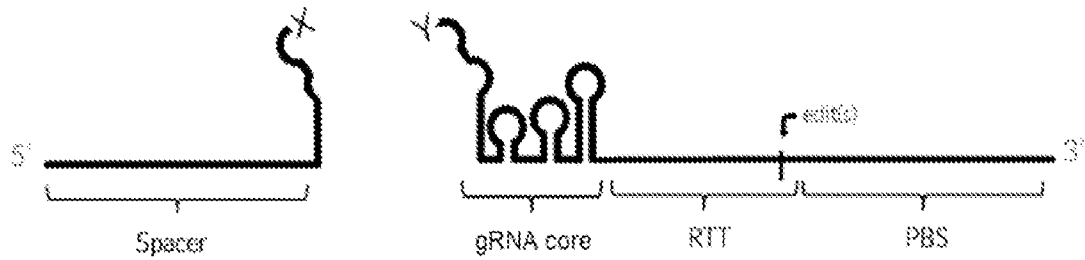


FIG. 3A

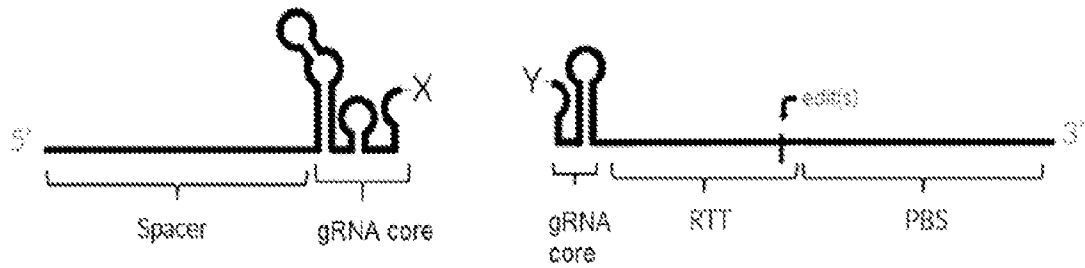


FIG. 3B

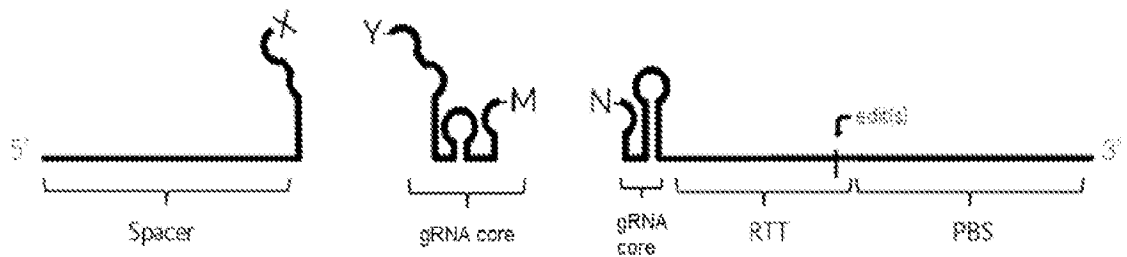


FIG. 4A

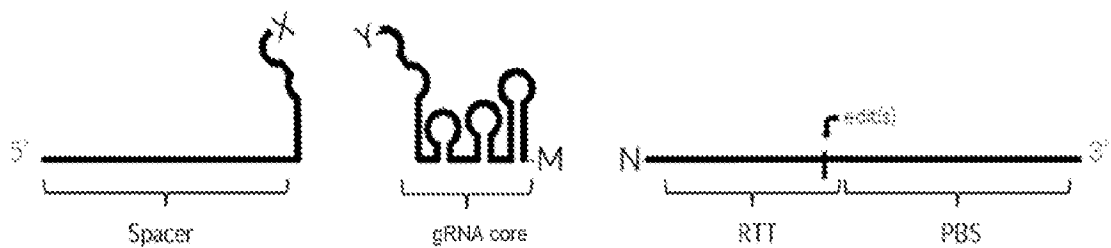


FIG. 4B

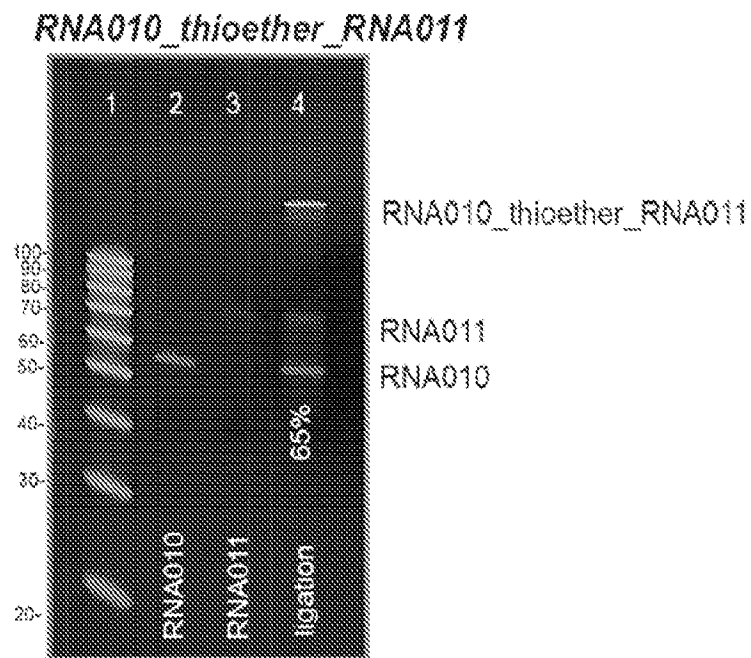


FIG. 6

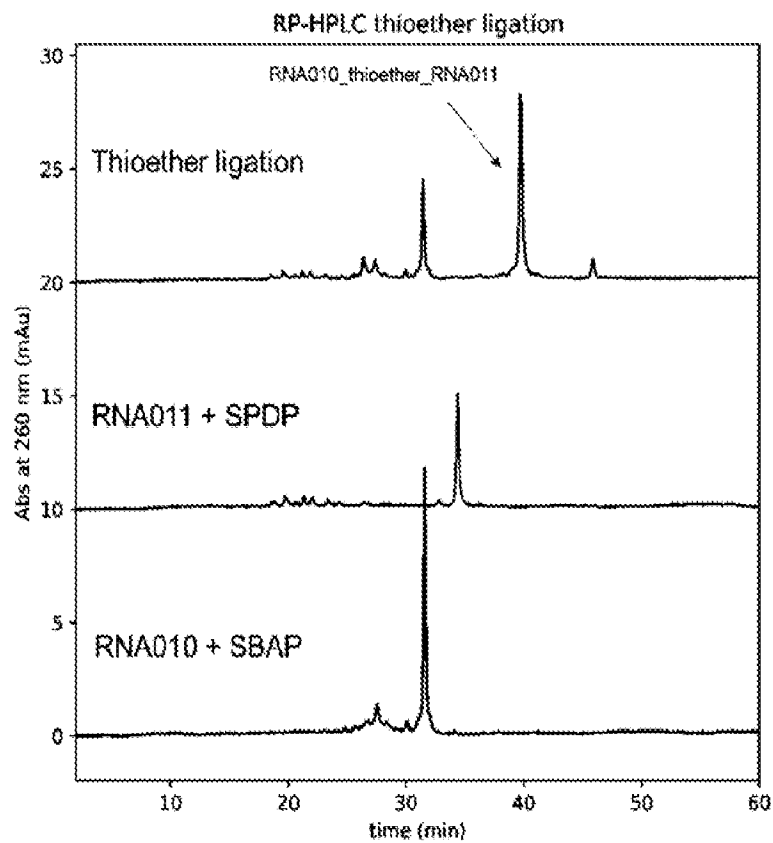


FIG. 7

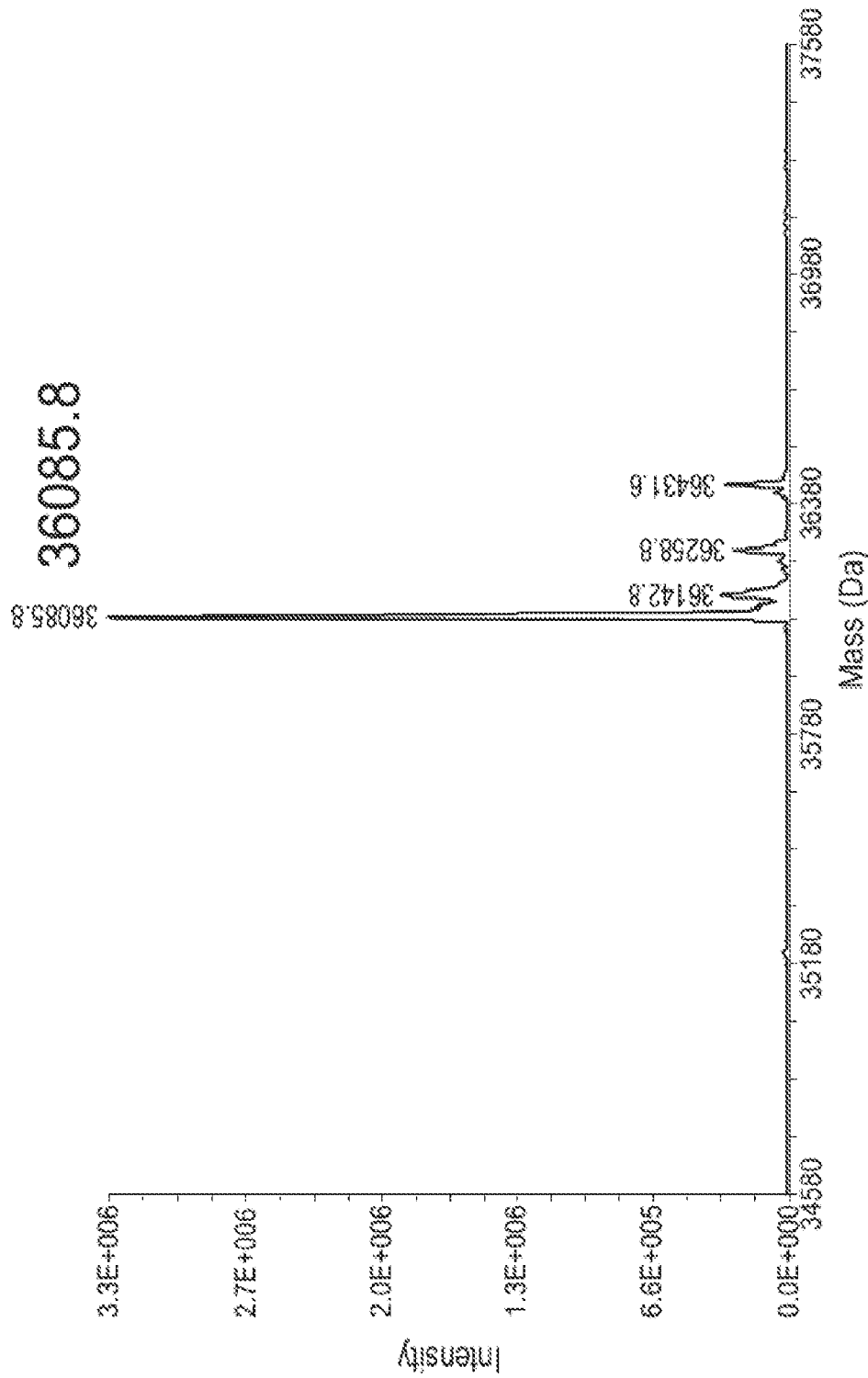


FIG. 8

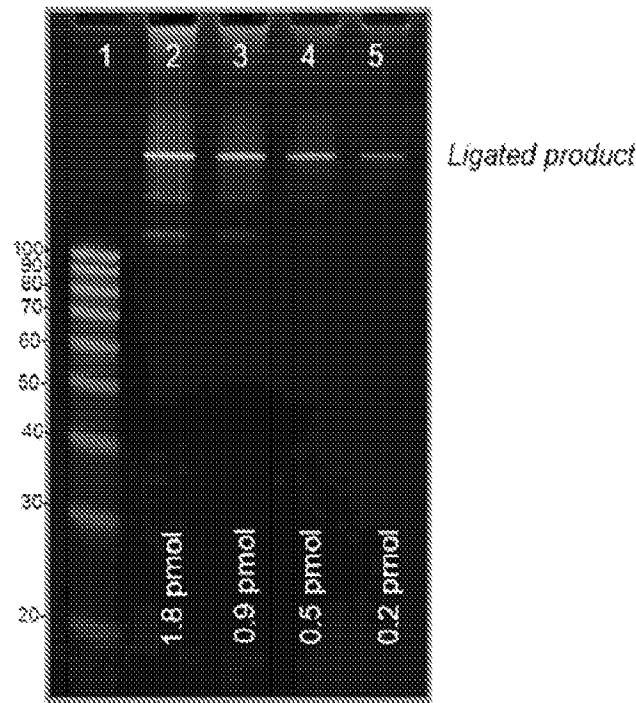


FIG. 9A

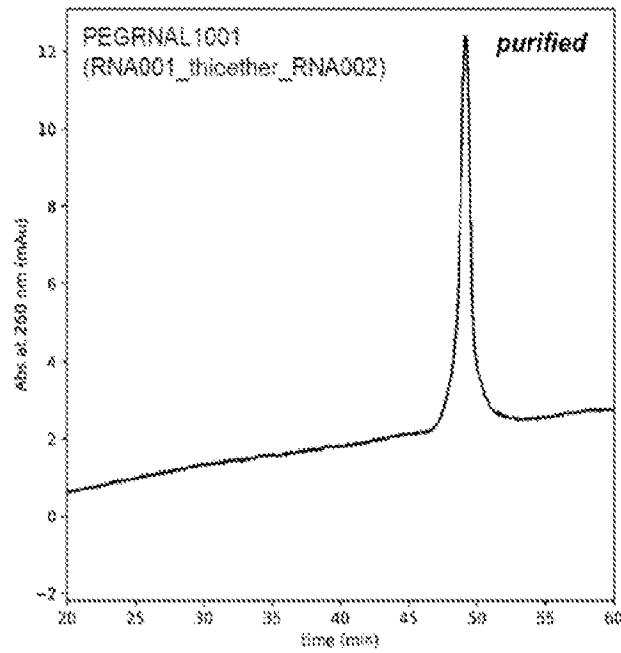


FIG. 9B

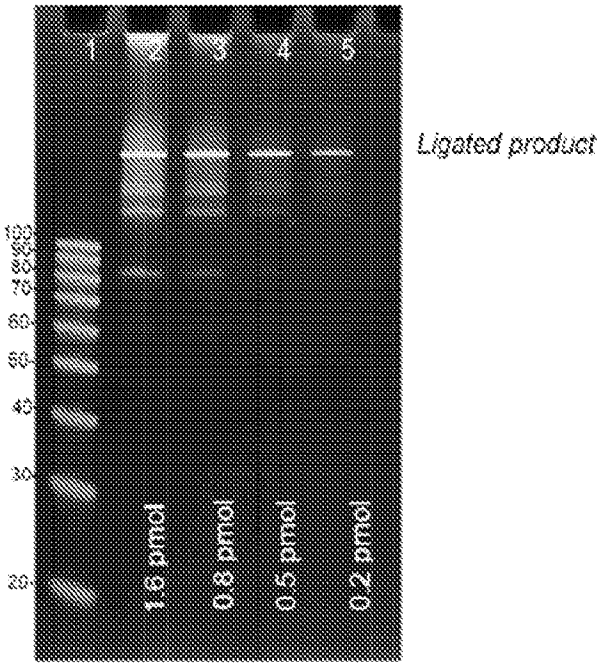


FIG. 10A

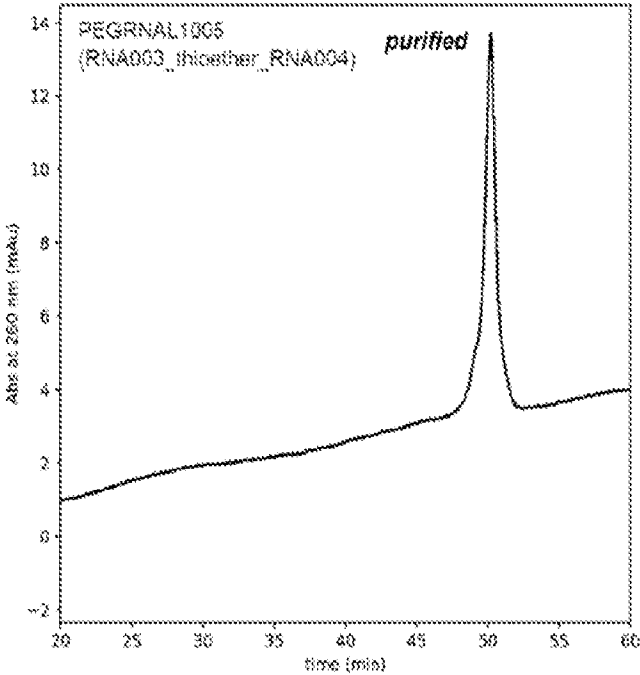


FIG. 10B

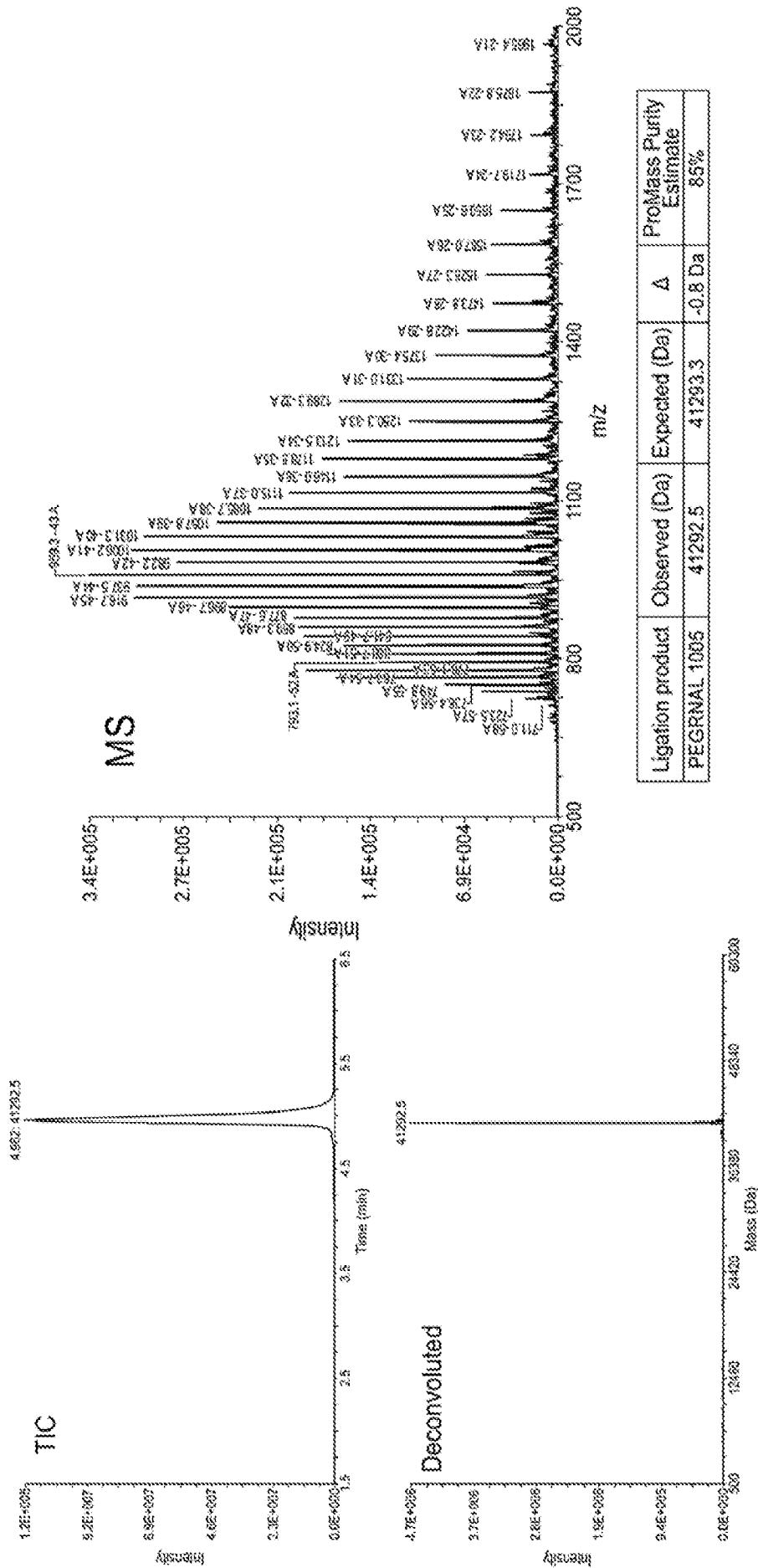


FIG. 11

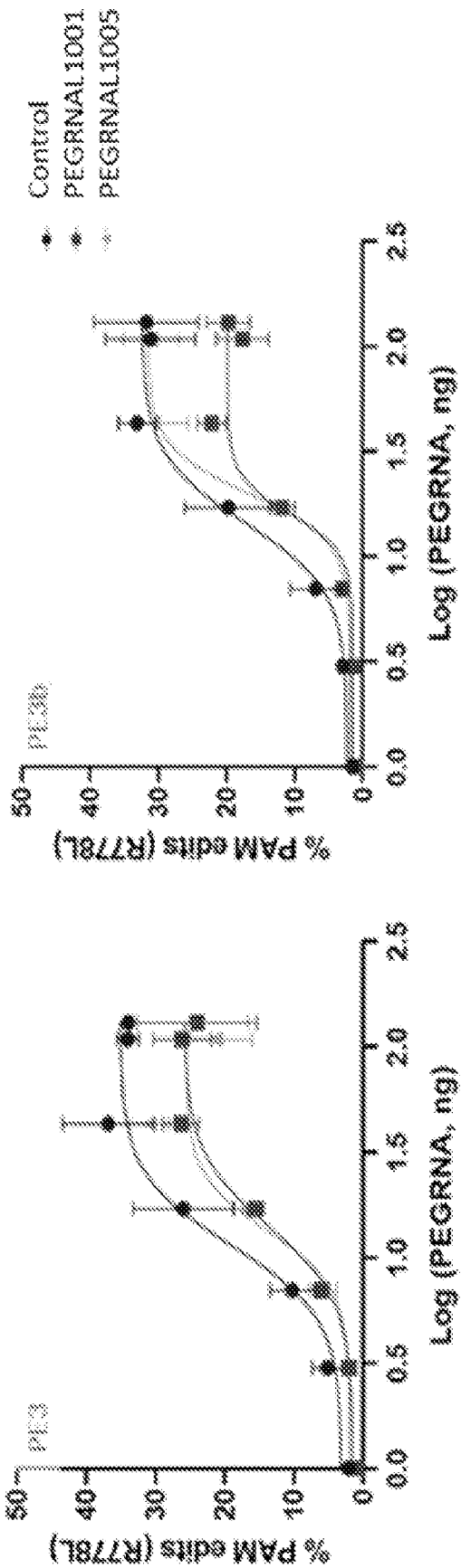


FIG. 12

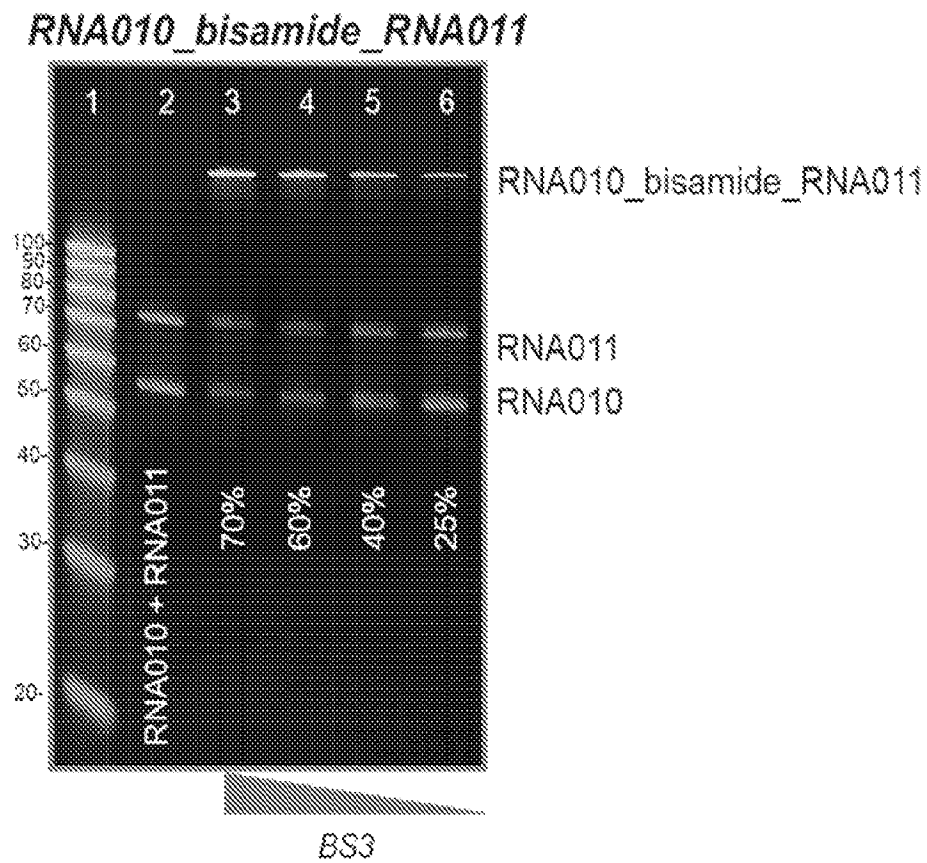


FIG. 13

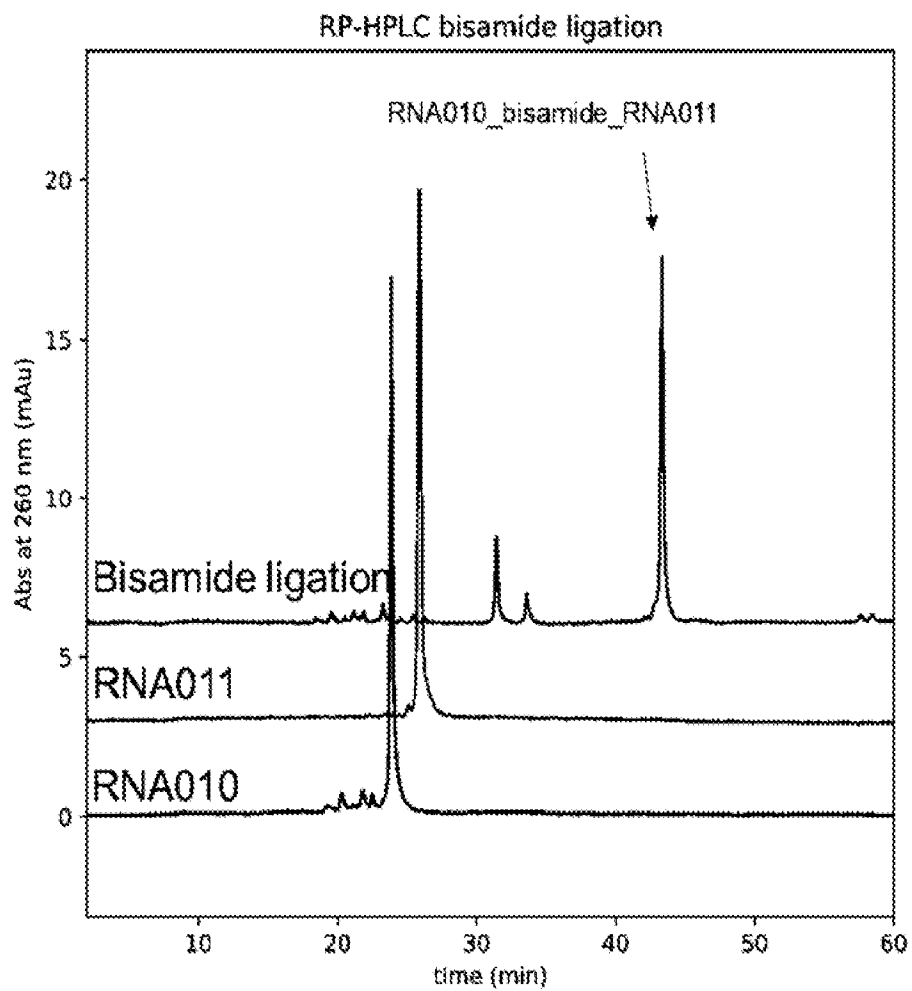


FIG. 14

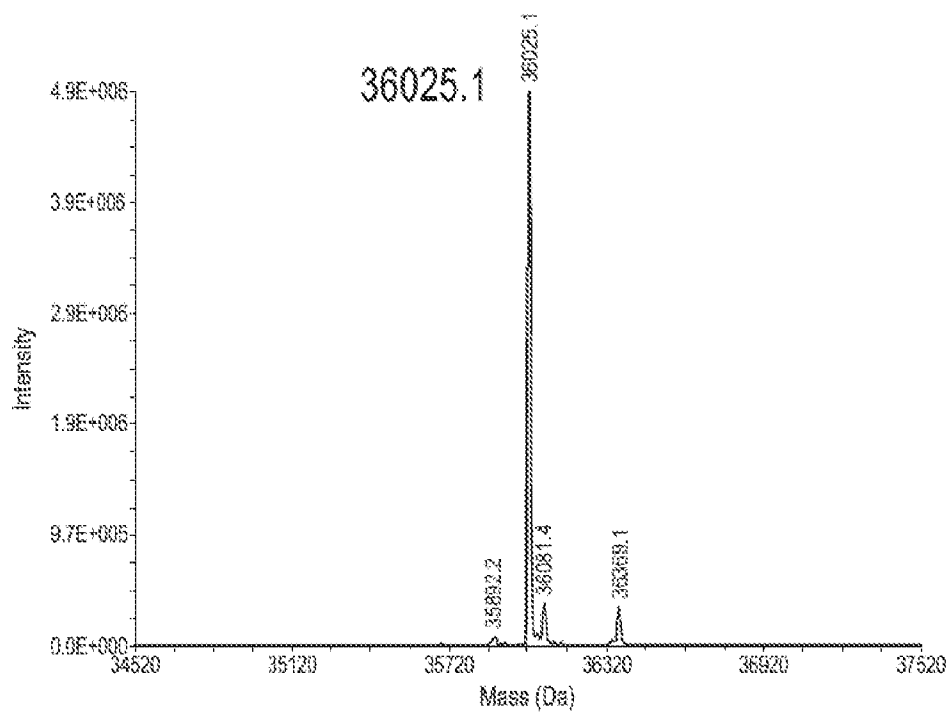


FIG. 15

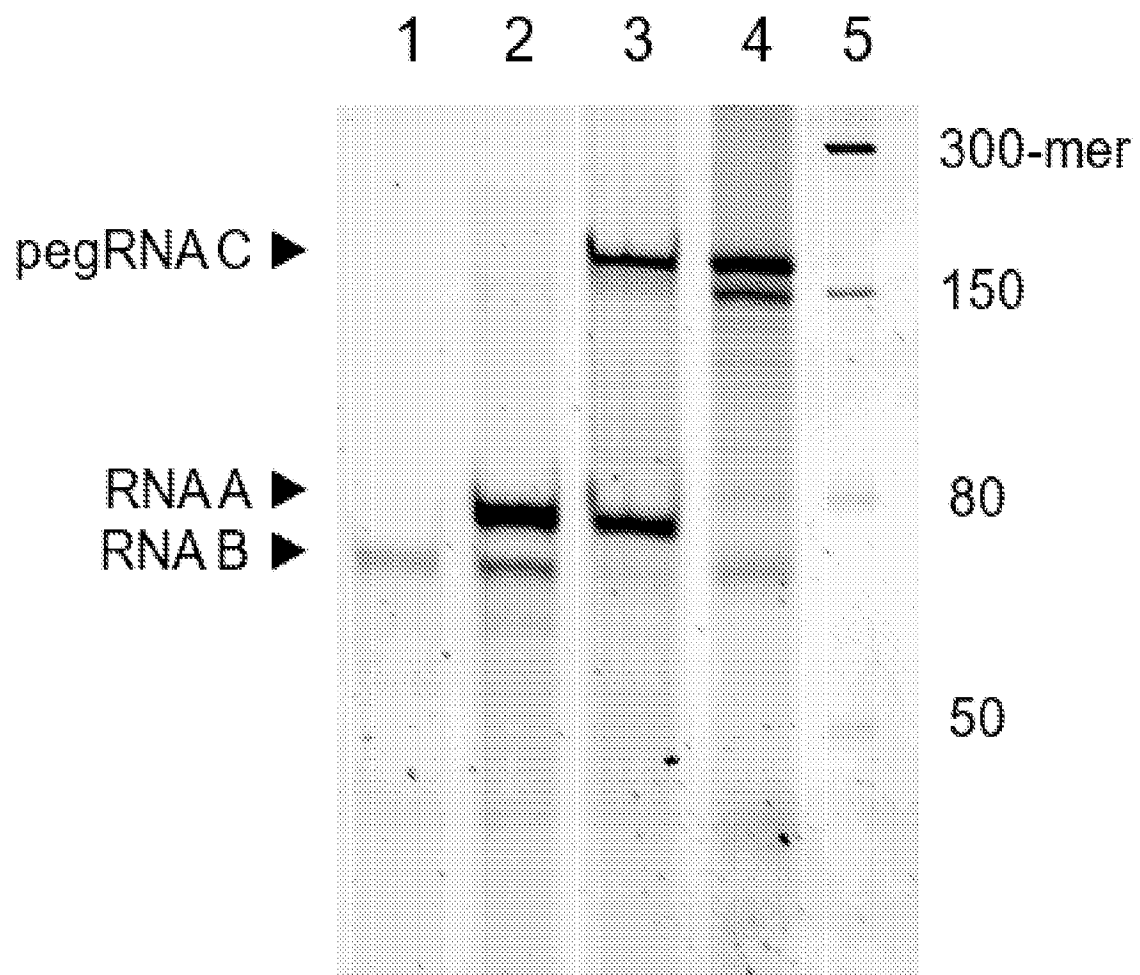


FIG. 16

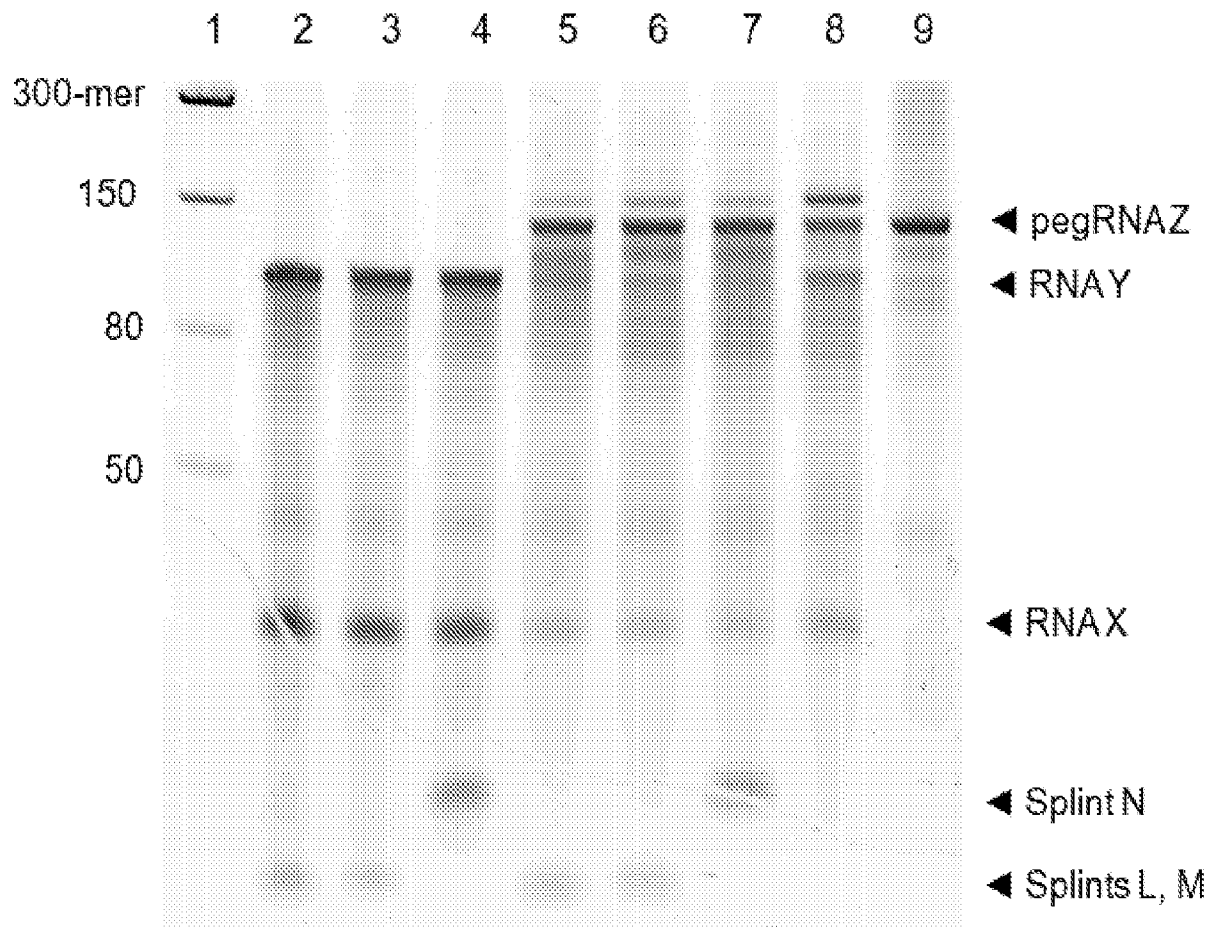


FIG. 17

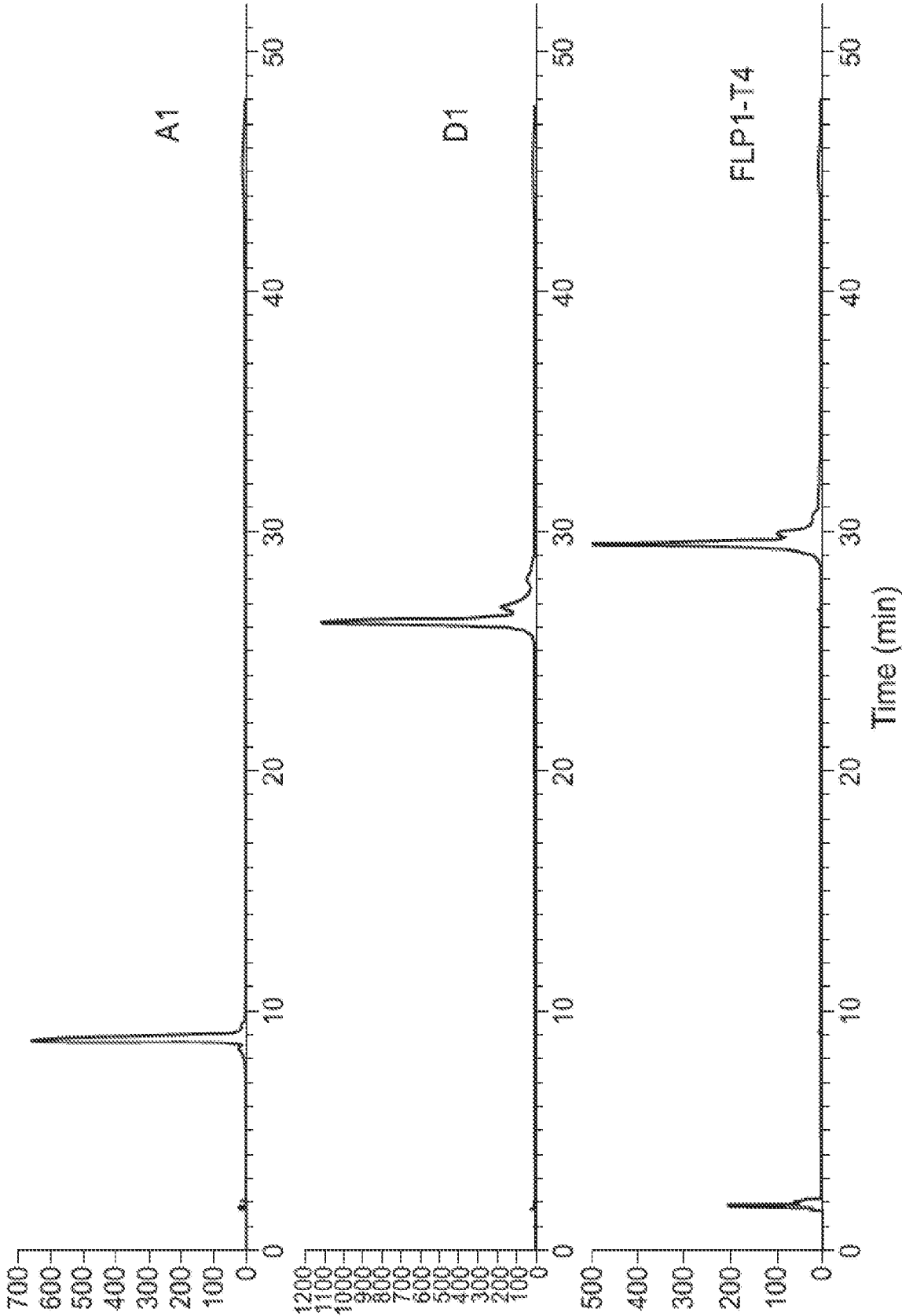


FIG. 18

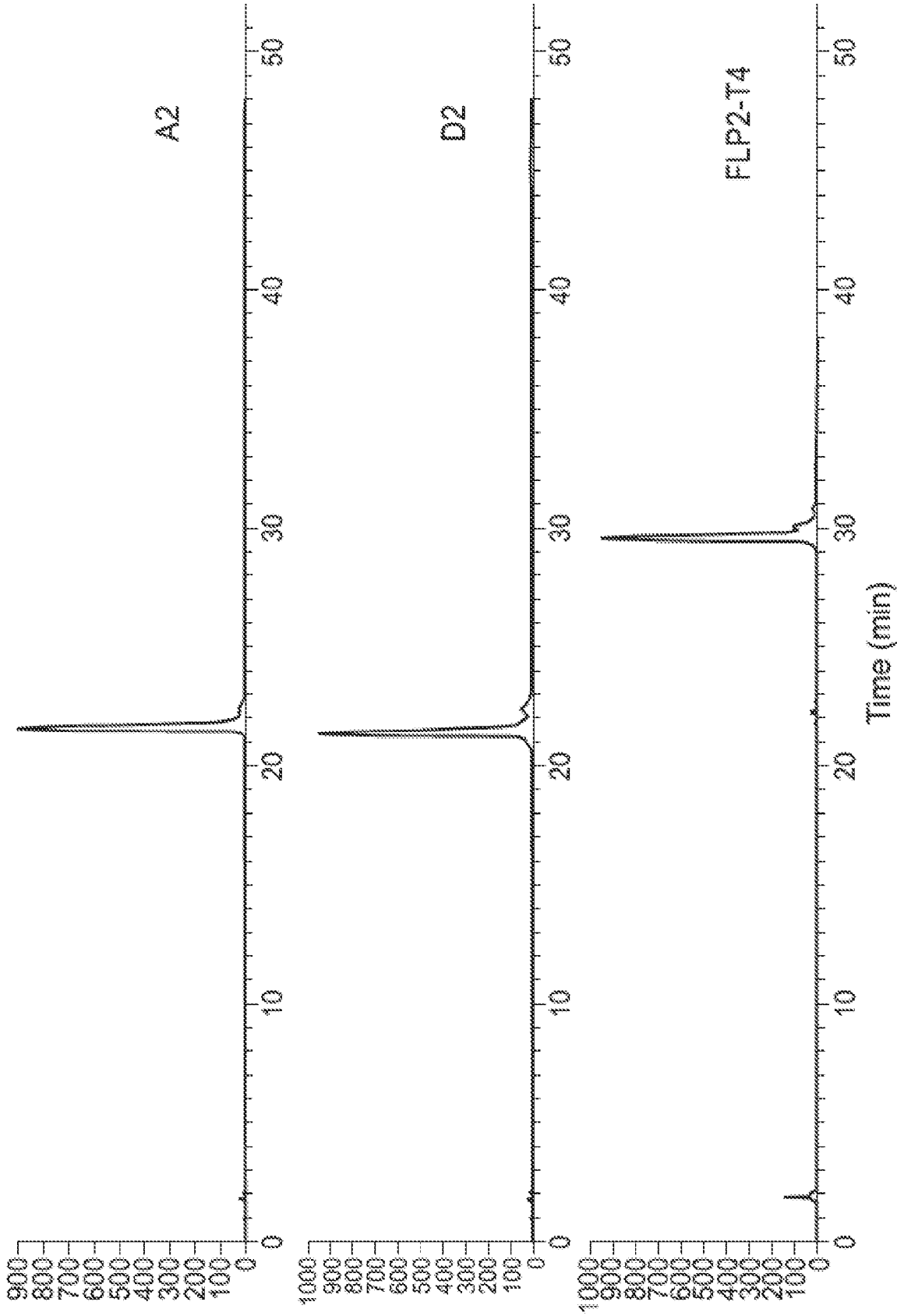


FIG. 19

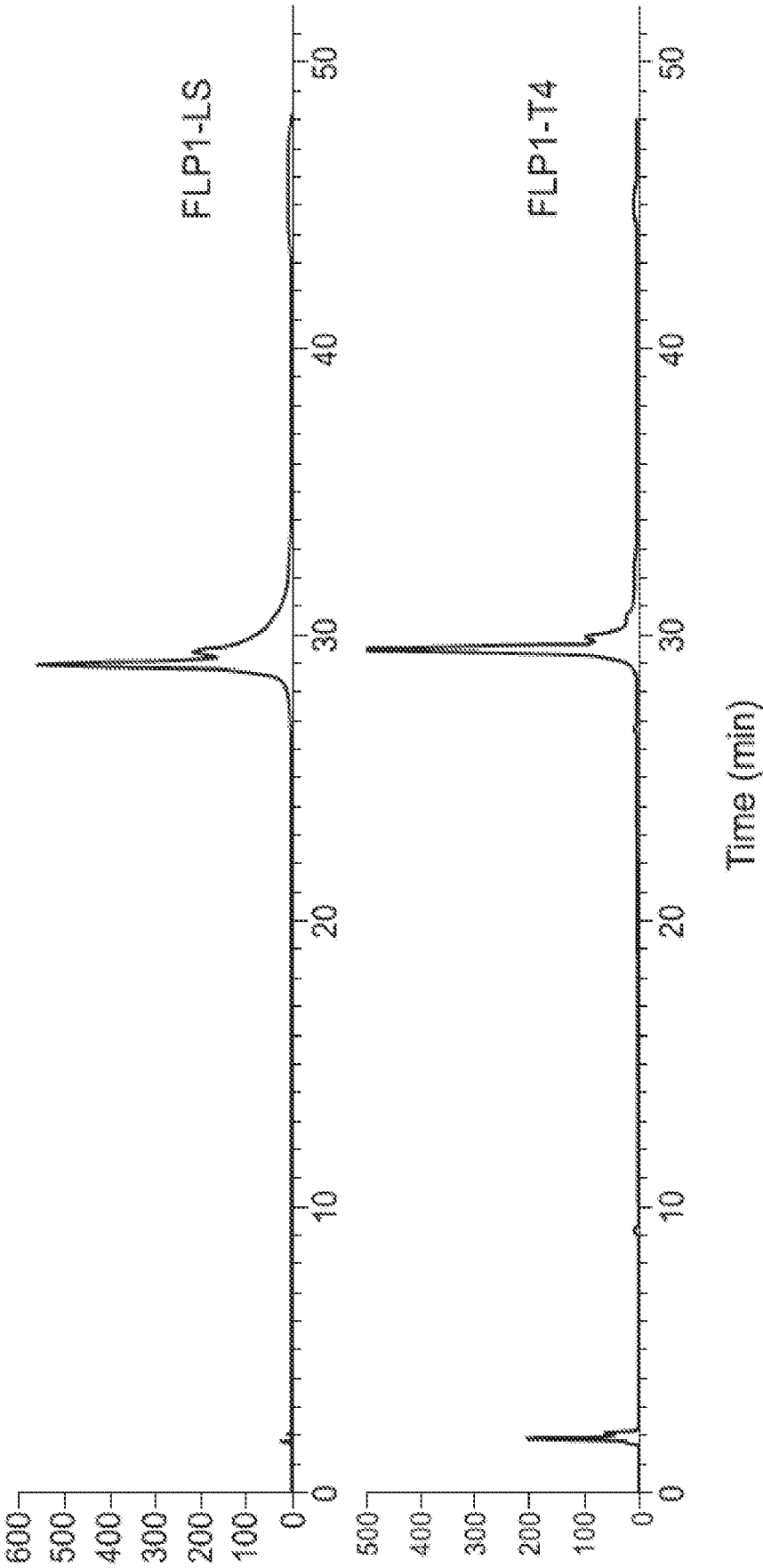


FIG. 20

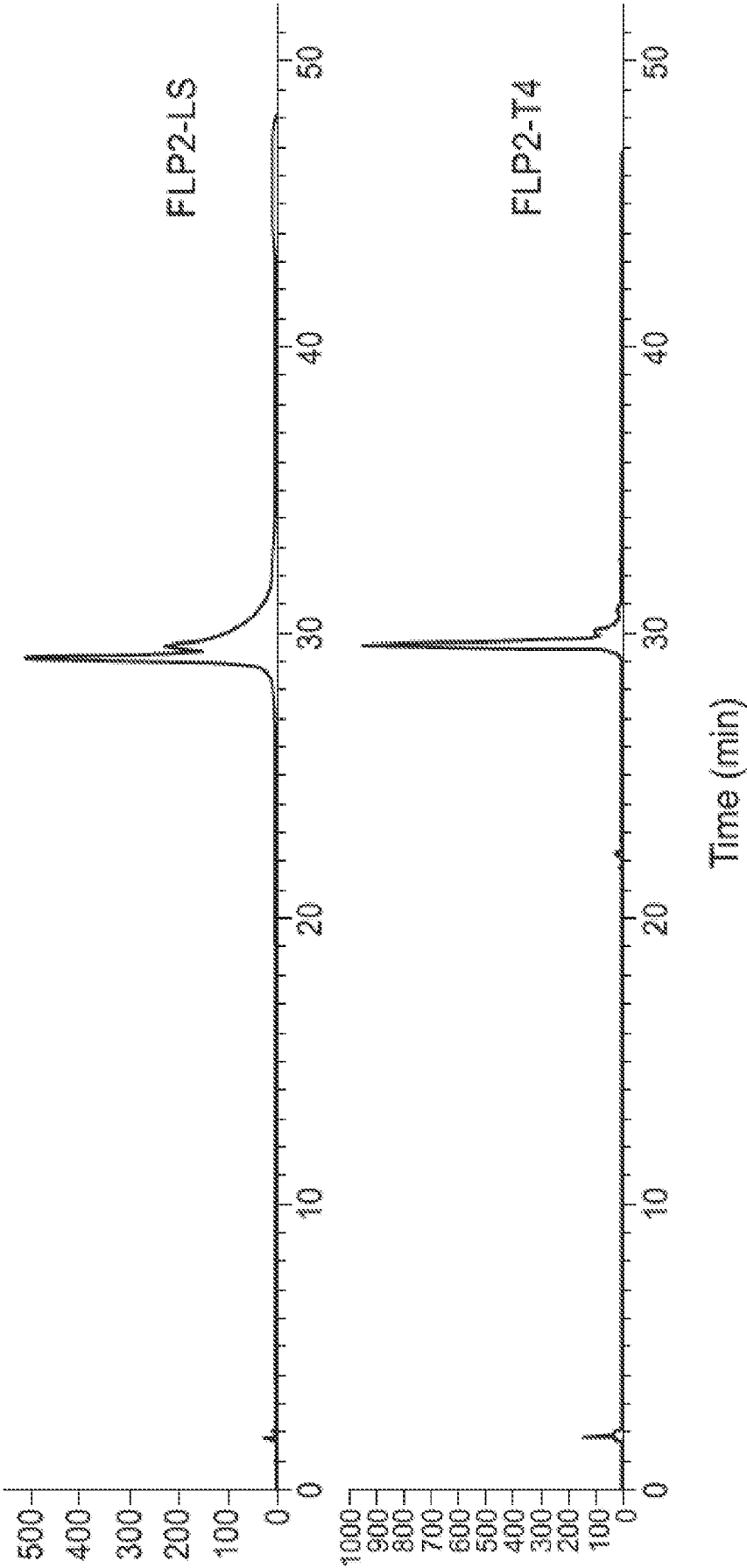


FIG. 21

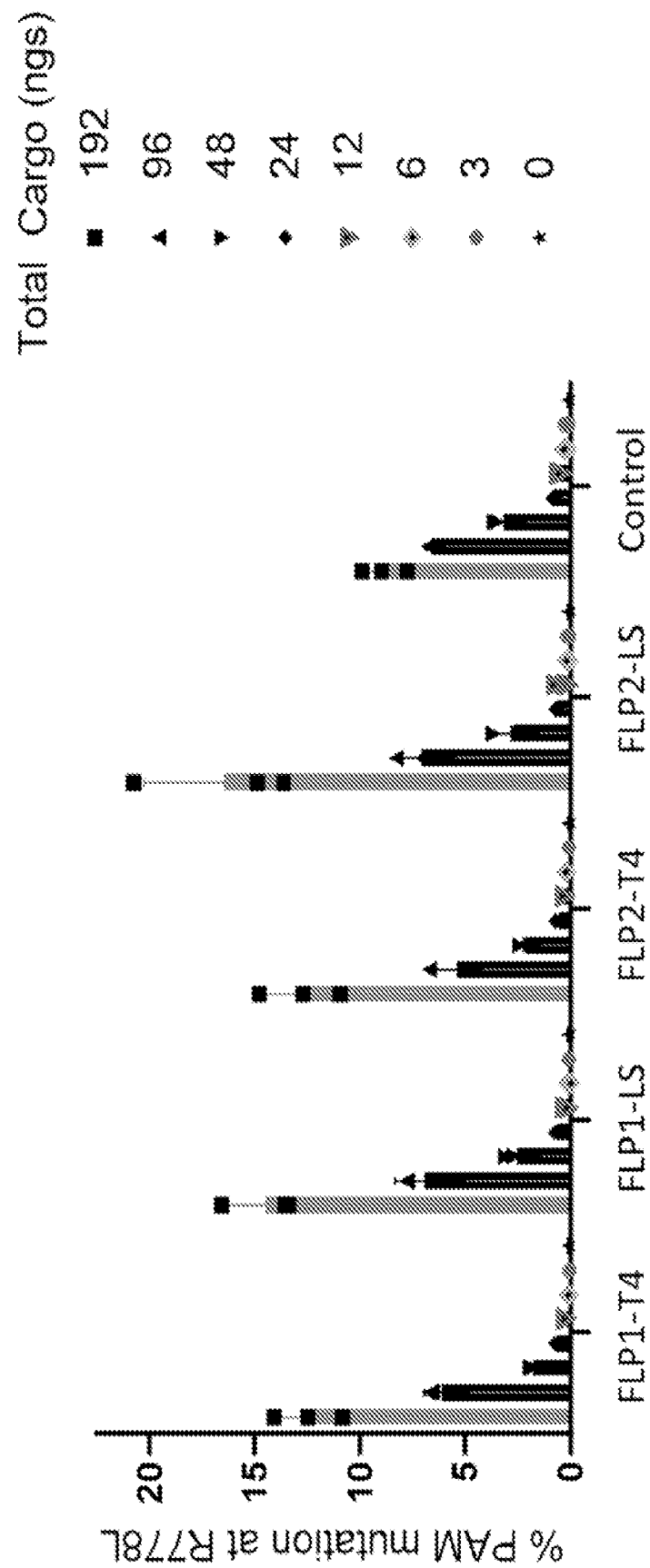


FIG. 22

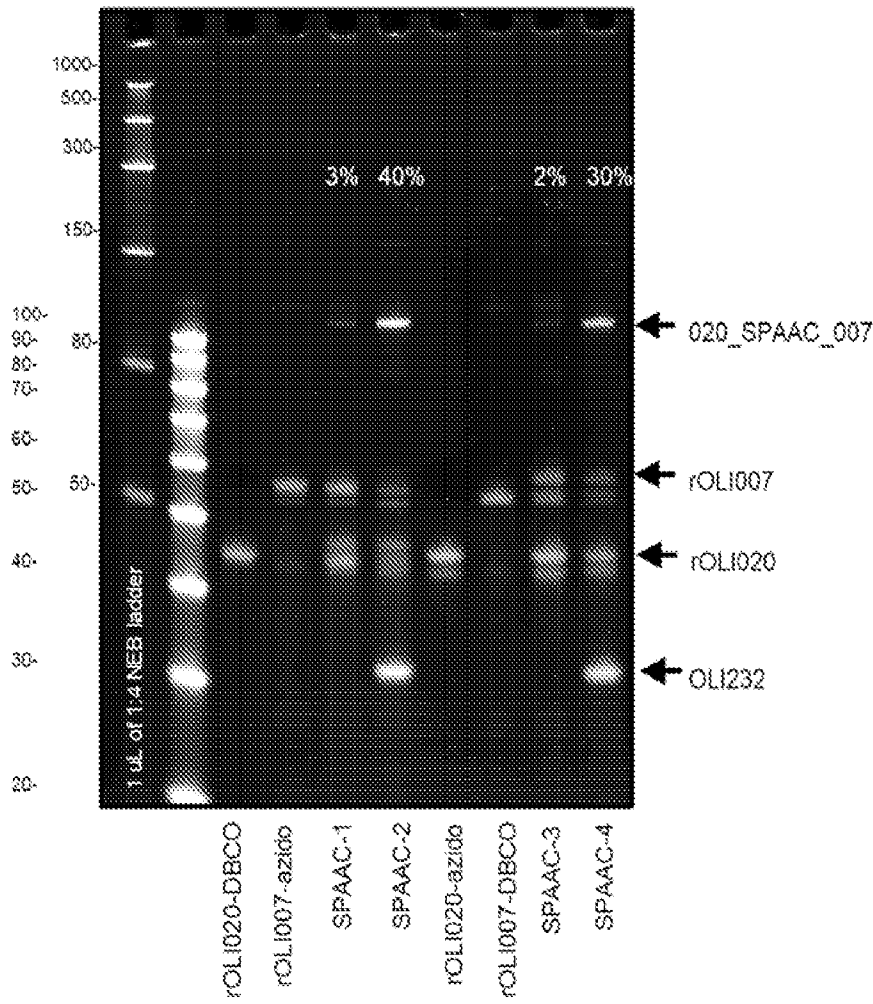


FIG. 23

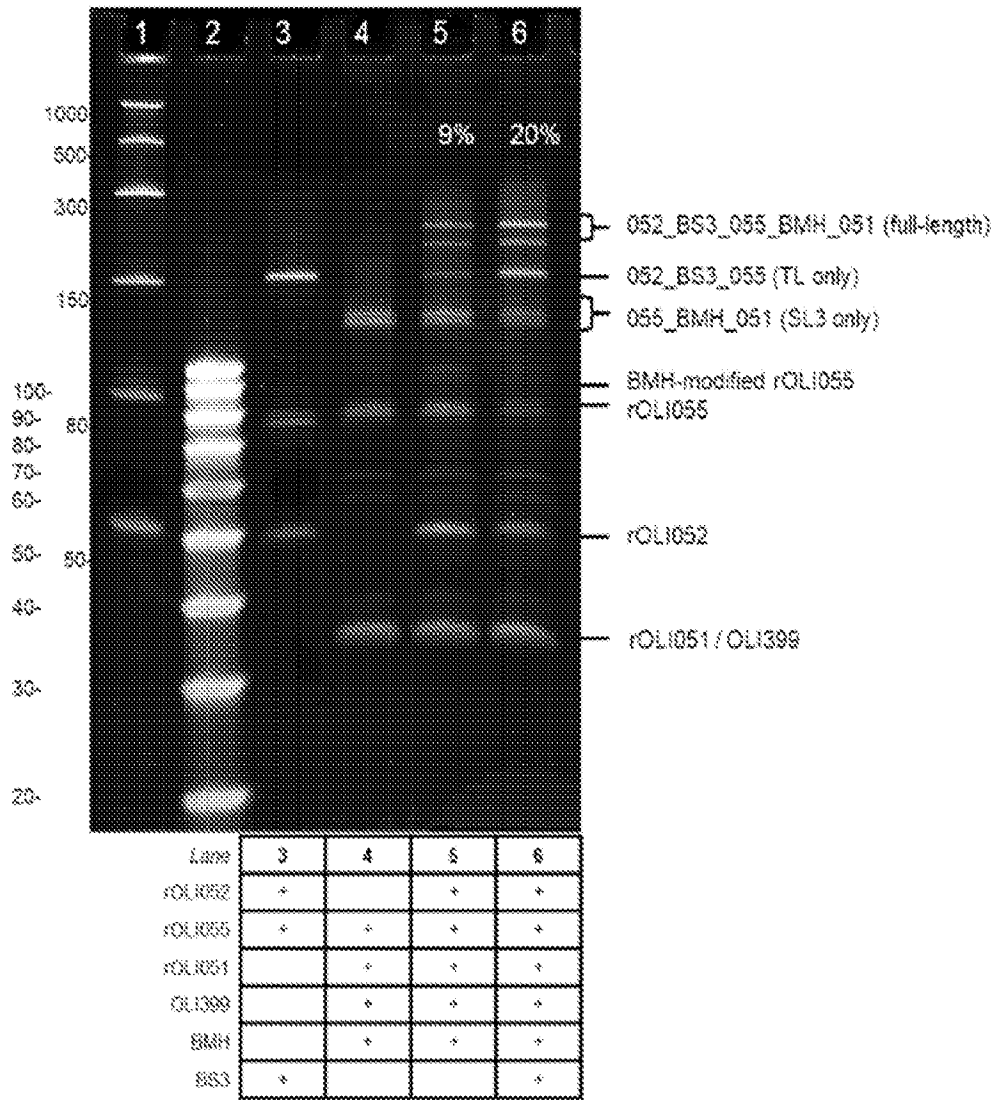


FIG. 24

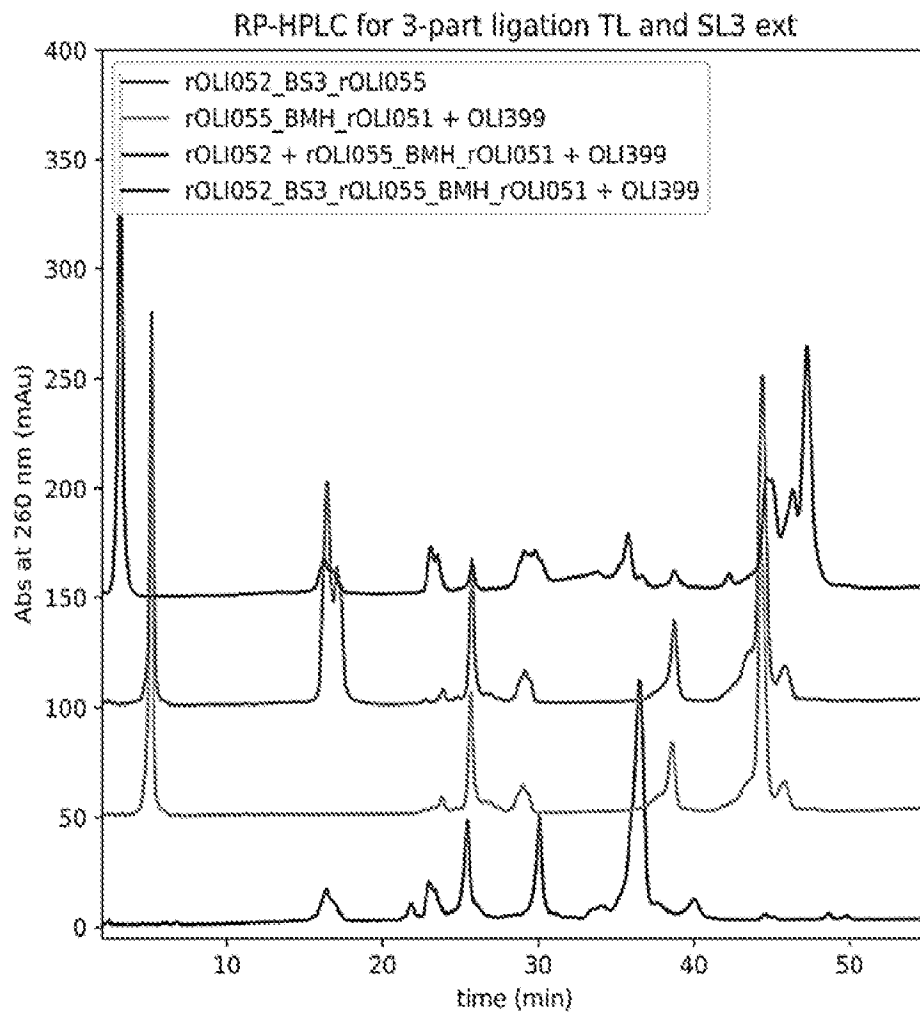


FIG. 25

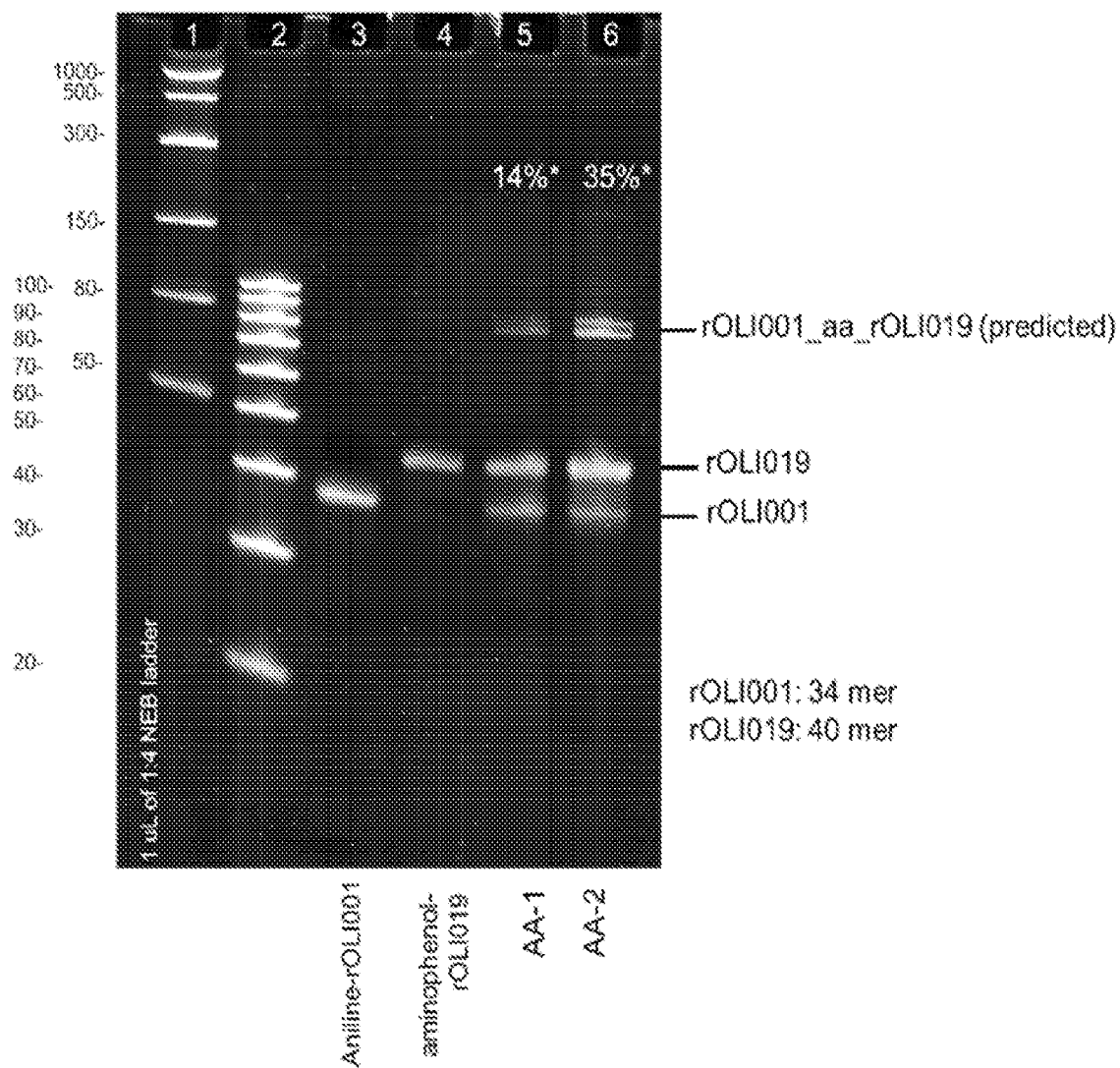


FIG. 26

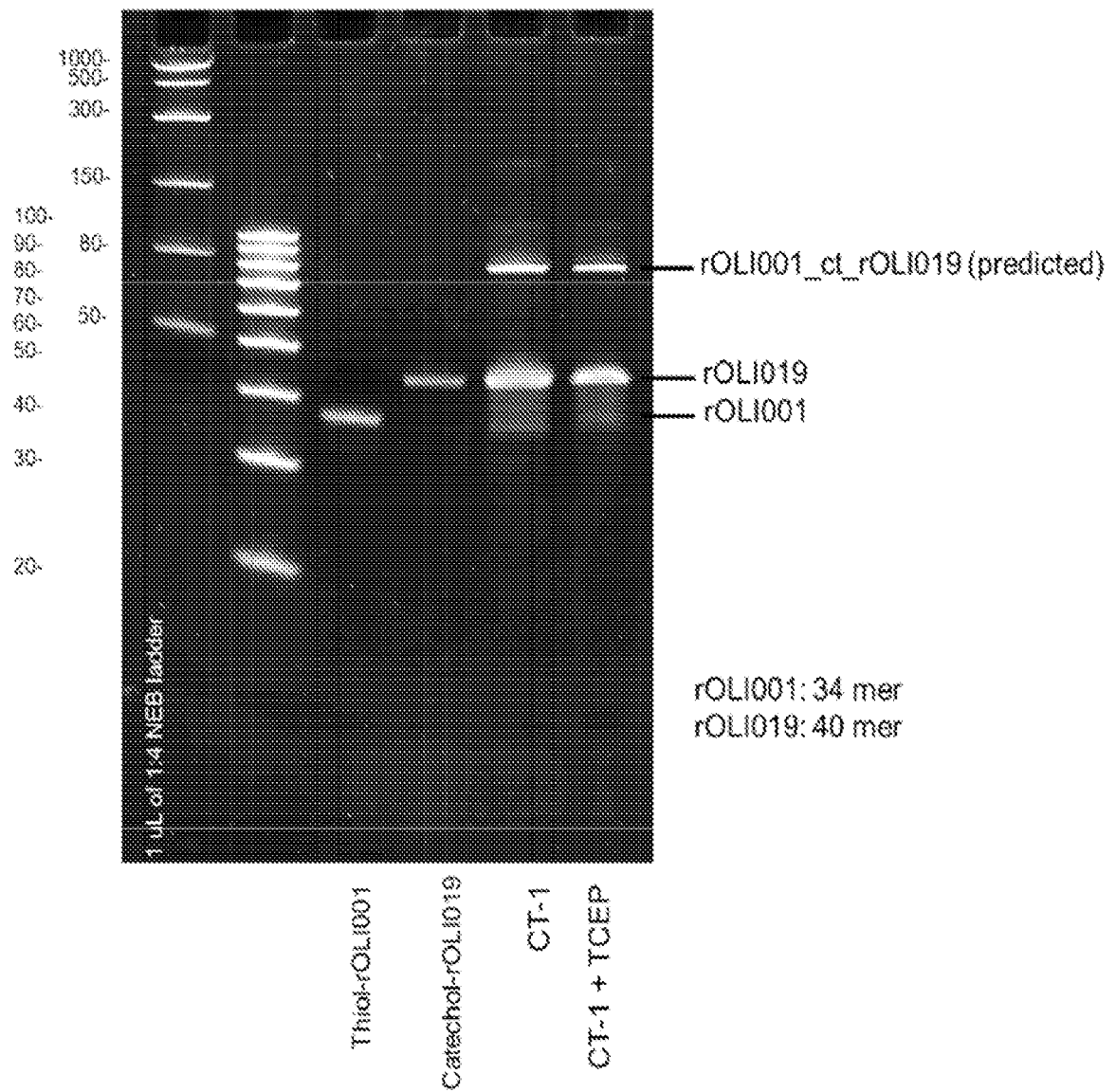


FIG. 27