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(54) Title: NOVEL THERAPEUTIC DNA FORMS

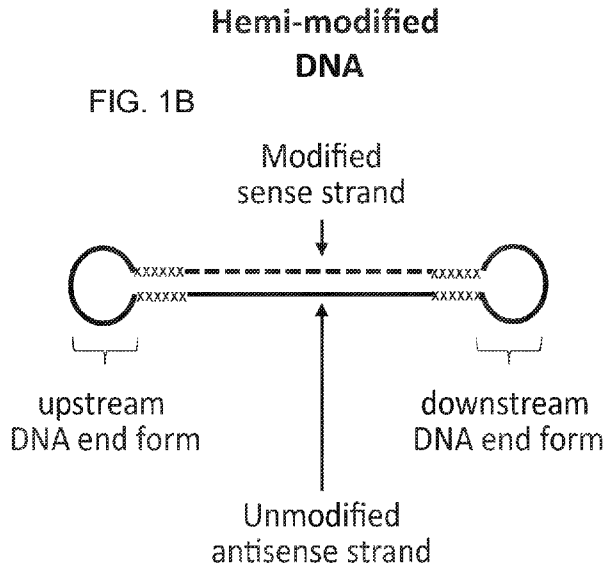

FIG. 1B

(57) Abstract: The disclosure provides, for example, a double stranded DNA (dsDNA) molecule comprising chemically modified nucleotides. In some embodiments, the dsDNA molecule comprises an upstream DNA end form which is a closed end, a downstream DNA end form which is a closed end, and a double stranded region comprising a sense strand and an antisense strand, in which the sense strand comprises one or more chemically modified nucleobases, and the antisense strand is free of chemically modified nucleobases.

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NOVEL THERAPEUTIC DNA FORMS

RELATED APPLICATIONS

This application claims priority to U.S. Serial No.: 63/594,814, filed October 31, 2023; U.S. Serial No.: 63/594,838, filed October 31, 2023; and U.S. Serial No.: 63/685,075, filed August 20, 2024, the entire contents of each of which are incorporated herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on October 7, 2024, is named F2128-7017WO_SL.xml and is 92,046 bytes in size.

BACKGROUND

There is a need for novel therapeutic modalities to address unmet medical need.

SUMMARY OF THE INVENTION

Described herein are pharmaceutical DNA compositions comprising chemically modified nucleotides, constructs, preparations, methods of using such compositions, constructs and preparations, and methods of making the same.

Enumerated Embodiments

1. A DNA molecule, e.g., a double stranded DNA (dsDNA) molecule.

2. A double stranded DNA (dsDNA) molecule comprising:
   a) an upstream DNA end form which is a closed end;
   b) a double stranded region comprising a sense strand and an antisense strand, wherein:
      the sense strand comprises one or more chemically modified nucleobases, and
      the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and
   c) a downstream DNA end form which is a closed end.

3. A double stranded DNA (dsDNA) molecule comprising:
   a) an upstream DNA end form which is a closed end;
   b) a double stranded region comprising a sense strand and an antisense strand; and

c) a downstream DNA end form which is a closed end,

wherein the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases, and

wherein the sense strand comprises one or more (e.g., at least 3) backbone modifications, e.g., phosphorothioate linkages, wherein optionally:

the one or more backbone modifications are situated between the 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides adjacent to the upstream DNA end form; and/or

the one or more backbone modifications are situated between the 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides adjacent to the downstream DNA end form.

4. The dsDNA molecule of any of the preceding embodiments, wherein the closed end of the upstream DNA end form is a single stranded loop.

5. The dsDNA molecule of any of the preceding embodiments, wherein the closed end of the downstream DNA end form is a single stranded loop.

6. The dsDNA molecule of any of the preceding embodiments, wherein the backbone modifications are phosphorothioate linkages.

7. The dsDNA molecule of any of embodiments 1-5, wherein the backbone modifications are boranophosphate linkages.

8. The dsDNA molecule of any of the preceding embodiments, wherein the antisense strand is substantially free of (e.g., is free of) phosphorothioate linkages.

9. The dsDNA molecule of any of the preceding embodiments, wherein the antisense strand is substantially free of (e.g., is free of) boranophosphate linkages.

10. The dsDNA molecule of any of the preceding embodiments, wherein the antisense strand is substantially free of (e.g., is free of) backbone modifications.

11. The dsDNA molecule of any of the preceding embodiments, wherein the 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides of the sense strand that are adjacent to the downstream end form are substantially free of (e.g., are free of) phosphorothioate linkages.

12. The dsDNA molecule of any of the preceding embodiments, wherein the 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides of the sense strand that are adjacent to the downstream end form are substantially free of (e.g., are free of) boranophosphate linkages.

13. The dsDNA molecule of any of the preceding embodiments, wherein the 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides of the sense strand that are adjacent to the downstream end form are substantially free of (e.g., are free of) backbone modifications.

14. The dsDNA molecule of any of the preceding embodiments, which has no chemically modified nucleobases between the downstream-most phosphorothioate linkage and the upstream DNA end form.

15. The dsDNA molecule of any of the preceding embodiments, which comprises at least 1, at least 2, at least 3, at least 4, at least 5, or at least 6, and optionally no more than 10, chemically modified backbone modifications adjacent to the upstream DNA end form.

16. The dsDNA molecule of any of the preceding embodiments, wherein the sense strand comprises, in an upstream to downstream direction starting from the nucleotide adjacent to the upstream DNA end form: one backbone modification (e.g., phosphorothioate linkage), one or more canonical phosphodiester linkages, and a second backbone modification (e.g., a second phosphorothioate linkage).

17. The dsDNA molecule of any of embodiments 1-15, wherein the sense strand comprises, in an upstream to downstream direction starting from the nucleotide adjacent to the upstream DNA end form: one or more (e.g., 1-5 or 1-10) canonical phosphodiester linkages, and a backbone modification (e.g., a phosphorothioate linkage).

18. The dsDNA molecule of any of the preceding embodiments, wherein the sense strand comprises a backbone modification at a position (the "backbone modification position") such that there are 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides between the backbone modification position and the upstream DNA end form.

19. The dsDNA molecule of any of the preceding embodiments, which is substantially free of (e.g., is free of) backbone modifications in a region of the sense strand that is adjacent to the downstream DNA end form.

20. The dsDNA molecule of embodiment 19, wherein the region is at least 50, at least 100, at least 200, at least 300, at least 400, at least 500, or at least 1,000 nucleotides in length.

21. The dsDNA molecule of embodiment 19 or 20, wherein the region is 50-100, 100-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800, 800-900, 900-1000, 1000-1500, or 1500-2000 nucleotides in length.

22. The dsDNA molecule of any of the preceding embodiments, wherein the double stranded region encodes a protein, and wherein the dsDNA molecule, when contacted to human cells, results in expression at a level at least 100%, at least 120%, at least 140%, at least 160%, or at least 180% the expression of an unmodified control DNA molecule, wherein the unmodified control DNA molecule comprises the same sequence and same closed end double stranded form as the dsDNA molecule, but comprises no chemically modified nucleobases.

23. A double stranded DNA (dsDNA) molecule comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises one or more chemically modified nucleobases, and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end,

wherein the double stranded region encodes a protein, and

wherein the dsDNA molecule, when contacted to human cells, results in expression at a level at least 100%, at least 120%, at least 140%, at least 160%, or at least 180% the expression of an unmodified control DNA molecule, wherein the unmodified control DNA molecule comprises the same sequence and same closed end double stranded form as the dsDNA molecule, but comprises no chemically modified nucleobases.

24. The dsDNA molecule of any of the preceding embodiments, wherein the double stranded region encodes a protein, wherein the dsDNA molecule, when contacted to human cells, results in expression at a level at least the expression of a modified control DNA molecule, wherein the modified control DNA molecule comprises the same sequence, same closed end double stranded form, and same degree of sense strand nucleobase modification as the dsDNA molecule, but comprises antisense strand nucleobase modification at the same degree as sense strand nucleobase modification.

25. A double stranded DNA (dsDNA) molecule comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises one or more chemically modified nucleobases, and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end,

wherein the double stranded region encodes a protein, and

wherein the dsDNA molecule, when contacted to human cells, results in expression at a level at least the expression of a modified control DNA molecule, wherein the modified control DNA molecule comprises the same sequence, same closed end double stranded form, and same degree of sense strand nucleobase modification as the dsDNA molecule, but comprises antisense strand nucleobase modification at the same degree as sense strand nucleobase modification.

26. The dsDNA molecule of any of the preceding embodiments, wherein the dsDNA molecule, when contacted to human cells, results in a lower level of IL6 or CXCL10 mRNA compared to a control DNA molecule (e.g., lower by at least 10%, at least 20%, at least 30%, at least 40%, or at least 50%), wherein the control DNA molecule comprises the same sequence and same closed end double stranded form as the dsDNA molecule, but comprises no chemically modified nucleobases.

27. A double stranded DNA (dsDNA) molecule comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises one or more chemically modified nucleobases, and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end,

wherein the dsDNA molecule, when contacted to human cells, results in a lower level of IL6 or CXCL10 mRNA compared to a control DNA molecule (e.g., lower by at least 10%, at least 20%, at least 30%, at least 40%, or at least 50%), wherein the control DNA molecule comprises the same sequence and same closed end double stranded form as the dsDNA molecule, but comprises no chemically modified nucleobases.

28. A population comprising a plurality of double stranded DNA (dsDNA) molecules, each dsDNA molecule of the plurality comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises one or more chemically modified nucleobases, and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end,

wherein:

at least 50%, at least 60%, or at least 70% of the dsDNA molecules in the plurality have substantially the same length;

at least 50%, at least 60%, or at least 70% of the dsDNA molecules in the plurality have a length in a predetermined range; or

at least 50%, at least 60%, or at least 70% of the dsDNA molecules in the plurality have a length of between 100, 200, 300, 400, or 500 nucleotides of each other.

29. A dsDNA molecule comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises a first type of chemically modified nucleobase and a second type of chemically modified nucleobase; and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end.

30. The dsDNA molecule of embodiment 29, wherein the first type of chemically modified nucleobase is a chemically modified cytosine nucleobase.

31. The dsDNA molecule of embodiment 29 or 30, wherein the second type of chemically modified nucleobase is a different chemically modified cytosine nucleobase, a uridine nucleobase, or an inosine nucleobase.

32. The dsDNA molecule of embodiment 29, wherein the first type of chemically modified nucleobase is a uridine nucleobase.

33. The dsDNA molecule of embodiment 29 or 32, wherein the second type of chemically modified nucleobase is a different chemically modified uridine nucleobase or an inosine nucleobase.

34. The dsDNA molecule of embodiment 29, wherein the first type of chemically modified nucleobase is an inosine nucleobase.

35. The dsDNA molecule of embodiment 29 or 34, wherein the second type of chemically modified nucleobase is a chemically modified cytosine nucleobase or a uridine nucleobase.

36. The dsDNA molecule or population of any of embodiments 1-35, wherein the sense strand comprises a chemically modified cytosine nucleotide.

37. The dsDNA molecule or population of embodiment 36, wherein the chemically modified cytosine nucleotide comprises 5-hydroxycytosine.

38. A dsDNA molecule comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises a uridine nucleotide; and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end.

39. The dsDNA molecule or population of any of embodiments 1-37, wherein the sense strand comprises a uridine nucleotide.

40. The dsDNA molecule or population of embodiment 38 or 39, wherein the uridine nucleotide is a canonical uridine nucleotide or a chemically modified uridine nucleotide.

41. The dsDNA molecule or population of any of embodiments 38-40, wherein the uridine nucleotide is a chemically modified uridine nucleotide.

42. The dsDNA molecule or population of embodiment 41, wherein the chemically modified uridine nucleotide comprises 5-azidomethyluridine, 5-formyluridine, 5-hydroxymethyluridine, or 5-methylthiouridine.

43. The dsDNA molecule or population of any of embodiments 38-40, wherein the uridine nucleotide is a canonical uridine nucleotide.

44. The dsDNA molecule or population of any of embodiments 38-43, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%, at least 90%, or at least 99% of thymine or uridine positions in the sense strand of the dsDNA molecule comprise a uridine nucleotide.

45. The dsDNA molecule or population of any of embodiments 38-40 or 43, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%, at least 90%, or at least 99% of thymine or udidine positions in the sense strand of the dsDNA molecule comprise a canonical uridine nucleotide.

46. The dsDNA molecule or population of any of embodiments 1-45, wherein the sense strand comprises an inosine nucleotide.

47. A dsDNA molecule comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises an inosine nucleotide; and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end.

48. A dsDNA molecule comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises a 5-methylthiouridine nucleotide; and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end.

49. A method of making a dsDNA molecule, the method comprising:

a) providing a double stranded linear DNA having a sense strand, an antisense strand, an upstream open end, and a downstream open end;

b) converting both open ends to closed ends (thereby producing an upstream closed end and downstream closed end) such that the sense strand comprises one or more backbone modifications adjacent to or within 10, 20, or 30 nucleotides of the upstream closed end;

c) producing a nick in the sense strand of the DNA adjacent to or within 10, 20, or 30 nucleotides of the downstream closed end and/or the upstream closed end;

d) subjecting the DNA of c) to conditions having exonuclease activity, such that at least 90%, at least 95%, at least 99%, or 100% of the region of the sense strand between the nick and the one or more backbone modifications are removed; and

e) contacting the DNA of d) with a DNA polymerase, unmodified deoxyribose nucleotides, and nucleotides comprising chemically modified nucleobases under conditions such that a chemically modified sense strand is produced;

thereby producing the dsDNA molecule.

50. A method of making a DNA molecule, the method comprising:

a) providing a double stranded linear DNA having a sense strand, an antisense strand, an upstream open end, and a downstream open end;

b) converting both open ends to closed ends (thereby producing an upstream closed end and downstream closed end) such that the sense strand comprises one or more backbone modifications adjacent to or within 10, 20, or 30 nucleotides of the upstream closed end;

c) producing a nick in the sense strand of the DNA adjacent to or within 10, 20, or 30 nucleotides of the downstream closed end and/or the upstream closed end; and

d) subjecting the DNA of c) to conditions having exonuclease activity, such that at least 90%, at least 95%, at least 99%, or 100% of the region of the sense strand between the nick and the one or more backbone modifications are removed;

thereby producing the DNA molecule.

51. The method of embodiment 49 or 50, wherein the DNA of a) was produced by PCR, optionally followed by endonuclease digestion (e.g., with one or two endonucleases).

52. The method of any of embodiments 49-51, wherein a) comprises performing a polymerase chain reaction on a composition comprising a DNA template, e.g., a plasmid, a forward primer, a reverse primer, a DNA polymerase, and deoxyribose nucleotides, e.g., unmodified or chemically modified deoxyribose nucleotides, wherein optionally the unmodified deoxyribose nucleotides comprise dATP, dCTP, dTTP, and/or dGTP.

53. The method of embodiment 49 or 50, wherein the DNA of a) was produced by rolling circle amplification followed by endonuclease digestion (e.g., with one or two endonucleases).

54. The method of any of embodiments 49-53, wherein the DNA of a) is free of chemically modified nucleotides.

55. The method of any of embodiments 49-54, wherein the DNA of a) is free of chemically modified nucleobases.

56. The method of any of embodiments 49-55, wherein the DNA of a) is free of backbone modifications.

57. The method of any of embodiments 49-56, wherein the DNA of a) is free of chemically modified sugars.

58. The method of any of embodiments 49-57, wherein each open end of the DNA of a) is independently a blunt end or a sticky end.

59. The method of any of embodiments 49-58, wherein a) comprises incubating a dsDNA molecule with a restriction enzyme that cleaves a restriction enzyme recognition sequence in the dsDNA molecule, thereby making the double stranded linear DNA having a sense strand, an antisense strand, an upstream open end, and a downstream open end.

60. The method of any of embodiments 49-59, wherein converting both open ends to closed ends comprises contacting the DNA of a) with a ligase and a population of end adaptors, wherein the end adaptors comprise closed ends (e.g., loops or no-loop ends), under conditions that allow ligation of the end adaptors to the open ends.

61. The method of embodiment 60, wherein the end adaptors of the population have the same sequence and chemical modifications.

62. The method of embodiment 60, wherein the end adaptors of the population have different chemical modifications.

63. The method of embodiment 60 or 62, wherein the population of end adaptors comprises:
a first sub-population of end adaptors comprising phosphorothioate; and
a second sub-population of end adaptors that are free of backbone modifications.

64. The method of embodiment 63, wherein:
the first sub-population of end adaptors is configured to ligate to the upstream open end (e.g., wherein the upstream open end has a sticky end that is compatible with a sticky end of end adaptors of the first sub-population); and
the second sub-population of end adaptors is configured to ligate to the downstream open end (e.g., wherein the downstream open end has a sticky end that is compatible with a sticky end of end adaptors of the second sub-population).

65. The method of any of embodiments 49-64, which comprises a step of removing or inactivating the ligase (e.g., heat-inactivating the ligase) between steps b) and c).

66. The method of any of embodiments 49-65, wherein step c) comprises contacting the DNA of b) with a nickase.

67. The method of embodiment 66, wherein the nickase is Nb.BsrDI (e.g., NEB, R0648), Nt.BspQI (e.g., NEB, R0644), or Nb.BtsI (e.g., NEB, R0707).

68. The method of any of embodiments 49-67, wherein step d) comprises contacting the DNA of c) with an exonuclease.

69. The method of embodiment 68, wherein the exonuclease is an exonclease that initiates at a 3' terminus of DNA, e.g., Exonuclease III (e.g., NEB, M0206).

70. The method of embodiment 68, wherein the exonuclease is an exonuclease that initiates at the 5' terminus of DNA, e.g., T7 Exonuclease (e.g., NEB, M0263).

71. The method of any of embodiments 49-70, wherein the DNA polymerase is a KME polymerase or Taq DNA Polymerase (e.g., NEB, M0495).

72. The method of any of embodiments 49-71, wherein the method comprises, after step e), contacting the DNA with a ligase, e.g., to join the chemically modified sense strand to the downstream closed end.

73. The method of any of embodiments 49-72, which further comprises a step of enriching the DNA molecule (e.g., dsDNA molecule), e.g., after step a), b), c), d), or e).

74. The method of embodiment 73, wherein enrichment comprises use of a DNA enrichment column or agarose gel enrichment.

75. The method of any of embodiments 49-74, which is performed *in vitro*, e.g., in a cell-free system.

76. The method of any of embodiments 49-75, wherein the dsDNA molecule is a dsDNA molecule of any of embodiments 1-27 or 29-48.

77. A DNA molecule (e.g., dsDNA molecule) produced by the method of any of embodiments 49-76.

78. A composition comprising a plurality of double stranded DNA (dsDNA) molecules comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises one or more chemically modified nucleobases, and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end,

wherein the composition comprises at least 0.5 mg, at least 1 mg, at least 2 mg, at least 5 mg, at least 10 mg, at least 20 mg, or at least 50 mg of the dsDNA molecules, or wherein the composition comprises 0.5-1, 1-2, 2-5, 5-10, 10-20, 20-50, or 50-100 mg of the dsDNA molecules.

79. The dsDNA molecule, population, method, or composition of any of embodiments 1-37 or 49-78, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%, at least 90%, or at least 99% of cytosine positions in the sense strand of the dsDNA molecule comprise a chemically modified cytosine nucleotide.

80. The dsDNA molecule, population, method, or composition of any of embodiments 1-37 or 49-79, wherein 1%-100% (e.g., 1%-5%, 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%) of cytosine positions in the sense strand of the dsDNA molecule comprise a chemically modified cytosine nucleotide.

81. The dsDNA molecule, population, method, or composition of embodiment 80 or 81, wherein the chemically modified cytosine nucleotide comprises 5-hydroxycytosine.

82. The dsDNA molecule, population, method, or composition of any of embodiments 1-35, 38-45, or 48-78, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%, at least 90%, or at least 99% of thymine and uridine positions in the sense strand of the dsDNA molecule comprise a uridine nucleotide (e.g., a canonical uridine nucleotide).

83. The dsDNA molecule, population, method, or composition of any of embodiments 1-35, 38-45, 48-78, or 82, wherein 1%-100% (e.g., 1%-5%, 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%) of thymine and uridine positions in the sense strand of the dsDNA molecule comprise a uridine nucleotide (e.g., a canonical uridine nucleotide).

84. The dsDNA molecule, population, method, or composition of any of embodiments 1-35, 38-42, 44, 48-78, 82, or 83, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%, at least 90%, or at least 99% of thymine and uridine positions in the sense strand of the dsDNA molecule comprise a chemically modified uridine nucleotide.

85. The dsDNA molecule, population, method, or composition of any of embodiments 1-35, 38-42, 44, 48-78, or 82-84, wherein 1%-100% (e.g., 1%-5%, 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-

75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%) of thymine and uridine positions in the sense strand of the dsDNA molecule comprise a chemically modified uridine nucleotide.

86. The dsDNA molecule, population, method, or composition of embodiment 84 or 85, wherein the chemically modified uridine nucleotide comprises 5-azidomethyluridine, 5-formyluridine, 5-hydroxymethyluridine, or 5-methylthiouridine.

87. The dsDNA molecule, population, method, or composition of any of embodiments 1-35, 46, 47, or 49-78, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, or at least 75% of guanine and inosine positions in the sense strand of the dsDNA molecule comprise an inosine nucleotide.

88. The dsDNA molecule, population, method, or composition of any of embodiments 1-35, 46, 47, 49-78 or 87, wherein 1%-75% (e.g., 1%-5%, 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, or 70%-75%) of guanine and inosine positions in the sense strand of the dsDNA molecule comprise an inosine nucleotide.

89. The dsDNA molecule, population, method, or composition of any of embodiments 46, 47, 87 or 88, wherein the dsDNA molecule further comprises a canonical uridine nucleotide.

90. The dsDNA molecule, population, method, or composition of embodiment 89, wherein:

(a) wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, or at least 50% of guanine and inosine positions in the sense strand of the dsDNA molecule comprise an inosine nucleotide; and

(b) wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, or at least 50% of thymine and uridine positions in the sense strand of the dsDNA molecule comprise a canonical uridine nucleotide.

91. The dsDNA molecule, population, method, or composition of embodiment 89 or 90, wherein:

(a) wherein 1%-50% (e.g., 1%-5%, 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, or 45%-50%) of guanine and inosine positions in the sense strand of the dsDNA molecule comprise an inosine nucleotide; and

(b) wherein 1%-50% (e.g., 1%-5%, 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, or 45%-50%) of thymine and uridine positions in the sense strand of the dsDNA molecule comprise a canonical uridine nucleotide.

92. A DNA molecule comprising, in an upstream to downstream direction:

a) an upstream DNA end form which is a closed end;

b) a first double stranded region comprising a first fragment of a sense strand and a first region of an antisense strand, wherein optionally the first fragment of the sense strand comprises one or more backbone modifications;

c) a second region of the antisense strand, which region is single stranded;

d) a second double stranded region comprising a second fragment of the sense strand and a third region of the antisense strand; and

e) a downstream DNA end form which is a closed end.

93. The DNA molecule of embodiment 92, wherein the first double stranded region has a length of 5-10, 10-20, or 20-30 base pairs.

94. The DNA molecule of embodiment 92 or 93, wherein the second double stranded region has a length of 5-10, 10-20, or 20-30 base pairs.

95. The DNA molecule of any of embodiments 92-94, wherein the first fragment of the sense strand comprises one or more (e.g., 2, 3, 4, 5, or 6) nucleotides having backbone modifications.

96. The DNA molecule of any of embodiments 92-95, wherein the second fragment of the sense strand comprises one or more (e.g., 2, 3, 4, 5, or 6) nucleotides having backbone modifications.

97. The DNA molecule of embodiment 95 or 96, wherein the one or more backbone modifications comprise phosphorothioate linkages or boranophosphate linkages.

98. The DNA molecule of any of embodiments 92-97, wherein the closed end of the upstream DNA end form is a single stranded loop, e.g., a loop having a length of 1-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, or 45-50 nucleotides.

99. The DNA molecule of any of embodiments 92-98, wherein the closed end of the downstream DNA end form is a single stranded loop, e.g., a loop having a length of 1-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, or 45-50 nucleotides.

100. The DNA molecule of any of embodiments 92-99, wherein the second region of the antisense strand has a length of 50-100, 100-200, 200-500, 500-1000, 1000-2000, 2000-3000, 3000-4000, 4000-5000, 5000-6000, 6000-7000, 7000-8000, 8000-9000, 9000-10000, 10000-11000, or 11000-12000 nucleotides.

101. The DNA molecule of any of embodiments 92-100, which comprises one or more chemically modified nucleobases.

102. The DNA molecule of any of embodiments 92-100, which is substantially free of (e.g., is free of) chemically modified nucleobases.

103. A linear DNA molecule comprising, in a 5' to 3' direction:

a) a first annealing sequence,

b) a first looping sequence,

c) a second annealing sequence complementary to the first annealing sequence,

d) an antisense effector sequence,

e) a third annealing sequence,

f) a second looping sequence, and

g) a fourth annealing sequence complementary to the third annealing sequence.

104. The DNA molecule of embodiment 103, wherein the first annealing sequence has a length of 5-10, 10-20, or 20-30 nucleotides.

105. The DNA molecule of embodiment 103 or 104, wherein the second annealing sequence has a length of 5-10, 10-20, or 20-30 nucleotides.

106. The DNA molecule of any of embodiments 103-105, wherein the first annealing sequence and the second annealing sequence have the same length and are perfectly complementary along that length.

107. The DNA molecule of any of embodiments 103-106, wherein the first annealing sequence and the second annealing sequence pair with each other to form a double stranded region.

108. The DNA molecule of any of embodiments 103-107, wherein the first annealing sequence comprises one or more (e.g., 2, 3, 4, 5, or 6) nucleotides having backbone modifications.

109. The DNA molecule of any of embodiments 103-108, wherein the second annealing sequence comprises one or more (e.g., 2, 3, 4, 5, or 6) nucleotides having backbone modifications.

110. The DNA molecule of any of embodiment 103-109, wherein the third annealing sequence has a length of 5-10, 10-20, or 20-30 nucleotides.

111. The DNA molecule of any of embodiments 103-110, wherein the fourth annealing sequence has a length of 5-10, 10-20, or 20-30 nucleotides.

112. The DNA molecule of any of embodiments 103-111, wherein the third annealing sequence and the fourth annealing sequence have the same length and are perfectly complementary along that length.

113. The DNA molecule of any of embodiments 103-112, wherein the third annealing sequence and the fourth annealing sequence pair with each other to form a double stranded region.

114. The DNA molecule of any of embodiments 103-113, wherein the third annealing sequence comprises one or more (e.g., 2, 3, 4, 5, or 6) nucleotides having backbone modifications.

115. The DNA molecule of any of embodiments 103-114, wherein the fourth annealing sequence comprises one or more (e.g., 2, 3, 4, 5, or 6) nucleotides having backbone modifications.

116. The DNA molecule of any of embodiments 108-115 wherein the one or more backbone modifications comprise phosphorothioate linkages or boranophosphate linkages.

117. The DNA molecule of any of embodiments 103-116, wherein the first looping sequence has a length of 1-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, or 45-50 nucleotides.

118. The DNA molecule of any of embodiments 103-117, wherein the first looping sequence does not comprise more than 5, more than 6, more than 7, more than 8, more than 9, or more than 10 consecutive nucleotides of perfect complementarity to any region of the linear DNA molecule.

119. The DNA molecule of any of embodiments 103-118, wherein the second looping sequence has a length of 1-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, or 45-50 nucleotides.

120. The DNA molecule of any of embodiments 103-119, wherein the second looping sequence does not comprise more than 5, more than 6, more than 7, more than 8, more than 9, or more than 10 nucleotides of perfect complementarity to any region of the linear DNA molecule.

121. The DNA molecule of any of embodiments 103-120, wherein the antisense effector sequence is single stranded.

122. The DNA molecule of any of embodiments 103-121, wherein the antisense effector sequence does not comprise more than 5, more than 10, more than 15, or more than 20 consecutive nucleotides of perfect complementarity to any region of the linear DNA molecule.

123. The DNA molecule of any of embodiments 103-122, which comprises one or more chemically modified nucleobases.

124. The DNA molecule of any of embodiments 103-123, which is substantially free of (e.g., is free of) chemically modified nucleobases.

125. The DNA molecule of any of embodiments 103-124, wherein the antisense effector sequence is substantially free of (e.g., is free of) chemically modified nucleobases.

126. A double stranded DNA (dsDNA) molecule comprising an inosine nucleotide.

127. The dsDNA molecule of embodiment 126, wherein the dsDNA molecule is circular or linear.

128. The dsDNA molecule of embodiment 126 or 127, wherein the dsDNA molecule is closed-ended linear.

129. The dsDNA molecule of any of embodiments 126-128, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, or at least 75% of guanosine and inosine positions in the dsDNA molecule comprise the inosine nucleotide.

130. A double stranded DNA (dsDNA) molecule comprising an inosine nucleotide, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, or at least 75% of guanosine and inosine positions in the dsDNA molecule comprise the inosine nucleotide.

131. The dsDNA molecule of any of embodiments 126-130, wherein 1%-75% (e.g., 1%-5%, 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, or 70%-75%) of guanosine and inosine positions in the dsDNA molecule comprise the inosine nucleotide.

132. A double stranded DNA (dsDNA) molecule comprising an inosine nucleotide, wherein 1%-75% (e.g., 1%-5%, 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, or 70%-75%) of guanosine and inosine positions in the dsDNA molecule comprise the inosine nucleotide.

133. The dsDNA molecule of any of embodiments 126-132, wherein at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% of the sugars of the inosine nucleotides of the dsDNA molecule are deoxyribose sugars.

134. The dsDNA molecule of any of embodiments126-133, wherein the dsDNA molecule is linear and can be circularized.

135. The dsDNA molecule of any of embodiments 126-133, wherein the dsDNA molecule is linear and cannot be circularized.

136. The dsDNA molecule of any of embodiments 126-135, wherein the dsDNA molecule or a portion thereof can be integrated into the genome.

137. The dsDNA molecule of any of embodiments 126-135, wherein the dsDNA molecule or a portion thereof cannot be integrated into the genome.

138. The dsDNA molecule of any of embodiments 126-137, wherein the dsDNA molecule can be concatemerized.

139. The dsDNA molecule of any of embodiments 126-137, wherein the dsDNA molecule cannot be concatemerized.

140. The dsDNA molecule of any of embodiments 126-139, which comprises a sense strand and an antisense strand.

141. The dsDNA molecule of embodiment 140, wherein the antisense strand comprises one or more chemically modified nucleotides.

142. The dsDNA molecule of embodiment 140 or 141, wherein the sense strand does not comprise any chemically modified nucleotides.

143. The dsDNA molecule of embodiment 140 or 141, wherein the sense strand comprises one or more chemically modified nucleotides.

144. The dsDNA molecule of any of embodiments 126-143, which, when contacted to HEKa cells, e.g., in an assay as described herein, results in one or both of:

a lower level of CXCL10 mRNA compared to a control DNA molecule (e.g., at least 50%, at least 40%, at least 30%, at least 20%, or at least 10% lower), or

a lower level of IL6 mRNA compared to a control DNA molecule (e.g., at least 40%, at least 30%, at least 20%, or at least 10% lower),

wherein the control DNA molecule comprises the same sequence, same strandedness, and same circular or linear character as the dsDNA molecule, but comprises guanosine nucleotides in place of the inosine nucleotides.

145. The dsDNA molecule of any of embodiments 126-144, which encodes a protein, and which, when contacted to HEKa cells, e.g., in an assay as described herein, results in expression at a level at least 80%, at least 90%, at least 100%, at least 110%, or at least 120% of the expression of a control DNA, wherein the control DNA molecule comprises the same sequence, same strandedness, and same circular or linear character as the dsDNA molecule, but comprises guanosine nucleotides in place of the inosine nucleotides.

146. The dsDNA molecule of any of embodiments 126-145, wherein the dsDNA molecule is a TDSC.

147. The dsDNA molecule of embodiment 146, wherein the TDSC comprises:

a) an upstream exonuclease-resistant DNA end form;

b) a double stranded region; and

c) a downstream exonuclease-resistant DNA end form.

148. The dsDNA molecule of embodiment 147, wherein one or both of the upstream exonuclease-resistant DNA end form and downstream exonuclease-resistant DNA end form are open ends.

149. The dsDNA molecule of embodiment 147 or 148, wherein the upstream DNA end form comprises a Y-adaptor configuration, and the downstream DNA end form comprises a Y-adaptor configuration.

150. The dsDNA molecule of embodiment 147 or 148, wherein one or both of the upstream exonuclease-resistant DNA end form and downstream exonuclease-resistant DNA end form are blunt ends or sticky ends.

151. The dsDNA molecule of embodiment 147 or 148, wherein the upstream DNA end form is double stranded and blunt-ended and comprises a phosphorothioate modification on each strand, and the downstream DNA end form is double stranded and blunt-ended and comprises a phosphorothioate modification on each strand.

152. The dsDNA molecule of embodiment 147, wherein one or both of the upstream exonuclease-resistant DNA end form and downstream exonuclease-resistant DNA end form are closed ends.

153. The dsDNA molecule of embodiment 147 or 152, wherein one or both of the upstream exonuclease-resistant DNA end form and downstream exonuclease-resistant DNA end form comprise a loop.

154. The dsDNA molecule of any of embodiments 147, 152, or 153, wherein the upstream DNA end form is a closed end, and the downstream DNA end form is a closed end.

155. The dsDNA molecule of any of embodiments 147-154, wherein the upstream DNA end form (e.g., upstream exonuclease-resistant DNA end form) comprises one or more chemically modified nucleotides.

156. The dsDNA molecule of any of embodiments 147-155, wherein the downstream DNA end form (e.g., downstream exonuclease-resistant DNA end form) comprises one or more chemically modified nucleotides.

157. The dsDNA molecule of any of embodiments 147-156, wherein the upstream exonuclease-resistant DNA end form comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 terminal nucleotides of the upstream exonuclease-resistant DNA end form, e.g., on the first strand, the second strand, or both of the first and second strands).

158. The dsDNA molecule of any of embodiments 147-157, wherein the upstream exonuclease-resistant DNA end form comprises at least 3 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 terminal nucleotides of the upstream exonuclease-resistant DNA end form, e.g., on the first strand, the second strand, or both of the first and second strands).

159. The dsDNA molecule of any of embodiments 147-158, wherein the upstream exonuclease-resistant DNA end form comprises at least 6 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 terminal nucleotides of the upstream exonuclease-resistant DNA end form, e.g., on the first strand, the second strand, or both of the first and second strands).

160. The dsDNA molecule of any of embodiments 147-159, wherein the downstream exonuclease-resistant DNA end form comprises at least 1, at least 2, at least 3, at least 4, at least at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 terminal nucleotides of the downstream exonuclease-resistant DNA end form, e.g., on the first strand, the second strand, or both of the first and second strands).

161. The dsDNA molecule of any of embodiments 147-160, wherein the downstream exonuclease-resistant DNA end form comprises at least 3 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 terminal nucleotides of the downstream exonuclease-resistant DNA end form, e.g., on the first strand, the second strand, or both of the first and second strands).

162. The dsDNA molecule of any of embodiments 147-161, wherein the downstream exonuclease-resistant DNA end form comprises at least 6 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 terminal nucleotides of the downstream exonuclease-resistant DNA end form, e.g., on the first strand, the second strand, or both of the first and second strands).

163. The dsDNA molecule of any of embodiments 147-162, wherein the upstream and downstream exonuclease-resistant DNA end form each comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 terminal nucleotides of the upstream and downstream exonuclease-resistant DNA end forms, e.g., on the first strand, the second strand, or both of the first and second strands).

164. The dsDNA molecule of any of embodiments 147-163, wherein the upstream and downstream exonuclease-resistant DNA end form each comprises at least 3 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 terminal nucleotides of the upstream and downstream exonuclease-resistant DNA end forms, e.g., on the first strand, the second strand, or both of the first and second strands).

165. The dsDNA molecule of any of embodiments 147-164, wherein the upstream and downstream exonuclease-resistant DNA end form each comprises at least 6 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 terminal nucleotides of the upstream and downstream exonuclease-resistant DNA end forms, e.g., on the first strand, the second strand, or both of the first and second strands).

166. The dsDNA molecule of embodiment 147, wherein one or both of the upstream exonuclease-resistant DNA end form and the downstream exonuclease-resistant DNA end form comprises a Y-adaptor configuration.

167. The dsDNA molecule of embodiment 166, wherein every nucleotide in the Y-adaptor is a chemically modified nucleotide.

168. The dsDNA molecule of any of embodiments 147-167, wherein one or both of the upstream exonuclease-resistant DNA end form and the downstream exonuclease-resistant DNA end form

comprises one or more of: a nuclear targeting sequence, a maintenance sequence, or a sequence that binds an endogenous polypeptide in a target cell.

169. The dsDNA molecule of any of embodiments 147-168, wherein every nucleotide in the TDSC binds another nucleotide in the TDSC.

170. The dsDNA molecule of any of embodiments 147-169, wherein the upstream exonuclease-resistant DNA end form and the downstream exonuclease-resistant DNA end form have the same structure.

171. The dsDNA molecule of any of embodiments 147-169, wherein the upstream exonuclease-resistant DNA end form and the downstream exonuclease-resistant DNA end form have different structures.

172. The dsDNA molecule of embodiment 147, wherein one or both of the upstream exonuclease-resistant DNA end form and downstream exonuclease-resistant DNA end form are open ends (e.g., blunt ends, sticky ends, or Y-adaptors).

173. The dsDNA molecule of embodiment 147, wherein the upstream DNA end form, the downstream DNA end form, or both, comprises a Y-adaptor.

174. The dsDNA molecule of embodiment 147, wherein one or both of the upstream exonuclease-resistant DNA end form and downstream exonuclease-resistant DNA end form are closed ends (e.g., hairpins).

175. The dsDNA molecule of embodiment 174, wherein the closed end comprises one or more (e.g., at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, or at least 50) nucleotides that are not hybridized (e.g., are not part of a double-stranded region).

176. The dsDNA molecule of embodiment 174, wherein the closed end does not comprise any nucleotides that are not hybridized (e.g., wherein all nucleotides of the closed end are hybridized to another nucleotide).

177. The dsDNA molecule of any of embodiments 147-176, wherein the upstream DNA end form, the downstream DNA end form, or both, comprise at least one chemically modified nucleotide.

178. The dsDNA molecule of any of embodiments 147-177, wherein both of the upstream DNA end form and the downstream DNA end form comprise at least one chemically modified nucleotide on the sense strand and at least one chemically modified nucleotide on the antisense strand.

179. The dsDNA molecule of any of embodiments 147-178, wherein both the upstream DNA end form and the downstream DNA end form comprise chemically modified nucleotides at every sense strand position and every antisense strand position.

180. The dsDNA molecule of any of embodiments 147-179, wherein the upstream DNA end form, the downstream DNA end form, or both, comprises an inverted terminal repeat (ITR).

181. The dsDNA molecule of any of embodiments 147-180, wherein the upstream DNA end form, the downstream DNA end form, or both, comprises a protelomerase sequence.

182. The dsDNA molecule of any of embodiments 147-181, wherein one or both of the upstream exonuclease-resistant DNA end form and the downstream exonuclease-resistant DNA end form comprises at least one chemically modified nucleotide (e.g., comprises a chemical modification on every sense strand nucleotide and every antisense strand nucleotide).

183. The dsDNA molecule of any of embodiments 147-182, wherein one or both of the upstream exonuclease-resistant DNA end form and the downstream exonuclease-resistant DNA end form comprises one or more chemically modified nucleotides (e.g., phosphorothioate modified nucleotides).

184. The dsDNA molecule of any of embodiments 147-183, wherein the double-stranded region comprises one or more chemically modified nucleotides (e.g., phosphorothioate modified nucleotides).

185. The dsDNA molecule of any of embodiments 147-184, wherein the double-stranded region comprises an effector sequence encoding an effector, and wherein the antisense strand for the effector sequence comprises one or more chemically modified nucleotides (e.g., phosphorothioate modified nucleotides).

186. The dsDNA molecule of any of embodiments 147-185, wherein the double-stranded region comprises an effector sequence encoding an effector, and wherein the sense strand for the effector sequence comprises one or more chemically modified nucleotides (e.g., phosphorothioate modified nucleotides).

187. The dsDNA molecule of any of embodiments 126-186, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, or at least 75% of guanosine and inosine positions in the sense strand of the dsDNA molecule comprise the inosine nucleotide.

188. The dsDNA molecule of any of embodiments 126-187, wherein 1%-75% (e.g., 1%-5%, 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, or 70%-75%) of guanosine and inosine positions in the sense strand of the dsDNA molecule comprise the inosine nucleotide.

189. A method of making or manufacturing a double stranded DNA (dsDNA) molecule, the method comprising:

(a) providing a composition comprising a DNA template (e.g., a plasmid), a forward primer, a reverse primer, a DNA polymerase, unmodified deoxyribose nucleotides, and inosine nucleotides; and

(b) performing a polymerase chain reaction on the composition of (a),

thereby making or manufacturing the dsDNA molecule, wherein optionally the dsDNA molecule is a dsDNA molecule of any of enumerated embodiments 126-188.

190. The method of embodiment 189, wherein the method further comprises purification of the dsDNA molecule, e.g., wherein purification comprises use of a DNA purification column or agarose gel purification.

191. The method of embodiment 189 or 190, wherein the DNA polymerase comprises a KOD polymerase, a KODX polymerase, a Deep Vent polymerase, or a KOD -Multi & Epi- polymerase.

192. The method of any of embodiments 189-191, wherein the unmodified deoxyribose nucleotides comprise dATP, dCTP, dTTP, and/or dGTP.

193. The method of any of embodiments 189-192, wherein the percentage of guanosine or inosine nucleotides that are inosine nucleotides in the composition of (a) is 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, or 70%-75%.

194. The method of any of embodiments 189-193, wherein the forward primer, the reverse primer, or both contains a protelomerase recognition sequence, e.g., a TelN protelomerase recognition sequence.

195. The method of embodiment 194, wherein the method further comprises (e.g., after step (b)):

(c) incubating the dsDNA molecule with a protelomerase, e.g., a TelN protelomerase.

196. The method of any of embodiments 189-195, wherein the forward primer, the reverse primer, or both contains a restriction enzyme recognition sequence.

197. The method of any of embodiments 189-196, wherein the method further comprises:

(i) incubating the dsDNA molecule with a restriction enzyme that cleaves the restriction enzyme recognition sequence, thereby making a cleaved dsDNA molecule;

(ii) incubating the cleaved dsDNA molecule with a DNA ligase, e.g., a T3 DNA ligase, thereby making a ligated dsDNA molecule; and/or

(iii) optionally, incubating the ligated dsDNA molecule with an exonuclease, e.g., a T5 exonuclease.

198. The method of any of embodiments 189-197, the method further comprising:

(c) ligating the dsDNA molecule to a hairpin DNA molecule comprising: a loop region and a double-stranded region comprising one or more chemically modified nucleotides.

199. The method of any of embodiments 189-198, the method further comprising ligating the dsDNA molecule to a self-annealed DNA molecule comprising a first region and a second region, wherein the first region is hybridized to the second region.

200. The method of embodiment 199, wherein the self-annealed DNA molecule further comprises a loop between the first region and the second region.

201. The method of embodiment 200, wherein the loop comprises a heterologous functional sequence, e.g., a nuclear targeting sequence (e.g., a CT3 sequence), or a regulatory sequence.

202. The method of embodiment 199, wherein the self-annealed DNA molecule does not comprise any nucleotides that are not hybridized (e.g., wherein all nucleotides of the self-annealed DNA molecule are hybridized to another nucleotide).

203. The method of any of embodiments 189-202, which further comprises ligating a second hairpin DNA molecule to the dsDNA molecule, wherein the second hairpin DNA molecule comprises a loop region and a double-stranded region, wherein optionally the second hairpin DNA molecule comprises one or more chemically modified nucleotides in one or both of the loop region or the double stranded region.

204. A method of making or manufacturing a TDSC, the method comprising:

a) providing the dsDNA molecule made by a method of any of embodiments 189-203, wherein the dsDNA molecule comprises closed ends;

b) incubating the TDSC with a double stranded DNA exonuclease, e.g., Exonuclease III, e.g., e.g., 1 µL of Exonuclease III per 5 µg of DNA in 50 µL, for 1 hour at 37 °C, e.g., as described in Example 2;

c) optionally, purifying the TDSC treated in step b), e.g., by Silica membrane column, e.g., as described in Example 2,

thereby making or manufacturing the TDSC.

205. A dsDNA molecule produced by the method of any of embodiments 189-204.

206. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the sense strand comprises one or more (e.g., at least 3) backbone modifications, e.g., phosphorothioate linkages, wherein optionally the one or more backbone modifications are situated between the 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides adjacent to the upstream DNA end form.

207. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule, when contacted to human cells, results in expression at a level at least 100%, at least 120%, at least 140%, at least 160%, or at least 180% the expression of a control DNA molecule (e.g., an unmodified control DNA molecule), wherein the control DNA molecule comprises the same sequence and same closed end form as the DNA molecule, but comprises no chemically modified nucleobases.

208. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the dsDNA molecule, when contacted to human cells, results in expression at a level at least the expression of a modified control DNA molecule, wherein the modified control DNA molecule comprises the same sequence, same closed end double stranded form, and same degree of sense strand nucleobase modification as the dsDNA molecule, but comprises antisense strand nucleobase modification at the same degree as sense strand nucleobase modification.

209. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the sense strand comprises a first type of chemically modified nucleobase and a second type of chemically modified nucleobase.

210. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the sense strand comprises a uridine nucleotide.

211. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 1-209, wherein the sense strand comprises an inosine nucleotide.

212. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 1-210, wherein the sense strand comprises a 5-methylthiouridine nucleotide.

213. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule has a length of at least 500, at least 1000, at least 2000, at least 3000, at least 4000, at least 5000, at least 6000, at least 7000, at least 8000, at least 9000, at least 10000, at least 11000, or at least 12000 nucleotides.

214. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule has a length of between 500-1000, 1000-2000, 2000-3000, 3000-4000, 4000-5000, 5000-6000, 6000-7000, 7000-8000, 8000-9000, 9000-10000, 10000-11000, or 11000-12000 nucleotides.

215. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule has a length of at least 15 nucleotides, at least 30 nucleotides, at least 50 nucleotides, at least 75 nucleotides, 100 nucleotides, at least 200 nucleotides, at

least 300 nucleotides, at least 500 nucleotides, at least 750 nucleotides, at least 1,000 nucleotides, at least 2,000 nucleotides, at least 3,000 nucleotides, at least 4,000 nucleotides, at least 5,000 nucleotides, at least 6,000 nucleotides, at least 7,000 nucleotides, at least 8,000 nucleotides, at least 9,000 nucleotides, at least 10,000 nucleotides, at least 11,000 nucleotides, at least 12,000 nucleotides, at least 15,000 nucleotides, at least 20,000 nucleotides, at least 25,000 nucleotides, at least 30,000 nucleotides, at least 35,000 nucleotides, at least 40,000 nucleotides at least 45,000 nucleotides, at least 50,000 nucleotides, at least 60,000 nucleotides, or more.

216. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule has a length of between 20 and 1000 nucleotides, between 20 and 50 nucleotides, between 100 and 500 nucleotides, between 500 and 50,000 nucleotides, between 1,000 and 50,000 nucleotides, between 2,000 and 40,000 nucleotides, between 5,000 and 50,000 nucleotides, between 500 and 50,000 nucleotides, between 500 and 25,000 nucleotides, between 1,000 and 20,000 nucleotides, between 1,000 and 10,000 nucleotides, between 10,000 and 60,000 nucleotides, between 1,000 and 20,000 nucleotides, between 1,000 and 40,000 nucleotides, between 500 and 1000 nucleotides, between 1000 and 2,000 nucleotides, between 2,000 and 3,000 nucleotides, between 3,000 and 4,000 nucleotides, between 4,000 and 5,000 nucleotides, between 5,000 and 6,000 nucleotides, between 6,000 and 7,000 nucleotides, between 7,000 and 8,000 nucleotides, between 8,000 and 9,000 nucleotides, between 9,000 and 10,000 nucleotides, between 10,000 and 11,000 nucleotides, or between 11,000 and 12,000 nucleotides.

217. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule is resistant to endonuclease digestion.

218. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule is resistant to immune sensor recognition.

219. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the upstream DNA end form is a single stranded region 1-5, 5-10, or 10-15 nucleotides in length.

220. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the downstream DNA end form is a single stranded region 1-5, 5-10, or 10-15 nucleotides in length.

221. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein one or both of the upstream DNA end form and the downstream DNA end form have one or more of the following characteristics:

i) the upstream DNA end form has a loop size of less than about 28 or less than about 56 nucleotides in length or greater than about 28 or greater than about 56 nucleotides in length; or

ii) the downstream DNA end form has a loop size of less than about 28 or less than about 56 nucleotides in length or greater than about 28 or greater than about 56 nucleotides in length.

222. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the upstream DNA end form is resistant to endonuclease digestion.

223. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the upstream DNA end form is resistant to immune sensor recognition.

224. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the downstream DNA end form is resistant to endonuclease digestion.

225. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the downstream DNA end form is resistant to immune sensor recognition.

226. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the double stranded region is resistant to endonuclease digestion.

227. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the double stranded region is resistant to immune sensor recognition.

228. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the upstream DNA end form and the downstream DNA end form have the same nucleotide sequence.

229. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the upstream DNA end form and the downstream DNA end form have different nucleotide sequences.

230. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the upstream DNA end form and the downstream DNA end form have the same length in nucleotides.

231. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the upstream DNA end form and the downstream DNA end form have different lengths in nucleotides.

232. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein one or both of the upstream DNA form and downstream DNA form comprises one or more (e.g., at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, or at least 50) nucleotides that are not hybridized to another nucleotide.

233. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the one or both of the upstream DNA form and downstream DNA form do not comprise any nucleotides that are not hybridized (e.g., wherein all nucleotides of the closed end are hybridized to another nucleotide).

234. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the sense strand of the dsDNA molecule comprises one or more phosphorothioate linkages.

235. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the antisense strand comprises no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 chemically modified nucleotides.

236. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the antisense strand comprises no more than 10, no more than 9, no

more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 chemically modified nucleobases.

237. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the antisense strand comprises no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 backbone modifications.

238. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the antisense strand comprises no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 nucleotides having a chemically modified sugar.

239. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% of the sugars of the DNA molecule are deoxyribose sugars.

240. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein all positions in the DNA molecule comprise a deoxyribose sugar.

241. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule comprises a chemical modification of a phosphate group.

242. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule comprises a chemically modified sugar, e.g., a 2'-deoxy-2'-fluoro (2'-F) nucleotide or a 2'-O-methyl (2'-O-Me) nucleotide.

243. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule further comprises one or more additional chemically modified nucleotide, wherein the additional chemically modified nucleotide comprises a modification in the backbone, sugar, or nucleobase.

244. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 243, wherein one or more of the chemically modified nucleotides is conjugated to a peptide or protein.

245. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 243 or 244, wherein one or more of the chemically modified nucleotides comprises a phosphorothioate linkage.

246. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 243-245, wherein the DNA molecule is a dsDNA molecule, wherein each of the first and second strands of the dsDNA molecule comprises one or more chemically modified nucleotides.

247. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 243-246, wherein the DNA molecule is a dsDNA molecule, wherein each of the first and second strands of the dsDNA molecule comprises one or more phosphorothioate linkages.

248. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the upstream DNA end form comprises one or more chemically modified nucleotides.

249. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the downstream DNA end form comprises one or more chemically modified nucleotides.

250. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the sense strand comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides adjacent to the upstream DNA end form).

251. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the sense strand comprises at least 3 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides adjacent to the upstream DNA end form).

252. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the sense strand comprises at least 6 phosphorothioate linkages (e.g., between the 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides adjacent to the upstream DNA end form.

253. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the double stranded region or the antisense strand comprises an effector sequence that encodes an effector (e.g., a therapeutic effector).

254. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 253, wherein the effector is a polypeptide (e.g., a protein).

255. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 253, wherein the effector is a functional RNA (e.g., a miRNA, siRNA, or tRNA).

256. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the double stranded region or the antisense strand comprises a promoter sequence, e.g., wherein the sequence that encodes an effector is operably linked to the promoter sequence.

257. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule comprises one or more of:

i) a promoter sequence (wherein optionally the promoter sequence is in the double stranded region);

ii) an effector sequence that encodes an effector, operably linked to the promoter sequence;

iii) a heterologous functional sequence, e.g., a nuclear targeting sequence or a regulatory sequence;

iv) a maintenance sequence; and/or

v) an origin of replication.

258. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 257, which comprises:

i, ii, and iii;

i, ii, and iv;

i, ii, and v;

i, ii, iii, and iv;

i, ii, iii, and v;

i, ii, iv, and v; or

i, ii, iii, iv, and v.

259. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 257 or 258, wherein the nuclear targeting sequence comprises a CT3 sequence (e.g., a sequence of AATTCTCCTCCCCACCTTCCCCACCCTCCCCA (SEQ ID NO: 40)), or a nucleic acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto.

260. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 257 or 258, wherein the nuclear targeting sequence binds to a hnRNPK protein (e.g., a human hnRNPK protein).

261. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 257-260, wherein the effector comprises a polypeptide (e.g., a protein).

262. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 257-260, wherein the effector comprises an RNA (e.g., an mRNA, a tRNA, lncRNA, miRNA, rRNA, snRNA, microRNA, siRNA, piRNA, snoRNA, snRNA, exRNA, scaRNA, Y RNA, or hnRNA), wherein optionally the effector comprises a functional RNA (e.g., a miRNA, siRNA, or tRNA).

263. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 1-261, wherein the DNA molecule does not comprise a sequence encoding an RNA.

264. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the DNA molecule can be replicated (e.g., by a DNA polymerase native to a cell comprising the DNA molecule).

265. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 1-263, wherein the DNA molecule cannot be replicated.

266. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 253-265, wherein the effector is heterologous to a target cell.

267. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein one or both of the upstream DNA end form and the downstream DNA end form comprises one or more of: a nuclear targeting sequence, a maintenance sequence, or a sequence that binds an endogenous polypeptide in a target cell.

268. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein one or both of the upstream DNA end form and the downstream DNA end form does not comprise the nucleic acid sequences TATCAGCACACAATTGCCCATTATACGC (SEQ ID NO: 41) and GCGTATAATGGGCAATTGTGTGCTGATA (SEQ ID NO: 42), or nucleic acid sequences having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity thereto; and/or the nucleic acid sequences TATCAGCACACAATAGTCCATTATACGC (SEQ ID NO: 43) and GCGTATAATGGACTATTGTGTGCTGATA (SEQ ID NO: 44).

269. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of the preceding embodiments, wherein the upstream DNA end form, the downstream DNA end form, or both, comprises a protelomerase sequence.

270. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 269, wherein one or more of the protelomerase sequences comprise (e.g., in 5'-to-3' order) the nucleic acid sequences TATCAGCACACAATTGCCCATTATACGC (SEQ ID NO: 41) and GCGTATAATGGGCAATTGTGTGCTGATA (SEQ ID NO: 42), or nucleic acid sequences having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity thereto.

271. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 269 or 270, wherein one or more of the protelomerase sequences comprise (e.g., in 5'-to-3' order) the nucleic acid sequences TATCAGCACACAATAGTCCATTATACGC (SEQ ID NO: 43) and GCGTATAATGGACTATTGTGTGCTGATA (SEQ ID NO: 44), or nucleic acid sequences having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity thereto.

272. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 269, wherein one or more of the protelomerase sequences comprise (e.g., in 5'-to-3' order) the nucleic

acid sequences ACCTATTTCAGCATACTACGC (SEQ ID NO: 45) and GCGTAGTATGCTGAAATAGGT (SEQ ID NO: 46), or nucleic acid sequences having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity thereto.

273. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 269, wherein one or more of the protelomerase sequences comprise (e.g., in 5'-to-3' order) the nucleic acid sequence CACACAATTGCCCATTATACGCGCGTATAATGGGCAATTGTGTG (SEQ ID NO: 47), or a nucleic acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity thereto.

274. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 269, wherein one or more of the protelomerase sequences comprise (e.g., in 5'-to-3' order) the nucleic acid sequences:

(i) TAAATATAATTTAA (SEQ ID NO: 48) and TTAAATTATATTTA (SEQ ID NO: 49),

(ii) AATATATAATCTAA (SEQ ID NO: 50) and TTAGATTATATATT (SEQ ID NO: 51),

(iii) TATTTATTATCTTT (SEQ ID NO: 52) and AAAGATAATAAATA (SEQ ID NO: 53),

(iv) ATATAATTTTTAATTAGTATAGAATATGTTAA (SEQ ID NO: 54) and TTAACATACTCTATACTAATTAAAAATTATAT (SEQ ID NO: 55),

(v) TATAATTTGATATTAGTACAAATCCC (SEQ ID NO: 56) and GGGATTTGTACTAATATCAAATTATA (SEQ ID NO: 57),

(vi) ATATAATATTTATTTAGTACAAAGTTC (SEQ ID NO: 58) and GAACTTTGTACTAAATAAATATTATAT (SEQ ID NO: 59),

(vii) ATATAATTTTTTATTAGTATAGAGTAT (SEQ ID NO: 60) and ATACTCTATACTAATAAAAAATTATAT (SEQ ID NO: 61),

(viii) TAAATATAATTTAA (SEQ ID NO: 48) and TTAAATTATATTTA (SEQ ID NO: 49); or nucleic acid sequences having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity thereto.

275. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 269, wherein one or more of the protelomerase sequences further comprise (e.g., in 5'-to-3' order) the nucleic acid sequences:

(i) TAGTATAAAAAACTGT (SEQ ID NO: 62) and ACAGTTTTTTATACTA (SEQ ID NO: 63),

(ii) TAGTATACAAAAGATT (SEQ ID NO: 64) and AATCTTTTGTATACTA (SEQ ID NO: 65),

(iii) TAGTATATATATCTCT (SEQ ID NO: 66) and AGAGATATATATACTA (SEQ ID NO: 67), or

(iv) TAGTATAAAAAAAATT (SEQ ID NO: 68) and AATTTTTTTTATACTA (SEQ ID NO: 69);

or nucleic acid sequences having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity thereto.

276. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 269-275, wherein the protelomerase sequences are produced by TelN protelomerase, ResT protelomerase, Tel PY54 protelomerase, or TelK protelomerase digestion.

277. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 269-275, wherein the protelomerase sequences are not produced by TelN protelomerase digestion.

278. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 269-275, wherein the protelomerase sequences are not produced by Tel PY54 protelomerase digestion.

279. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 269-275, wherein the protelomerase sequences are not produced by TelK protelomerase digestion.

280. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 269-275, wherein the protelomerase sequences are not produced by ResT protelomerase digestion.

281. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 269-280, wherein the protelomerase sequences are about 28 or 56 nucleotides in length.

282. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 269-281, wherein the protelomerase sequences are less than 28 (e.g., less than 15, less than 20, less than 25, less than 26, less than 27, or less than 28) nucleotides in length.

283. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 269-282, wherein the protelomerase sequences are between about 28 (e.g., 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides and about 56 (e.g., 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60) nucleotides in length.

284. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of any of embodiments 269-283, wherein the protelomerase sequences are greater than about 56 (e.g., greater than 50, greater than 51, greater than 52, greater than 53, greater than 54, greater than 55, greater than 56, greater than 57, greater than 58, greater than 59, greater than 60, greater than 65, greater than 70, greater than 75, greater than 80, greater than 90, or greater than 100) nucleotides in length.

285. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 269, wherein the protelomerase sequence is produced from a first protelomerase recognition sequence (PRS) and a second PRS that are recognized by a TelN protelomerase or ResT protelomerase.

286. The DNA molecule (e.g., dsDNA molecule), population, method, or composition of embodiment 269, wherein the protelomerase sequence is produced from a first protelomerase recognition sequence (PRS) and a second PRS that are recognized by a Tel PY54 protelomerase or TelK protelomerase.

287. A composition comprising a plurality of DNA molecules (e.g., dsDNA molecules) of any of the preceding embodiments.

288. A composition comprising a comprising a plurality of DNA molecules (e.g., dsDNA molecules) of any of the preceding embodiments, wherein the composition comprises at least 0.5 mg, at least 1 mg, at least 2 mg, at least 5 mg, at least 10 mg, at least 20 mg, or at least 50 mg of the DNA molecules, or wherein the composition comprises 0.5-1, 1-2, 2-5, 5-10, 10-20, 20-50, or 50-100 mg of the DNA molecules.

289. A plurality of DNA molecules (e.g., dsDNA molecules) of any of the preceding embodiments.

290. The composition of embodiment 287 or 288, or the plurality of DNA molecules of embodiment 289, wherein:

at least 50%, at least 60%, or at least 70% of the DNA molecules in the plurality have substantially the same length;

at least 50%, at least 60%, or at least 70% of the DNA molecules in the plurality have a length in a predetermined range; or

at least 50%, at least 60%, or at least 70% of the DNA molecules in the plurality have a length of between 100, 200, 300, 400, or 500 nucleotides of each other.

291. The composition of embodiment 287 or 288, or the plurality of DNA molecules of embodiment 289, wherein:

40%-50%, 50%-60%, or 60%-70% of the DNA molecules in the plurality have substantially the same length;

40%-50%, 50%-60%, or 60%-70% of the DNA molecules in the plurality have a length in a predetermined range; or

40%-50%, 50%-60%, or 60%-70% of the DNA molecules in the plurality have a length of between 100, 200, 300, 400, or 500 nucleotides of each other.

292. A pharmaceutical composition comprising the DNA molecule (e.g., the dsDNA molecule) of any of the preceding embodiments.

293. The pharmaceutical composition of embodiment 292, wherein the DNA molecule lacks a material portion of vector backbone, or does not comprise a non-human (e.g., bacterial) origin of replication.

294. The pharmaceutical composition of embodiment 292 or 293, wherein the DNA molecule is unencapsidated.

295. The pharmaceutical composition of any of embodiments 292-294, wherein the DNA molecule does not comprise a viral packaging signal.

296. The pharmaceutical composition of any of embodiments 292-295, wherein the DNA molecule does not comprise a viral ITR.

297. The pharmaceutical composition of any of embodiments 292-296, which is essentially free of viral proteins.

298. The pharmaceutical composition of any of embodiments 292-297, wherein the DNA molecule is comprised in a lipid nanoparticle (LNP).

299. The pharmaceutical composition of any of embodiments 292-298, further comprising an electroporation buffer.

300. The pharmaceutical composition of any of embodiments 292-299, further comprising a transfection reagent.

301. A method of expressing an effector in a target cell, the method comprising:

(i) introducing into a target cell the DNA molecule (e.g., dsDNA molecule), population, or composition (e.g., pharmaceutical composition) of any of the preceding embodiments, or a DNA molecule (e.g., dsDNA molecule) made by the method of any of the preceding embodiments, wherein the DNA molecule (e.g., the double stranded region or antisense strand of the DNA molecule) comprises an effector sequence encoding an effector; and

(ii) maintaining (e.g., incubating) the cell under conditions suitable for expressing the effector from the dsDNA molecule;

thereby expressing the effector in the target cell.

302. A method of delivering an effector to target cell, the method comprising:

introducing into a target cell the DNA molecule (e.g., dsDNA molecule), population, or composition (e.g., pharmaceutical composition) of any of the preceding embodiments, or a DNA molecule (e.g., dsDNA molecule) made by the method of any of the preceding embodiments, wherein the DNA molecule (e.g., the double stranded region or antisense strand of the DNA molecule) comprises an effector sequence encoding an effector;

thereby delivering the DNA molecule to the target cell.

303. A method of modulating (e.g., increasing or decreasing) a biological activity in a target cell, the method comprising:

(i) providing a target cell comprising the DNA molecule (e.g., dsDNA molecule), population, or composition (e.g., pharmaceutical composition) of any of the preceding embodiments, or a DNA

molecule (e.g., dsDNA molecule) made by the method of any of the preceding embodiments, wherein the DNA molecule (e.g., the double stranded region or antisense strand of the DNA molecule) comprises an effector sequence encoding an effector that modulates a biological activity in the target cell; and

(ii) maintaining (e.g., incubating) the cell under conditions suitable for expressing the effector from the DNA molecule;

thereby modulating the biological activity in the target cell.

304. The method of embodiment 303, wherein the effector increases the biological activity in the target cell.

305. The method of embodiment 303, wherein the effector decreases the biological activity in the target cell.

306. The method of any of embodiments 303-305, wherein the biological activity comprises cell growth, cell metabolism, cell signaling, cell movement, specialization, interactions, division, transport, homeostasis, osmosis, or diffusion.

307. The method of any of embodiments 301-306, wherein the cell is an animal cell, e.g., a mammalian cell, e.g., a human cell.

308. A method of treating a cell, tissue, or subject in need thereof, the method comprising:

administering to the cell, tissue, or subject the DNA molecule (e.g., dsDNA molecule), population, or composition (e.g., pharmaceutical composition) of any of the preceding embodiments, or a DNA molecule (e.g., dsDNA molecule) made by the method of any of the preceding embodiments, wherein the DNA molecule (e.g., the double stranded region or antisense strand of the DNA molecule) comprises an effector sequence encoding an effector;

thereby treating the cell, tissue, or subject.

309. The method of any of embodiments 301-308, which is performed *ex vivo* or *in vivo*.

Definitions

As used herein, a first nucleotide being "*adjacent*" to a second nucleotide means that there are no nucleotides situated between the first and second nucleotide. The two nucleotides may be connected by,

for instance, a phosphate linkage or a phosphorothioate linkage. When a plurality of nucleotides is described as being adjacent to a given nucleotide, it is understood that one nucleotide of the plurality (either the 5' most or the 3' most nucleotide of the plurality) is adjacent to the given nucleotide, and that each nucleotide of the plurality is adjacent to at least one other nucleotide in the plurality.

As used herein, the term "*annealing sequence*" refers to a part of a DNA molecule having a sequence that is complementary to (e.g., perfectly complementary to) another annealing sequence in the same DNA molecule. In some embodiments, a first annealing sequence hybridizes to a second annealing sequence to create a hairpin structure.

As used herein, the term "*looping sequence*" refers to a part of a DNA molecule having a sequence that lacks perfect complementarity (e.g., lacks substantial complementarity) to any other region of the DNA molecule, and wherein the looping sequence is situated between an annealing sequence and a second annealing sequence complementary to the first annealing sequence.

As used herein, the term "*antibody*" refers to a molecule that specifically binds to, or is immunologically reactive with, a particular antigen and includes at least the variable domain of a heavy chain, and normally includes at least the variable domains of a heavy chain and of a light chain of an immunoglobulin. Antibodies and antigen-binding fragments, variants, or derivatives thereof include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized, primatized, or chimeric antibodies, heteroconjugate antibodies (e.g., bi- tri- and quad-specific antibodies, diabodies, triabodies, and tetrabodies), single-domain antibodies (sdAb), epitope-binding fragments, e.g., Fab, Fab' and F(ab').sub.2, Fd, Fvs, single-chain Fvs (scFv), rlgG, single-chain antibodies, disulfide-linked Fvs (sdFv), nanobody, fragments including either a VL or VH domain, fragments produced by an Fab expression library, and anti-idiotypic (anti-Id) antibodies. Antibodies described herein can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. Moreover, unless otherwise indicated, the term "monoclonal antibody" (mAb) is meant to include both intact molecules as well as antibody fragments (such as, for example, Fab and F(ab')2 fragments) that are capable of specifically binding to a target protein. Fab and F(ab')2 fragments lack the Fc fragment of an intact antibody.

As used herein, the term "*backbone modification*" refers to a chemical modification to the backbone of a DNA molecule. In some embodiments, the backbone modification is a chemical modification to a phosphate group, e.g., phosphorothioate. In some embodiments, the backbone modification is a chemical modification to deoxyribose.

As used herein, the term "*carrier*" means a compound, composition, reagent, or molecule that facilitates or promotes the transport or delivery of a composition (e.g., a dsDNA molecule described herein) into a cell. For example, a carrier may be a partially or completely encapsulating agent.

As used herein, the term "*chemically modified nucleotide*" as used herein with respect to DNAs, refers to a nucleotide comprising one or more structural differences relative to the canonical deoxyribonucleotides (i.e., G, T, C, and A). A chemically modified nucleotide may have (relative to a canonical nucleotide) a chemically modified nucleobase, a chemically modified sugar, a chemically modified phosphodiester linkage, or a combination thereof. No particular process of making is implied; for instance, a chemically modified nucleotide can be produced directly by chemical synthesis, or by covalently modifying a canonical nucleotide.

As used herein, the term "*chemically modified cytosine nucleotide*," as used herein with respect to DNAs, refers to a chemically modified nucleotide wherein the nucleobase comprises a monocyclic 6-member ring in which carbon 4 is covalently bound to a nitrogen that is not one of the six members of the ring, wherein the nucleobase of the chemically modified cytosine nucleotide comprises one or more structural differences relative to canonical cytosine nucleobase. In some embodiments, the C-5 position of the nucleobase can have a substitution other than H. For example, the C-5 position of the nucleobase can have a substitution of –OH; -aldehyde; -carboxylic acid; -alkyl; $-(CH2)_mOR_3$, m=1-3 and R_3 = H or a sugar molecule; or –propargylamino. In some embodiments, the chemically modified cytosine nucleotide further comprises a chemical modification on the sugar or phosphodiester linkage. No particular process of making is implied.

As used herein, the term "*chemically modified uridine nucleotide*" as used herein with respect to DNAs, refers to a chemically modified nucleotide wherein the nucleobase comprises a monocyclic 6-member ring in which carbon 4 is covalently bound to an oxygen through a double bond, wherein the nucleobase of the chemically modified uridine nucleotide comprises one or more structural differences relative to canonical uracil and thymine nucleobases. In some embodiments, the C-5 position of the nucleobase can have a substitution other than H or a methyl group. For example, the C-5 position of the nucleobase can have a substitution of $-(CH_2)_mOH$, m=1-10; -halogen; $-(CH_2)_n$-CHO, n=0-10; $-(CH_2)_pCOOH$, p=0-10; -aminoallyl; or -propargylamino. In some embodiments, the chemically modified uridine nucleotide further comprises a chemical modification on the sugar or phosphodiester linkage. No particular process of making is implied.

As used herein, the term "*uridine nucleotide*" as used herein encompasses both canonical uridine nucleotides and chemically modified uridine nucleotides.

As used herein, the term "*closed end*" refers to a portion of a DNA molecule positioned at one end of a double-stranded region, in which all nucleotides within the portion of the DNA molecule are covalently attached to adjacent nucleotides on either side. A closed end may, in some embodiments, include a loop comprising one or more nucleotides that are not hybridized to another nucleotide. In some embodiments (involving "no-loop" ends), the DNA end form is simply a covalent bond between the 5'

most nucleotide of the sense strand and the 3' most nucleotide of the antisense strand, in the case of an upstream closed end, or the 3' most nucleotide of the sense strand and the 5' most nucleotide of the antisense strand, in the case of a downstream closed end. In some embodiments, a dsDNA molecule comprises a first closed end (e.g., upstream of a heterologous object sequence) and a second closed end (e.g., downstream of a heterologous object sequence).

As used herein, the term "*open end*" refers to a portion of a DNA molecule positioned at one end of a double-stranded region, in which at least one nucleotide (a "terminal nucleotide") is covalently attached to exactly one other nucleotide. In some embodiments, the terminal nucleotide comprises a free 5' phosphate. In some embodiments, the terminal nucleotide comprises a free 3' OH. In some embodiments, in a dsDNA molecule comprising a first DNA strand and a second DNA strand, the open end comprises a first terminal nucleotide on the first DNA strand and a second terminal nucleotide on the second DNA strand. In some embodiments, a dsDNA molecule comprises a first open end (e.g., upstream of a heterologous object sequence) and a second open end (e.g., downstream of a heterologous object sequence). In some embodiments, the open end comprises a blunt end, a sticky end, or a Y-adaptor.

As used herein, the term "*DNA*" refers to any compound and/or substance that comprises at least two (e.g., at least 10, at least 20, at least 50, at least 100) covalently linked deoxyribonucleotides. In some embodiments, the DNA is a single oligonucleotide chain, while in other embodiments, the DNA comprises a plurality of oligonucleotide chains, while in yet other embodiments the DNA is a portion of an oligonucleotide chain. In some embodiments, DNA is a compound and/or substance that is or can be incorporated into an oligonucleotide chain via a phosphodiester linkage. In some embodiments, the DNA comprises solely canonical nucleotides. In some embodiments, the DNA comprises one or more chemically modified nucleotides. In some embodiments, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% of the sugars of the DNA are deoxyribose sugars. In some embodiments, the DNA was prepared by one or more of: isolation from a natural source, enzymatic synthesis by polymerization based on a complementary template (*in vivo* or *in vitro*), reproduction in a recombinant cell or system, and chemical synthesis.

As used herein, the term "*DNA end form*" refers to a structure comprising a bond between nucleotides (e.g., wherein the DNA end form comprises DNA), that is situated at an end of a dsDNA molecule (e.g., a TDSC). In some embodiments, the DNA end form comprises a closed end, and in other embodiments, the DNA end form comprises an open end. In embodiments where the DNA end form is a closed end, the DNA end form is the bond or single stranded DNA region that connects the two strands of the dsDNA molecule (e.g., the DNA end form connects the sense strand to the antisense strand). In some embodiments, the DNA end form comprises a loop, a Y-adaptor, a blunt end, or a sticky end. The DNA

end form may comprise canonical nucleotides, chemically modified nucleotides, or a combination thereof. In some embodiments, the DNA end form comprises between 3-100 nucleotides. In some embodiments, the dsDNA molecule comprises a first DNA end form at a first end and a second DNA end form at a second end. In some embodiments, the first DNA end form and the second DNA end form of a dsDNA molecule are the same type. In some embodiments, the first DNA end form and the second DNA end form of a dsDNA molecule are different types.

As used herein, the term "*effector sequence*" refers to the part of a DNA molecule that exerts a function on a cell, either directly (wherein the effector sequence is a functional DNA sequence) or by encoding a functional RNA or protein. The encoded functional RNA or protein is referred to as the "*effector*".

As used herein, the term "*end adaptor*" refers to a DNA molecule that can be joined to a dsDNA molecule to produce a DNA end form. For instance, the end adaptor may comprise a hairpin, such that when the hairpin end adaptor is ligated to the dsDNA molecule, the loop of the hairpin becomes a DNA end form which is a closed end, while the stem of the hairpin becomes parts of the sense and antisense strands. As another example, an end adaptor may comprise two short DNA strands hybridized to each other, and may be used to produce an DNA end form which is an open end.

As used herein, the term "*exonuclease-resistant*", when used to describe a DNA, means that the DNA, if it comprises closed ends, is resistant to the exonuclease assay described in Example 2, and if it comprises an open end (e.g., two open ends), is resistant to the exonuclease assay described in Example 3.

As used herein, the term "*heterologous*", when used to describe a first element in reference to a second element means that the first element and second element do not exist in nature disposed as described. For example, a heterologous polypeptide, nucleic acid molecule, construct or sequence refers to (a) a polypeptide, nucleic acid molecule or portion of a polypeptide or nucleic acid molecule sequence that is not native to a cell in which it is expressed, (b) a polypeptide or nucleic acid molecule or portion of a polypeptide or nucleic acid molecule that has been altered or mutated relative to its native state, or (c) a polypeptide or nucleic acid molecule with an altered expression as compared to the native expression levels under similar conditions. For example, a heterologous regulatory sequence (e.g., promoter, enhancer) may be used to regulate expression of a gene or a nucleic acid molecule in a way that is different than the gene or a nucleic acid molecule is normally expressed in nature. In another example, a heterologous domain of a polypeptide or nucleic acid sequence (e.g., a DNA binding domain of a polypeptide or nucleic acid encoding a DNA binding domain of a polypeptide) may be disposed relative to other domains or may be a different sequence or from a different source, relative to other domains or portions of a polypeptide or its encoding nucleic acid. In certain embodiments, a heterologous nucleic acid molecule may exist in a native host cell genome, but may have an altered expression level or have a

different sequence or both. In other embodiments, heterologous nucleic acid molecules may not be endogenous to a host cell or host genome but instead may have been introduced into a host cell by transformation (e.g., transfection, electroporation), wherein the added molecule may integrate into the host genome or can exist as extra-chromosomal genetic material either transiently (e.g., mRNA) or semi-stably for more than one generation (e.g., episomal viral vector, plasmid or other self-replicating vector).

As used herein, the term "*heterologous functional sequence*" refers to a nucleic acid sequence that is heterologous to a nearby (e.g., adjacent) nucleic acid sequence and has one or more biological function. In some embodiments, the biological function comprises targeting to an organelle, e.g., nuclear targeting. In some embodiments, the heterologous functional sequence comprises a nuclear targeting sequence or a regulatory sequence.

As used herein, the terms "*increasing*" and "*decreasing*" refer to modulating resulting in, respectively, greater or lesser amounts, of function, expression, or activity of a metric relative to a reference. For example, subsequent to administration of a dsDNA molecule in a method described herein, the amount of the metric described herein (e.g., the level of gene expression, or a marker of innate immunity) may be increased or decreased in a subject by at least 5%, 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% or at least 98% relative to the amount of the marker prior to administration, or relative to administration of a control dsDNA molecule, such as a dsDNA molecule comprising chemically modified nucleotides compared to a control dsDNA molecule having only unmodified nucleotides. Generally, the metric is measured subsequent to administration at a time that the administration has had the recited effect, e.g., at least one day, one week, one month, 3 months, or 6 months, after a treatment regimen has begun.

As used herein, the term "*inosine nucleotide*," as used herein with respect to DNAs, refers to a nucleotide wherein the nucleobase is hypoxanthine.

As used herein the term "*linear*" in reference to a dsDNA molecule (e.g., TDSC) described herein, means a nucleic acid comprising two DNA strands or portions of strands which hybridize with each other (thereby forming a double stranded region), wherein the structure comprises two ends. An end may be a closed end or an open end. The two strands that hybridize with each other may be partially or completely complementary. In some embodiments, a linear dsDNA molecule consists of a single strand of DNA that is circular under denaturing conditions, wherein under physiological conditions a first portion of the strand hybridizes to a second portion of the strand (thereby forming a double stranded region), and the linear dsDNA molecule comprises a first closed end comprising a first loop and a second closed end comprising a second loop.

As used herein, the term "*loop*" refers to a nucleic acid sequence that is single stranded. A loop is connected at both ends by a double stranded region referred to as a "stem", to form a "stem-loop".

As used herein, the term "*maintenance sequence*" is a DNA sequence or motif that enables or facilitates retention of a DNA molecule in the nucleus through cell division. A maintenance sequence typically enables replication and/or transcription of DNA in the nucleus by interacting with proteins that facilitate chromatin looping. An example of a maintenance sequence is a scaffold/matrix attached region (S/MAR element).

As used herein, a "*nuclear targeting sequence*" is a DNA sequence that enables or facilitates DNA entry into a target cell nucleus.

As used herein, a "*pharmaceutical composition*" or "*pharmaceutical preparation*" is a composition or preparation which is indicated for animal, e.g., human or veterinary pharmaceutical use, for example, non-human animal or human prophylactic or therapeutic use. A pharmaceutical preparation comprises an active agent having a biological effect on a cell or tissue of a subject, e.g., having pharmacological activity or an effect in the mitigation, treatment, or prevention of disease, in combination with a pharmaceutically acceptable excipient or diluent. A pharmaceutical composition also means a finished dosage form or formulation of a prophylactic or therapeutic composition.

As used herein, the terms "*peptide*," "*polypeptide*," and "*protein*" are used interchangeably and refer to a compound comprising amino acid residues covalently linked by peptide bonds, or by means other than peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or by means other than peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. In some embodiments, a polypeptide comprises a non-canonical amino acid residue.

As used herein, the term "*protelomerase sequence*" refers to a nucleotide sequence capable of being generated by a protelomerase that joins a first protelomerase recognition sequence (PRS) to a second PRS. In some embodiments, the protelomerase sequence was produced by a process involving protelomerase, and in other embodiments the protelomerase sequence was produced by a process that does not involve protelomerase (e.g., by solid phase synthesis).

As used herein, a "*sense strand*" of a dsDNA is a strand which has the same sequence as an mRNA or pre-mRNA which encodes for a functional protein, and does not serve as a template for transcription of the protein. An "*antisense strand*" of a dsDNA is a strand that has a sequence

complementary to an mRNA or pre-mRNA which encodes for a functional protein and/or can serve as a template for transcription.

As used herein, the term "*double stranded DNA molecule*" or dsDNA molecule means a DNA composition comprising two complementary chains of deoxyribonucleotides that base pair to each other. The two complementary strands may have perfect complementarity or may have one or more mismatches, e.g., forming bulges. Either of the two strands may, in some embodiments, have paired regions of self-complementarity that form intramolecular/intrastrand double stranded motifs in a folded configuration, for example, may form hairpin loops, junctions, bulges or internal loops. In some embodiments, the dsDNA molecule is circular or linear. In some embodiments, the dsDNA molecule comprises one or two closed ends. In some embodiments (e.g., in a dsDNA molecule with closed ends) the two complementary chains of deoxyribonucleotides are covalently linked. In some embodiments, the dsDNA molecule is a TDSC.

As used herein, the term "*therapeutic double stranded construct*" ("*TDSC*") refers to a linear construct comprising DNA, wherein the construct is at least partially double stranded. A TDSC does not comprise a plasmid backbone sequence (e.g., does not comprise a bacterial origin of replication). A TDSC does not comprise a viral capsid or a viral envelope. In some embodiments, the TDSC comprises a closed end or an open end (e.g., a blunt end or a sticky end). In some embodiments, the TDSC is suitable for administration to a human subject.

As used herein, the term "*terminal nucleotide*" refers to a nucleotide that is covalently attached to exactly one other nucleotide. In some embodiments, the terminal nucleotide comprises a free 5' phosphate. In some embodiments, the terminal nucleotide comprises a free 3' OH.

As used herein, "*treatment*" and "*treating*" refer to the medical management of a subject with the intent to improve, ameliorate, stabilize (i.e., not worsen), prevent or cure a disease, pathological condition, or disorder. This term includes active treatment (treatment directed to improve the disease, pathological condition, or disorder), causal treatment (treatment directed to the cause of the associated disease, pathological condition, or disorder), palliative treatment (treatment designed for the relief of symptoms), preventative treatment (treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder); and supportive treatment (treatment employed to supplement another therapy). Treatment also includes diminishment of the extent of the disease or condition; preventing spread of the disease or condition; delay or slowing the progress of the disease or condition; amelioration or palliation of the disease or condition; and remission (whether partial or total), whether detectable or undetectable. "Ameliorating" or "palliating" a disease or condition means that the extent and/or undesirable clinical manifestations of the disease, disorder, or condition are lessened and/or time course of the progression is slowed or lengthened, as compared to the extent or time

course in the absence of treatment. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder, as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

As used herein, the term "*Y-adaptor*" refers to a nucleic acid structure comprising a first nucleic acid region and a second nucleic acid region which are complementary (e.g., perfectly complementary) to each other; the first and second regions may hybridize to form a double stranded region. The first nucleic acid region is covalently linked to a third nucleic acid region, and the second nucleic acid region is covalently linked to a fourth nucleic acid region, and the third and fourth nucleic acid regions are not substantially complementary to each other; the third and fourth regions may be single stranded. The first nucleic acid region is 3' of the third nucleic acid region and the second nucleic acid region is 5' of the fourth nucleic acid region. As a result, the third and fourth regions may be situated on the same side of the double stranded regions. The Y-adaptor may be part of a dsDNA molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1B illustrate a looped-end DNA (leDNA) in **FIG. 1A**, and hemi-modified dsDNA in **FIG. 1B**. In more detail, the leDNA of **FIG. 1A** includes, in an upstream to downstream direction, an upstream DNA end form which is a loop, a short double stranded region comprising phosphorothioate linkages (shown as Xs) on the sense strand and antisense strand, a single stranded antisense region, another double stranded region comprising phosphorothioate linkages on the sense strand and antisense strand, and a downstream DNA end form which is a loop. In some embodiments, the antisense strand is substantially free of chemically modified nucleobases. Although the leDNA shown in this figure has phosphorothioate linkages in four regions, in other embodiments, one or more of those regions may lack phosphorothioate. In some embodiments, the leDNA is suitable for administration to a subject, to deliver an effector encoded by the antisense strand. In some embodiments, the leDNA is a manufacturing intermediate used to make hemi-modified DNA, by using a polymerase to produce a sense strand having chemically modified nucleobases. In particular, one of the looped ends can serve as a primer for synthesis of the sense strand. In the hemi-modified construct shown in **FIG. 1B**, the top strand is the sense strand and comprises chemically modified nucleobases, and the bottom line is the antisense strand and is substantially free of chemically modified nucleobases. Without wishing to be bound by theory, in some embodiments the hemi-modified DNA achieves both immune stealth and transcription by virtue of the chemically modified nucleobases in the sense strand leading to immune stealth, and the canonical nucleobases in the antisense strand leading to effective transcription.

FIGS. 2A-2B are a series of diagrams showing exemplary covalently-closed DNA end forms that can be included in a dsDNA molecule, e.g., therapeutic double-stranded construct (TDSC), as described herein (e.g., at one or both ends of the dsDNA molecule). Shown in (A) are exemplary dsDNA molecules, e.g., TDSCs, comprising no loop ends (e.g., protelomerase sequences), inverted terminal repeats (ITRs), or loops at the ends, which can be made up of unmodified nucleotides (white symbols) or may comprise chemically modified nucleotides (gray symbols). (A hairpin configuration is another way to describe a construct in which the closed end is a loop, and the ends of the sense and antisense strands form a stem of the hairpin.) Chemically modified nucleotides can include nucleotides modified, for example, in the backbone, sugar, or base, or nucleotides that are conjugated to a peptide or protein. In some instances, both of the DNA strands are unmodified. In some instances, both of the DNA strands are chemically modified. In some instances, the antisense strand is chemically modified. In some instances, the sense strand is chemically modified. The solid-line box indicates a dsDNA molecule that is covalently closed with hairpins at the ends, e.g., a linear, covalently closed dsDNA molecule with end forms comprising phosphorothioate modifications. The dashed-line box indicates a dsDNA molecule that is covalently closed with no loop ends, e.g., a linear, covalently closed dsDNA molecule with TelN end forms.

FIG. 3 is a series of diagrams showing double-stranded DNA constructs, including exemplary dsDNA molecules, e.g., TDSCs, comprising exemplary DNA end forms (e.g., at one or both ends) that are not covalently closed. Such exemplary dsDNA molecules, e.g., TDSCs, can comprise a Y end (e.g., a Y adaptor, e.g., as described herein). The DNA end forms can, in some instances, be made up of unmodified nucleotides (white symbols). In some instances, the DNA end forms comprise chemically modified nucleotides (gray symbols). Chemically modified nucleotides can include nucleotides modified, for example, in the backbone, sugar, or base, or nucleotides that are conjugated to a peptide or protein. In some instances, both of the DNA strands are unmodified. In some instances, both of the DNA strands are chemically modified. In some instances, the antisense strand is chemically modified. In some instances, the sense strand is chemically modified. Also shown in the upper right is an exemplary DNA construct lacking DNA end forms or chemical modifications (i.e., an unmodified double-stranded DNA molecule).

FIG. 4 is a diagram depicting production of covalently closed linear dsDNA molecules with end forms comprising phosphorothioate modifications. **FIG. 4** discloses SEQ ID NOS 87-88, respectively, in order of appearance.

FIG. 5 is a diagram depicting production of covalently closed linear dsDNA molecules with TelN end forms. **FIG. 5** discloses SEQ ID NOS 89-90, respectively, in order of appearance.

FIG. 6 is a diagram depicting circular dsDNA molecules with or without chemical modifications.

FIG. 7 is a diagram depicting an exemplary method of production of circular dsDNA molecules. A linear dsDNA molecule may be contacted with a restriction enzyme (e.g., KpnI) that creates compatible sticky ends which may then be joined to each other by ligation, producing a circular dsDNA. **FIG. 7** discloses SEQ ID NOS 91-92, respectively, in order of appearance.

FIG. 8 is a pair of graphs showing the immune steath characteristics of various hemi-modified dsDNAs and looped-end DNA (leDNA), based on IL6 levels (left panel) or CXCL10 levels (right panel). In **FIGs. 8** and **9**, P6 unmod indicates a closed end dsDNA with phosphorothioate but lacking modified nucleobases, MC stands for method control, and cdsDNA indicates circular double stranded DNA.

FIG. 9 is a pair of graphs showing the function (here, ability to produce a protein, in this case mCherry) of various hemi-modified dsDNAs and looped-end DNA (leDNA), measured as total fluorescence over background (left panel) or % mCherry+ cells (right panel).

DETAILED DESCRIPTION

This disclosure relates to compositions and methods for providing an effector sequence to a cell, tissue or subject, e.g., in vivo or in vitro. The effector may be a DNA sequence. In some embodiments, the effector sequence encodes a polypeptide, e.g., a therapeutic protein. In some embodiments, the effector sequence encodes an RNA, e.g., a regulatory RNA or an mRNA.

Chemically modified (e.g., hemi-modified) DNA molecules

The dsDNA molecules described herein may have chemical modifications of the nucleobases, sugars, and/or the phosphate backbone (e.g., as shown in **FIGS. 2A-3**). While not wishing to be bound by theory, such modifications can be useful for protecting a DNA from degradation (e.g., from exonucleases) or from the immune system of a host tissue or subject. In general, a chemically modified nucleotide has the same base-pairing specificity as the unmodified nucleotide, e.g., a chemically modified adenine "A" can base-pair with thymine "T". In certain embodiments, chemical modifications (e.g., one or more modifications) are present in each of the sugar and the internucleoside linkage.

Note that the phosphate group (or chemically modified analog thereof) of a nucleotide is considered to be the phosphate 5' of the sugar of that nucleotide. Therefore, in a configuration such as phosphate - nucleoside 1 - phosphorothioate - nucleoside 2, the phosphorothioate is considered to be part of the same nucleotide as nucleoside 2, while the phosphate is considered to be part of the same nucleotide as nucleoside 1. Therefore, when nucleotide X is positioned at the 5' end of the sense strand and adjacent to an upstream DNA end form, the phosphate (or chemically modified analog thereof) of nucleotide X, which is 5' of the sugar of nucleotide X, is considered to be part of the sense strand rather than part of the upstream DNA end form. Similarly, when nucleotide Y is positioned at the 5' end of the

upstream DNA end form which is a loop, the phosphate (or chemically modified analog thereof) of nucleotide Y, which is 5' of the sugar of nucleotide Y is considered to be part of the upstream DNA end form rather than part of the antisense strand. Examples of chemical modifications to DNA useful in the methods described herein include, e.g., phosphorothioate; or S and R phsophorothioate linkages; . See, e.g., *Pu et al. 2020. An in-vitro DNA phosphorothioate modification reaction. Mol Microbiol. 113: 452– 463; Zheng & Sheng. 2021.*

In some embodiments, a dsDNA molecule as described herein may comprise a phosphorothioate-modified nucleotide. In some embodiments, a DNA end form (e.g., an exonuclease-resistant DNA end form) as described herein may comprise a phosphorothioate-modified nucleotide. In some embodiments, the dsDNA molecules described herein may include S and R phosphorothioate modified nucleotide linkages. In one embodiment, the phosphorothioate linkages are made according to Iwamoto et al, 2017, Nature Biotechnology, Volume 35:845-851. Briefly, monomers of nucleoside 3'-oxazaphospholidine derivates undergo stereocontrolled oligonucleotide synthesis with iterative capping and sulfurization to create stereocontrolled phosphorothioate linkages. The final sample is analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) and Ultraperformance liquid chromatography mass spectrometry (UPLC/MS) to determine stereochemistry of the modification. Nucleic acids containing phosphorothioate linkages are also commercially available.

In embodiments, a dsDNA molecule described herein, or one strand (e.g., the sense strand or the antisense strand) of the dsDNA molecule, comprises between 1%-100% chemically modified nucleotides, between 1%-90% chemically modified nucleotides, between 1%-80% chemically modified nucleotides, between 1%-70% chemically modified nucleotides, between 1%-60% chemically modified nucleotides, between 1%-50% chemically modified nucleotides, between 1%-40% chemically modified nucleotides, between 1%-30% chemically modified nucleotides, between 1%-20% chemically modified nucleotides, between 1%-15% chemically modified nucleotides, between 1%-10% chemically modified nucleotides, between 20%-90% chemically modified nucleotides, between 20%-80% chemically modified nucleotides. In embodiments, a dsDNA molecule described herein, or one strand (e.g., the sense strand or the antisense strand) of the dsDNA molecule, comprises at least 1% chemically modified nucleotides, at least 5% chemically modified nucleotides; at least 10% chemically modified nucleotides; at least 15% chemically modified nucleotides; at least 20% chemically modified nucleotides; at least 25% chemically modified nucleotides; at least 30% chemically modified nucleotides; at least 40% chemically modified nucleotides; at least 50% chemically modified nucleotides; at least 60% chemically modified nucleotides; at least 70% chemically modified nucleotides; at least 80% chemically modified nucleotides; at least 85% chemically modified nucleotides; at least 90% chemically modified nucleotides; at least 92% chemically modified

nucleotides; at least 95% chemically modified nucleotides; or at least 97% chemically modified nucleotides..

In embodiments, chemically modified nucleotides, e.g., modifications described herein, can be introduced in the dsDNA molecules described herein throughout the entire sequence; within an element of a sequence, e.g., an element described herein; at a 5'- or 3'- end; and/or between the last 10, 8, 6, 5, 4, 3, or 2 nucleotides at the 5'- or 3'- end.

In some embodiments, a dsDNA molecule as described herein comprises chemically modified nucleobases on only one strand (e.g., as shown in **FIG. 1B or 2A**). Such asymmetrically modified dsDNA molecules may be called "hemi-modified." Note that, in some embodiments, the hemi-modified DNA may be completely free of chemically modified nucleotides on the antisense strand, and in other embodiments, the hemi-modified DNA may comprise a few chemical modificaitons (such as backbone modifications) on the antisense strand. In some embodiments, a dsDNA molecule as described herein comprises chemically modified nucleotides on the antisense strand. In some embodiments, a dsDNA molecule as described herein comprises chemically modified nucleotides on the sense strand.

In embodiments, a dsDNA molecule as described herein comprises one or more DNA end forms (e.g., exonuclease-resistant DNA end forms, e.g., covalently closed DNA end forms or non-covalently closed DNA end forms, e.g., as described herein) that each comprise one or more chemically-modified nucleotides (e.g., on one or both strands of the DNA end form). In embodiments, a dsDNA molecule comprises a double-stranded region flanked by non-covalently closed exonuclease-resistant DNA end forms comprising chemically-modified nucleotides, e.g., as described herein (e.g., in **FIG. 3**).

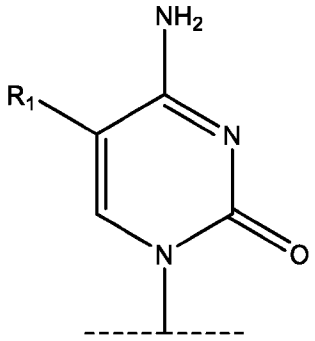
In embodiments, a dsDNA molecule described herein has one or more chemical modification that disrupts the ability of a portion of the dsDNA molecule to form a double stranded structure, e.g., a dsDNA molecule described herein has one or more chemical modification on a nucleotide present in a region having intramolecular complementarity. In embodiments, a dsDNA molecule described herein has one or more chemical modification that disrupts base pairing of regions of intramolecular complementarity relative to the unmodified sequence of the dsDNA molecule. In some embodiments the chemically modified nucleotides used herein have a reduced propensity to base-pair with chemically modified nucleotides compared to the propensity of unmodified nucleotides to base pair with unmodified nucleotides. In some embodiments the chemically modified nucleotides used herein have an increased propensity to base-pair with unmodified nucleotides compared to modified nucleotides.

In some embodiments, a chemically modified dsDNA molecule described herein exhibits decreased recognition by DNA sensors in a host tissue or subject compared to an unmodified dsDNA molecule of the same sequence, e.g., at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or more decreased recognition by DNA

sensors in a host tissue or subject compared to an unmodified dsDNA molecule of the same sequence. In some embodiments, a chemically modified dsDNA molecule described herein exhibits decreased degradation by DNA nucleases compared to an unmodified dsDNA molecule of the same sequence, e.g., at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or more decreased degradation by DNA nucleases in a host tissue or subject compared to an unmodified dsDNA molecule. In some embodiments, a chemically modified dsDNA molecule described herein shows decreased activation of the innate immune system in a target/host tissue or subject compared to an unmodified dsDNA molecule of the same sequence, e.g., at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or more decreased activation of the innate immune system in a target/host tissue or subject compared to an unmodified dsDNA molecule of the same sequence.

In some embodiments, a dsDNA molecule comprising chemically modified nucleotides described herein exhibits any of the following properties in a target/host tissue or subject compared to dsDNA of the same sequence that does not comprise chemically modified nucleotides (unmodified dsDNA): increased integration of exogenous construct in genome of target cell; increased retention in a target cell through replication; reduced secondary or tertiary structure formation; reduced interaction with innate immune sensors; reduced interaction with nucleases; enhanced stability; enhanced longevity; reduced toxicity; enhanced delivery; increased expression; increased transport across membranes; or increased binding to DNA binding moieties such as nuclear DNA binding proteins, transcription factors, chaperones, or DNA polymerases. In embodiments, any of the above listed properties is modulated at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95% in a target/host tissue or subject compared to an unmodified dsDNA of the same sequence.

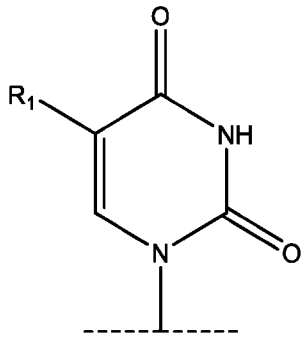
In some embodiments, a dsDNA molecule described herein comprises a chemically modified cytosine nucleotide. In some embodiments, the chemically modified cytosine nucleotide comprises a substitution other than hydrogen at the carbon 5 (C-5) position of the nucleobase. In some embodiments, the chemically modified cytosine nucleotide comprises the structure of Formula I:



Formula I,

wherein R_1 is selected from the group consisting of -OH; -aldehyde; -carboxylic acid; -alkyl; $-(CH_2)_mOR_2$, m=1-3 and R_2 = H or a sugar molecule; and –propargylamino. In some embodiments, R_1 is selected from the group consisting of -OH; -CHO; -COOH; -alkyl; $-(CH_2)_mOR_2$, m=1-3 and R_2 = H or a sugar molecule; and -propargylamino, wherein the alkyl group includes one to six carbons. In some embodiments, R_1 is selected from the group consisting of -OH; -CHO; -COOH; $-CH_2OR_3$, R_3 = H or glucose; -methyl; and -propargylamino. In some embodiments, the chemically modified cytosine nucleotide comprises 5-formylcytosine, 5-hydroxycytosine, 5-carboxycytosine, 5-propargylaminocytosine, 5-methylcytosine, 5-hydroxymethylcytosine, or glucosyl-5-hydroxymethylcytosine. Chemically modified cytosine nucleotides are further described in International Application WO/2024/173836, which is herein incorporated by reference in its entirety.

In some embodiments, a dsDNA molecule described herein comprises a chemically modified uridine nucleotide. In some embodiments, the chemically modified uridine nucleotide comprises a substitution other than hydrogen or a methyl group at the carbon 5 (C-5) position of the nucleobase. In some embodiments, the chemically modified uridine nucleotide comprises the structure of Formula II:

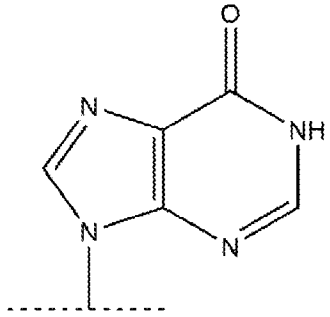


Formula II,

wherein R_1 is selected from the group consisting of $-(CH_2)_mOH$, m=1-10; -halogen; $-(CH_2)_n$-CHO, n=0-10; $-(CH_2)_pCOOH$, p=0-10; -aminoallyl; -S-(C1-C6)alkyl; and -propargylamino. In some embodiments, R_1 is selected from the group consisting of $-(CH_2)_mOH$, m=1-6; -halogen; $-(CH_2)_n$-CHO, n=0-6; $-(CH_2)_pCOOH$, p=0-6; -aminoallyl; -S-(C1-C3)alkyl; and -propargylamino. In some embodiments, R_1 is selected from the group consisting of $-(CH_2)OH$; -I; -Br; -CHO; -COOH; -aminoallyl; -S-methyl; and -propargylamino. In some embodiments, the chemically modified uridine nucleotide comprises 5-hydroxymethyluridine, 5-aminoallyluridine, 5-bromouridine, 5-iodouridine, 5-propargylaminouridine, 5-formyluridine, 5-carboxyuridine, or 5-methylthiouridine. Chemically modified uridine nucleotides are further described in International Application WO2024/173828, which is herein incorporated by reference in its entirety.

A DNA molecule, e.g., a dsDNA molecule, described herein may comprise a chemically modified nucleotide, such as an inosine nucleotide. In some embodiments, a guanosine nucleotide in a position of a DNA molecule can be replaced with an inosine nucleotide. In some embodiments, a deoxyguanosine triphosphate (dGTP) can be replaced with a deoxyinosine triphosphate in a reaction, e.g., a polymerase chain reaction. Typically, an inosine on one strand will pair with a cytosine on the opposite strand. Without wishing to be bound by theory, in some embodiments the inosine nucleotide described herein increases the "stealth" of a dsDNA molecule to an immune response, while supporting expression of a gene on the dsDNA molecule.

A nucleobase comprised by an inosine nucleotide is shown below as Formula III.



Formula III

In some embodiments, the dsDNA molecule comprises at least one chemical modification. In some embodiments, a dsDNA molecule as described herein comprises chemically modified nucleotides on only one strand. In some embodiments, a dsDNA molecule as described herein comprises chemically modified nucleotides on the antisense strand. In some embodiments, a dsDNA molecule as described herein comprises chemically modified nucleotides on the sense strand. In embodiments, a dsDNA molecule described herein, or one strand (e.g., the sense strand or the antisense strand) of the dsDNA molecule, comprises inosine at between 0%-100%, 10%-75%, 20%-75%, 30%-75%, 40%-75%, 50%-75%, 60%-75%, or 10%-50% of guanosine and inosine positions.

Other chemically modified (e.g., substantially or fully modified) DNA molecules

While many of the DNA molecules described herein are hemi-modified, other configurations are also contemplated. For instance, in some embodiments, a dsDNA comprises chemically modified nucleotides on both strands. In some embodiments, each of the sense strand and the antisense strand comprises some chemically modified nucleotides and other nucleotides that are not chemically modified.

In some embodiments, the present disclosure provides a DNA molecule (e.g., a dsDNA molecule) comprising two or more chemically modified nucleobases described herein. For instance, in some embodiments, the first chemically modified nucleobase is an inosine and the second chemically modified

nucleobase is a uridine nucleotide (e.g., as described herein) or a chemically modified cytosine nucleotide (e.g., as described herein). In some embodiments, the first chemically modified nucleobase comprises a canonical uridine nucleobase and the second chemically modified nucleotide is an inosine nucleotide or a chemically modified cytosine nucleotide (e.g., as described herein). In some embodiments, the first chemically modified nucleotide is a 5-methylthiouridine nucleotide and the second chemically modified nucleotide is an inosine nucleotide, a chemically modified cytosine nucleotide (e.g., as described herein), or a different uridine nucleotide (e.g., as described herein). In some embodiments, the first chemically modified nucleotide is a 5-azidomethyluridine nucleotide, and the second chemically modified nucleotide is an inosine nucleotide, a chemically modified cytosine nucleotide (e.g., as described herein), or a different uridine nucleotide (e.g., as described herein).

In some embodiments, a dsDNA molecule as described herein comprises chemically modified nucleotides on both strands (e.g., as shown in **FIGS. 2A and 3**). In certain embodiments, both strands comprise chemical modifications at the same positions (e.g., chemically modified nucleotides on one strand are base-paired with chemically modified nucleotides on the opposite strand, and/or non-chemically modified nucleotides on one strand are base-paired with non-chemically modified nucleotides on the opposite strand). In embodiments, the entirety of both strands are composed of chemically modified nucleotides. In other embodiments, the two strands of a dsDNA molecule as described herein comprise different chemical modification patterns (e.g., one or more chemically modified nucleotides on one strand are base-paired with non-chemically modified nucleotides on the other strand). In embodiments, a dsDNA molecule as described herein comprises one or more double-stranded regions in which both strands are chemically modified, and/or one or more double-stranded regions in which neither strand is chemically modified. In embodiments, a dsDNA molecule as described herein comprises one or more double-stranded regions in which one strand is chemically modified and the other is not.

Elements of DNA constructs

The dsDNA molecules or nucleic acids comprising dsDNA described herein contain elements sufficient to deliver an effector sequence to a target cell, tissue or subject. In some embodiments, the effector sequence is a DNA sequence. In some embodiments, the dsDNA molecule drives expression of an effector, e.g., comprises a promoter and a sequence encoding an RNA or a polypeptide, e.g., a therapeutic RNA or polypeptide. In some embodiments, the DNA constructs described herein further contain one or both of: a nuclear targeting sequence and a maintenance sequence. While some of the embodiments herein refer to a TDSC, it is understood that as applicable an embodiment that refers to a TDSC may also apply to a nucleic acid comprising dsDNA.

Exonuclease-resistant DNA end forms

The dsDNA molecules described herein comprise a DNA end form at each end of the double-stranded DNA molecule. The DNA end forms described herein can, in some instances, comprise a closed end, wherein every nucleotide of the DNA end form is covalently attached to two other nucleotides of the DNA end form. In other instances, the DNA end forms described herein comprise an open end comprising at least one nucleotide that is only covalently attached to one other nucleotide of the DNA end form. The DNA end forms are generally exonuclease resistant. In some instances, a DNA end form comprising a closed end (e.g., a covalently closed end) is resistant to the exonuclease assay described in Example 2. In some instances, a DNA end form comprising an open end (e.g., such as a Y adaptor, blunt end, or sticky end, e.g., as described herein) is resistant to the exonuclease assay described in Example 3. In some embodiments, every nucleotide of the closed end is hybridized to another nucleotide.

Loops

In some embodiments, a DNA end form comprises a loop.

The DNA end form comprising a loop may be produced, e.g., by ligating an end adaptor which is a hairpin to a dsDNA molecule. A hairpin generally comprises a single-stranded loop region covalently attached at both the 5' and 3' ends to a double-stranded stalk region. In certain embodiments, the single-stranded loop region comprises one or more nucleotides (e.g., 1-2, 2-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, or 35-40 nucleotides) that are not hybridized to another nucleotide. Exemplary loop structures, and exemplary dsDNA molecules comprising loops, are shown in **FIG. 2A**.

In certain embodiments, the single-stranded loop region comprises one or more functional elements (e.g., a nuclear import sequence (e.g., a CT3 ssDNA sequence), or a regulatory sequence. In embodiments, a functional element comprised in the single-stranded loop region is heterologous to one or more other elements of the DNA end form and/or a dsDNA molecule comprising the DNA end form. In certain embodiments, the single-stranded loop region of the end form is less than about 5, less than 10, less than 15, less than 20, less than 25, less than 26, less than 27, less than 28, less than 29, or less than 30 nucleotides in length. In certain embodiments, the single-stranded loop region of the end form is less than about 5, less than about 10, less than about 15, less than about 20, less than about 25, less than about 26, less than about 27, less than about 28, less than about 29, or less than about 30 nucleotides in length.

In embodiments, the loop is comprised in a dsDNA molecule having a doggybone conformation. In embodiments, the dsDNA molecule comprises a protelomerase sequence (e.g., as described herein). In embodiments, the protelomerase sequence is produced by TelN protelomerase, ResT protelomerase, Tel PY54 protelomerase, or TelK protelomerase digestion. In embodiments, the protelomerase sequence is less than about 15, less than about 20, less than about 25, less than about 26, less than about 27, less

than about 28, less than about 29, or less than about 30 nucleotides in length. In embodiments, the protelomerase sequences are between about 28 (e.g., 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides and about 56 (e.g., 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60) nucleotides in length. In embodiments, the protelomerase sequences are greater than about 56 (e.g., greater than 50, greater than 51, greater than 52, greater than 53, greater than 54, greater than 55, greater than 56, greater than 57, greater than 58, greater than 59, greater than 60, greater than 65, greater than 70, greater than 75, greater than 80, greater than 90, or greater than 100) nucleotides in length.

A loop can be attached to one or both ends of a double-stranded DNA molecule, for example, by ligation of a hairpin (e.g., as described herein). In some embodiments, a dsDNA molecule as described herein comprises, at one or both ends, a loop. In some embodiments, the upstream exonuclease-resistant DNA end form of a dsDNA molecule as described herein comprises a loop. In some embodiments, the downstream exonuclease-resistant DNA end form of a dsDNA molecule as described herein comprises a loop.

In certain embodiments, a loop comprises one or more unmodified nucleotides. In embodiments, a loop consists entirely of unmodified nucleotides. In certain embodiments, a loop comprises one or more chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, a loop consists entirely of chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein).

In embodiments, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, or at least 99% of the nucleotides in the single-stranded loop region are chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein).

In some embodiments, a DNA molecule described herein is produced by a ligating a DNA hairpin loop to a double stranded region. In certain embodiments, the single-stranded loop region of a DNA hairpin loop comprises one or more chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, or at least 99% of the nucleotides in the single-stranded loop region are chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, the single-stranded loop region of a DNA hairpin loop consists entirely of chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In certain embodiments, the single-stranded loop region of a DNA hairpin loop comprises one or more unmodified nucleotides. In embodiments, the single-stranded loop region of a DNA hairpin loop consists entirely of unmodified nucleotides.

In certain embodiments, the double-stranded stalk region of a DNA hairpin loop comprises one or more unmodified nucleotides. In embodiments, the double-stranded stalk region of a DNA hairpin loop consists entirely of unmodified nucleotides. In certain embodiments, the double-stranded stalk region of a DNA hairpin loop comprises one or more chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, or at least 99% of the nucleotides in the double-stranded stalk region are modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, the double-stranded stalk region of a DNA hairpin loop consists entirely of chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein).

In embodiments, the single-stranded loop region of a DNA hairpin loop comprises one or more chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein) and the double-stranded stalk region comprises one or more unmodified nucleotides. In embodiments, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, or at least 99% of the nucleotides in the single-stranded loop region are chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, the single-stranded loop region of a DNA hairpin loop consists entirely of chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein) and the double-stranded stalk region consists entirely of unmodified nucleotides.

Y-Adaptors

In some embodiments, an exonuclease-resistant DNA end form as described herein comprises a Y-adaptor. As described herein, a Y-adaptor generally comprises a pair of single-stranded DNA regions, each attached at one end to a strand of a double-stranded DNA region, thereby forming a "Y" shape (wherein the base of the "Y" represents the double-stranded DNA region, and each of the upper prongs of the "Y" represents the two single-stranded DNA region). Exemplary Y-adaptor structures and exemplary dsDNA molecules comprising Y-adaptors are shown in **FIG. 3**.

In some embodiments, a Y-adaptor is produced by attaching a hairpin loop comprising a single-stranded region comprising a cleavable moiety to the end of a double-stranded DNA region (e.g., via ligation). The cleavable moiety can then be cleaved to produce the two single-stranded DNA regions of the Y-adaptor.

In certain embodiments, a single-stranded DNA region (e.g., one or both single-stranded DNA regions) of a Y-adaptor comprises one or more chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, at least 50%, at least 60%, at least 70%,

at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, or at least 99% of the nucleotides in the single-stranded DNA region are chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, a single-stranded DNA region (e.g., one or both single-stranded DNA regions) of a Y-adaptor consists entirely of chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In certain embodiments, a single-stranded DNA region (e.g., one or both single-stranded DNA regions) of a Y-adaptor comprises one or more unmodified nucleotides.

In embodiments, a single-stranded DNA region (e.g., one or both single-stranded DNA regions) of a Y-adaptor comprises one or more chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein) and a double-stranded DNA region of the Y-adaptor comprises one or more unmodified nucleotides. In embodiments, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, or at least 99% of the nucleotides in the single-stranded DNA region or regions are chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, a single-stranded DNA region (e.g., one or both single-stranded DNA regions) of a Y-adaptor consists entirely of chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein) and the double-stranded DNA region of the Y-adaptor consists entirely of unmodified nucleotides.

No Loop Closed DNA End Forms

In some embodiments, a dsDNA molecule as described herein comprises an exonuclease-resistant DNA end form that is covalently closed but does not include a single stranded loop. For example, in certain embodiments, every nucleotide of a covalently-closed DNA molecule is complementary to another nucleotide. Accordingly, the DNA end form may be a bond between the endmost nucleotide of the sense strand and the nucleotide of the antisense strand which base pairs with the nucleotide of the sense strand. In embodiments, a covalently-closed DNA end form as described herein can be attached to one end of a dsDNA molecule as described herein, e.g., by ligation.

Open DNA End Forms

In some embodiments, a dsDNA molecule as described herein comprises an exonuclease-resistant DNA end form that is not covalently closed. In certain embodiments, the DNA end form comprises a blunt end (e.g., a blunt end comprising one or more chemical modifications as described herein) or a sticky end (e.g., a sticky end comprising one or more chemical modifications as described herein).

In certain embodiments, the open DNA end form is produced by nuclease digestion of a covalently closed DNA end form, such as a loop. In embodiments, the dsDNA region near the loop

comprises a double-stranded stalk region comprising a cleavable moiety on each strand, and the DNA hairpin is then contacted with an enzyme capable of cleaving the cleavable moieties. In embodiments, this results in the formation of a sticky end comprising an overhang. In embodiments, the overhang is digested with an enzyme (e.g., a single-stranded specific nuclease, e.g., a Mung Bean nuclease) to form a blunt end.

In certain embodiments, a DNA end form comprising a blunt end comprises one or more chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, or at least 99% of the nucleotides in the DNA end form comprising a blunt end are chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, the DNA end form comprising a blunt end consists entirely of chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, the terminal base pair of the DNA end form comprising a blunt end comprises a chemically modified nucleotide (e.g., one or both nucleotides of the base pair are chemically modified), e.g., a phosphorothioate-modified nucleotide, e.g., as described herein. In embodiments, a plurality of base pairs (e.g., 2, 3, 4, 5, or 6 base pairs) at the terminal end of the DNA end form comprise chemically modified nucleotides (e.g., one or both nucleotides of the base pair are chemically modified), e.g., phosphorothioate-modified nucleotides, e.g., as described herein. In an embodiment, the three base pairs at the terminal end of the DNA end form comprise chemically modified nucleotides (e.g., one or both nucleotides of the base pair are chemically modified), e.g., phosphorothioate-modified nucleotides, e.g., as described herein. In an embodiment, the six base pairs at the terminal end of the DNA end form comprise chemically modified nucleotides (e.g., one or both nucleotides of the base pair are chemically modified), e.g., phosphorothioate-modified nucleotides, e.g., as described herein.

In certain embodiments, a DNA end form comprising a sticky end comprises one or more chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, or at least 99% of the nucleotides in the DNA end form comprising a sticky end are chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, the DNA end form comprising a sticky end consists entirely of chemically modified nucleotides (e.g., phosphorothioate-modified nucleotides, e.g., as described herein). In embodiments, a terminal nucleotide of the DNA end form comprising a sticky end comprises a chemically modified nucleotide (e.g., one or both nucleotides of the base pair are chemically modified), e.g., a phosphorothioate-modified nucleotide, e.g., as described herein. In embodiments, the

overhang region of the sticky end of a DNA end form comprises one or more chemically modified nucleotide, e.g., phosphorothioate-modified nucleotides, e.g., as described herein.

Inverted Terminal Repeats (ITRs)

In some embodiments, a dsDNA molecule as described herein comprises an exonuclease-resistant DNA end form comprising an inverted terminal repeat (ITR). In some embodiments, the ITR is an ITR from a virus, e.g., an adenovirus or an adeno-associated virus (AAV). In some embodiments, the ITR comprises a nucleic acid sequence having at least 50%, at least 60%, 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% or 100% sequence identity to an ITR sequence from a virus, e.g., an adenovirus or an adeno-associated virus (AAV). In certain embodiments, the ITR comprises an origin of replication (e.g., a viral origin of replication). In embodiments, a dsDNA molecule as described herein comprises an exonuclease-resistant DNA end form comprising an ITR (e.g., as described herein) at each end. In some embodiments, a dsDNA molecule does not comprise an ITR.

Promoters and Other Regulatory Sequences

A DNA molecule (e.g., dsDNA molecule) described herein may contain a promoter (a DNA sequence at which RNA polymerase and transcription factors bind to, directly or indirectly, to initiate transcription) operably linked to an effector sequence. A promoter may be found in nature operably linked to the effector sequence, or may be heterologous to the effector sequence. A promoter described herein may be native to the target cell or tissue, or heterologous to the target cell or tissue. A promoter may be constitutive, inducible and/or tissue-specific.

Examples of constitutive promoters include the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) (see, e.g., Boshart et al, Cell, 41:521-530 (1985), the SV40 promoter, the dihydrofolate reductase promoter, the beta-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1alpha promoter.

Inducible promoters allow regulation of expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, e.g., acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of sources. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al., Proc. Natl.

Acad. Sci. USA, 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen et al., Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al., Science, 268:1766-1769 (1995), see also Harvey et al, Curr. Opin. Chem. Biol., 2:512-518 (1998)), the RU486-inducible system (Wang et al., Nat. Biotech., 15:239-243 (1997) and Wang et al., Gene Ther., 4:432-441 (1997)) and the rapamycin-inducible system (Magari et al., J. Clin. Invest., 100:2865-2872 (1997)).

In some embodiments, the native promoter for the sequence encoding the effector can be used.

In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (e.g., promoters, enhancers, etc.) are known in the art. Exemplary tissue-specific regulatory sequences include, but are not limited to the following tissue specific promoters: a liver-specific thyroxin binding globulin (TBG) promoter, an insulin promoter, a glucagon promoter, a somatostatin promoter, a pancreatic polypeptide (PPY) promoter, a synapsin-1 (Syn) promoter, a creatine kinase (MCK) promoter, a mammalian desmin (DES) promoter, a alpha-myosin heavy chain (a-MHC) promoter, or a cardiac Troponin T (cTnT) promoter. Other exemplary promoters include Beta-actin promoter, hepatitis B virus core promoter, Sandig et al., Gene Ther., 3:1002-9 (1996); alpha-fetoprotein (AFP) promoter, Arbuthnot et al., Hum. Gene Ther., 7:1503-14 (1996)), bone osteocalcin promoter (Stein et al., Mol. Biol. Rep., 24:185-96 (1997)); bone sialoprotein promoter (Chen et al., J. Bone Miner. Res., 11:654-64 (1996)), CD2 promoter (Hansal et al., J. Immunol., 161:1063-8 (1998); immunoglobulin heavy chain promoter; T cell receptoralpha.-chain promoter, neuronal such as neuron-specific enolase (NSE) promoter (Andersen et al., Cell. Mol. Neurobiol., 13:503-15 (1993)), neurofilament light-chain gene promoter (Piccioli et al., Proc. Natl. Acad. Sci. USA, 88:5611-5 (1991)), and the neuron-specific vgf gene promoter (Piccioli et al., Neuron, 15:373-84 (1995)), among others which will be known to the skilled artisan.

Examples of tissue/cell specific promoters are listed in **Table 1**:

Table 1: Tissue or cell specific promoters

Tissue/Cell	Promoter	Accession Number; Human Genome Coordinate (hg38)
Skeletal muscle	ACTA1	NM_001100; chr1:229,439,090-229,432,090
Melanoma	TYR	NM_000372; chr11:89,300,750-89,293,750
Hepatoma	a-fetoprotein	NM_001354717; chr4:73,461,175-73,454,175
Mammary carcinoma	Mucin 1	NM_001371720; chr1:155,197,900-155,190,900

Tissue/Cell	Promoter	Accession Number; Human Genome Coordinate (hg38)
Prostate Cancer	KLK3	NM_001648; chr19: 50,865,760-50,858,760
Neuronal cells	ENO2	NM_001975; chr12:6,928,700-6,921,700
Response to Hypoxia	HIF-1alpha	NM_001530; chr14:61,753,200-61,746,200
Retinoblastoma	E2F1	NM_005225; chr20: 33,691,380-33,684,380
Ionizing radiation	EGR-1	NM_001964; chr5:138,474,303-138,467,303
Oncogene	ErbB2	NM_004448; chr17:39,735,530-39,728,530
Endothelial cells	vWF	NM_000552; chr12:6,129,670-6,122,670
Endothelial cells	FLT-1	NM_002019; chr13:28,500,100-28,493,100
Endothelial cells	ICAM-2	NM_001099786; chr17:64,025,630-64,018,630
Retinal pigment epithelium	VMD2	NM_004183; chr11:61,972,630-61,965,630
Rod cells	RHO	NM_000539; chr3:129,540,350-129,533,350
Cone cells	Red/green opsin (OPN1LW)	NM_020061; chrX:154,164,030-154,157,030
Ganglion cells	Thymocyte antigen (Thy1)	NM_006288; chr11:119,428,150-119,421,150
T cells	TIM3	NM_032782; chr5:157,114,050-157,107,050
T cells	FOXP3	NM_014009; chrX:49,269,700-49,262,700
PBMCs	Vβ6.7	ENST00000390373.2; chr7:142,493,295-142,486,295
Cell cycle	Cdk1	NM_001786; chr10:60,799,850-60,792,850

The constructs described herein may also include other native or heterologous expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences.

Effector sequence

The effector sequence of a DNA molecule (e.g., dsDNA molecule) described herein may be, e.g., a functional DNA sequence, e.g., a therapeutically functional DNA sequence; a DNA sequence encoding a therapeutic peptide, polypeptide or protein; or a DNA sequence encoding a therapeutic RNA (e.g., a non-coding RNA).

DNA effector sequences:

A therapeutically functional DNA sequence may be a DNA sequence that forms a functional structure, e.g., a DNA sequence comprising a DNA aptamer, DNAzyme or allele-specific oligonucleotide (a DNA ASO). A therapeutically functional DNA sequence may not have a promoter operably linked. In embodiments, a dsDNA molecule described herein may include one or a plurality of functional DNA sequences, e.g., 2, 3, 4, 5, 6, or more sequences, which may be the same or different.

Polypeptide effectors:

A DNA sequence encoding a therapeutic polypeptide may be a DNA sequence encoding one or more effector which is a peptide, protein, or combinations thereof. For example, the DNA sequence encodes an mRNA. The peptide or protein may be: a DNA binding protein; an RNA binding protein; a transporter; a transcription factor; a translation factor; a ribosomal protein; a chromatin remodeling factor; an epigenetic modifying factor; an antigen; a hormone; an enzyme (such as a nuclease, e.g., an endonuclease, e.g., a nuclease element of a CRISPR system, e.g., a Cas9, dCas9, aCas9-nickase, Cpf/Cas12a); a Crispr-linked enzyme, e.g. a base editor or prime editor; a mobile genetic element protein (e.g., a transposase, a retrotransposase, a recombinase, an integrase); a gene writer; a polymerase; a methylase; a demethylase; an acetylase; a deacetylase; a kinase; a phosphatase; a ligase; a deubiquitinase; a protease; an integrase; a recombinase; a topoisomerase; a gyrase; a helicase; a lysosomal acid hydrolase); an antibody (e.g., an intact antibody, a fragment thereof, or a nanobody); a signaling peptide; a receptor ligand; a receptor; a clotting factor; a coagulation factor; a structural protein; a caspase; a membrane protein; a mitochondrial protein; a nuclear protein; an engineered binder such as a centyrin, darpin, or adnectin. See, e.g., Gebauer & Skerra. 2020. Annual Review of Pharmacology and Toxicology 60:1, 391-415.

In embodiments, a dsDNA molecule described herein may include one or a plurality of sequences encoding a polypeptide, e.g., 2, 3, 4, 5, 6, or more sequences encoding a polypeptide. Each of the plurality may encode the same or different protein. For example, a dsDNA molecule described herein may include multiple sequences encoding multiple proteins, e.g., a plurality of proteins in a biological pathway.

In some embodiments, a dsDNA molecule may include a plurality of sequences encoding a polypeptide, e.g., 2, 3, 4, 5, 6, or more sequences encoding a polypeptide, separated by a self-cleaving peptide, e.g., P2A, T2A, E2A or F2A. Self-cleaving peptides are 18-22 amino acids long, and can induce ribosomal skipping during protein translation so that two polypeptides can be encoded in the same transcript. Each of the polypeptides may encode the same or different protein. In one embodiment, a dsDNA molecule may include a promoter followed by a sequence encoding a first polypeptide of interest, a sequence encoding a 2A self-cleaving peptide, a sequence encoding a second polypeptide of interest,

and a polyA site. In another embodiment, a dsDNA molecule may include a promoter followed by a sequence encoding the first polypeptide of interest, a first 2A self-cleaving peptide, a second polypeptide of interest, a sequence encoding a second 2A self-cleaving peptide, a sequence encoding a third polypeptide of interest, and a polyA site.

In some embodiments, the effector comprises a cell penetrating polypeptide. In some embodiments, the effector is a fusion protein that comprises a cell penetrating polypeptide and a second amino acid sequence.

RNA effectors:

An effector sequence may be a DNA sequence encoding a non-coding RNA, e.g., one or more of a short interfering RNA (siRNA), a microRNA (miRNA), long non-coding RNA, a piwi-interacting RNA (piRNA), a small nucleolar RNA (snoRNA), a small Cajal body-specific RNA (scaRNA), a transfer RNA (tRNA), a ribosomal RNA (rRNA), an RNA aptamer, and a small nuclear RNA (snRNA).

In some embodiments, a DNA molecule (e.g., dsDNA molecule) disclosed herein comprises one or more expression sequences that encode a regulatory RNA, e.g., an RNA that modifies expression of an endogenous gene and/or an exogenous gene. In some embodiments, the dsDNA molecule or sequence disclosed herein can comprise a sequence that is antisense to a regulatory nucleic acid like a non-coding RNA, such as, but not limited to, tRNA, lncRNA, miRNA, rRNA, snRNA, microRNA, siRNA, piRNA, snoRNA, snRNA, exRNA, scaRNA, Y RNA, and hnRNA. In one embodiment, the regulatory nucleic acid targets a host gene. A regulatory nucleic acid may include, but is not limited to, a nucleic acid that hybridizes to an endogenous gene, e.g., an antisense RNA, a guide RNA, a nucleic acid that hybridizes to an exogenous nucleic acid such as a viral DNA or RNA, nucleic acid that hybridizes to an RNA, nucleic acid that interferes with gene transcription, nucleic acid that interferes with RNA translation, nucleic acid that stabilizes RNA or destabilizes RNA such as through targeting for degradation, and nucleic acid that modulates a DNA or RNA binding factor. In one embodiment, the sequence is an miRNA. In some embodiments, the regulatory nucleic acid targets a sense strand of a host gene. In some embodiments, the regulatory nucleic acid targets an antisense strand of a host gene.

In some embodiments, the DNA molecule (e.g., dsDNA molecule) encodes a guide RNA. Guide RNA sequences are generally designed to have a sequence having a length of between 15-30 nucleotides (e.g., 17, 19, 20, 21, 24 nucleotides) that is complementary to the targeted nucleic acid sequence, and a region that facilitates complex formation (e.g., with a tracrRNA or a nuclease). Custom gRNA generators and algorithms are available commercially for use in the design of effective guide RNAs. Gene editing has also been achieved using a chimeric "single guide RNA" ("sgRNA"), an engineered (synthetic) single RNA molecule that mimics a naturally occurring crRNA-tracrRNA complex and contains both a

tracrRNA (for binding the nuclease) and at least one crRNA (to guide the nuclease to the sequence targeted for editing). Chemically modified sgRNAs have also been demonstrated to be effective in genome editing; see, for example, Hendel et al. (2015) Nature Biotechnol., 985-991. The gRNA may recognize specific DNA sequences (e.g., sequences adjacent to or within a promoter, enhancer, silencer, or repressor of a gene). In one embodiment, the gRNA is used as part of a CRISPR system for gene editing. For the purposes of gene editing, the dsDNA molecule or sequence disclosed herein may be designed to include one or multiple sequences encoding guide RNA sequences corresponding to a desired target DNA sequence; see, for example, Cong et al. (2013) Science, 339:819-823; Ran et al. (2013) Nature Protocols, 8:2281-2308.

A DNA molecule (e.g., dsDNA molecule) or sequence disclosed herein may encode certain regulatory nucleic acids that can inhibit gene expression through the biological process of RNA interference (RNAi). RNAi molecules comprise RNA or RNA-like structures typically containing 15-50 base pairs (such as about 18-25 base pairs) and having a nucleobase sequence identical (complementary) or nearly identical (substantially complementary) to a coding sequence in an expressed target gene within the cell. Such RNAi molecules include, but are not limited to: short interfering RNAs (siRNAs), double-strand RNAs (dsRNA), micro RNAs (miRNAs), short hairpin RNAs (shRNA), meroduplexes, and dicer substrates (U.S. Pat. Nos. 8,084,599 8,349,809 and 8,513,207), RNA antisense oligonucleotides (RNA ASOs).

In one embodiment, the DNA molecule (e.g., dsDNA molecule) or sequence disclosed herein comprises a sequence comprising a sense strand of a lncRNA. In one embodiment, the dsDNA molecule or sequence disclosed herein comprises a sequence encoding an antisense strand of a lncRNA.

The DNA molecule (e.g., dsDNA molecule) or sequence disclosed herein may encode a regulatory nucleic acid substantially complementary, or fully complementary, to a fragment of an endogenous gene or gene product (e.g., mRNA). The regulatory nucleic acids may complement sequences at the boundary between introns and exons, in between exons, or adjacent to exon, to prevent the maturation of newly-generated nuclear RNA transcripts of specific genes into mRNA for transcription. The regulatory nucleic acids that are complementary to specific genes can hybridize with the mRNA for that gene and prevent its translation. The antisense regulatory nucleic acid can be DNA, RNA, or a derivative or hybrid thereof. In some embodiments, the regulatory nucleic acid comprises a protein-binding site that can bind to a protein that participates in regulation of expression of an endogenous gene or an exogenous gene.

The length of a DNA molecule (e.g., dsDNA molecule) or sequence disclosed herein that may encode a regulatory nucleic acid that hybridizes to a transcript of interest and may be, for instance, between about 5 to 30 nucleotides, between about 10 to 30 nucleotides, or about 11, 12, 13, 14, 15, 16,

17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotides. The degree of identity of the regulatory nucleic acid to the targeted transcript should be at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%.

A DNA molecule (e.g., dsDNA molecule) or sequence disclosed herein may encode a micro-RNA (miRNA) molecule identical to about 5 to about 30 contiguous nucleotides of a target gene. In some embodiments, the miRNA sequence targets a mRNA and commences with the dinucleotide AA, comprises a GC-content of about 30-70% (about 30-60%, about 40-60%, or about 45%-55%), and does not have a high percentage identity to any nucleotide sequence other than the target in the genome of the mammal in which it is to be introduced, for example as determined by standard BLAST search. In some embodiments, the dsDNA molecule or sequence disclosed herein encodes at least one miRNA, e.g., 2, 3, 4, 5, 6, or more. In some embodiments, the dsDNA molecule or sequence disclosed herein comprises a sequence that encodes an miRNA having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, or at least 99% nucleotide sequence identity to any one of the nucleotide sequences or a sequence that is complementary to a target sequence. In some embodiments, the dsDNA molecule or sequence disclosed herein comprises a sequence that encodes an miRNA having at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or 100% nucleotide sequence identity to any one of the nucleotide sequences or a sequence that is complementary to a target sequence. Lists of known miRNA sequences can be found in databases maintained by research organizations, such as Wellcome Trust Sanger Institute, Penn Center for Bioinformatics, Memorial Sloan Kettering Cancer Center, and European Molecule Biology Laboratory, among others. Known effective siRNA sequences and cognate binding sites are also well represented in the relevant literature. RNAi molecules are readily designed by technologies known in the art. In addition, there are computational tools that increase the chance of finding effective and specific sequence motifs (see, e.g., Lagana et al., Methods Mol. Bio., 2015, 1269:393-412).

The dsDNA molecule or sequence disclosed herein may modulate expression of RNA encoded by a gene. Because multiple genes can share some degree of sequence homology with each other, in some embodiments, the dsDNA molecule or sequence disclosed herein can be designed to target a class of genes with sufficient sequence homology. In some embodiments, the dsDNA molecule or sequence disclosed herein can contain a sequence that has complementarity to sequences that are shared amongst different gene targets or are unique for a specific gene target. In some embodiments, the dsDNA molecule or sequence disclosed herein can be designed to target conserved regions of an RNA sequence having homology between several genes thereby targeting several genes in a gene family (e.g., different gene isoforms, splice variants, mutant genes, etc.). In some embodiments, the dsDNA molecule or sequence

disclosed herein can be designed to target a sequence that is unique to a specific RNA sequence of a single gene.

In embodiments, the effector sequence encoding a regulatory RNA has a length less than 5000 bps (e.g., less than about 5000 bps, less than about 4000 bps, less than about 3000 bps, less than about 2000 bps, less than about 1000 bps, less than about 900 bps, less than about 800 bps, less than about 700 bps, less than about 600 bps, less than about 500 bps, less than about 400 bps, less than about 300 bps, less than about 200 bps, less than about 100 bps, less than about 50 bps, less than about 40 bps, less than about 30 bps, less than about 20 bps, less than about 10 bps, or less). In some embodiments, the effector sequence has, independently or in addition to, a length greater than 10 bps (e.g., at least 10 bps, at least 20 bps, at least 30 bps, at least 40 bps, at least 50 bps, at least 60 bps, at least 70 bps, at least 80 bps, at least 90 bps, at least 100 bps, at least 200 bps, at least 300 bps, at least 400 bps, at least 500 bps, at least 600 bps, at least 700 bps, at least 800 bps, at least 900 bps, at least 1000 kb, at least 1.1 kb, at least 1.2 kb, at least 1.3 kb, at least 1.4 kb, at least 1.5 kb, at least 1.6 kb, at least 1.7 kb, at least 1.8 kb, at least 1.9 kb, at least 2 kb, at least 2.1 kb, at least 2.2 kb, at least 2.3 kb, at least 2.4 kb, at least 2.5 kb, at least 2.6 kb, at least 2.7 kb, at least 2.8 kb, at least 2.9 kb, at least 3 kb, at least 3.1 kb, at least 3.2 kb, at least 3.3 kb, at least 3.4 kb, at least 3.5 kb, at least 3.6 kb, at least 3.7 kb, at least 3.8 kb, at least 3.9 kb, at least 4 kb, at least 4.1 kb, at least 4.2 kb, at least 4.3 kb, at least 4.4 kb, at least 4.5 kb, at least 4.6 kb, at least 4.7 kb, at least 4.8 kb, at least 4.9 kb, at least 5 kb or greater). In some embodiments, the effector sequence has, independently or in addition to, a length greater than 10 bps (e.g., at least about 10 bps, at least about 20 bps, at least about 30 bps, at least about 40 bps, at least about 50 bps, at least about 60 bps, at least about 70 bps, at least about 80 bps, at least about 90 bps, at least about 100 bps, at least about 200 bps, at least about 300 bps, at least about 400 bps, at least about 500 bps, at least about 600 bps, at least about 700 bps, at least about 800 bps, at least about 900 bps, at least about 1000 kb, at least about 1.1 kb, at least about 1.2 kb, at least about 1.3 kb, at least about 1.4 kb, at least about 1.5 kb, at least about 1.6 kb, at least about 1.7 kb, at least about 1.8 kb, at least about 1.9 kb, at least about 2 kb, at least about 2.1 kb, at least about 2.2 kb, at least about 2.3 kb, at least about 2.4 kb, at least about 2.5 kb, at least about 2.6 kb, at least about 2.7 kb, at least about 2.8 kb, at least about 2.9 kb, at least about 3 kb, at least about 3.1 kb, at least about 3.2 kb, at least about 3.3 kb, at least about 3.4 kb, at least about 3.5 kb, at least about 3.6 kb, at least about 3.7 kb, at least about 3.8 kb, at least about 3.9 kb, at least about 4 kb, at least about 4.1 kb, at least about 4.2 kb, at least about 4.3 kb, at least about 4.4 kb, at least about 4.5 kb, at least about 4.6 kb, at least about 4.7 kb, at least about 4.8 kb, at least about 4.9 kb, at least about 5 kb or greater).

In some embodiments, a DNA molecule (e.g., dsDNA molecule) or sequence disclosed herein comprises one or more of the features described hereinabove, e.g., one or more structural DNA sequence, a sequence encoding one or more peptides or proteins, a sequence encoding one or more regulatory

element, a sequence encoding one or more regulatory nucleic acids, e.g., one or more non-coding RNAs, other expression sequences, and any combination of the aforementioned. A construct described herein may have one or a plurality of effector sequences, e.g., 2, 3, 4, 5 or more effector sequences. In the case of a plurality of effector sequences in a single construct, the effector sequences may be the same or different. In some emobodiments, a dsDNA molecule can include an effector sequence that is a structural DNA and a second effector sequence that is a DNA sequence encoding a functional RNA or polypeptide.

In one embodiment, the DNA molecule (e.g., dsDNA molecule) includes a therapeutically functional, structural DNA sequence. In one embodiment, the DNA molecule (e.g., dsDNA molecule) includes a promoter and a sequence encoding a therapeutic peptide, polypeptide, or protein described herein. In one embodiment, the DNA molecule (e.g., dsDNA molecule) includes a promoter and a sequence encoding a regulatory RNA described herein.

In some embodiments, the effector sequence that encodes a polypeptide or protein is codon optimized, e.g., codon optimized for expression in a mammal, e.g., a human. In general, codon optimization means modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g., one or more, e.g., 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons; e.g., at least 1%, at least 5%, at least 10%, at least 20%, at least 25%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or 100%) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Codon usage tables are available, for example, at the "Codon Usage Database" available at http://www.kazusa.or.jp/codon/. These tables can be adapted in a number of ways, see, e.g., Nakamura et al., 2000, Nucl. Acids Res. 28:292. Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge.

Nuclear targeting sequences (NTS)

A DNA molecule (e.g., dsDNA molecule) or nucleic acid comprising dsDNA (e.g., as disclosed herein) may include a nuclear targeting sequence (NTS) that facilitates transport of DNA from the cytoplasm into the nucleus of a cell. An NTS includes binding sites to proteins (e.g., transcription factors, chaperones, etc.) which bind to importin which transports cargo into the nucleus via the nuclear pore complex. In embodiments, an NTS may function generally (e.g. SV40 enhancer NTS). In other embodiments, NTS's may be cell or tissue specific, e.g., containing binding sites for transcription factors expressed in unique cell types that may target a DNA molecule (e.g., dsDNA molecule) described herein to the nucleus in a cell-specific manner (e.g., SRF, Nkx3). An NTS can be functional in multiple locations in a dsDNA molecule described herein, e.g., before the promoter and/or after the effector sequence.

An NTS may be viral or non-viral derived. NTSs are described, e.g., in Le Guen et al. 2021. Nucleic Acids Vol. 24: 477-486. Examples of NTS's are disclosed in **Table 2**:

Table 2: Exemplary nuclear targeting sequences

Viral/Non-viral	Name	Sequence
Viral	SV40	5'-cccaagaagaagaggaaagtc-3' (SEQ ID NO: 1)
Non-viral	3NF	5'-ctggggactttccagcctggggactttccagctgggactttccagg-3' (SEQ ID NO: 70)

In some embodiments, the NTS has a sequence according to **Table 2**, or a functional sequence having at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity thereto.

Nuclear import proteins

In some embodiments, a DNA molecule (e.g., dsDNA molecule) is capable of being imported into the nucleus, e.g., by a nuclear import protein. In some embodiments, the DNA molecule (e.g., dsDNA molecule) can be bound by a nuclear import protein. In some embodiments, a DNA molecule (e.g., dsDNA molecule) comprises a recognition sequence for a nuclear import protein. In some embodiments, an exonuclease-resistant DNA end form (e.g., comprised in a dsDNA molecule) comprises a recognition sequence for a nuclear import protein.

Exemplary import proteins include, e.g., basic helix–loop–helix (bHLH) proteins, heterogeneous nuclear ribonucleoprotein (hnRNP) isoforms, or nuclear factor I (NFI) proteins. In some embodiments, the import protein comprises an importin.

In some embodiments, the import protein comprises a Ran binding protein. In some embodiments, the import protein comprises a homeobox transcription factor. In some embodiments, the import factor specifically binds an E-box, a DTS, a promoter, a telomere, an ATTT motif, a cell cycle regulatory unit (CCRU), a CT3 sequence, an S/MAR, a topoisomerase II consensus sequence, an ARS consensus sequence, a 3NF, a viral ori.

Maintenance sequence

A DNA molecule (e.g., dsDNA molecule) disclosed herein may include a maintenance sequence that supports or enables sustained gene expression through successive rounds of cell division and/or progenitor differentiation in a host cell for a DNA molecule (e.g., dsDNA molecule) of the invention. In embodiments, a maintenance sequence is a nuclear scaffold/matrix attachment region (S/MAR). S/MAR elements are diverse, AT-rich sequences ranging from 60-500 bp that are conserved across species,

thought to anchor chromatin to nuclear matrix proteins during interphase (Bode et al. *2003.* Chromosome Res 11, 435–445). An S/MAR can be incorporated into a DNA molecule (e.g., dsDNA molecule) described herein to facilitate long-term transgene expression and extra-chromosomal maintenance. In one embodiment, the maintenance sequence is human interferon-beta MAR (5'tataattcactggaatttttttgtgtgtatggtatgacatatgggttccctttttatttttacatataaatatatttccctgtttttctaaaaaagaaaaagatcatca ttttcccattgtaaaatgccatatttttttcataggtcacttacata-3' (SEQ ID NO: 39)), or a functional sequence having at least 80%, at least 90%, at least 95%, or at least 98% identity thereto. In one embodiment, the maintenance sequence is human interferon-beta MAR (5'tataattcactggaatttttttgtgtgtatggtatgacatatgggttccctttttatttttacatataaatatatttccctgtttttctaaaaaagaaaaagatcatca ttttcccattgtaaaatgccatatttttttcataggtcacttacata-3' (SEQ ID NO: 39)), or a functional sequence having at least 80%, 90%, 95%, or 98% identity thereto. In embodiments, S/MARs useful in the constructs described herein can be found by searching the MARome at http://bioinfo.net.in/MARome, described also by Narwade et al. 2019. Nucleic Acids Research. Volume 47, Issue 14: 7247–7261.

In embodiments, a DNA molecule (e.g., dsDNA molecule) described herein is capable of replicating in a mammalian cell, e.g., human cell. In some embodiments, a DNA molecule (e.g., dsDNA molecule) described herein is maintained in a host cell, tissue or subject through at least one cell division. For example, a DNA molecule (e.g., dsDNA molecule) described herein is maintained in a host cell, tissue or subject through at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 10, at least 15, at least 20, at least 40, or at least 50 cell divisions. A dsDNA molecule described herein is maintained in a host cell, tissue or subject through at least 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, 40, 50 or more cell divisions. In vitro, cell division may be tracked by flow cytometry or microscopy. In vivo, cell division may be tracked by intravital microscopy.

Other elements

A DNA molecule (e.g., dsDNA molecule) disclosed herein may also include other control elements operably linked to the effector sequence, e.g., the sequence encoding an effector, in a manner which permits its transport, localization, transcription, translation and/or expression in a target cell, or which promotes its degradation or repression of expression in a non-target cell. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the sequence encoding the effector and expression control sequences that act in trans or at a distance to control the sequence encoding the effector. The precise nature of regulatory sequences needed for gene expression in host cells may vary between species, tissues or cell types, but in general may include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, enhancer elements and

the like. Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The constructs described herein may optionally include 5' leader or signal sequences. In some embodiments, a DNA molecule described herein does not encode a viral protein.

Structure of DNA constructs

In some embodiments, the DNA molecule (e.g., dsDNA molecule) disclosed herein is at least about 20 nucleotides, at least about 30 nucleotides, at least about 40 nucleotides, at least about 50 nucleotides, at least about 75 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, at least about 300 nucleotides, at least about 500 nucleotides, at least about 1000 nucleotides, at least about 2000 nucleotides, at least about 3000 nucleotides, at least about 4000 nucleotides, at least about 5000 nucleotides, at least about 6000 nucleotides, at least about 7000 nucleotides, at least about 8000 nucleotides, at least about 9000 nucleotides, at least about 10,000 nucleotides, at least about 11,000 nucleotides, at least about 12,000 nucleotides, at least about 20,000 nucleotides, at least about 30,000 nucleotides, at least about 40,000 nucleotides, or at least about 50,000 nucleotides in length. In some embodiments, the DNA molecule (e.g., dsDNA molecule) disclosed herein is between 20-30, 30-40, 40-50, 50-75, 75-100, 100-200, 200-300, 300-500, 500-1000, 1000-2000, 2000-3000, 3000-4000, 4000-5000, 5000-6000, 6000-7000, 7000-8000, 8000-9000, 9000-10,000, 10,000-11,000, 11,000-12,000, 10,000-20,000, 20,000-30,000, 30,000-40,000, or 40,000-50,000 nucleotides in length. In some embodiments, the size of a DNA molecule (e.g., dsDNA molecule) disclosed herein is a length sufficient to encode useful polypeptides or RNAs. It is understood that when the length of a linear closed-ended dsDNA molecule is discussed herein, the length refers to the number of nucleotides starting with and including the upstream end, through the downstream end. For example, a no-loop dsDNA molecule having 100 base pairs would have a length of 100 nucleotides.

In some embodiments, a DNA molecule (e.g., dsDNA molecule) comprises a DNA end form (e.g., as described herein). In some embodiments, the DNA end form is at least 2, at least 3, at least 4, at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100 nucleotides in length. In some embodiments, the DNA end form is less than 10, less than 15, less than 20, less than 25, less than 30, less than 40, less than 50, less than 60, less than 70, less than 80, less than 90, or less than 100 nucleotides in length. In some embodiments, the DNA end form is 2-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-70, 70-80, 80-90, or 90-100 nucleotides in length.

In some embodiments, a DNA molecule (e.g., dsDNA molecule) comprises a double stranded region encoding an effector (e.g., a polypeptide or RNA, e.g., as described herein), e.g., positioned between two exonuclease-resistant DNA end forms. In some embodiments, the double stranded region is

at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 200, at least 300, at least 400, at least 500, at least 600, at least 700, at least 800, at least 900, at least 1000, at least 2000, at least 3000, at least 4000, at least 5000, at least 6000, at least 7000, at least 8000, at least 9000, at least 10,000, at least about 11,000, at least about 12,000, at least 20,000, at least 30,000, at least 40,000, or at least 50,000 nucleotides in length. In some embodiments, the double stranded region is less than 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 20,000, 30,000, 40,000, or 50,000 nucleotides in length. In some embodiments, the double stranded region is less than 50, less than 60, less than 70, less than 80, less than 90, less than 100, less than 200, less than 300, less than 400, less than 500, less than 600, less than 700, less than 800, less than 900, less than 1000, less than 2000, less than 3000, less than 4000, less than 5000, less than 6000, less than 7000, less than 8000, less than 9000, less than 10,000, less than 11,000, less than 12,000, less than 20,000, less than 30,000, less than 40,000, or less than 50,000 nucleotides in length. In some embodiments, the double stranded region is 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-70, 70-80, 80-90, 90-100, 100-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800, 800-900, 900-1000, 1000-2000, 2000-3000, 3000-4000, 4000-5000, 5000-6000, 6000-7000, 7000-8000, 8000-9000, 9000-10,000, 10,000-11,000, 11,000-12,000, 10,000-20,000, 20,000-30,000, 30,000-40,000, or 40,000 to 50,000 nucleotides in length.

A dsDNA molecule described herein may have less than a threshold level of single stranded structures. In one embodiment, the dsDNA molecule does not comprise more than 20, more than 18, more than 16, more than 14, more than 12, more than 10, more than 8, more than 7, more than 5, more than 4, more than 3, more than 2, or more than 1 single stranded region longer than 100, longer than 80, longer than 70, longer than 60, longer than 50, longer than 40, longer than 30, longer than 20 or longer than 10 bases, e.g., does not comprise single stranded regions longer than 100, longer than 80, longer than 70, longer than 60, longer than 50, longer than 40, longer than 30, longer than 20 or longer than 10 bases. In one embodiment, double stranded regions formed by a dsDNA molecule described herein is determined as described by Xayaphoummine et al. 2005. *Kinefold web server for RNA/DNA folding path and structure prediction including pseudoknots and knots*. Nucleic Acids Research, Volume 33:W605-610. In one embodiment, the Kinefold website (http://kinefold.curie.fr/cgi-bin/form.pl) is used to predict double stranded regions of a construct described herein, using the following parameters:

- Sequence to fold: enter and select "DNA sequence"
- Stochastic Simulation: Co-transcriptional fold, 3 milliseconds
- Simulated molecular time: default
- Pseudoknots: not allowed
- Entanglements: non crossing

- Random seed: 11453

In some aspects, the present disclosure provides looped-end DNA (leDNA) molecules. A leDNA molecule has a long single stranded region, and each end is folded to produce a hairpin. In some embodiments, the leDNA comprises an upstream DNA end form which is a closed end. In some embodiments, the leDNA comprises, adjacent to the upstream DNA end form, a first double stranded region comprising a first fragment of a sense strand and a first region of an antisense strand. In some embodiments, the first double stranded region comprises backbone modifications in the sense strand, the antisense strand, or both of the sense and antisense strands. In some embodiments, the leDNA comprises, adjacent to the first double stranded region, a second region of the antisense strand, which region is single stranded. In some embodiments, the leDNA comprises, adjacent to the single stranded region of the antisense strand, a second double stranded region comprising a second fragment of the sense strand and a third region of the antisense strand. In some embodiments, the second double stranded region comprises backbone modifications in the sense strand, the antisense strand, or both of the sense and antisense strands. In some embodiments, the leDNA comprises, adjacent to the second double stranded region, a downstream DNA end form which is a closed end.

In some embodiments, a dsDNA form described herein is asymmetrically modified, where one strand comprises chemically modified nucleobases and the other strand is substantially free of chemically modified nucleobases. In some embodiments, the hemi-modified DNA may be completely free of chemically modified nucleotides on the antisense strand, and in other embodiments, the hemi-modified DNA may comprise a few chemical modifications (such as backbone modifications, e.g., phosphorothioate) on the antisense strand. In some embodiments, the hemi-modified DNA molecule comprises chemically modified nucleotides (e.g., nuclotides comprising chemically modified nucleobases) on the sense strand. In some embodiments, the hemi-modified DNA molecule comprises inosine nucleotides on the sense strand.

Production

In some embodiments, a hemi-modified dsDNA may be produced as follows. First, a double stranded linear DNA having a sense strand, an antisense strand, an upstream open end, and a downstream open end is provided, e.g., using routine methods. Next, both open ends are converted to closed ends (thereby producing an upstream closed end and downstream closed end), e.g., by ligating end adaptors. The end adaptors may comprise backbone modifications that are exonuclease-resistant, such as phosphorothioate. As a result of the ligation, the sense strand may comprise one or more backbone modifications adjacent to or within 10, 20, or 30 nucleotides of the upstream closed end and/or the downstream closed end. Next, a nick may be produced in the sense strand of the DNA adjacent to or

within 10, 20, or 30 nucleotides of the downstream closed end or the upstream closed end. Next, the nicked DNA may be subjected to conditions having exonuclease activity (e.g., the nicked DNA may be contacted with an exonuclease), such that the region of the sense strand between the nick and the one or more backbone modifications are removed. This results in a looped end DNA (leDNA) having a hairpin at each end, separated by a single stranded antisense region. If desired, the leDNA can be converted to dsDNA by contacting the leDNA with a DNA polymerase and nucleotides. For instance, a mixture of unmodified deoxyribose nucleotides, and nucleotides comprising chemically modified nucleobases may be used, such that a chemically modified sense strand is produced, and the leDNA is converted into dsDNA. Finally, a ligase may be used to connect the newly synthesized sense strand to the neighboring DNA end form.

Additional methods of making dsDNAs are described, for instance, in International Application WO/2023/220729, which is hereby incorporated by reference in its entirety. See, for instance, the Examples therein.

In some embodiments, a dsDNA molecule comprising chemical modifications on one strand is produced by amplification of one strand (e.g., from a plasmid template) using a dNTP mixture comprising one or more chemically modified nucleotides and a primer that can amplify one strand of the dsDNA molecule sequence. In certain embodiments, the opposite strand (e.g., an unmodified strand or a differently chemically modified strand, e.g., as described herein, for example, in **FIGS. 2A-3**) is produced in a separate amplification reaction, e.g., using a dNTP mixture comprising unmodified nucleotides or a different set of chemically modified nucleotides, and a primer that can amplify the opposite strand of the dsDNA molecule sequence.

In some embodiments, a dsDNA molecule as described herein is produced from a plasmid assembled to contain the desired elements described herein. The plasmid template can be assembled, for example, using Golden Gate cloning for assembly of multiple DNA fragments in a defined linear order in a recipient vector using a one-pot assembly procedure. Golden Gate cloning is described in Marillonnet & Grützner, 2020, *Synthetic DNA assembly using golden gate cloning and the hierarchical modular cloning pipeline*, Current Protocols in Molecular Biology, 130:e115. In some embodiments, a plasmid template is linearized, for example, by digestion with a nuclease (e.g., a restriction endonuclease) or by PCR amplification of a linear nucleic acid sequence from the plasmid template.

In some embodiments, a dsDNA molecule comprising the same chemical modification(s) on both strand is produced by amplification of the dsDNA molecule strands (e.g., from a plasmid template) using a dNTP mixture comprising one or more chemically modified nucleotides and primers that can amplify both strand of the dsDNA molecule sequence.

In some embodiments, an exonuclease-resistant DNA end form (e.g., as described herein) is introduced (e.g., attached) to one or both ends of a dsDNA molecule. In certain embodiments, the DNA end form is attached to an end of the dsDNA molecule by ligation. In embodiments, attachment (e.g., ligation) of the DNA end form (e.g., a covalently closed DNA end form) to the dsDNA molecule produces the final dsDNA molecule. In certain embodiments, exonuclease resistance of the attached DNA end form is confirmed, for example, by incubating the dsDNA molecule in the presence of an exonuclease (e.g., Exonuclease III and/or Mung Bean Nuclease), e.g., as described in Examples 2 and 3. In embodiments, exonuclease resistance of the attached DNA end form is confirmed, for example, by incubating the dsDNA molecule in the presence of Exonuclease III. In embodiments, the DNA end form comprises a blunt end, sticky end, or Y-adaptor (e.g., as described herein), and the exonuclease resistance of the attached DNA end form is confirmed by incubating the dsDNA molecule in the presence of Exonuclease III and (e.g., subsequently, prior to, or concurrently) Mung Bean nuclease.

In certain embodiments, the DNA end form is attached to the end of the dsDNA molecule in a nascent form (e.g., a non-covalently closed DNA end form may be attached to the dsDNA molecule as a hairpin. In a subsequent step, the nascent form of the DNA end form may be further modified (e.g., cleaved) to produce the final DNA end form. For example, a non-covalently closed DNA end form may be produced by cleavage of a nascent form, e.g., by a nuclease. In some embodiments, a nascent form comprising an overhang or sticky end can be converted to a blunt end by digestion with a single strand-specific nuclease, e.g., a Mung Bean nuclease. In some embodiments, a nascent form comprising a hairpin comprising a cleavable moiety in its single-stranded loop region is converted to a Y-adaptor by cleavage of the cleavable moiety.

In an embodiment, the method further comprises formulating the enriched dsDNA molecule for pharmaceutical use, e.g., formulating the dsDNA molecule with a pharmaceutically acceptable excipient and/or with a carrier, e.g., an LNP.

In an embodiment, a method described herein comprises enriching the dsDNA molecule. In an embodiment, the enriching includes substantially removing from the dsDNA molecule one or more impurity selected from: endotoxin, process impurities such as mononucleotides, chemically modified mononucleotides, single stranded DNA, DNA fragments or truncations, and proteins (e.g., enzymes, e.g., ligases, restriction enzymes).

The dsDNA molecule may be enriched from impurities or byproducts selected from the group consisting of: endotoxin, process impurities such as mononucleotides, chemically modified mononucleotides, single stranded DNA, circular DNA, proteins (e.g., enzymes, e.g., ligases, restriction enzymes), DNA fragments or truncations. In some embodiments, the enriched dsDNA molecule is substantially free of process byproducts and impurities, e.g., process byproducts or impurities described

herein. In embodiments, the pharmaceutical composition is substantially free of impurities or process byproducts, e.g., selected from the group consisting of: endotoxin, mononucleotides, chemically modified mononucleotides, DNA fragments or truncations, and proteins (e.g., enzymes, e.g., ligases, restriction enzymes). In some embodiments, the pharmaceutical composition is substantially free of circular DNA.

In some embodiments, enrichment involves a partial reduction of one or more contaminants.

In some embodiments, a dsDNA molecule is formulated with a lipid based carrier, e.g., a lipid nanoparticle (LNP), e.g., as described in Example 1.

The dsDNA molecule may be sequenced to confirm the desired, designed sequence. In embodiments, other structural analysis of the dsDNA molecule (e.g., restriction enzyme analysis) may be performed to confirm or verify its sequence.

A chemically modified dsDNA molecule described herein may be produced by a number of methods, including methods routine in the art. For instance, a chemically modified dsDNA molecule can be produced by performing polymerase chain reaction on a DNA template in the presence of unmodified and chemically modified nucleotides and a suitable polymerase. Exemplary suitable polymerases are described in Example 9 and include KOD polymerase (710864, Sigma Aldrich), KODX polymerase (719753, Sigma Aldrich), Deep Vent polymerase (M0258, NEB), and KOD -Multi & Epi- polymerase (TYB-KME-101, Diagnocine). A wide variety of polymerases are available, e.g., from commercial sources. Various polymerases can be used so long as they incorporate chemically modified nucleotides with a sufficiently high efficiency.

A chemically modified dsDNA molecule may also be produced by a method that does not comprise performing polymerase chain reaction. For instance, direct chemical synthesis may be used.

A chemically modified dsDNA molecule may be produced by providing a dsDNA molecule and chemically modifying nucleotides of the dsDNA molecule. For instance, a dsDNA molecule may be contacted with an enzyme, resulting in a chemically modified dsDNA molecule. In some embodiments, the enzyme converts an unmodified nucleotide into a chemically modified nucleotide. In some embodiments, the enzyme converts a chemically modified nucleotide into a differently modified nucleotide.

Pharmaceutical compositions

The present disclosure includes a DNA molecule (e.g., dsDNA molecule) and related compositions in combination with one or more pharmaceutically acceptable excipients and/or carriers.

Pharmaceutical compositions may optionally comprise one or more additional active substances, e.g., therapeutically and/or prophylactically active substances. Pharmaceutical compositions of the present invention are generally sterile and/or pyrogen-free.

A DNA molecule (e.g., dsDNA molecule) described herein may be formulated without a carrier, e.g., the DNA molecule (e.g., dsDNA molecule) described herein may be administered to a host cell, tissue or subject "naked". A naked formulation may include pharmaceutical excipients or diluents but lacks a carrier.

Pharmaceutically acceptable excipients or diluents may comprise an inactive substance that serves as a vehicle or medium for the compositions described herein, such as any one of the inactive ingredients approved by the United States Food and Drug Administration (FDA) and listed in the Inactive Ingredient Database, which is incorporated by reference herein. Non-limiting examples of pharmaceutically acceptable excipients or diluents include solvents, aqueous solvents, non-aqueous solvents, tonicity agents, dispersion media, cryoprotectants, diluents, suspension aids, surface active agents, isotonic agents, thickening agents, emulsifying agents, preservatives, hyaluronidases, dispersing agents, preservatives, lubricants, granulating agents, disintegrating agents, binding agents, antioxidants, buffering agents (e.g., phosphate buffered saline (PBS)), lubricating agents, oils, and mixtures thereof.

General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in Remington: The Science and Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference).

Carriers

A DNA molecule (e.g., dsDNA molecule) described herein may also be formulated, or included, with a carrier. General considerations of carriers and delivery of pharmaceutical agents may be found, for example, in *Delivery Technologies for Biopharmaceuticals: Peptides, Proteins, Nucleic Acids and Vaccines* (Lene Jorgensen and Hanne Morck Nielson, Eds.) Wiley; 1st edition (December 21, 2009); and Vargason et al. 2021. Nat Biomed Eng 5, 951–967.

Non-limiting examples of carriers include carbohydrate carriers (e.g., an anhydride- modified phytoglycogen or glycogen-type material, GalNAc), nanoparticles (e.g., a nanoparticle that encapsulates or is covalently linked to the dsDNA molecule, gold nanoparticles, silica nanoparticles), lipid particles (e.g., liposomes, lipid nanoparticles), cationic carriers (e.g., a cationic lipopolymer or transfection reagent), fusosomes, non-nucleated cells (e.g., ex vivo differentiated reticulocytes), nucleated cells, exosomes, protein carriers (e.g., a protein covalently linked to the DNA molecule (e.g., dsDNA molecule)), peptides (e.g., cell-penetrating peptides), materials (e.g., graphene oxide), single pure lipids (e.g., cholesterol), DNA origami (e.g., DNA tetrahedron).

In one embodiment, the DNA molecule (e.g., dsDNA molecule) compositions, constructs and systems described herein can be formulated in liposomes or other similar vesicles. Liposomes are spherical vesicle structures composed of a uni- or multilamellar lipid bilayer surrounding internal aqueous

compartments and a relatively impermeable outer lipophilic phospholipid bilayer. Liposomes may be anionic, neutral or cationic. Liposomes are biocompatible, nontoxic, can deliver both hydrophilic and lipophilic drug molecules, protect their cargo from degradation by plasma enzymes, and transport their load across biological membranes and the blood brain barrier (BBB) (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

Vesicles can be made from several different types of lipids; however, phospholipids are most commonly used to generate liposomes as drug carriers. Methods for preparation of multilamellar vesicle lipids are known in the art (see for example U.S. Pat. No. 6,693,086, the teachings of which relating to multilamellar vesicle lipid preparation are incorporated herein by reference). Although vesicle formation can be spontaneous when a lipid film is mixed with an aqueous solution, it can also be expedited by applying force in the form of shaking by using a homogenizer, sonicator, or an extrusion apparatus (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review). Extruded lipids can be prepared by extruding through filters of decreasing size, as described in Templeton et al., Nature Biotech, 15:647-652, 1997, the teachings of which relating to extruded lipid preparation are incorporated herein by reference.

Exosomes can also be used as drug delivery vehicles for the compositions and systems described herein. For a review, see Ha et al. July 2016. Acta Pharmaceutica Sinica B. Volume 6, Issue 4, Pages 287-296; https://doi.org/10.1016/j.apsb.2016.02.001.

Ex vivo differentiated red blood cells can also be used as a carrier for an agent (e.g., a dsDNA molecule) described herein. See, e.g., WO2015073587; WO2017123646; WO2017123644; WO2018102740; WO2016183482; WO2015153102; WO2018151829; WO2018009838; Shi et al. 2014. Proc Natl Acad Sci USA. 111(28): 10131–10136; US Patent 9,644,180; Huang et al. 2017. Nature Communications 8: 423; Shi et al. 2014. Proc Natl Acad Sci USA. 111(28): 10131–10136.

Fusosome compositions, e.g., as described in WO2018208728, can also be used as carriers to deliver the dsDNA molecules described herein.

Lipid Nanoformulations/Lipid-based carriers

In some embodiments, compositions or compounds described herein are formulated into a lipid-based carrier (or lipid nanoformulation). In some embodiments, the lipid-based carrier (or lipid nanoformulation) is a liposome or a lipid nanoparticle (LNP). In one embodiment, the lipid-based carrier is an LNP.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises a cationic lipid (e.g., an ionizable lipid), a non-cationic lipid (e.g., phospholipid), a structural lipid (e.g., cholesterol),

and a PEG-modified lipid. In some embodiments, the lipid-based carrier (or lipid nanoformulation) contains one or more compounds described herein, or a pharmaceutically acceptable salt thereof.

As described herein, suitable compositions or compounds to be used in the lipid-based carrier (or lipid nanoformulation) include all the isomers and isotopes of the compositions or compounds described above, as well as all the pharmaceutically acceptable salts, solvates, or hydrates thereof, and all crystal forms, crystal form mixtures, and anhydrides or hydrates.

In addition to one or more compositions or compounds described herein, the lipid-based carrier (or lipid nanoformulation) may further include a second lipid. In some embodiments, the second lipid is a cationic lipid, a non-cationic (*e.g.*, neutral, anionic, or zwitterionic) lipid, or an ionizable lipid.

One or more naturally occurring and/or synthetic lipid compounds may be used in the preparation of the lipid-based carrier (or lipid nanoformulation).

The lipid-based carrier (or lipid nanoformulation) may contain positively charged (cationic) lipids, neutral lipids, negatively charged (anionic) lipids, or a combination thereof.

Cationic Lipids (Positively Charged) and Ionizable Lipids

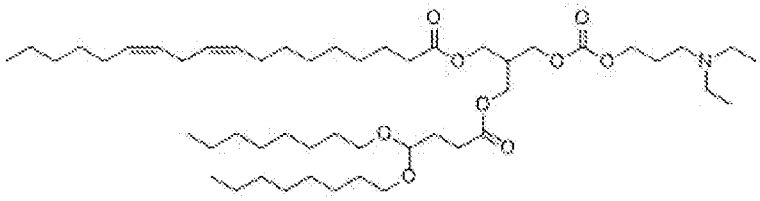
In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises one or more cationic lipids, *e.g.*, a cationic lipid that can exist in a positively charged or neutral form depending on pH, or an amine-containing lipid that can be readily protonated. In some embodiments, the cationic lipid is a lipid capable of being positively charged, *e.g.*, under physiological conditions.

Exemplary cationic lipids include one or more amine group(s) which bear the positive charge. Examples of positively charged (cationic) lipids include, but are not limited to, N,N'-dimethyl-N,N'-dioctacyl ammonium bromide (DDAB) and chloride DDAC), N-(l-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), 3β-[N-(N',N'-dimethylaminoethyl)carbamoyl) cholesterol (DC-chol), 1,2-dioleoyloxy-3-[trimethylammonio]-propane (DOTAP), 1,2-dioctadecyloxy-3-[trimethylammonio]-propane (DSTAP), and 1,2-dioleoyloxypropyl-3-dimethyl-hydroxy ethyl ammonium chloride (DORI), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), N,N-dimethyl-2,3-dioleyloxy)propylamine (DODMA), 1,2-Dioleoyl-3-Dimethylammonium-propane (DODAP), 1,2-Dioleoylcarbamyl-3-Dimethylammonium-propane (DOCDAP), 1,2-Dilinoeyl-3-Dimethylammonium-propane (DLINDAP), 3-Dimethylamino-2-(Cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5′-(cholest-5-en-3-beta-oxy)-3′-oxapentoxy)-3-dimethyl-1-(cis, cis-9′,12′-octadecadienoxy)propane (CpLin DMA), N,N-Dimethyl-3,4-dioleyloxybenzylamine (DMOBA), and the cationic lipids described in *e.g.* Martin et al., *Current Pharmaceutical Design*, pages 1-394, which is herein incorporated by reference in its entirety. In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises more than one cationic lipid.

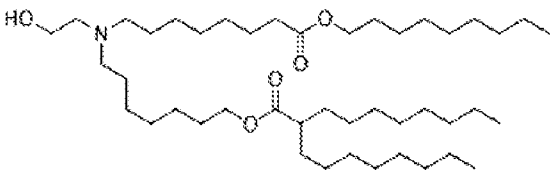
In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises a cationic lipid having an effective pKa over 6.0. In some embodiments, the lipid-based carrier (or lipid nanoformulation) further comprises a second cationic lipid having a different effective pKa (*e.g.*, greater than the first effective pKa) than the first cationic lipid.

In some embodiments, cationic lipids that can be used in the lipid-based carrier (or lipid nanoformulation) include, for example those described in Table 4 of WO 2019/217941, which is incorporated by reference.

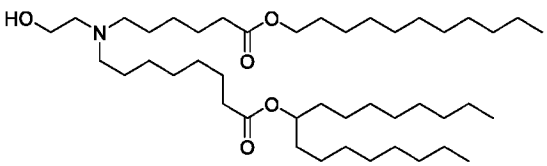
In some embodiments, the cationic lipid is an ionizable lipid (*e.g.*, a lipid that is protonated at low pH, but that remains neutral at physiological pH). In some embodiments, the lipid-based carrier (or lipid nanoformulation) may comprise one or more additional ionizable lipids, different than the ionizable lipids described herein. Exemplary ionizable lipids include, but are not limited to,



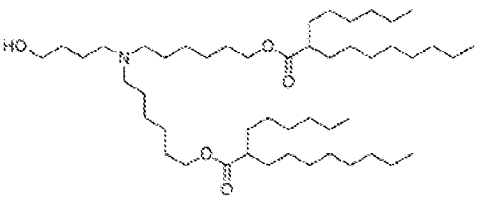
(LP01),



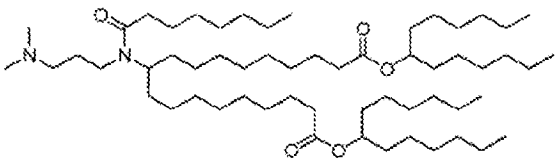
(SM-086),



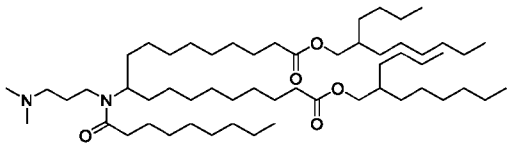
(SM-102),



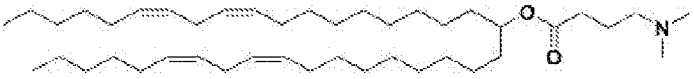
(ALC-0315),



(Lipid 10),



(Lipid A9), and



(DLin-MC3-DMA),

(see WO 2017/004143A1, which is incorporated herein by reference in its entirety).

In some embodiments, the lipid-based carrier (or lipid nanoformulation) further comprises one or more compounds described by WO 2021/113777 (*e.g.*, a lipid of Formula (3) such as a lipid of Table 3 of WO 2021/113777), which is incorporated herein by reference in its entirety.

In one embodiment, the ionizable lipid is a lipid disclosed in Hou, X., et al. Nat Rev Mater 6, 1078–1094 (2021). https://doi.org/10.1038/s41578-021-00358-0 (*e.g.*, L319, C12-200, and DLin-MC3-DMA), (which is incorporated by reference herein in its entirety).

Examples of other ionizable lipids that can be used in lipid-based carrier (or lipid nanoformulation) include, without limitation, one or more of the following formulas: X of US 2016/0311759; I of US 20150376115 or in US 2016/0376224; Compound 5 or Compound 6 in US 2016/0376224; I, IA, or II of US 9,867,888; I, II or III of US 2016/0151284; I, IA, II, or IIA of US 2017/0210967; I-c of US 2015/0140070; A of US 2013/0178541; I of US 2013/0303587 or US 2013/0123338; I of US 2015/0141678; II, III, IV, or V of US 2015/0239926; I of US 2017/0119904; I or II of WO 2017/117528; A of US 2012/0149894; A of US 2015/0057373; A of WO 2013/116126; A of US 2013/0090372; A of US 2013/0274523; A of US 2013/0274504; A of US 2013/0053572; A of WO 2013/016058; A of WO 2012/162210; I of US 2008/042973; I, II, III, or IV of US 2012/01287670; I or II of US 2014/0200257; I, II, or III of US 2015/0203446; I or III of US 2015/0005363; I, IA, IB, IC, ID, II, IIA, IIB, IIC, IID, or III-XXIV of US 2014/0308304; of US 2013/0338210; I, II, III, or IV of WO 2009/132131; A of US 2012/01011478; I or XXXV of US 2012/0027796; XIV or XVII of US 2012/0058144; of US 2013/0323269; I of US 2011/0117125; I, II, or III of US 2011/0256175; I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII of US 2012/0202871; I, II, III, IV, V, VI, VII, VIII, X, XII, XIII, XIV, XV, or XVI of US 2011/0076335; I or II of US 2006/008378; I of WO2015/074085 (*e.g.*, ATX-002); I of US 2013/0123338; I or X-A-Y-Z of US 2015/0064242; XVI, XVII, or XVIII of US 2013/0022649; I, II, or III of US 2013/0116307; I, II, or III of US 2013/0116307; I or II of US 2010/0062967; I-X of US 2013/0189351; I of US 2014/0039032; V of US 2018/0028664; I of US 2016/0317458; I of US 2013/0195920; 5, 6, or 10 of US 10,221,127; III-3 of WO 2018/081480; I-5 or I-8 of WO 2020/081938; I of WO 2015/199952 (*e.g.*, compound 6 or 22) and Table 1 therein; 18 or 25 of US 9,867,888; A of US 2019/0136231; II of WO 2020/219876; 1 of US 2012/0027803; OF-02 of US

2019/0240349; 23 of US 10,086,013; cKK-E12/A6 of Miao et al (2020); C12-200 of WO 2010/053572; 7C1 of Dahlman et al (2017); 304-O13 or 503-O13 of Whitehead et al; TS-P4C2 of U S9,708,628; I of WO 2020/106946; I of WO 2020/106946; (1), (2), (3), or (4) of WO 2021/113777; and any one of Tables 1-16 of WO 2021/113777, all of which are incorporated herein by reference in their entirety.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) further includes biodegradable ionizable lipids, for instance, (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3- ((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate). *See, e.g.*, lipids of WO 2019/067992, WO 2017/173054, WO 2015/095340, and WO 2014/136086, which are incorporated herein by reference in their entirety.

Non-Cationic Lipids (e.g., Phospholipids)

In some embodiments, the lipid-based carrier (or lipid nanoformulation) further comprises one or more non-cationic lipids. In some embodiments, the non-cationic lipid is a phospholipid. In some embodiments, the non-cationic lipid is a phospholipid substitute or replacement. In some embodiments, the non-cationic lipid is a negatively charged (anionic) lipid.

Exemplary non-cationic lipids include, but are not limited to, distearoyl-sn-glycero-phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl- phosphatidylethanolamine (such as 16-O-dimethyl PE), 18-l-trans PE, 1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), hydrogenated soy phosphatidylcholine (HSPC), egg phosphatidylcholine (EPC), dioleoylphosphatidylserine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), dierucoylphosphatidylcholine (DEPC), palmitoyloleyolphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), 1,2-dilauroyl- sn-glycero-3-phosphocholine (DLPC), Sodium 1,2- ditetradecanoyl-sn-glycero-3-phosphate (DMPA), phosphatidylcholine (lecithin), phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), phosphatidylethanolamine (cephalin), cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, lysophosphatidylcholine,

dilinoleoylphosphatidylcholine, or mixtures thereof. It is understood that other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C_{10}-C_{24} carbon chains, *e.g.*, lauroyl, myristoyl, paimitoyl, stearoyl, or oleoyl. Additional exemplary lipids, in certain embodiments, include, without limitation, those described in Kim et al. (2020) dx.doi.org/10.1021/acs.nanolett.0c01386, which is incorporated herein by reference. Such lipids include, in some embodiments, plant lipids found to improve liver transfection with mRNA (*e.g.*, DGTS).

In some embodiments, the lipid-based carrier (or lipid nanoformulation) may comprise a combination of distearoylphosphatidylcholine/cholesterol, dipalmitoylphosphatidylcholine/cholesterol, dimyrystoylphosphatidylcholine/cholesterol, 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC)/cholesterol, or egg sphingomyelin/cholesterol.

Other examples of suitable non-cationic lipids include, without limitation, nonphosphorous lipids such as, *e.g.*, stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerol ricinoleate, hexadecyl stearate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyl dimethyl ammonium bromide, ceramide, sphingomyelin, and the like. Other non-cationic lipids are described in WO 2017/099823 or US 2018/0028664, which are incorporated herein by reference in their entirety.

In one embodiment, the lipid-based carrier (or lipid nanoformulation) further comprises one or more non-cationic lipid that is oleic acid or a compound of Formula I, II, or IV of US 2018/0028664, which is incorporated herein by reference in its entirety.

The non-cationic lipid content can be, for example, 0-30% (mol) of the total lipid components present. In some embodiments, the non-cationic lipid content is 5-20% (mol) or 10-15% (mol) of the total lipid components present.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) further comprises a neutral lipid, and the molar ratio of an ionizable lipid to a neutral lipid ranges from about 2:1 to about 8:1 (*e.g.*, about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, or 8:1).

In some embodiments, the lipid-based carrier (or lipid nanoformulation) does not include any phospholipids.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) can further include one or more phospholipids, and optionally one or more additional molecules of similar molecular shape and dimensions having both a hydrophobic moiety and a hydrophilic moiety (*e.g.*, cholesterol).

Structural Lipids

The lipid-based carrier (or lipid nanoformulation) described herein may further comprise one or

more structural lipids. As used herein, the term "structural lipid" refers to sterols (*e.g.*, cholesterol) and also to lipids containing sterol moieties.

Incorporation of structural lipids in the lipid nanoparticle may help mitigate aggregation of other lipid in the particle. Structural lipids can be selected from the group including but not limited to, cholesterol or cholesterol derivative, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, tomatine, ursolic acid, alpha-tocopherol, hopanoids, phytosterols, steroids, and mixtures thereof. In some embodiments, the structural lipid is a sterol. In certain embodiments, the structural lipid is a steroid. In certain embodiments, the structural lipid is cholesterol. In certain embodiments, the structural lipid is an analog of cholesterol. In certain embodiments, the structural lipid is alpha-tocopherol.

In some embodiments, structural lipids may be incorporated into the lipid-based carrier at molar ratios ranging from about 0.1 to 1.0 (cholesterol phospholipid).

In some embodiments, sterols, when present, can include one or more of cholesterol or cholesterol derivatives, such as those described in WO 2009/127060 or US 2010/0130588, which are incorporated herein by reference in their entirety. Additional exemplary sterols include phytosterols, including those described in Eygeris et al. (2020), Nano Lett. 2020;20(6):4543-4549, incorporated herein by reference.

In some embodiments, the structural lipid is a cholesterol derivative. Non-limiting examples of cholesterol derivatives include polar analogues such as 5a-cholestanol, 53-coprostanol, cholesteryl-(2'-hydroxy)-ethyl ether, cholesteryl-(4'- hydroxy)-butyl ether, and 6-ketocholestanol; non-polar analogues such as 5a-cholestane, cholestenone, 5a-cholestanone, 5p-cholestanone, and cholesteryl decanoate; and mixtures thereof. In some embodiments, the cholesterol derivative is a polar analogue, *e.g.*, cholesteryl-(4'-hydroxy)-butyl ether. Exemplary cholesterol derivatives are described in WO 2009/127060 and US 2010/0130588, each of which is incorporated herein by reference in its entirety.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) further comprises sterol in an amount of 0-50 mol% (*e.g.*, 0-10 mol %, 10-20 mol %, 20-50 mol%, 20-30 mol %, 30-40 mol %, or 40-50 mol %) of the total lipid components.

Polymers and Polyethylene Glycol (PEG) - Lipids

In some embodiments, the lipid-based carrier (or lipid nanoformulation) may include one or more polymers or co-polymers, *e.g.*, poly(lactic-co-glycolic acid) (PFAG) nanoparticles.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) may include one or more polyethylene glycol (PEG) lipid. Examples of useful PEG-lipids include, but are not limited to, 1,2-Diacyl-sn-Glycero-3- Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-350] (mPEG 350 PE);

1,2-Diacyl-sn- Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-550] (mPEG 550 PE); 1,2- Diacyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-750] (mPEG 750 PE); 1,2-Diacyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-1000] (mPEG 1000 PE); 1,2-Diacyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (mPEG 2000 PE); 1,2-Diacyl-sn-Glycero-3-Phosphoethanolamine-N- [Methoxy(Polyethylene glycol)-3000] (mPEG 3000 PE); 1,2-Diacyl-sn-Glycero-3- Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-5000] (mPEG 5000 PE); N-Acyl- Sphingosine-1-[Succinyl(Methoxy Polyethylene Glycol) 750] (mPEG 750 Ceramide); N-Acyl- Sphingosine-1-[Succinyl(Methoxy Polyethylene Glycol) 2000] (mPEG 2000 Ceramide); and N- Acyl-Sphingosine-1-[Succinyl(Methoxy Polyethylene Glycol) 5000] (mPEG 5000 Ceramide). In some embodiments, the PEG lipid is a polyethyleneglycol-diacylglycerol (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB) conjugate.

In some embodiments, the lipid-based carrier (or nanoformulation) includes one or more conjugated lipids (such as PEG-conjugated lipids or lipids conjugated to polymers described in Table 5 of WO 2019/217941, which is incorporated herein by reference in its entirety). In some embodiments, the one or more conjugated lipids is formulated with one or more ionic lipids (*e.g.*, non-cationic lipid such as a neutral or anionic, or zwitterionic lipid); and one or more sterols (*e.g.*, cholesterol).

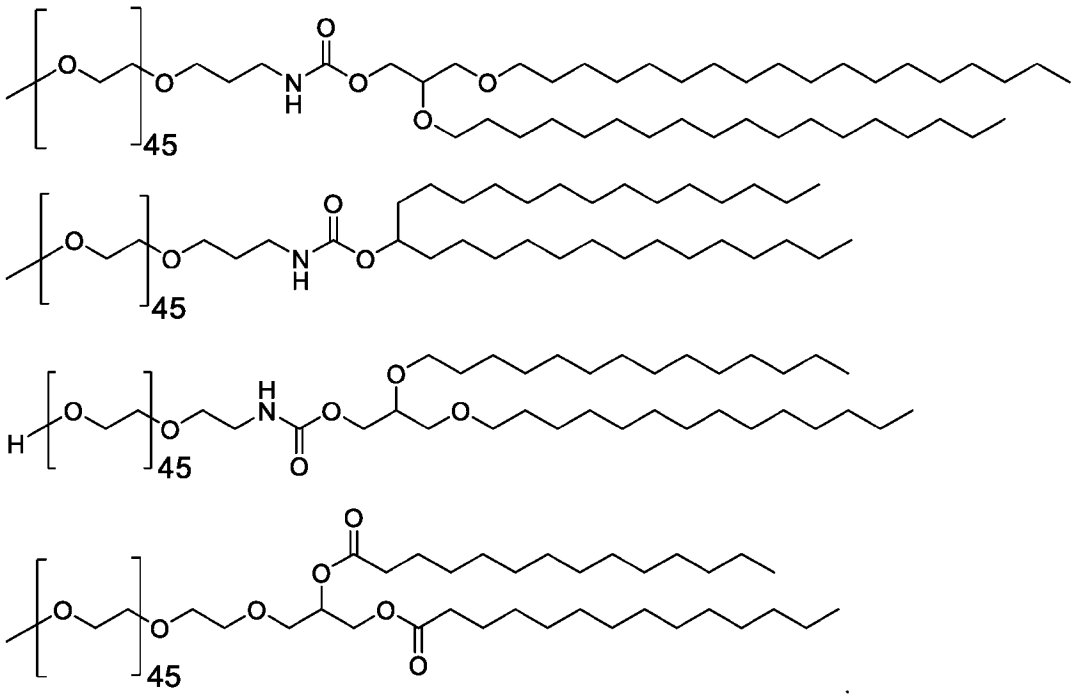
The PEG conjugate can comprise a PEG-dilaurylglycerol (C12), a PEG-dimyristylglycerol (C14), a PEG-dipalmitoylglycerol (C16), a PEG-disterylglycerol (C18), PEG-dilaurylglycamide (C12), PEG-dimyristylglycamide (C14), PEG-dipalmitoylglycamide (C16), and PEG-disterylglycamide (C18).

In some embodiments, conjugated lipids, when present, can include one or more of PEG-diacylglycerol (DAG) (such as l-(monomethoxy-polyethyleneglycol)-2,3- dimyristoylglycerol (PEG-DMG)), PEG-dialkyloxypropyl (DAA), PEG-phospholipid, PEG- ceramide (Cer), a pegylated phosphatidylethanoloamine (PEG-PE), PEG succinate diacylglycerol (PEGS-DAG) (such as 4-0-(2',3'-di(tetradecanoyloxy)propyl-l-0-(w- methoxy(polyethoxy)ethyl) butanedioate (PEG-S-DMG)), PEG dialkoxypropylcarbam, N- (carbonyl-methoxypolyethylene glycol 2000)- 1 ,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt, and those described in Table 2 of WO 2019/051289 (which is herein incorporated by reference in its entirety), and combinations of the foregoing.

Additional exemplary PEG-lipid conjugates are described, for example, in US 5,885,613, US 6,287,591, US 2003/0077829, US 2003/0077829, US 2005/0175682, US 2008/0020058, US 2011/0117125, US 2010/0130588, US 2016/0376224, US 2017/0119904, US 2018/0028664, and WO 2017/099823, all of which are incorporated herein by reference in their entirety.

In some embodiments, the PEG-lipid is a compound of Formula III, III-a-I, III-a-2, III-b-1, III-b-2, or V of US 2018/0028664, which is incorporated herein by reference in its entirety. In some embodiments, the PEG-lipid is of Formula II of US 2015/0376115 or US 2016/0376224, both of which

are incorporated herein by reference in their entirety. In some embodiments, the PEG-DAA conjugate can be, for example, PEG-dilauryloxypropyl, PEG- dimyristyloxypropyl, PEG-dipalmityloxypropyl, or PEG-distearyloxypropyl. In some embodiments, the PEG-lipid includes one of the following:



In some embodiments, lipids conjugated with a molecule other than a PEG can also be used in place of PEG-lipid. For example, polyoxazoline (POZ)-lipid conjugates, polyamide-lipid conjugates (such as ATTA-lipid conjugates), and cationic-polymer lipid (GPL) conjugates can be used in place of or in addition to the PEG-lipid.

Exemplary conjugated lipids, *e.g.*, PEG-lipids, (POZ)-lipid conjugates, ATTA-lipid conjugates and cationic polymer-lipids, include those described in Table 2 of WO 2019/051289A9, which is incorporated herein by reference in its entirety.

In some embodiments, the conjugated lipid (*e.g.*, the PEGylated lipid) can be present in an amount of 0-20 mol% of the total lipid components present in the lipid-based carrier (or lipid nanoformulation). In some embodiments, the conjugated lipid (*e.g.*, the PEGylated lipid) content is 0.5-10 mol% or 2-5 mol% of the total lipid components.

When needed, the lipid-based carrier (or lipid nanoformulation) described herein may be coated with a polymer layer to enhance stability *in vivo* (*e.g.*, sterically stabilized LNPs).

Examples of suitable polymers include, but are not limited to, poly(ethylene glycol), which may form a hydrophilic surface layer that improves the circulation half-life of liposomes and enhances the amount of lipid nanoformulations (*e.g.*, liposomes or LNPs) that reach therapeutic targets. See, *e.g.*,

Working et al. *J Pharmacol Exp Ther*, 289: 1128-1133 (1999); Gabizon et al., *J Controlled Release* 53: 275-279 (1998); Adlakha Hutcheon et al., *Nat Biotechnol* 17: 775-779 (1999); and Koning et al., *Biochim Biophys Acta* 1420: 153-167 (1999), which are incorporated herein by reference in their entirety.

Percentages of Lipid Nanoformulation Components

In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises one of more of the compounds described herein, optionally a non-cationic lipid (*e.g.*, a phospholipid), a sterol, a neutral lipid, and optionally conjugated lipid (*e.g.*, a PEGylated lipid) that inhibits aggregation of particles. In some embodiments, the lipid-based carrier (or lipid nanoformulation) further comprises a payload (e.g., a DNA molecule described herein). The amounts of these components can be varied independently and to achieve desired properties. For example, in some embodiments, the ionizable lipid including the lipid compounds described herein is present in an amount from about 20 mol% to about 100 mol% (*e.g.*, 20-90 mol%, 20-80 mol%, 20-70 mol%, 25-100 mol%, 30-70 mol%, 30-60 mol%, 30-40 mol%, 40-50 mol%, or 50-90 mol%) of the total lipid components; a non-cationic lipid (*e.g.*, phospholipid) is present in an amount from about 0 mol% to about 50 mol% (*e.g.*, 0-40 mol%, 0-30 mol%, 5-50 mol%, 5-40 mol%, 5-30 mol%, or 5-10 mol%) of the total lipid components, a conjugated lipid (*e.g.*, a PEGylated lipid) in an amount from about 0.5 mol% to about 20 mol% (*e.g.*, 1-10 mol% or 5-10%) of the total lipid components, and a sterol in an amount from about 0 mol % to about 60 mol% (*e.g.*, 0-50 mol%, 10-60 mol%, 10-50 mol%, 15-60 mol%, 15-50 mol%, 20-50 mol%, 20-40 mol%) of the total lipid components, provided that the total mol% of the lipid component does not exceed 100%.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises about 25-100 mol% of the ionizable lipid including the lipid compounds described herein, about 0-50 mol% phospholipid, about 0-50 mol% sterol, and about 0-10 mol% PEGylated lipid.

In some embodiments, the lipid-based carrier comprises a payload (e.g., a DNA molecule described herein) that is formulated in a lipid nanoparticle, wherein the lipid nanoparticle comprises about 25-100 mol% of the ionizable lipid including the lipid compounds described herein, about 0-50 mol% phospholipid, about 0-50 mol% sterol, and about 0-10 mol% PEGylated lipid. In some embodiments, the encapsulation efficiency of the payload may be at least 70%.

In one embodiment, the lipid-based carrier (or lipid nanoformulation) comprises about 25-100 mol% of the ionizable lipid including the lipid compounds described herein; about 0-40 mol% phospholipid (*e.g.*, DSPC), about 0-50 mol% sterol (*e.g.*, cholesterol), and about 0-10 mol% PEGylated lipid.

In some embodiments, the lipid-based carrier comprises a payload (e.g., a DNA molecule described herein) that is formulated in a lipid nanoparticle, wherein the lipid nanoparticle comprises about

25-100 mol% of the ionizable lipid including the lipid compounds described herein; about 0-40 mol% phospholipid (*e.g.*, DSPC), about 0-50 mol% sterol (*e.g.*, cholesterol), and about 0-10 mol% PEGylated lipid. In some embodiments, the encapsulation efficiency of the payload may be at least 70%.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises about 30-60 mol% (*e.g.*, about 35-55 mol%, or about 40-50 mol%) of the ionizable lipid including the lipid compounds described herein, about 0-30 mol% (*e.g.*, 5-25 mol%, or 10-20 mol%) phospholipid, about 15-50 mol% (*e.g.*, 18.5-48.5 mol%, or 30-40 mol%) sterol, and about 0-10 mol% (*e.g.*, 1-5 mol%, or 1.5-2.5 mol%) PEGylated lipid.

In some embodiments, the lipid-based carrier comprises a payload (e.g., a DNA molecule described herein) that is formulated in a lipid nanoparticle, wherein the lipid nanoparticle comprises about 30-60 mol% (*e.g.*, about 35-55 mol%, or about 40-50 mol%) of the ionizable lipid including the lipid compounds described herein, about 0-30 mol% (*e.g.*, 5-25 mol%, or 10-20 mol%) phospholipid, about 15-50 mol% (*e.g.*, 18.5-48.5 mol%, or 30-40 mol%) sterol, and about 0-10 mol% (*e.g.*, 1-5 mol%, or 1.5-2.5 mol%) PEGylated lipid. In some embodiments, the encapsulation efficiency of the payload may be at least 70%.

In some embodiments, molar ratios of ionizable lipid/sterol/phospholipid (or another structural lipid)/PEG-lipid/additional components is varied in the following ranges: ionizable lipid (25-100%); phospholipid (DSPC) (0-40%); sterol (0-50%); and PEG lipid (0-5%).

In some embodiments, the lipid-based carrier comprises a payload (e.g., a DNA molecule described herein) that is formulated in a lipid nanoparticle, wherein the lipid nanoparticle comprises molar ratios of ionizable lipid/sterol/phospholipid (or another structural lipid)/PEG-lipid/additional components in the following ranges: ionizable lipid (25-100%); phospholipid (DSPC) (0-40%); sterol (0-50%); and PEG lipid (0-5%). In some embodiments, the encapsulation efficiency of the payload may be at least 70%.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises, by mol% or wt% of the total lipid components, 50-75% ionizable lipid (including the lipid compound as described herein), 20-40% sterol (*e.g.*, cholesterol or derivative), 0 to 10% non-cationic-lipid, and 1-10% conjugated lipid (*e.g.*, the PEGylated lipid).

In some embodiments, the lipid-based carrier comprises a payload (e.g., a DNA molecule described herein) that is formulated in a lipid nanoparticle, wherein the lipid nanoparticle comprises, by mol% or wt% of the total lipid components, 50-75% ionizable lipid (including the lipid compound as described herein), 20-40% sterol (*e.g.*, cholesterol or derivative), 0 to 10% non-cationic-lipid, and 1-10% conjugated lipid (*e.g.*, the PEGylated lipid). In some embodiments, the encapsulation efficiency of the payload may be at least 70%.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises (i) a DNA molecule described herein; (ii) a cationic lipid comprising from 50 mol% to 65 mol% of the total lipid present in the lipid-based carrier; (iii) a non-cationic lipid comprising a mixture of a phospholipid and a cholesterol derivative thereof, wherein the phospholipid comprises from 3 mol% to 15 mol% of the total lipid present in the lipid-based carrier and the cholesterol or derivative thereof comprises from 30 mol% to 40 mol% of the total lipid present in the lipid-based carrier; and (iv) a conjugated lipid comprising 0.5 mol% to 2 mol% of the total lipid present in the particle.

In some embodiments, the lipid-based carrier (or lipid nanoformulation) comprises (i) a DNA molecule described herein; (ii) a cationic lipid comprising from 50 mol % to 85 mol % of the total lipid present in the lipid-based carrier; (iii) a non-cationic lipid comprising from 13 mol % to 49.5 mol % of the total lipid present in the lipid-based carrier; and (d) a conjugated lipid comprising from 0.5 mol % to 2 mol % of the total lipid present in the lipid-based carrier.

In some embodiments, the phospholipid component in the mixture may be present from 2 mol% to 20 mol%, from 2 mol% to 15 mol%, from 2 mol% to 12 mol%, from 4 mol% to 15 mol%, from 4 mol% to 10 mol%, from 5 mol% to 10 mol%, (or any fraction of these ranges) of the total lipid components. In some embodiments, the lipid-based carrier (or lipid nanoformulation) is phospholipid-free.

In some embodiments, the sterol component (*e.g.* cholesterol or derivative) in the mixture may comprise from 25 mol% to 45 mol%, from 25 mol% to 40 mol%, from 25 mol% to 35 mol%, from 25 mol% to 30 mol%, from 30 mol% to 45 mol%, from 30 mol% to 40 mol%, from 30 mol% to 35 mol%, from 35 mol% to 40 mol%, from 27 mol% to 37 mol%, or from 27 mol% to 35 mol% (or any fraction of these ranges) of the total lipid components.

In some embodiments, the non-ionizable lipid components in the lipid-based carrier (or lipid nanoformulation) may be present from 5 mol% to 90 mol%, from 10 mol% to 85 mol%, or from 20 mol% to 80 mol% (or any fraction of these ranges) of the total lipid components.

The ratio of total lipid components to the payload (*e.g.*, an encapsulated therapeutic agent such as a DNA molecule described herein can be varied as desired. For example, the total lipid components to the payload (mass or weight) ratio can be from about 10:1 to about 30:1. In some embodiments, the total lipid components to the payload ratio (mass/mass ratio; w/w ratio) can be in the range of from about 1:1 to about 25:1, from about 10:1 to about 14:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1. The amounts of total lipid components and the payload can be adjusted to provide a desired N/P ratio, for example, N/P ratio of 3, 4, 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, or higher. Generally, the lipid-based carrier (or lipid nanoformulation's) overall lipid content can range from about 5 mg/ml to about 30

mg/mL. Nitrogen:phosphate ratios (N:P ratio) is evaluated at values between 0.1 and 100.

The efficiency of encapsulation of a payload such as a protein and/or nucleic acid, describes the amount of protein and/or nucleic acid that is encapsulated or otherwise associated with a lipid nanoformulation (*e.g.*, liposome or LNP) after preparation, relative to the initial amount provided. The encapsulation efficiency is desirably high (*e.g.*, at least 70%, at least 80%, at least 90%, at least 95%, or close to 100%). In some embodiments, the encapsulation efficiency is at least 70%, 80%, 90%, 95%, or close to 100%. The encapsulation efficiency may be measured, for example, by comparing the amount of protein or nucleic acid in a solution containing the liposome or LNP before and after breaking up the liposome or LNP with one or more organic solvents or detergents. An anion exchange resin may be used to measure the amount of free protein or nucleic acid (*e.g.*, RNA) in a solution. Fluorescence may be used to measure the amount of free protein and/or nucleic acid (*e.g.*, RNA) in a solution. For the lipid-based carrier (or lipid nanoformulation) described herein, the encapsulation efficiency of a protein and/or nucleic acid may be 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 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. For the lipid-based carrier (or lipid nanoformulation) described herein, the encapsulation efficiency of a protein and/or nucleic acid may be at least 50%, for example 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the encapsulation efficiency may be at least 70%. In some embodiments, the encapsulation efficiency may be at least 80%. In some embodiments, the encapsulation efficiency may be at least 90%. In some embodiments, the encapsulation efficiency may be at least 95%.

Route of administration

A dsDNA molecule described herein is introduced into a cell, tissue or subject by any suitable route.

Administration to a target cell or tissue (e.g., ex vivo) may be by methods known in the art such as transfection, e.g., transient or stable transfection using reagents (e.g., liposomal, calcium phosphate) or physical means (e.g., electroporation, gene gun, microinjection, microfluidic fluid shear, cell squeezing). Other methods are described, e.g., in Rad et al. 2021. Adv. Mater. 33:2005363, which is incorporated herein by reference.

Administration to a subject, e.g., a mammal, e.g., a human subject, may be by parenteral (e.g., intravenous, intramuscular, intraperitoneal, subcutaneous, or intracranial) route; by topical administration, transdermal administration or transcutaneous administration. Other suitable routes include oral, rectal, transmucosal, intranasal, inhalation (e.g., via an aerosol), buccal (e.g., sublingual), vaginal, intrathecal,

intraocular, transdermal, intraendothelial, in utero (or in ovo), intrapleural, intracerebral, intraarticular, topical, intralymphatic. Also included is direct tissue or organ injection (e.g., to liver, eye, skeletal muscle, cardiac muscle, diaphragm, muscle or brain).

Applications

The DNA molecule (e.g., dsDNA molecule) described herein can be used in therapeutic or health applications for a subject, e.g., a human or non-human animal. Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal. The subject can be any animal, e.g., a mammal, e.g., a human or non-human mammal. In embodiments, the subject is a vertebrate animal (e.g., mammal, bird, fish, reptile, or amphibian). In embodiments, the subject is a human. In embodiments, the method subject is a non-human mammal. In embodiments, the subject is a non-human mammal is such as a non-human primate (e.g., monkeys, apes), ungulate (e.g., cattle, buffalo, sheep, goat, pig, camel, llama, alpaca, deer, horses, donkeys), carnivore (e.g., dog, cat), rodent (e.g., rat, mouse), or lagomorph (e.g., rabbit). In embodiments, the subject is a bird, such as a member of the avian taxa Galliformes (e.g., chickens, turkeys, pheasants, quail), Anseriformes (e.g., ducks, geese), Paleaognathae (e.g., ostriches, emus), Columbiformes (e.g., pigeons, doves), or Psittaciformes (e.g., parrots). In embodiments, the subject is an invertebrate such as an arthropod (e.g., insects, arachnids, crustaceans), a nematode, an annelid, a helminth, or a mollusk.

In some embodiments, a DNA described herein is provided at a dose of about 0.1-100 mg/kg of the DNA.

In some embodiments, a DNA molecule (e.g., dsDNA molecule) described herein imparts a biological effect of the effector, e.g., expression of a therapeutic polypeptide, on a host cell, tissue or subject over a time period of at least 2, at least 3, at least 4, at least 5, at least 6 days or at least a week; at least 8, at least 9, at least 10, at least 12, at least 14 days or at least two weeks; at least 16, at least 18, at least 20 days or at least 3 weeks; at least 22, at least 24, at least 25, at least 27, at least 28 days or at least a month; at least 2 months, 3 months, 4 months, 5 months, 6 months or more; between one week and 6 months, between 1 month to 6 months, between 3 months to 6 months. In some embodiments, a dsDNA molecule described herein imparts a biological effect of the effector, e.g., expression of a therapeutic polypeptide, on a host cell, tissue or subject over a time period of at least 2, at least 3, at least 4, at least 5, at least 6 days or at least a week; at least at least 8 days, at least 9 days, at least 10 days, at least 12 days, at least 14 days or two weeks; at least 16 days, at least 18 days, at least 20 days or at least 3 weeks; at least 22 days, at least 24 days, at least 25 days, at least 27 days, at least 28 days or at least a month; at

least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months or more; between one week and 6 months, between 1 month to 6 months, between 3 months to 6 months.

In some embodiments, a DNA molecule (e.g., dsDNA molecule) described herein imparts a biological effect of the effector, e.g., expression of a therapeutic polypeptide, on a host cell, tissue or subject over a time period of at least 1 cell divisions of the host cell.

In embodiments, a DNA molecule (e.g., dsDNA molecule) described herein can be used to deliver an effector, e.g., an effector described herein, to a cell, tissue or subject.

In embodiments, a DNA molecule (e.g., dsDNA molecule) described herein can be used to modulate (e.g., increase or decrease) a biological parameter in a cell, tissue or subject. The biological parameter may be an increase or decrease in gene expression of a subject gene in a target cell, tissue or subject.

In embodiments, a DNA molecule (e.g., dsDNA molecule) described herein can be used to treat a cell, tissue or subject in need thereof by administering a DNA molecule (e.g., dsDNA molecule) described herein to such cell, tissue or subject. In an embodiment, the subject has or has been diagnosed with a condition that can be treated with an effector encoded in the dsDNA.

In some embodiments, the present disclosure provides a method of modulating (e.g., increasing or decreasing) a biological activity in a target cell, the method comprising: (i) providing a target cell comprising a DNA molecule (e.g., dsDNA molecule) as described herein, wherein the DNA molecule (e.g., dsDNA molecule) encodes an effector that modulates a biological activity in the target cell; and (ii) maintaining (e.g., incubating) the cell under conditions suitable for expressing the heterologous effector from the DNA molecule (e.g., dsDNA molecule); thereby modulating the biological activity in the target cell. In some embodiments, the present disclosure provides a method of modulating (e.g., increasing or decreasing) a biological activity in a target cell, the method comprising: (i) providing a target cell comprising a DNA molecule (e.g., dsDNA molecule) as described herein, wherein the DNA molecule (e.g., dsDNA molecule) comprises an effector sequence encoding an effector that modulates a biological activity in the target cell; and (ii) maintaining (e.g., incubating) the cell under conditions suitable for expressing the effector from the DNA molecule (e.g., dsDNA molecule); thereby modulating the biological activity in the target cell.

In embodiments, the DNA molecule (e.g., dsDNA molecule) delivers an effector to a cell, e.g., an epidermal cell (e.g., a keratinocyte).

EXAMPLES

Table of Contents

Example 1: Formulation of a dsDNA molecule (e.g., TDSC) with LNP

This example describes how to formulate the constructs made as described herein with a lipid nanoparticle (LNP).

Nucleic acid constructs are combined with lipid components via microfluidic devices according to the method of Chen et al. 2012. J Am Chem Soc. Volume 134, Issue 16:6948-6951. Briefly, the microfluidic devices are fabricated in polydimethylsiloxane (PDMS) according to standard lithographic procedures (McDonald & Whitesides. 2002. Accounts Chem Res Volume 35, Issue 7:491-499). The lipid components, typically containing cationic lipids, cholesterol, helper lipids, polyethylene glycol modified lipids, and lipids facilitating targeting moiety conjugation (optional), are combined and solubilized in 90% ethanol. The nucleic acid constructs are dissolved in buffer. The nucleic acid solution, the lipid solution, and phosphate buffer saline (PBS) are injected into the microfluidic device. The freshly prepared LNPs are dialyzed against PBS buffer using membranes with MWCO of 3.5kD to remove ethanol and exchange buffer.

The LNPs are characterized in terms of effective diameter, polydispersity, and zeta potential using dynamic light scattering (DLS) (ZetaPALS, Brookhaven Instruments, NY, 15-mW laser, incident beam 676 nm); and total nucleic acid concentration is determined by lysing the particles and using Quant-iT™ 1X dsDNA Assay Kits, High Sensitivity (HS) and Broad Range (BR) (ThermoFisher Scientific, Q33232).

Example 2: Determining exonuclease resistance for a dsDNA molecule comprising closed ends

This example describes how to test if a dsDNA molecule comprising closed ends (e.g., an adaptor-ligated linear dsDNA construct) is Exonuclease III (M0206, New England Biolabs Inc.) resistant. The dsDNA molecule is tested next to a non-nuclease control. The non-nuclease control contains DNA with the identical sequence to the dsDNA molecule of interest except that it underwent the adaptor ligation protocol that is used to add the exonuclease-resistant DNA end form to the dsDNA molecule, but without an adaptor oligonucleotide added to the mixture. 1 µL of Exonuclease III (at a starting concentration of 100 units/uL) is added per 5 µg of DNA in 50 µL. The tubes are mixed well and spun down. The tubes are run on the thermocycler for 1 hour at 37 °C, and heat inactivated at 70 °C for 30 minutes.

The samples are concentrated via the Nucleospin® Gel and PCR Clean-up kit (catalog # 740609, Macherey-Nagel) using a vacuum manifold. Briefly, the elution buffer is warmed to 70 °C. 2x volumes of NTI binding buffer are added to 1x volume of Exo III-treated DNA. The samples are mixed until evenly distributed and left at room temperature for 5 minutes. The column on the vacuum manifold is secured, valve opened, and vacuum turned on. 375 µL DNA-NTI mix is added to 2x columns and allowed to fully pass through each column. 700 µL of NTC wash buffer is added twice. The column is removed from the vacuum manifold and placed into a collection tube. The assembly is centrifuged at 11,000 xg for 1 minute. The column is placed into a new low bind microcentrifuge tube, 25 µL of prewarmed buffer is added, and the assembly is incubated at 70 °C for 5 min. The assembly is centrifuged at 11,000 xg for 1 min. The incubation and elution steps are repeated a second time. The collected DNA is quantified by dsDNA BR Qubit (Q32850, Thermo Fisher Scientific) on the Qubit 4 Fluorometer (Q33226, Thermo Fisher Scientific).

The samples are loaded into E-Gel EX, 1% Agarose Gel (G402021, Thermo Fisher Scientific) in individual wells at an amount of 16 ng of DNA per well. The ladder (10488090, Thermo Fisher Scientific) is loaded at 2 µl into the left most lane of the gel. The gel is run through the E-Gel Power Snap Electrophoresis System (G8100, G8200, Thermo Fisher Scientific). After the gel is run, the exonuclease-resistant dsDNA molecule is visible at the molecular weight corresponding to the full-length DNA plus closed-adapter sequence. A dsDNA molecule will be considered exonuclease-resistant in this assay if at least 95% of the product that appears in the gel in that lane corresponds to the full-length dsDNA molecule.

Example 3: Determining exonuclease resistance for a dsDNA molecule comprising an open end (e.g., two open ends)

This example describes how to test if a dsDNA molecule comprising an open end (e.g., an adaptor-ligated linear dsDNA construct) is Exonuclease III (M0206, New England Biolabs Inc.) resistant. The dsDNA molecule is tested next to a non-nuclease control. The non-nuclease control contains DNA with the identical sequence to the dsDNA molecule of interest except that it underwent the adaptor ligation protocol that is used to add the exonuclease-resistant DNA end form to the dsDNA molecule, but without an adaptor oligonucleotide added to the mixture. 2 units of Exonuclease III are added per 200 ng of DNA (at 10 ng/ul), in a 20 ul reaction. The tubes are mixed well and spun down. The tubes are run on the thermocycler for 30 min at 37 °C.

The samples are loaded into E-Gel EX, 1% Agarose Gel (G402021, Thermo Fisher Scientific) in individual wells at an amount of 20 ng of DNA per well. The ladder (10488090, Thermo Fisher Scientific) is loaded at 2 µl into the left most lane of the gel. The gel is run through the E-Gel Power Snap Electrophoresis System (G8100, G8200, Thermo Fisher Scientific). After the gel is run, the exonuclease-resistant dsDNA molecule is visible at the molecular weight corresponding to the full-length DNA plus closed-adapter sequence. A dsDNA molecule will be considered exonuclease-resistant in this assay if at least 95% of the product that appears in the gel in that lane corresponds to the full-length dsDNA molecule.

Example 4: Design and assembly of a plasmid template for production of double-stranded DNA (dsDNA) molecules

This example describes production of a plasmid template for a dsDNA molecule. In this example, a construct template was designed with the following specific sequence components.

- Promoter Ef1a:

5'ggctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccgagaagttgggggggagggtcggcaattgaaccggtgc
ctagagaaggtggcgcggggtaaactgggaaagtgatgtcgtgtactggctccgccttttcccgagggtgggggagaaccgtatataagt
gcagtagtcgccgtgaacgttcttttcgcaacgggtttgccgccagaacacaggtaagtgccgtgtgtggttcccgcgggcctggcctcttta
cgggttatggcccttgcgtgccttgaattacttccacctggctgcagtacgtgattcttgatcccgagcttcgggttggaagtgggtgggagag
ttcgaggccttgcgcttaaggagccccttcgcctcgtgcttgagttgaggcctggcctgggcgctggggccgccgcgtgcgaatctggtgg
cacctttcgcgcctgtctcgctgctttcgataagtctctagccatttaaaatttttgatgacctgctgcgacgctttttttctggcaagatagtcttgta
aatgcgggccaagatctgcacactggtatttcggttttttgggggccgcgggcggcgacggggcccgtgcgtcccagcgcacatgttcggcg
aggcggggcctgcgagcgcggccaccgagaatcggacgggggtagtctcaagctggccggcctgctctggtgcctggcctcgcgccgc
cgtgtatcgccccgccctgggcggcaaggctggcccggtcggcaccagttgcgtgagcggaaagatggccgcttcccggccctgctgca
gggagctcaaaatggaggacgcggcgctcgggagagcgggcgggtgagtcacccacacaaaggaaaagggcctttccgtcctcagccg
tcgcttcatgtgactccacggagtaccgggcgccgtccaggcacctcgattagttctcgagcttttggagtacgtcgtctttaggttgggggga
ggggttttatgcgatggagtttccccacactgagtgggtggagactgaagttaggccagcttggcacttgatgtaattctccttggaatttgccct
ttttgagtttggatcttggttcattctcaagcctcagacagtggttcaaagttttttttcttccatttcaggtgtcgtga-3' (SEQ ID NO: 37)

- Effector sequence encoding a model/marker protein (mCherry):

5'atggtgagcaagggcgaggaggataacatggccatcatcaaggagttcatgcgcttcaaggtgcacatggagggctccgtgaacggcc
acgagttcgagatcgagggcgagggcgagggccgcccctacgagggcacccagaccgccaagctgaaggtgaccaagggtggcccc
ctgcccttcgcctgggacatcctgtcccctcagttcatgtacggctccaaggcctacgtgaagcaccccgccgacatccccgactacttgaag
ctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtgaccgtgacccaggactcctccctgcag
gacggcgagttcatctacaaggtgaagctgcgcggcaccaacttccctccgacggccccgtaatgcagaagaagaccatgggctggga
ggcctcctccgagcggatgtaccccgaggacggcgccctgaagggcgagatcaagcagaggctgaagctgaaggacggcggccacta
cgacgctgaggtcaagaccacctacaaggccaagaagcccgtgcagctgccccggcgcctacaacgtcaacatcaagttggacatcacctc
ccacaacgaggactacaccatcgtggaacagtacgaacgcgccgagggccgccactccaccggcggcatggacgagctgtacaagtaa
-3' (SEQ ID NO: 38)

Optional:

- NTS: SV40 enhancer: 5'-cccaagaagaagaggaaagtc-3' (SEQ ID NO: 1)
- Maintenance sequence: human interferon-β MAR
  5'tataattcactggaatttttttgtgtgtatggtatgacatatgggttccctttattttttacatataaatatatttccctgtttttctaaaaaagaaaaag
  atcatcattttcccattgtaaaatgccatatttttttcataggtcacttacata3' (SEQ ID NO: 39)
- Second strand motif: AAV2 wildtype ITR
  5'aggaacccctagtgatggagttggccactccctctctgcgcgctcgctcgctcactgaggccgggcgaccaaaggtcgcccgacgccc
  gggctttgcccgggcggcctcagtgagcgagcgagcgcgcagctgcctgcagg-3' (SEQ ID NO: 26)

A plasmid template was designed with these elements using standard DNA design manipulation software. Once designed, plasmids were ordered from a commercial supplier (GenScript) for use as a template in PCR amplification.

Example 5: Quantification of DNA chemical modifications in vitro

This example describes the quantification of modified nucleotides within chemically modified dsDNA molecules.

Enriched dsDNA molecules with chemically modified nucleotides are prepared from a plasmid template as described herein. The proportion of modified nucleotides is quantified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as previously described in Bachman et al., 2014, *Nature Chemistry* volume 6 issue 12 pages 1049-1055). Briefly, DNA is degraded to nucleosides via incubation with DNA Degradase Plus (Zymo Research). Following enzymatic digestion, LC-MS/MS analysis is conducted on a mass spectrometer fitted with a liquid chromatography system. Calibration curves are generated using a mixture of synthetic standards in the ranges of 0.01-100 μM for deoxycytosine and 0.0001-1 μM for chemically modified nucleotides, respectively. Samples and synthetic standards are spiked with an isotopically labeled mix of deoxycytosine and chemically modified derivatives as an internal standard. The mass spectrometer is operated in multiple reaction monitoring (MRM) mode. The ion source is electrospray in positive mode. Results are expressed as a percentage of total nucleotides of that type.

Example 6: Validation of chemically modified DNA sequences in cells

This example describes the sequence validation of chemically modified DNAs delivered to cells.

Enriched dsDNA constructs with native or chemically modified nucleotides are prepared and delivered to cells as described herein. Following transfection, DNA and RNA are simultaneously extracted from cells (AllPrep DNA/RNA Kit, Qiagen) and converted into libraries for next-generation sequencing (Illumina). Demultiplexed reads are mapped to the sequence of the chemically modified dsDNA constructs as described in Langmead and Salzberg, 2012, Nature Methods volume 9 pages 357-359. Mutations are identified via standard variant calling programs.

Example 7: Design and assembly of a plasmid template for production of double-stranded DNA (dsDNA) molecules

This example describes production of a plasmid template for a dsDNA molecule. In this example, a construct template was designed with the following specific sequence components.

- Promoter Ef1a:

5'ggctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccgagaagttgggggggagggtcggcaattgaaccggtgc
ctagagaaggtggcgcggggtaaactgggaaagtgatgtcgtgtactggctccgcctttttcccgagggtgggggagaaccgtatataagt
gcagtagtcgccgtgaacgttcttttcgcaacgggtttgccgccagaacacaggtaagtgccgtgtgtggttcccgcgggcctggcctcttta
cgggttatggcccttgcgtgccttgaattacttccacctggctgcagtacgtgattcttgatcccgagcttcgggttggaagtgggtgggagag
ttcgaggccttgcgcttaaggagccccttcgcctcgtgcttgagttgaggcctggcctgggcgctggggccgccgcgtgcgaatctggtgg
cacctttcgcgcctgtctcgctgctttcgataagtctctagccatttaaaatttttgatgacctgctgcgacgcttttttttctggcaagatagtcttgta
aatgcgggccaagatctgcacactggtatttcggttttgggggccgcgggcggcgacgggggccgtgcgtccccagcgcacatgttcggcg
aggcggggcctgcgagcgcggccaccgagaatcggacgggggtagtctcaagctggccggcctgctctggtgcctggcctcgcgccgcc
cgtgtatcgccccgccctgggcggcaaggctggcccggtcggcaccagttgcgtgagcggaaagatggccgcttcccggccctgctgca
gggagctcaaaatggaggacgcggcgctcgggagagcgggcgggtgagtcacccacacaaaggaaaagggcctttccgtcctcagccg
tcgcttcatgtgactccacggagtaccgggcgccgtccaggcacctcgattagttctcgagcttttggagtacgtcgtctttaggttgggggga
ggggttttatgcgatggagtttccccacactgagtgggtggagactgaagttaggccagcttggcacttgatgtaattctccttggaatttgccct
ttttgagtttggatcttggttcattctcaagcctcagacagtggttcaaagttttttcttccatttcaggtgtcgtga-3' (SEQ ID NO: 71)

- Effector sequence encoding a model protein (mCherry):

5'atggtgagcaagggcgaggaggataacatggccatcatcaaggagttcatgcgcttcaaggtgcacatggagggctccgtgaacggcc
acgagttcgagatcgagggcgagggcgagggccgcccctacgagggcacccagaccgccaagctgaaggtgaccaagggtggcccc
ctgcccttcgcctgggacatcctgtcccctcagttcatgtacggctccaaggcctacgtgaagcaccccgccgacatccccgactacttgaag
ctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtgaccgtgacccaggactcctccctgcag
gacggcgagttcatctacaaggtgaagctgcgcggcaccaacttccctccgacggccccgtaatgcagaagaagaccatgggctggga
ggcctcctccgagcggatgtaccccgaggacggcgccctgaagggcgagatcaagcagaggctgaagctgaaggacggcggccacta
cgacgctgaggtcaagaccacctacaaggccaagaagcccgtgcagctgcccggcgcctacaacgtcaacatcaagttggacatcacctc
ccacaacgaggactacaccatcgtggaacagtacgaacgcgccgagggccgccactccaccggcggcatggacgagctgtacaagtaa
-3' (SEQ ID NO: 72)

Optional:

- SV40 enhancer sequence:

5'-ggtgtggaaagtccccaggctccccagcaggcagaagtatgcaaagcatgcatctcaattagtcagcaacca-3'(SEQ ID NO: 73)

- Chimeric intron:
  5'gtaagtatcaaggttacaagacaggtttaaggaaaccaatagaaactgggcttgtcgagacagagaagactcttgcgtttctgataggcac
  ctattggtcttactgacatccactttgcctttctctccacag-3'(SEQ ID NO: 74)
- Woodchuck hepatitis virus post transcriptional element (WPRE)
  5'aatcaacctctggattacaaaatttgtgaaagattgactggtattcttaactatgttgctccttttacgctatgtggatacgctgctttaatgccttt
  gtatcatgctattgcttcccgtatggctttcattttctcctccttgtataaatcctggttgctgtctctttatgaggagttgtggcccgttgtcaggcaa
  cgtggcgtggtgtgcactgtgtttgctgacgcaacccccactggttggggcattgccaccacctgtcagctcctttccgggactttcgctttccc
  cctccctattgccacggcggaactcatcgccgcctgccttgcccgctgctggacaggggctcggctgttgggcactgacaattccgtggtgtt
  gtcggggaaatcatcgtcctttccttggctgctcgcctgtgttgccacctggattctgcgcgggacgtccttctgctacgtcccttcggccctca
  atccagcggaccttccttcccgcggcctgctgccggctctgcggcctcttccgcgtcttcgccttcgccctcagacgagtcggatctccctttg
  ggccgcctccccgc-3' (SEQ ID NO: 75)
- Bovine growth hormone poly-A sequence
  5'ctgtgccttctagttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctggaaggtgccactcccactgtcctttcctaataa
  aatgaggaaattgcatcgcattgtctgagtaggtgtcattctattctgggggggtggggtggggcaggacagcaagggggaggattgggaag
  acaatagcaggcatgctggggatgcggtgggctctatgg (SEQ ID NO: 76)

A plasmid template was designed with these elements using standard DNA design manipulation software. Once designed, plasmids were prepared according to standard methods for use as a template in PCR amplification.

Example 8: Design of adapter sequences for ligation to dsDNA molecules

This Example describes design of end adapters for ligation to dsDNA molecules to create covalently closed DNA with looped ends.

End adapters for DNA molecules were designed as shown below:

P-Pt6-AvrII-ADR:
/5Phos/CTAGTG*C*C*C*G*A*GCAGGATCGAGCCACACGTACTAC**GCTCGATCCTGC*T*C*G*G*G*CA** (SEQ ID NO: 77)

P-Pt6-INV-A2-ADR:
/5Phos/GATCTG*C*C*C*G*A*GCAGGATCGAGCCACACGTACTACGCTCGATCCTGC*T*C*G*G*G*CA (SEQ ID NO: 78)

In these sequences, /5Phos/ indicates that the following base is phosphorylated and the star (*) indicates a phosphorothioate linkage. DNA bases that form a dsDNA region are underlined. Bases that will be used as a primer for second-strand synthesis are in bold.

Adapters were made as DNA oligonucleotides and synthesized and purified per the standard methods.

Example 9: Production of looped-end DNA (leDNA) molecules

This Example demonstrates preparation of ssDNA molecules with termini comprising loops, i.e., looped-end DNA (leDNA) (illustrated in **FIG. 1A**).

Plasmid DNA (2ng/100 ul PCR reaction), e.g., as described in Example 7, was used as a template for PCR amplification using KOD -Multi & Epi- polymerase (TYB-KME-101, Diagnocine). Other commercially available polymerases such as KOD Xtreme or Deep Vent may also be used. PCR reaction conditions include:

a. PCR Buffer for KOD FX (TYB-KFX-1B, Diagnocine) at a 1x concentration.
b. Forward and reverse primers at a final concentration of 300 μM.
c. 2 units of KOD -Multi & Epi- polymerase/ 100 ul PCR reaction

For the synthesis of dsDNA molecules by PCR, in addition to containing sequences complementary to the plasmid, primers contained additional sequences useful in downstream processes:

a. Nicking enzyme(s) recognition sequence;
b. Restriction enzyme recognition sequence (e.g. BsaI), used to create sticky ends in the DNA after restriction enzyme digestion and facilitate adapter ligation; and
c. Additional bases (e.g., 5'- GGTCCTTC-3') to increase restriction enzyme digestion efficiency.

The following primers were used for PCR amplification:

- OUT-BsaI-UNIVER-F GGTCCTTCGGTCTCACTAGgctgcttcgcgatgtacgggccag (SEQ ID NO: 79)
- OUT-BsaI-INV-A2-R GGTCCTTCGGTCTCAGATCGCAATGAGCCATAGAGCCCACCGCATCCC CAG (SEQ ID NO: 80)

In these sequences, the bases specific for the template are in lower case, and the additional bases to create the sticky ends, including the BsaI recognition site, are shown in UPPER CASE.

Thermocycling was performed. For KOD -Multi & Epi- polymerase, the following reaction conditions were used: 1 cycle at 94°C for 2 minutes, and 40 repeats of the following cycles: 10 seconds at 98°C, 10 seconds at 63°C, and 80 seconds at 68°C.

The resulting PCR products were enriched using standard DNA concentration columns. DNA was then digested with the restriction enzyme(s) corresponding to the restriction site(s) encoded in the sequence (e.g., BsaI-HF-V2 (R3733, New England Biolabs), at 5 units per ug of DNA for 4-16 hours. After this incubation time, reactions were supplemented with 1 mM of ATP (P0756, New England Biolabs), T3 DNA ligase (M0317, New England Biolabs) at 18.75 units per ug of DNA, and 0.075 ul of each adapter (P-Pt6-AvrII-ADR and P-Pt6-INV-A2-ADR) at 100 uM per ug of DNA. These reactions

were incubated for 1 hour at 26°C to allow ligation, then 1 hour at 65°C to inactivate T3 DNA ligase. Following equilibration to room temperature, samples were treated with Exonuclease I (M0293, New England Biolabs) at 2 units/ug and Exonuclease III (M0206, New England Biolabs) at 10 units/ug. The resulting adapter-ligated products were concentrated using DNA concentration columns (740609, Takara). Thereafter, the synthesis of loop-ended ssDNA was performed in a single reaction, with a DNA nickase (e.g., Nb.BsrDI, R0648, New England Biolabs) added to the reaction at 2.5 units per ug of DNA to create a nick in the sense strand, while Exonuclease III (M0206, New England Biolabs), added at 10 units per ug DNA, facilitated the removal of the nicked strand as far as the phosphorothioate modifications present in the adapter.

Analysis of the composition of the resultant leDNA forms was performed on the Invitrogen E-gel system, using 1% EX gels (G401001, Invitrogen). leDNA forms were verified by color change from green (dsDNA) to orange (ssDNA). Upon validation, leDNA was concentrated from the exonuclease-treated samples using standard DNA concentration columns (740609, Takara). Formamide can optionally be added to E-gel wells to improve leDNA band migration uniformity.

Example 10: Production of hemi-modified end-closed dsDNA molecules

This Example demonstrates preparation of "hemi-modified" dsDNA molecules, i.e., dsDNA molecules containing chemically modified nucleobases on a single strand (illustrated in **FIG. 1B**). The starting material for hemi-modified dsDNA is the looped end DNA (leDNA) generated in Example 9.

To initiate 5' second-strand synthesis from the priming element of the end adapter (underlined nucleotides in Example 8), the polymerase requires a heat activation step. For this, KOD -Multi & Epi- polymerase was incubated at 94°C for two minutes. Thereafter, second-strand synthesis reactions were set up, consisting of 2 units of heat-activated KOD -Multi & Epi- polymerase, 1 microgram of looped-end DNA, dNTPs to a final concentration of 0.2 mM each, and KOD FX polymerase buffer (0.4-1x final concentration). Modified deoxynucleoside triphosphates were added at various ratios with their cognate dNTP, summing to a total of 200 μM. For instance, the cognate dNTP of 5-hydroxymethyluridine triphosphate, 5-formyluridine triphosphate, deoxyuridine triphosphate, 5-azidomethyluridine triphosphate, and 5-methylthiouridine is dTTP, the cognate dNTP of deoxyinosine triphosphate is dGTP, and the cognate dNTP of 5-hydroxycytosine is dCTP. A reaction designed for 25% incorporation would be 50 μM chemically modified dNTP and 150 μM cognate dNTP, whereas a reaction designed for 75% incorporation would be 150 μM chemically modified dNTP and 50 μM cognate dNTP. 100% incorporation entails complete replacement of the cognate dNTP with the modified dNTP.

Importantly, production of hemi-modified dsDNA entails isothermal extension of DNA from a primer, not a thermocycling process akin to polymerase chain reaction. To polymerize DNA starting from

the primer, reaction mixtures were incubated for 20 minutes at 68 °C. DNA was concentrated using standard DNA concentration columns.

Because KOD -Multi & Epi- polymerase does not have ligase activity, it does not connect the extended DNA to the short double stranded region near the downstream closed end; thus, there will be a nick in the sense DNA strand to be sealed in a ligation reaction. Therefore, ligation reactions consisting of 125 units of T4 DNA ligase (M0202M, New England Biolabs) per microgram of DNA, in a 20 µl 1x T4 DNA ligase buffer (B0202, New England Biolabs) volume, were incubated for 15 minutes at 25°C. To confirm that the nick was successfully repaired, 10 units of Exonuclease III (M0206, New England Biolabs) were added per microgram of DNA. Reactions were incubated for 30 minutes at 37 °C. Samples were run on the Invitrogen E-gel system with 1% EX gels (G401001, Invitrogen). Under standard conditions, bands corresponding to dsDNA appear green after approximately 20 minutes of incubation post-run, and bands corresponding to ssDNA appear orange. DNA was then concentrated using standard DNA concentration columns (740609, Takara).

Example 11: Assessment of reporter gene expression in vitro

This example demonstrates detection and quantification of gene expression using DNA molecules, e.g., looped end DNA (leDNA) or hemi-modified dsDNA molecules.

Experimental DNA molecules and controls were administered via lipid transfection (lipofection). Lipofection for DNA was performed using the Lipofectamine3000 transfection reagent (# L3000001, ThermoFisher) in HEKa cells. A 1:2:3 ratio of DNA:P3000:Lipofectamine3000 was used for all DNA constructs and controls. 10,000 to 30,000 cells were pre-seeded into each well of 96-well plates one day before transfection. Transfection was performed when cells reached roughly 80 to 90% confluence. For each well of a 96-well plate, 3X Lipofectamine3000 was first diluted in 5 uL of Opti-MEM™ I Reduced Serum Medium (#31985070, ThermoFisher). DNA was diluted in 5 uL Opti-MEM™ I Reduced Serum Medium with 2X P3000 reagent. The DNA was then added into the Lipofectamine3000 containing Opti-MEM™ I Reduced Serum Medium and mixed gently by pipetting. After incubating for 15 minutes at room temperature, the DNA-Lipofectamine3000 complex was added to target cells with full culture medium in a dropwise manner to different areas of the well. The plate was gently rocked back-and-forth and side-to-side to evenly distribute the DNA-Lipofectamine3000 complex. Following transfection, cells were incubated in a CO_2 tissue culture incubator, and culture medium was changed 6 to 8 hours after transfection.

To determine expression of constructs encoding the fluorescent reporter mCherry, cells were first washed with PBS before dissociation with 0.25% Trypsin (#25200056, ThermoFisher) to get single cell suspension. Cells were then stained with the live/dead fixable yellow dead cell stain kit (#L34959,

ThermoFisher) and fixed with 4% PFA (#J61899.AP, ThermoFisher). Cells were washed once with DPBS (#14190144, ThermoFisher) and then resuspended in DPBS. All flow cytometry was performed on Attune Nxt Flow Cytometer from ThermoFisher. For detection of mCherry signal, a yellow laser (wavelength 561 nm) was used for excitation and the YL2 620/15 emission filter was used. For live and death cell detection, a violet (405 nm) laser with the VL3 (603/48) filter was used. 10,000 events were recorded for each sample and data were analyzed using Flowjo V.9.0 software. Cells were first gated on FSC-A and SSC-A plot to remove cell debris. The population was further plotted on an FSC-A and FSC-H plot to circumscribe the single cell population. Cell viability was evaluated based on the signal intensity of the fixable live/dead yellow dye. Cells with low signal intensity were gated as live cells, while the population with high signal intensity was gated as dead cells. Finally, a bivariate plot between the fluorescent signal-expressing and non-expressing cells was used to determine the percentage of expressing cells in the live cell population. A distribution of expressing cells was used to determine the level of expression within each cell at 2 days post-transfection.

FIG. 9 shows that leDNA molecules and hemi-modified dsDNA molecules comprising various chemical modifications were functional, as defined by detectable expression of the reporter protein mCherry. Specifically, the proportion of mCherry+ cells and the total fluorescence intensity of cells transfected with leDNA molecules and hemi-modified dsDNA molecules were comparable to, and in some cases greater than, control covalently closed dsDNA (comprising phosphorothioate modifications but not chemically modified nucleobases; "P6 unmodified") and unmodified circular dsDNA. Moreover, hemi-modified dsDNA with 100% chemical modification (e.g., 5hmU) was considerably more functional than circular double-stranded DNA with that same 100% chemical modification. These results demonstrate that leDNA and hemi-modified DNAs comprising various chemically modified nucleotides can be efficiently transcribed and ultimately yield a protein product in cells.

Example 12: Assessment of innate immune response in cells in vitro.

This example demonstrates the effect of looped-end DNA (leDNA) and hemi-modified dsDNA molecules with various chemical modifications on the innate immune response of cultured cells.

Experimental constructs were prepared as in Examples 9 and 10 above, then administered to cells as in Example 11 above. qPCR was performed on cells to determine the RNA level of proinflammatory cytokines, including human IL6, CXCL10. Human GAPDH was used as an endogenous control for analysis. Primer sequences can be found in **Table 3**. Briefly, mRNA was extracted from cells using the PicoPure RNA Isolation Kit (ThermoFisher #KIT0204). cDNA was synthesized using the RNA to cDNA EcoDry™ Premix (Oligo dT) (Takara #639542) kit. The analyses were performed using the QuantStudio7 Flex Real-time PCR System with SYBR Select Master Mix from Life Technologies

Corporation. RNA expression was normalized to GAPDH and expressed as fold-change relative to the relevant vehicle control.

Table 3. Primer sequences used in qPCR quantification of immune markers.

Gene Name	SEQ ID NO:	5'-Sequence-3'
h-GAPDH-F	81	GTCTCCTCTGACTTCAACAGCG
h-GAPDH-R	82	ACCACCCTGTTGCTGTAGCCAA
h-IL6-F	83	AGACAGCCACTCACCTCTTCAG
h-IL6-R	84	TTCTGCCAGTGCCTCTTTGCTG
h-CXCL10-F	85	GGTGAGAAGAGATGTCTGAATCC
h-CXCL10-R	86	GTCCATCCTTGGAAGCACTGCA

FIG. 8 shows that leDNA and hemi-modified dsDNA molecules with various chemical modifications led to reduced innate immune response relative to control covalently closed dsDNA (comprising phosphorothioate modifications but not chemically modified nucleobases; "P6 unmodified") and unmodified circular dsDNA. For two key innate immune markers – CXCL10, a widely used marker of interferon response, and IL6, a prominent pro-inflammatory cytokine) – leDNA and hemi-modified DNAs with various chemical modifications were considerably less immunogenic than control covalently closed DNA. For CXCL10, looped-end and hemi-modified DNAs were also considerably less immunogenic than were circular dsDNA molecules. These results demonstrate that altering DNA structure – in this case, by creating looped-end DNA and hemi-modified DNA with diverse chemical modifications – blunts the innate immune response to DNA, and that this attenuation does not come at the cost of protein expression.

All publications, patents, and patent applications cited herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. In the event of a conflict between a term herein and a term in an incorporated reference, the term herein controls.

CLAIMS

1. A double stranded DNA (dsDNA) molecule comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand, wherein:

the sense strand comprises one or more chemically modified nucleobases, and

the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases; and

c) a downstream DNA end form which is a closed end.

2. The dsDNA molecule of claim 1, wherein the sense strand comprises one or more (e.g., at least 3) backbone modifications, e.g., phosphorothioate linkages, wherein optionally the one or more backbone modifications are situated between the 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides adjacent to the upstream DNA end form.

3. The dsDNA molecule of claim 1 or 2, wherein the sense strand comprises a uridine nucleotide.

4. The dsDNA molecule of claim 3, wherein the sense strand comprises a canonical uridine nucleotide or a chemically modified uridine nucleotide.

5. The dsDNA molecule of claim 4, wherein the chemically modified uridine nucleotide comprises 5-azidomethyluridine, 5-formyluridine, 5-hydroxymethyluridine, or 5-methylthiouridine.

6. The dsDNA molecule of any of claims 3-5, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%, at least 90%, or at least 99% of thymine and uridine positions in the sense strand comprise a uridine nucleotide.

7. The dsDNA molecule of claim 1 or 2, wherein the sense strand comprises a chemically modified cytosine nucleotide.

8. The dsDNA molecule of claim 7, wherein the chemically modified cytosine nucleotide comprises 5-hydroxycytosine.

9. The dsDNA molecule of claim 7 or 8, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 75%, at least 90%, or at least 99% of cytosine positions in the sense strand comprise a chemically modified cytosine nucleotide.

10. The dsDNA molecule of claim 1 or 2, wherein the sense strand comprises an inosine nucleotide.

11. The dsDNA molecule of claim 10, wherein at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, or at least 75% of guanine and inosine positions in the sense strand comprise an inosine nucleotide.

12. The dsDNA molecule of any of claims 1-11, wherein the double stranded region encodes a protein, wherein the dsDNA molecule, when contacted to human cells, results in expression at a level at least 100%, at least 120%, at least 140%, at least 160%, or at least 180% the expression of a control DNA molecule, wherein the control DNA molecule comprises the same sequence and same closed end form as the dsDNA molecule, but comprises no chemically modified nucleobases.

13. The dsDNA molecule of any of claims 1-12, wherein the dsDNA molecule, when contacted to human cells, results in a lower level of IL6 or CXCL10 mRNA compared to a control DNA molecule, wherein the control DNA molecule comprises the same sequence and same closed end double stranded form as the dsDNA molecule, but comprises no chemically modified nucleobases.

14. The dsDNA molecule of any of claims 1-13, wherein the sense strand comprises a first type of chemically modified nucleobase and a second type of chemically modified nucleobase.

15. A dsDNA molecule comprising:

a) an upstream DNA end form which is a closed end;

b) a double stranded region comprising a sense strand and an antisense strand; and

c) a downstream DNA end form which is a closed end,

wherein the antisense strand is substantially free of (e.g., is free of) chemically modified nucleobases, and

wherein the sense strand comprises one or more (e.g., at least 3) backbone modifications, e.g., phosphorothioate linkages, wherein optionally:

the one or more backbone modifications are situated between the 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides adjacent to the upstream DNA end form; and/or

the one or more backbone modifications are situated between the 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides adjacent to the downstream DNA end form.

16. A DNA molecule comprising, in an upstream to downstream direction:

a) an upstream DNA end form which is a closed end;

b) a first double stranded region comprising a first fragment of a sense strand and a first region of an antisense strand, wherein optionally the first fragment of the sense strand comprises one or more backbone modifications;

c) a second region of the antisense strand, which region is single stranded;

d) a second double stranded region comprising a second fragment of the sense strand and a third region of the antisense strand; and

e) a downstream DNA end form which is a closed end.

17. A linear DNA molecule comprising, in a 5' to 3' direction:

a) a first annealing sequence,

b) a first looping sequence,

c) a second annealing sequence complementary to the first annealing sequence,

d) an antisense effector sequence,

e) a third annealing sequence,

f) a second looping sequence, and

g) a fourth annealing sequence complementary to the third annealing sequence.

18. A dsDNA molecule comprising an inosine nucleotide.

19. A method of making a dsDNA molecule, the method comprising:

a) providing a double stranded linear DNA having a sense strand, an antisense strand, an upstream open end, and a downstream open end;

b) converting both open ends to closed ends (thereby producing an upstream closed end and downstream closed end) such that the sense strand comprises one or more backbone modifications adjacent to or within 10, 20, or 30 nucleotides of the upstream closed end;

c) producing a nick in the sense strand of the DNA adjacent to or within 10, 20, or 30 nucleotides of the downstream closed end and/or the upstream closed end;

d) subjecting the DNA of c) to conditions having exonuclease activity, such that at least 90%, at least 95%, at least 99%, or 100% of the region of the sense strand between the nick and the one or more backbone modifications are removed; and

e) contacting the DNA of d) with a DNA polymerase, unmodified deoxyribose nucleotides, and nucleotides comprising chemically modified nucleobases under conditions such that a chemically modified sense strand is produced;

thereby producing the dsDNA molecule.

20. A method of making a DNA molecule, the method comprising:

a) providing a double stranded linear DNA having a sense strand, an antisense strand, an upstream open end, and a downstream open end;

b) converting both open ends to closed ends (thereby producing an upstream closed end and downstream closed end) such that the sense strand comprises one or more backbone modifications adjacent to or within 10, 20, or 30 nucleotides of the upstream closed end;

c) producing a nick in the sense strand of the DNA adjacent to or within 10, 20, or 30 nucleotides of the downstream closed end and/or the upstream closed end; and

d) subjecting the DNA of c) to conditions having exonuclease activity, such that at least 90%, at least 95%, at least 99%, or 100% of the region of the sense strand between the nick and the one or more backbone modifications are removed;

thereby producing the DNA molecule.

21. A method of making or manufacturing a dsDNA molecule, the method comprising:

(a) providing a composition comprising a DNA template, e.g., a plasmid, a forward primer, a reverse primer, a DNA polymerase, unmodified deoxyribose nucleotides, and inosine nucleotides; and

(b) performing a polymerase chain reaction on the composition of (a),

thereby making or manufacturing the dsDNA molecule.

22. A DNA molecule produced by the method of any of claims 19-21.

23. A composition comprising a plurality of DNA molecules of any of claims 1-18.

24. A pharmaceutical composition comprising the DNA molecule of any of claims 1-18.

25. A method of expressing an effector in a target cell, the method comprising:

(i) introducing into a target cell the DNA molecule of any of claims 1-18, wherein the DNA molecule comprises an effector sequence encoding an effector; and

(ii) maintaining (e.g., incubating) the cell under conditions suitable for expressing the effector from the dsDNA molecule;

thereby expressing the effector in the target cell.

26. A method of delivering an effector to a target cell, the method comprising:

introducing into a target cell the DNA molecule of any of claims 1-18, wherein the DNA molecule comprises an effector sequence encoding an effector;

thereby delivering the effector to the target cell.

27. A method of modulating (e.g., increasing or decreasing) a biological activity in a target cell, the method comprising:

(i) introducing into a target cell the DNA molecule of any of claims 1-18, wherein the DNA molecule comprises an effector sequence encoding an effector that modulates a biological activity in the target cell; and

(ii) maintaining (e.g., incubating) the cell under conditions suitable for expressing the effector from the DNA molecule;

thereby modulating the biological activity in the target cell.

28. A method of treating a subject in need thereof, the method comprising:

administering to the subject the DNA molecule of any of claims 1-18, wherein the DNA molecule comprises an effector sequence encoding an effector;

thereby treating the subject.

Loop-end DNA
(leDNA)
upstream
DNA end form
downstream
DNA end form
Unmodified
antisense strand

FIG. 1A

Hemi-modified
DNA
Modified
sense strand
upstream
DNA end form
downstream
DNA end form
Unmodified
antisense strand

FIG. 1B

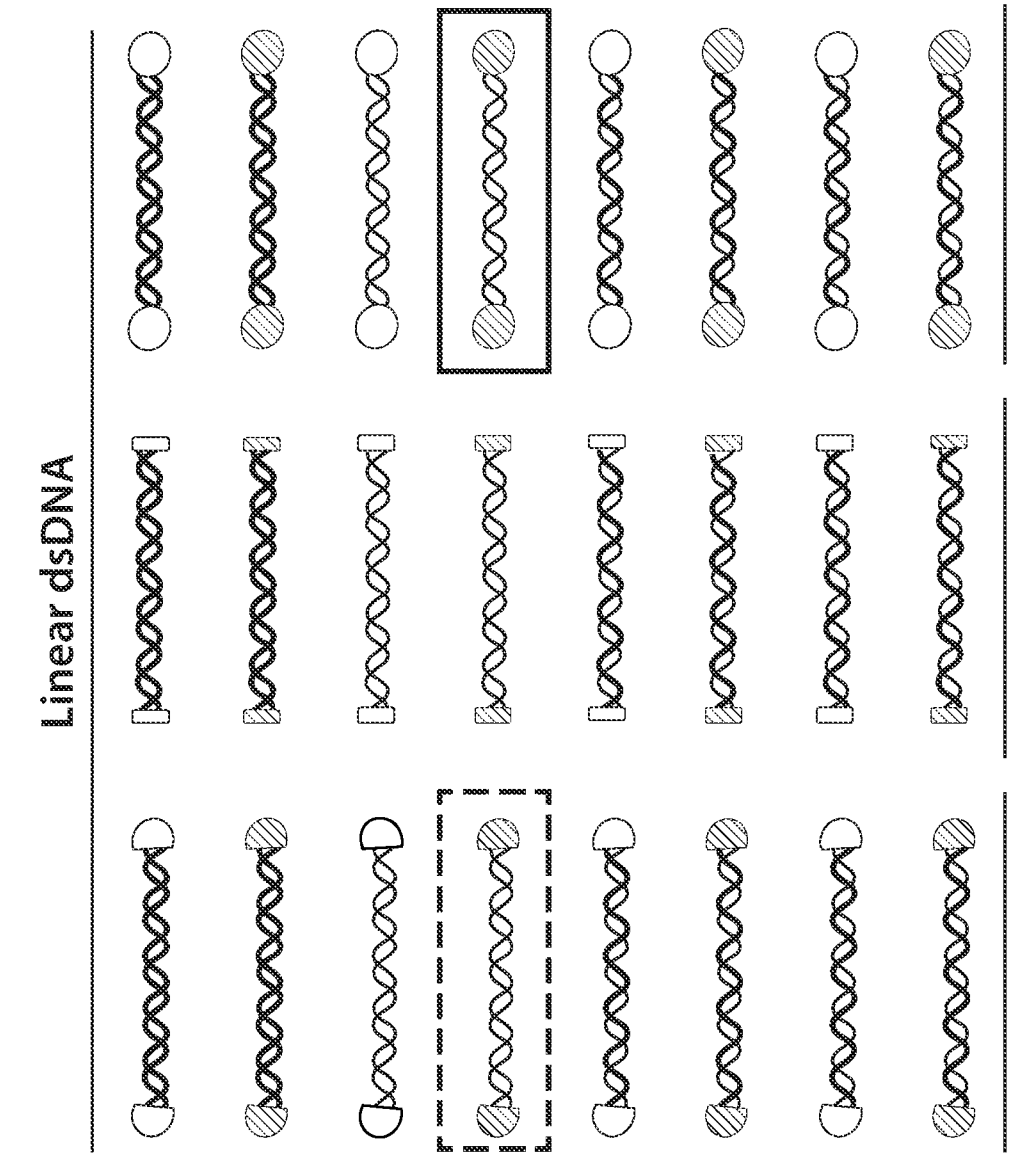

Linear dsDNA
Covalently Closed
Both Strands Unmodified
Both Strands Modified
Antisense Strand Modified
Sense Strand Modified
End Seq. Unmodified
End Seq. Modified
No Loop End
ITR
Hairpin

FIG. 2A

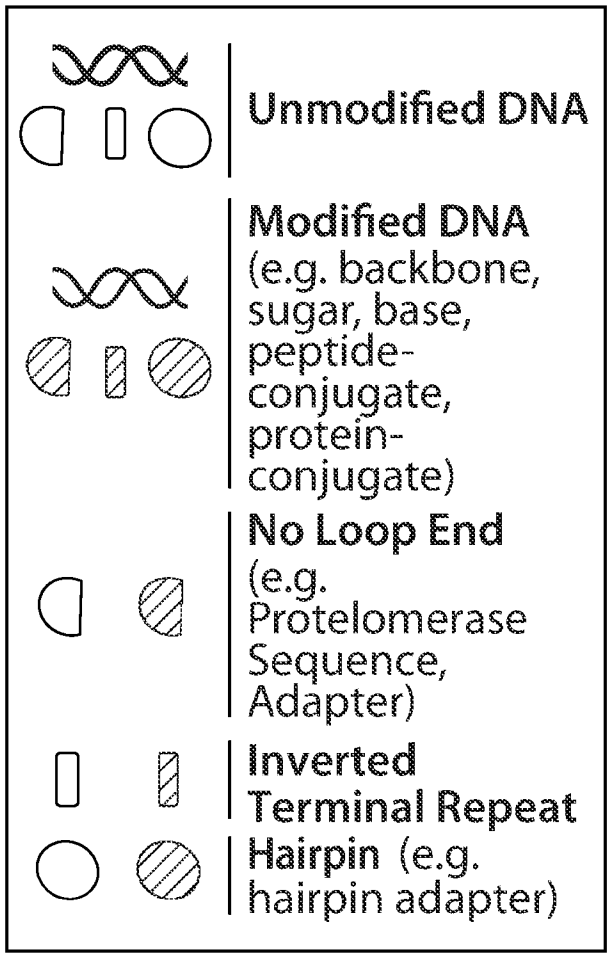

Unmodified DNA
Modified DNA (e.g. backbone, sugar, base, peptide-conjugate, protein-conjugate)
No Loop End (e.g. Protelomerase Sequence, Adapter)
Inverted Terminal Repeat
Hairpin (e.g. hairpin adapter)

FIG. 2B

Unmodified DNA
Modified DNA (e.g. backbone, sugar, base, peptide-conjugate, protein-conjugate)
Y End (e.g. Y adapter)
Linear dsDNA
Not Covalently Closed
Both Strands Unmodified
Both Strands Modified
Antisense Strand Modified
Sense Strand Modified
End Seq. Unmodified
End Seq. Modified
Y End
Nucleotide Mod. Only

FIG. 3

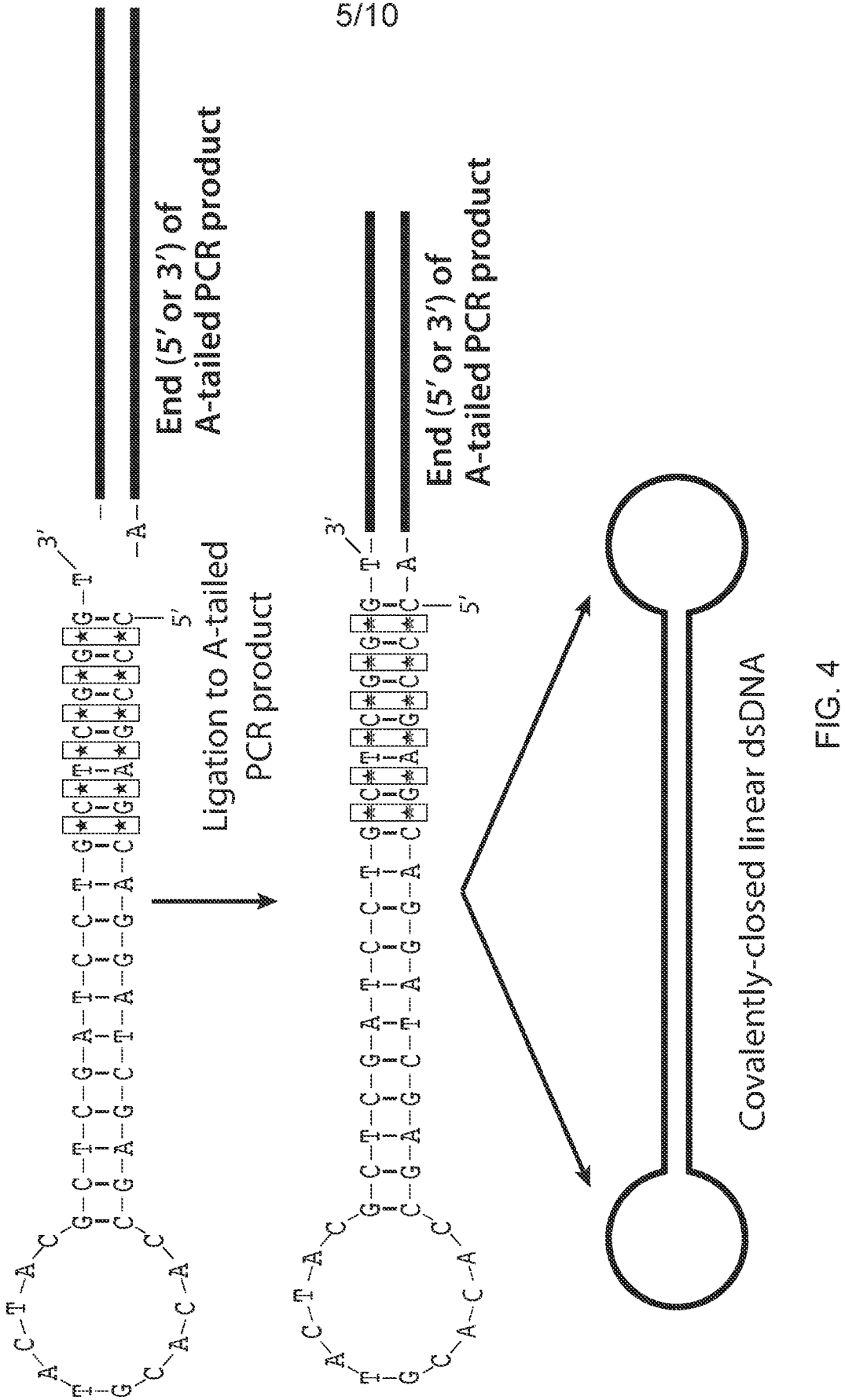

End (5′ or 3′) of
A-tailed PCR product
Ligation to A-tailed
PCR product
End (5′ or 3′) of
A-tailed PCR product
Covalently-closed linear dsDNA

FIG. 4

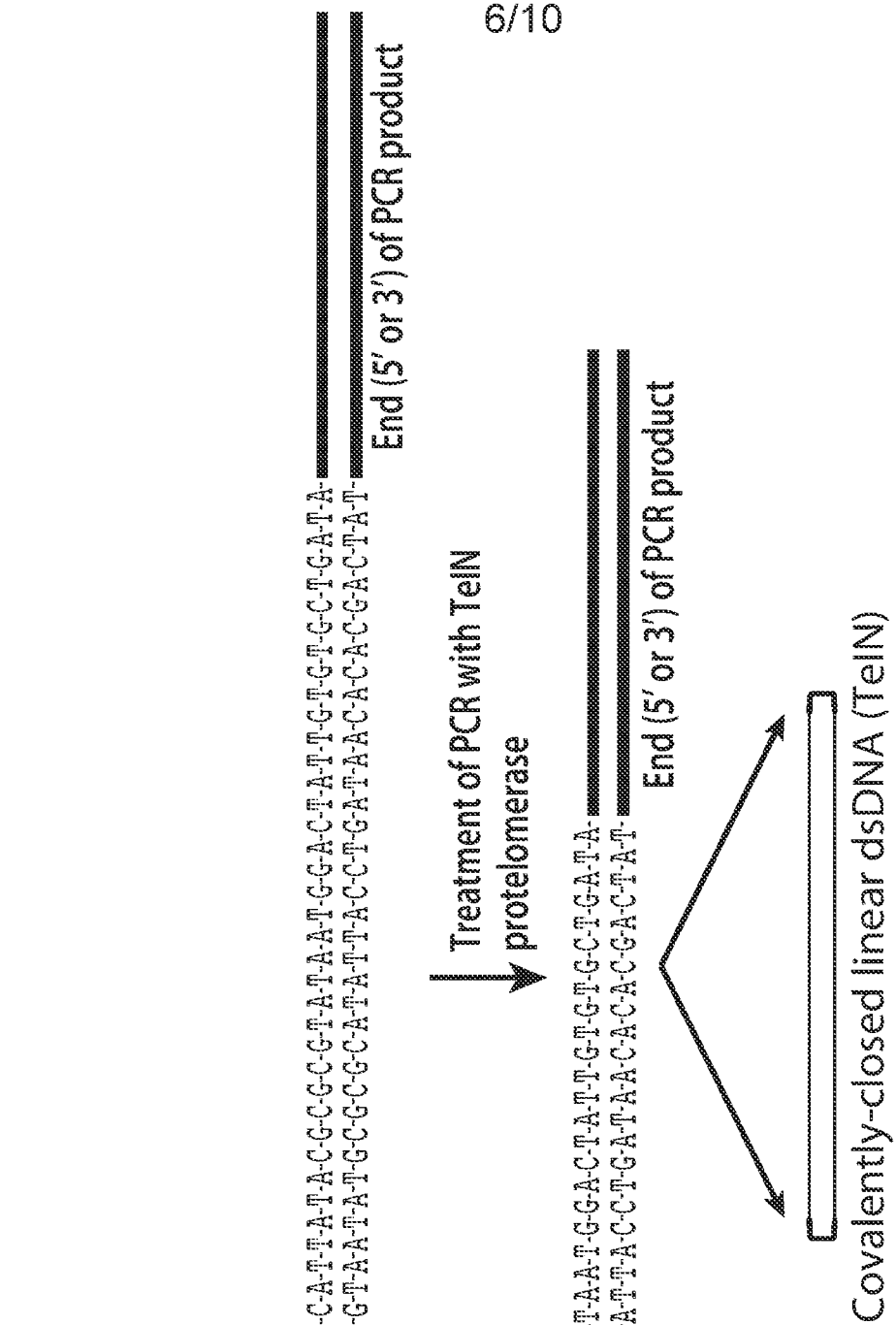

5′-T-A-T-C-A-G-C-A-C-A-C-A-A-T-T-G-C-C-C-A-T-T-A-T-A-A-T-G-G-A-C-T-A-T-T-G-T-G-T-G-C-T-G-A-T-A-
3′-A-T-A-G-T-C-G-T-G-T-G-T-T-A-A-C-G-G-G-T-A-A-T-T-A-T-G-C-G-C-G-C-A-T-T-A-A-C-A-C-G-A-C-T-A-T-
End (5′ or 3′) of PCR product
Treatment of PCR with TelN protelomerase
G-C-G-T-A-T-A-A-T-G-G-A-C-T-A-T-T-G-T-G-T-G-C-T-G-A-T-A-
C-G-C-A-T-A-T-T-A-C-C-T-G-A-T-A-A-C-A-C-G-A-C-T-A-T-
End (5′ or 3′) of PCR product
Covalently-closed linear dsDNA (TelN)

FIG. 5

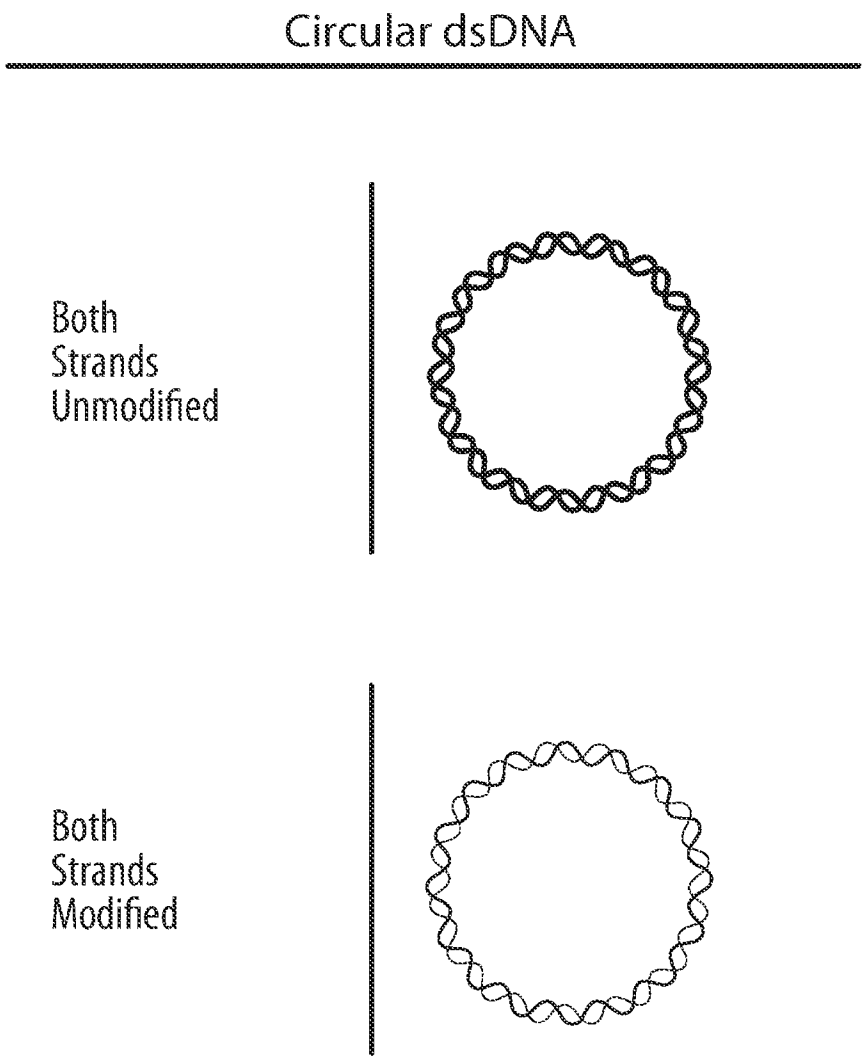

Circular dsDNA
Both Strands Unmodified
Both Strands Modified

FIG. 6

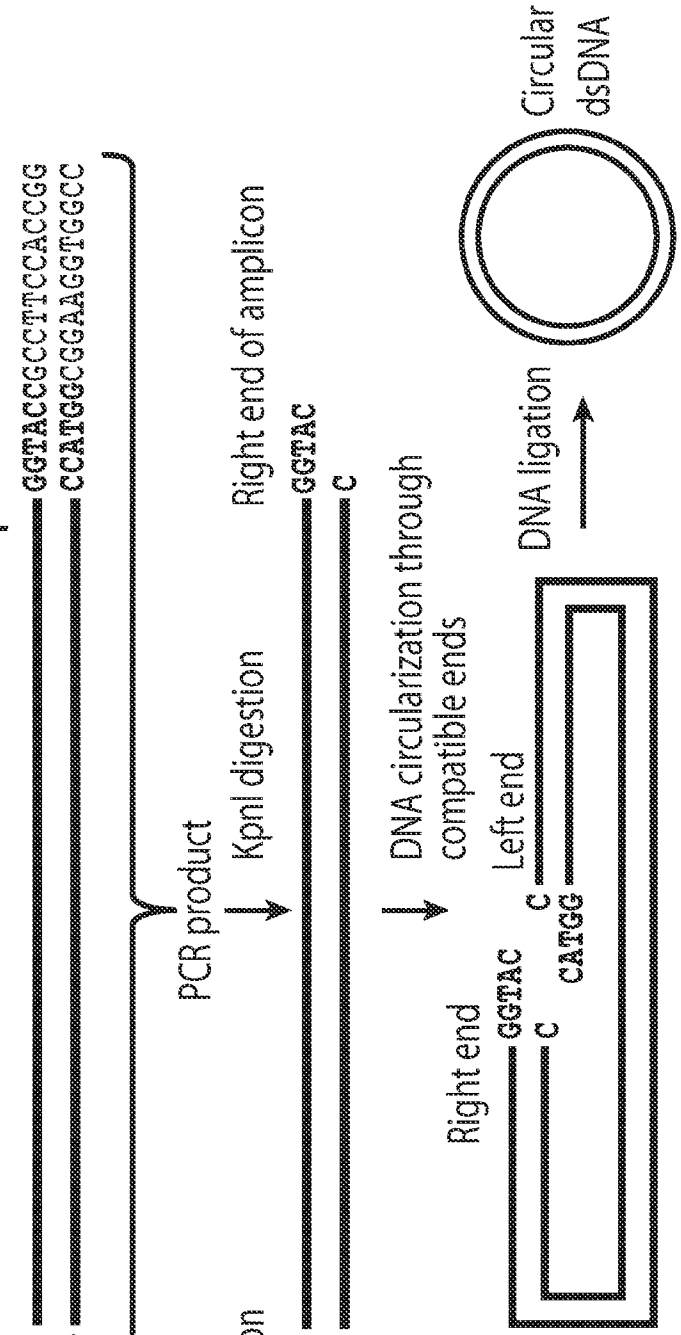

KpnI restriction site
KpnI restriction site
5′CCGTGGTCCTTCGGTACC
3′GGCACCAGGAAGCCATGG
GGTACCGCCTTCCACCGG
CCATGGCGGAAGGTGGCC
PCR product
Left end of amplicon
KpnI digestion
Right end of amplicon
GGTAC
C
C
CATGG
DNA circularization through compatible ends
Right end
GGTAC
C
Left end
C
CATGG
DNA ligation
Circular dsDNA

FIG. 7

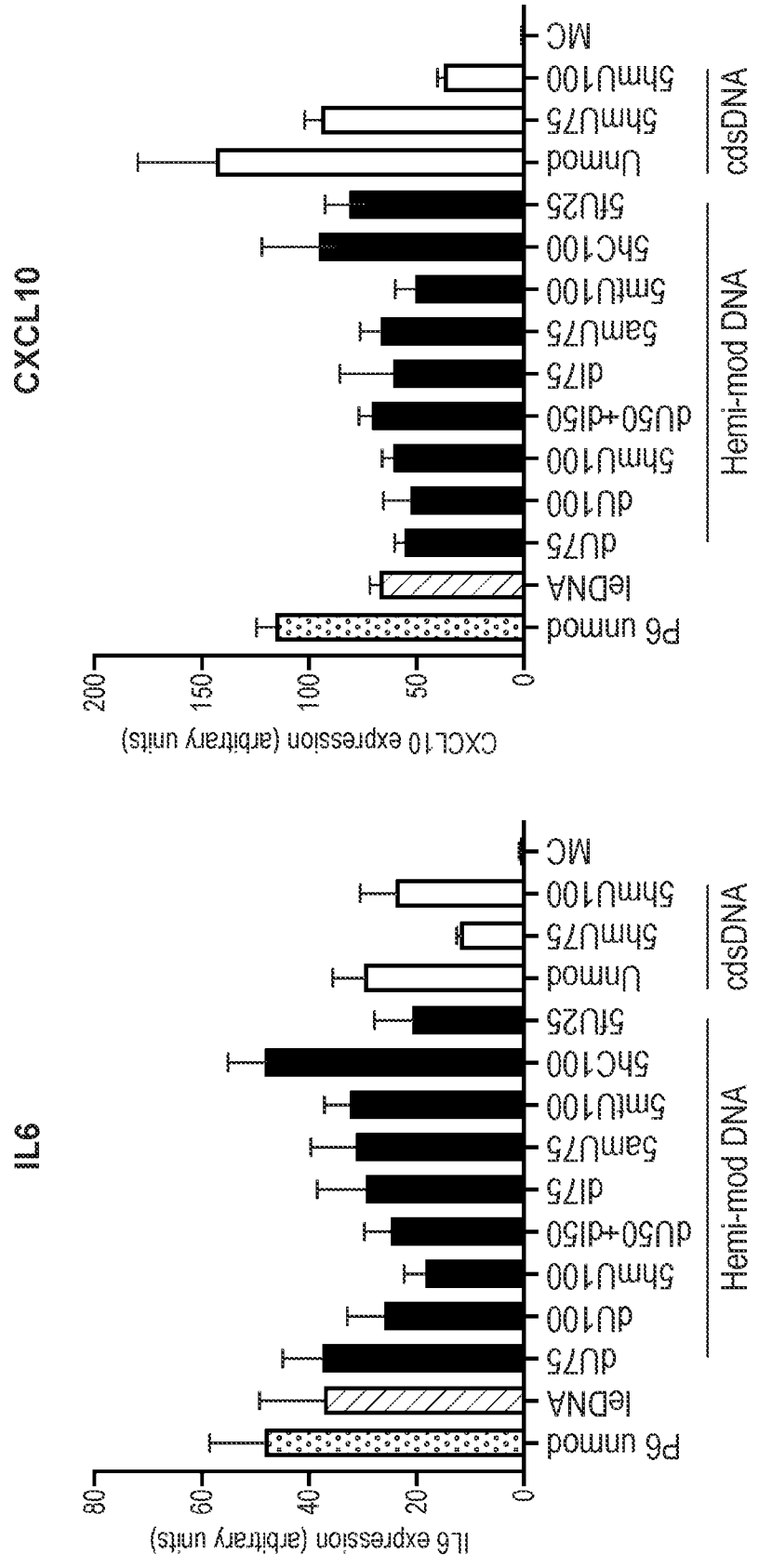
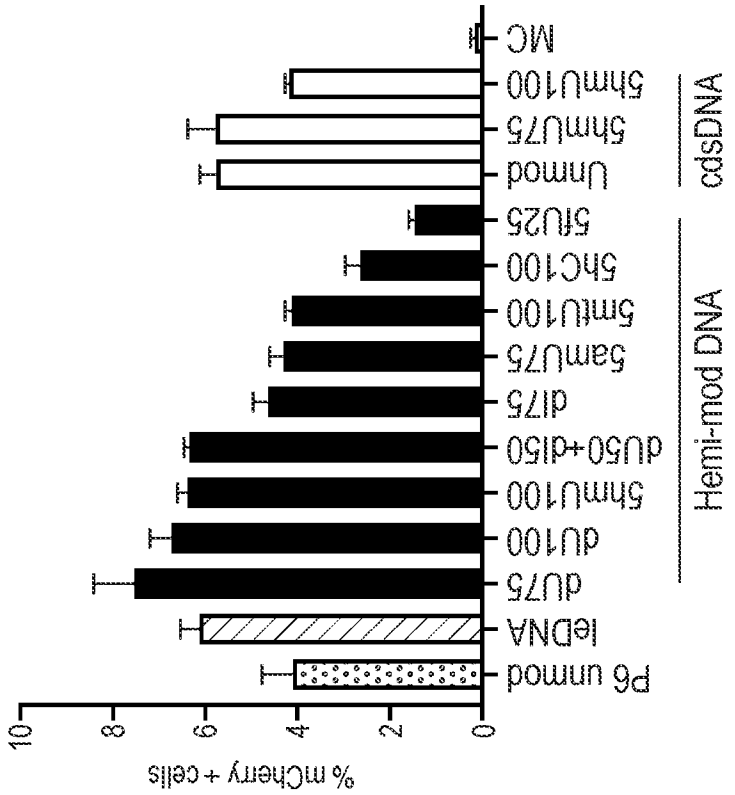

CXCL10
CXCL10 expression (arbitrary units)
0
50
100
150
200
P6 unmod
leDNA
dU75
dU100
5hmU100
dU50+dI50
dI75
5amU75
5mtU100
5hC100
5fU25
Unmod
5hmU75
5hmU100
MC
Hemi-mod DNA
cdsDNA
IL6
IL6 expression (arbitrary units)
0
20
40
60
80
P6 unmod
leDNA
dU75
dU100
5hmU100
dU50+dI50
dI75
5amU75
5mtU100
5hC100
5fU25
Unmod
5hmU75
5hmU100
MC
Hemi-mod DNA
cdsDNA

FIG. 8


% mCherry + cells
0
2
4
6
8
10
P6 unmod
leDNA
dU75
dU100
5hmU100
dU50+dI50
dI75
5amU75
5mtU100
5hC100
5fU25
Unmod
5hmU75
5hmU100
MC
Hemi-mod DNA
cdsDNA

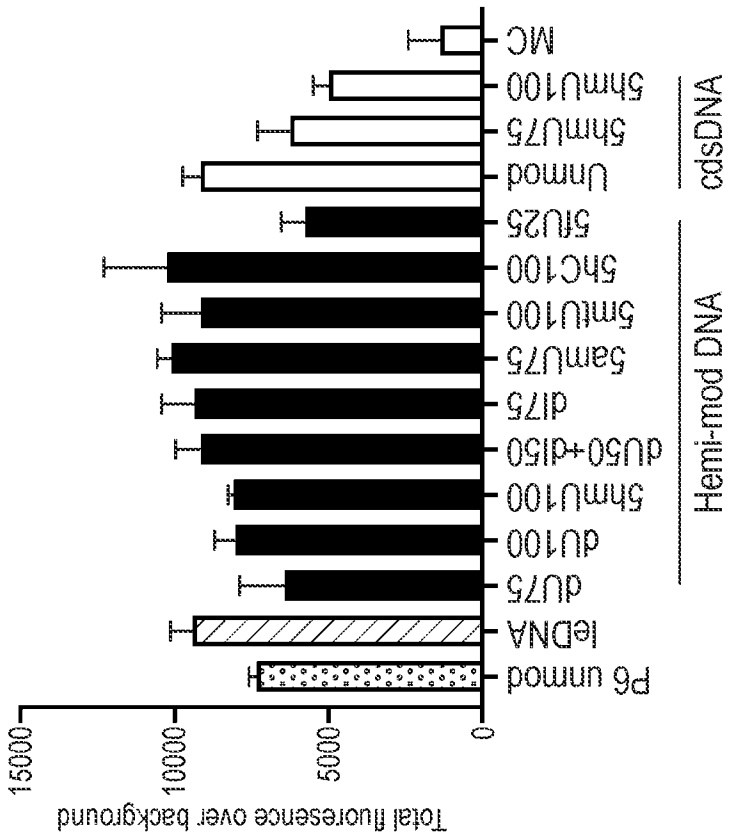

Total fluorescence over background
0
5000
10000
15000
P6 unmod
leDNA
dU75
dU100
5hmU100
dU50+dI50
dI75
5amU75
5mtU100
5hC100
5fU25
Unmod
5hmU75
5hmU100
MC
Hemi-mod DNA
cdsDNA

FIG. 9