DNA COMPOSITIONS AND RELATED METHODS

RELATED APPLICATIONS

This application claims priority to U.S. Serial No.: 63/262,690, filed October 18, 2021, U.S. Serial No.: 63/304,913, filed January 31, 2022, U.S. Serial No.: 63/373,293, filed August 23, 2022, and U.S. Serial No.: 63/402,772, filed August 31, 2022, the entire contents of each of which is incorporated herein by reference.

BACKGROUND

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

SUMMARY

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

In one aspect, the present disclosure refers to a pharmaceutical formulation comprising a lipid nanoparticle (LNP) that comprises a single stranded DNA (ssDNA), wherein the ssDNA: (a) encodes a therapeutic protein, (b) is covalently closed, (c) does not comprise a region of intramolecular complementarity longer than 100 base pairs or does not form a double stranded structure longer than 100 base pairs, (d) is at least 200 nucleotides in length, and (e) does not comprise a protelomerase target sequence; wherein the pharmaceutical formulation is substantially free of linear DNA and protein.

In another aspect, the present disclosure refers to the pharmaceutical formulation disclosed herein for use in therapy.

In some examples, the present disclosure provides a pharmaceutical formulation comprising: an LNP comprising a single stranded DNA (ssDNA), wherein the ssDNA: (a) encodes a therapeutic protein, (b) is covalently closed, (c) does not form a double stranded structure longer than 100 base pairs, (d) is more than 200 nucleotides in length, and (e) does not comprise a protelomerase target sequence; wherein the pharmaceutical formulation is substantially free of linear DNA and protein. In some examples, the ssDNA does not form a double stranded structure longer than 40 base pairs.

In some examples, the ssDNA comprises a promoter sequence operably linked to a sequence encoding the therapeutic polypeptide. In some examples, the therapeutic protein is selected from the group consisting of: a transcription factor, a chromatin remodeling factor, an antigen, a peptide, a hormone, an enzyme, an antibody, a receptor ligand, a receptor, a clotting factor, and a membrane protein. In some examples, the ssDNA has a GC content of 30-40%, 40-50%, 50-60%, or 60-70%.

In some examples, the ssDNA lacks one or both of a bacteriophage packaging site and a bacteriophage origin of replication, or wherein the ssDNA does not encode a bacteriophage

capsid gene. In some examples, the ssDNA was not produced by rolling circle amplification. In some examples, the ssDNA was not produced by strand displacement amplification.

In some examples, the ssDNA further comprises a nuclear targeting sequence (NTS). In some examples, the ssDNA further comprises a maintenance sequence. In some examples, the ssDNA further comprises a second strand motif (SSM).

In some examples, the ssDNA comprises between 200-3,000 nucleotides. In some examples, the ssDNA comprises between 500 and 2,000 nucleotides. In some examples, the ssDNA is a sense ssDNA strand. In some examples, the ssDNA is an antisense ssDNA strand.

In some examples, the ssDNA comprises at least one nucleotide modification. In some examples, the nucleotide modification is 5-formylcytosine.

In some examples, the pharmaceutical formulation is formulated for parenteral administration. In some examples, the pharmaceutical formulation is formulated for topical administration. In some examples, the pharmaceutical formulation is substantially free of one or more of: endotoxin, mononucleotides, modified mononucleotides, double stranded DNA.

In some examples, the present disclosure provides a method of delivering a therapeutic protein to a subject, comprising administering to the subject a pharmaceutical formulation described herein. In some examples, the method does not result in substantial integration of the ssDNA into the genome of the subject.

In one example the disclosure features a composition, e.g., a pharmaceutical composition, that includes a single stranded DNA (ssDNA) comprising an effector sequence, the single stranded DNA having one, two or three of the following characteristics: the ssDNA is covalently closed; the ssDNA does not form a double stranded structure longer than 100 base pairs; the ssDNA comprises at least one covalent modification.

In an example, the ssDNA has an effector sequence and one, two or three of: a nuclear targeting sequence (NTS), a maintenance sequence, and a second strand motif (SSM). In one example the ssDNA has a DNA effector sequence. In one example, the ssDNA has a DNA effector sequence and an NTS. In one example, the ssDNA has a DNA effector sequence, an NTS, and a SSM. In one example, the ssDNA has a DNA effector sequence, an NTS, a SSM and a maintenance sequence. In one example, the ssDNA has a DNA effector sequence, an NTS, and a maintenance sequence. In one example, the ssDNA has a DNA

effector sequence, an SSM and a maintenance sequence. In one example, the ssDNA has a promoter operably linked to a sequence encoding an RNA or protein (peptide or polypeptide) effector. In one example, the ssDNA has a promoter operably linked to a sequence encoding an RNA or protein (peptide or polypeptide) effector, and an NTS. In one example, the ssDNA has a promoter operably linked to a sequence encoding an RNA or protein (peptide or polypeptide) effector, an NTS, and a SSM. In one example, the ssDNA has a promoter operably linked to a sequence encoding an RNA or protein (peptide or polypeptide) effector, an NTS, a SSM and a maintenance sequence. In one example, the ssDNA has a promoter operably linked to a sequence encoding an RNA or protein (peptide or polypeptide) effector, an NTS, and a maintenance sequence. In one example, the ssDNA has a promoter operably linked to a sequence encoding an RNA or protein (peptide or polypeptide) effector, an SSM and a maintenance sequence. In some examples, a ssDNA described herein comprises an enhancer, e.g., an SV40 enhancer. In some examples, a ssDNA comprises two enhancers. In some examples, one or both of the enhancers is an SV40 enhancer. In some examples, a ssDNA described herein comprises a NTS, e.g., an NTS that binds a transcription factor, e.g., an NTS that binds NF-κB, e.g., a 3NF sequence. In some examples, a ssDNA comprises two, three, or four NTSs. In some examples, 1, 2, 3, or 4 of the NTSs is a 3NF sequence. In some examples, a ssDNA described herein comprises a SMM (e.g., an Anellovirus hairpin). In some examples, a ssDNA described herein comprises a polyA signal, e.g., a bGH polyA signal. In some examples, a ssDNA described herein comprises a promoter, e.g., an EF1a promoter.

In some examples, the enhancer is situated upstream of the promoter. The NTS may be situated, for example, upstream of the promoter or downstream of the polyA signal. In some examples, the SMM (e.g., Anellovirus hairpin) is situated upstream of the promoter.

In some examples, the promoter is situated between the enhancer and the effector sequence. In some examples, the NTS is situated between the SMM (e.g., anellovirus hairpin) and the promoter. In some examples, the SMM (e.g., anellovirus hairpin) is situated between the enhancer and the promoter. In some examples, the polyA signal is situated between the effector sequence and the NTS. In some examples, the polyA signal is situated between the effector sequence and the enhancer. In some examples, the polyA signal is situated between the SMM (e.g., anellovirus hairpin) and the effector sequence. In some

examples, the NTS is situated between the SMM (e.g., anellovirus hairpin) and the polyA signal. In some examples, a first NTS sequence is immediately adjacent to a second NTS sequence. In some examples, a ssDNA described herein comprises a series of components arranged as shown in Table 5 herein.

In an example, the ssDNA has 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 10,000 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.

In an example, the ssDNA has 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 200 and 1,000 nucleotides, between 200 and 2,000 nucleotides, between 200 and 3,000 nucleotides, between 500 and 1,000 nucleotides, between 500 and 2,000 nucleotides, between 500 and 3,000 nucleotides, between 1,000 and 2,000 nucleotides, between 1,000 and 3,000 nucleotides, or between 2,000 and 3,000 nucleotides. In some examples, the ssDNA comprises between 20-20,000 nucleotides. In some examples the ssDNA comprises between 50-50,000 nucleotides.

In an example, the ssDNA is a sense ssDNA strand. In an example, the ssDNA is an antisense ssDNA strand.

In an example, the ssDNA comprises at least one nucleotide modification, e.g., a covalent nucleotide modification, e.g., selected from: N6-Methyladenosine (m6A, 6mA); 5-Formylcytosine (5fC, f5C); 5-carboxylcytosine (ca5C, 5caC); 5-hydroxymethylcytosine (5hmC, hm5C); 5-methyldeoxycytosine (m5dC); 5-methylcytosine (5mC, m5C); 5'-methylcytosine; 3-methylcytosine (m3C); 5-methyl pyrimidine; 8-oxoguanine (8-oxoG); phosphorothioate; S and R

phsophorothioate linkages; methylthymine; N3'-P5' Phosphoroamidate (NP); cyclohexane nucleic acid (CeNA); and tricyclo-DNA (tcDNA). In some examples, the ssDNA comprises N^6-methyladenosine. In some examples, the majority of A positions in the ssDNA are N^6-methyladenosine. In some examples, the nucleotide modification is a covalent modification selected from phosphothioate; borano-phosphate; 1,5-disubstituted triazole; 2'-fluoro-2'deoxynucleoside 5'-triphosphate; and 7-mehtylguanine; 5-glucosylmethylcytosine.

In some examples, the nucleotide modification is a base modification. In some examples, the nucleotide modification is a backbone modification. In some examples, the nucleotide modification is a sugar modification. In some examples, the nucleotide modification comprises a peptide conjugate. In some examples, the nucleotide modification comprises a protein conjugate.

In an example, the effector sequence is a therapeutically functional sequence, e.g., a functional, structural DNA sequence such as a DNA aptamer, DNAzyme or allele-specific oligonucleotide (ASO).

In an example, the effector sequence is a DNA sequence encoding a therapeutic (e.g., regulatory) RNA, operably linked to a promoter. In an example, the RNA can be, e.g., a tRNA, lncRNA, miRNA, rRNA, snRNA, microRNA, siRNA, piRNA, snoRNA, exRNA, scaRNA, Y RNA, or hnRNA.

In some examples, the therapeutically functional sequence comprises a promoter sequence operably linked to a sequence encoding a therapeutic RNA or polypeptide. In an example, the effector sequence is a DNA sequence encoding a therapeutic peptide or polypeptide, operably linked to a promoter. The therapeutic peptide or polypeptide may be, e.g., 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; a protein scaffold binder such as a centyrin, darpin, or adnectin.

In examples, the ssDNA can include a plurality of effector sequences. The plurality may be the same or different types, e.g., a ssDNA 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 some examples, the ssDNA comprises a second effector sequence which is the same as or different than the first effector sequence. A ssDNA can include an effector sequence that is a DNA sequence encoding a functional RNA and a second effector sequence that is a DNA sequence encoding a functional polypeptide. The plurality of effector sequences may be the same or different sequences of the same type.

In some examples, when the ssDNA is introduced to a cell, the cell exhibits a lower cytokine mRNA level increase (e.g., normalized to GAPDH mRNA levels) compared to a control cell of the same type that was contacted with a dsDNA having the same sequence as the ssDNA at the same molar amount as the ssDNA. In some examples, the cytokine comprises cytokine IFN-b, IL-6, IL-1b, TNF-a, or CXCL10. In some examples, the cytokine increase in the control cells is less than 50%, 40%, 30%, 20%, or 10% of the cytokine increase in the control cells.

In some examples, a ssDNA described herein has an A260/A280 ratio of 1.6-1.7, 1.7-1.8. or 1.63-1.76. In some examples, a ssDNA described herein has an A230/A260 ratio of 0.3-1, 1-1.5, 1.5-1.8, or 0.34 - 1.79.

In examples, the ssDNA is not disposed in a carrier, e.g., it is formulated for naked administration.

In examples, the ssDNA is formulated with a carrier, e.g., a lipid-based carrier, e.g., an lipid nanoparticle (LNP). In examples, the pharmaceutical composition further comprises a carrier, e.g., a lipid-based carrier, e.g., an LNP.

In some examples, the composition comprising ssDNA is substantially free of (e.g., is free of) an LNP. In some examples, the composition comprising ssDNA is substantially free of (e.g., is free of) a lipid-based carrier. In some examples, the composition comprising ssDNA is substantially free of (e.g., is free of) a lipid.

In examples, the ssDNA is formulated with a pharmaceutical excipient.

In examples, the ssDNA is formulated for parenteral administration.

In examples, the pharmaceutical composition is formulated for topical administration.

In examples, the pharmaceutical composition is substantially free of impurities or process byproducts, e.g., selected from the group consisting of: endotoxin, mononucleotides, modified mononucleotides, double-stranded DNA, DNA fragments or truncations, and proteins (e.g., enzymes, e.g., ligases, restriction enzymes). In the case of circular, e.g., covalently closed, ssDNA, the pharmaceutical composition is substantially free of linear DNA.

In any example described herein, the ssDNA may be covalently closed, e.g., the ssDNA is circularized.

In another example, the disclosure includes a method of delivering an effector to a subject, e.g., a subject in need thereof. The method incudes administering to the subject a composition described herein, e.g., described in any example above. In an example, the subject has or has been diagnosed with a condition that can be treated with the effector.

In another example, the disclosure includes a method of modulating (e.g., increasing or decreasing) a biological parameter in a cell, tissue or subject. The method incudes administering to the subject a composition described herein, e.g., described in any example above. In examples, the biological parameter is an increase or decrease in gene expression of a subject gene in a target cell, tissue or subject, which increase or decrease is effected by an effector sequence described herein. In an example, the subject has or has been diagnosed with a condition that can be treated with the effector.

In another example, the disclosure includes a method of treating a cell, tissue or subject. The method includes administering to a cell, tissue or subject in need thereof an ssDNA or construct described herein, e.g., described in any example above. In an example, the subject has or has been diagnosed with a condition that can be treated with the effector.

In another example, the disclosure features a method of making a pharmaceutical composition that includes a ssDNA comprising an effector sequence. The method comprises: (a) providing or generating a plasmid comprising an effector sequence and, optionally, one or more

of (e.g., 2, 3, or 4 of) a promoter operably linked to the effector sequence, an NTS, a SSM and a maintenance sequence, (b) providing or generating a ssDNA comprising the effector sequence and, optionally, one or more of (e.g., 2, 3, or 4 of) a promoter operably linked to the effector sequence, an NTS, a SSM and a maintenance sequence, using the plasmid as a template, and (c) optionally, circularizing the ssDNA.

In one example, the method comprises circularizing (e.g., ligating the ends of) the ssDNA.

In examples the ssDNA is any ssDNA described herein, e.g., described in any example above.

In an example, step (a) comprises performing golden gate assembly of the recited sequence elements.

In an example, the method further comprises (d) enriching or purifying the ssDNA made in step (b) or the circularized, e.g., covalently closed, ssDNA made in step (c).

In an example, step (d) includes substantially removing from the ssDNA one or more impurity selected from: endotoxin, mononucleotides, modified mononucleotides, double stranded DNA, DNA fragments or truncations, and proteins (e.g., enzymes, e.g., ligases, restriction enzymes). In the case of circular, e.g., covalently closed, ssDNA, linear DNA is removed.

In an example, the method further comprises formulating the enriched or purified ssDNA or circularized ssDNA of step (d) for pharmaceutical use, e.g., formulating the enriched or purified ssDNA or circularized, e.g., covalently closed, ssDNA with a pharmaceutically acceptable excipient and/or with a carrier, e.g., an LNP.

In another example, the disclosure features a plasmid comprising an effector sequence described herein and 1, 2, 3, or 4 of the following elements: a promoter, a NTS, a SSM, and a maintenance sequence.

In some examples, the disclosure provides a composition (e.g., a pharmaceutical composition) comprising: a covalently closed, single stranded DNA (ssDNA) comprising an effector sequence, wherein one or more of: a) at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, or 98%, or 99% by mass of total DNA in the composition is the covalently closed ssDNA; b) at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% by mass of total DNA in the composition is full length; c) less than 10%, 5%, 4%, 3%, 2%, or 1% by mass of DNA in the

composition is double stranded DNA (dsDNA); d) less than 10%, 5%, 4%, 3%, 2%, or 1% by mass of DNA in the composition is linear DNA; or e) less than 10%, 5%, 4%, 3%, 2%, or 1% by mass of DNA in the composition is linear ssDNA.

In some examples, the disclosure provides a composition (e.g., a pharmaceutical composition) comprising: a covalently closed, single stranded DNA (ssDNA) comprising an effector sequence, wherein one or more of: a) the composition is substantially free of, e.g., is free of, chloroform; b) the composition is substantially free of, e.g., is free of, phenol; c) the composition is substantially free of, e.g., is free of, phenol and chloroform; d) the composition is substantially free of, e.g., is free of, organic solvents; or e) the composition is substantially free of, e.g., is free of, aromatic organic solvents.

In some examples, the disclosure provides a composition (e.g., a pharmaceutical composition) comprising: a covalently closed, single stranded DNA (ssDNA) comprising an effector sequence, wherein the ssDNA was produced by a method that does not comprise a phenol-chloroform extraction step.

In some examples, the disclosure provides a composition (e.g., a pharmaceutical composition) comprising: a covalently closed, single stranded DNA (ssDNA) comprising an effector sequence, wherein one or more of: a) the composition is substantially free of (e.g., is free of) Exonuclease III; b) the composition is substantially free of (e.g., is free of) T7 Exonuclease; or c) the composition is substantially free of (e.g., is free of) T5 Exonuclease.

In some examples, the disclosure provides a composition (e.g., a pharmaceutical composition) comprising at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mg of a covalently closed, single stranded DNA (ssDNA) comprising an effector sequence.

In some examples, the disclosure provides a composition (e.g., a pharmaceutical composition or a manufacturing intermediate) comprising: a circular double stranded DNA comprising an effector sequence and comprising at least one modified nucleotide, and Nb.BsrDI or Nt.BspQI.

In some examples, the ssDNA is not disposed in a carrier. In some examples, the composition further comprises a carrier. In some examples, the carrier is a lipid-based carrier. In some examples, the lipid-based carrier is a lipid nanoparticle (LNP). In some examples, the composition is formulated for naked administration. In some examples, the composition is formulated for parenteral administration. In some examples, the

composition is formulated for topical administration. In some examples, the composition is substantially free of impurities or byproducts selected from the group consisting of: endotoxin, mononucleotides, modified mononucleotides, double stranded DNA, DNA fragments or truncations, proteins (e.g., enzymes, e.g., ligases, restriction enzymes).

In some examples, the disclosure provides a method of making a circular, ssDNA, the method comprising: a) providing (e.g., producing or obtaining) a circular dsDNA, wherein the circular dsDNA: i) lacks a plasmid backbone; ii) lacks a bacterial origin of replication; iii) lacks a selectable marker, e.g., an antibiotic resistance marker; and/or iv) comprises a chemical modification, e.g., a chemical modification to a sugar, a chemical modification to a base, or a chemical modification to a nucleic acid backbone; b) introducing a discontinuity into one strand of the circular dsDNA (e.g., contacting the circular dsDNA with a nicking endonuclease (e.g., Nb.BsrDI or Nb.Bpu10I or Nt.BspQI) that recognizes a nicking recognition site in the dsDNA under conditions that allow the nicking endonuclease to nick the site in the dsDNA) thereby producing a dsDNA having a discontinuity; c) contacting the dsDNA having the discontinuity with an exonuclease (e.g., T7 exonuclease or Exonuclease III) under conditions that allow for degradation (e.g., complete degradation) of the nicked strand, thereby making the circular, ssDNA.

In some examples, the disclosure provides a method of making a circular, ssDNA, the method comprising: a) providing (e.g., producing or obtaining) a circular dsDNA; b) contacting the circular dsDNA with a nicking endonuclease chosen from Nb.BsrDI or Nt.BspQI that recognizes a nicking recognition site in the dsDNA under conditions that allow the nicking endonuclease to nick the site in the dsDNA, thereby producing a nicked dsDNA; c) contacting the nicked dsDNA with an exonuclease (e.g., T7 exonuclease or Exonuclease III) under conditions that allow for degradation (e.g., complete degradation) of the nicked strand, thereby making the circular, ssDNA.

In some examples, the disclosure provides a method of making a circular, ssDNA, the method comprising: a) providing (e.g., producing or obtaining) a circular dsDNA; b) introducing a discontinuity into one strand of the circular dsDNA (e.g., contacting the circular dsDNA with a nicking endonuclease (e.g., Nb.BsrDI or Nb.Bpu10I or Nt.BspQI) that recognizes a nicking recognition site in the dsDNA under conditions that allow the nicking endonuclease to nick the site in the dsDNA), thereby producing a nicked dsDNA having a discontinuity; c) contacting the

nicked dsDNA with a T7 exonuclease under conditions that allow for degradation (e.g., complete degradation) of the nicked strand, thereby making the circular, ssDNA.

In some examples, the disclosure provides a method of making a circular, ssDNA, the method comprising: a) providing (e.g., producing or obtaining) a circular dsDNA; b) introducing a discontinuity into one strand of the circular dsDNA (e.g., contacting the circular dsDNA with a nicking endonuclease (e.g., Nb.BsrDI or Nb.Bpu10I or Nt.BspQI) that recognizes a nicking recognition site in the dsDNA under conditions that allow the nicking endonuclease to nick the site in the dsDNA), thereby producing a nicked dsDNA having a discontinuity; c) contacting the nicked dsDNA with an exonuclease (e.g., T7 exonuclease or Exonuclease III) under conditions that allow for degradation (e.g., complete degradation) of the nicked strand, thereby making a circular, ssDNA, and d) performing gel purification on the circular, ssDNA.

In some examples, the circular dsDNA: i) lacks a plasmid backbone; ii) lacks a bacterial origin of replication; iii) lacks a selectable marker, e.g., an antibiotic resistance marker; and/or iv) comprises a chemical modification, e.g., a chemical modification to a sugar, a chemical modification to a base, or a chemical modification to a nucleic acid backbone.

In some examples, the method comprises contacting the circular dsDNA with a nicking endonuclease chosen from Nb.BsrDI or Nt.BspQI that recognizes a nicking recognition site in the dsDNA under conditions that allow the nicking endonuclease to nick the site in the dsDNA, thereby producing a nicked dsDNA. In some examples, the method comprises contacting the nicked dsDNA with a T7 exonuclease under conditions that allow for degradation (e.g., complete degradation) of the nicked strand. In some examples, the method comprises performing gel purification on the circular, ssDNA.

In some examples, the method comprises producing circular dsDNA, wherein producing the circular dsDNA comprises one or more of (e.g., 2, 3, 4, or all of): (i) providing a nucleic acid (e.g., a plasmid) comprising an effector sequence; (ii) performing PCR to amplify a region of the nucleic acid (e.g., plasmid) that comprises the effector sequence, wherein performing PCR comprises contacting the plasmid with a first primer comprising a first endonuclease recognition site and a second primer comprising a second endonuclease recognition site and a nicking recognition site, wherein the first primer and the second primer are positioned at locations on the plasmid suitable to amplify the effector sequence (and optionally, not amplify backbone of the plasmid), and contacting the plasmid with a DNA-dependent DNA

polymerase (e.g., a high fidelity polymerase, e.g., Q5), and applying thermal cycling conditions sufficient to amplify the region of the nucleic acid that comprises the effector sequence, thereby producing a linear dsDNA; (iii) digesting the linear dsDNA with an endonuclease (e.g., BsaI, KpnI, or NheI) that cleaves the endonuclease recognition sites, thereby producing a digested linear DNA, (iv) circularizing the digested linear dsDNA (e.g., by contacting the linear digested dsDNA with a ligase, e.g., T4 ligase); and (v) digesting residual linear DNA by contacting a composition comprising the digested linear dsDNA with an exonuclease (e.g., T5 exonuclease).

In some examples, the method comprises one or more purification steps, e.g., gel purification or use of a DNA purification column. In some examples, the purification step is performed on: the linear dsDNA; the digested linear dsDNA; the circular dsDNA; or the circular ssDNA. In some examples, the method does not comprise an organic extraction step (e.g., a phenol-chloroform extraction step).

In some examples, the method comprises contacting the nicked dsDNA with the exonuclease (e.g., the T7 exonuclease) for 15-120 minutes, e.g., 20-60 minutes, e.g., about 30 min. In some examples, introducing a discontinuity into one strand of the circular dsDNA comprises introducing a nick between two adjacent nucleotides. In some examples, introducing a discontinuity into one strand of the circular dsDNA comprises removing a nucleotide, e.g., wherein the nucleotide is a uracil.

In some examples, the disclosure provides a method of evaluating a sample of a composition comprising ssDNA, the method comprising determining whether a condition is satisfied, wherein the condition is chosen from: a) at least 70%, 80%, 85%, 90%, 95%, 97%, or 98%, or 99% by mass of total DNA in the composition is the covalently closed ssDNA; b) at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% by mass of total DNA in the composition is full length; c) less than 10%, 5%, 4%, 3%, 2%, or 1% by mass of DNA in the composition is double stranded DNA (dsDNA); d) less than 10%, 5%, 4%, 3%, 2%, or 1% by mass of DNA in the composition is linear DNA; e) less than 10%, 5%, 4%, 3%, 2%, or 1% by mass of DNA in the composition is linear ssDNA; f) the composition is substantially free of, e.g., is free of, chloroform; g) the composition is substantially free of, e.g., is free of, phenol; h) the composition is substantially free of, e.g., is free of, phenol and chloroform; i) the composition is substantially free of, e.g., is free of, organic solvents; j) the composition is substantially free of, e.g., is free of, aromatic organic solvents; k) the composition is substantially free of (e.g., is free

of) Exonuclease III; l) the composition is substantially free of (e.g., is free of) T7 Exonuclease; and/or m) the composition is substantially free of (e.g., is free of) T5 Exonuclease. In some examples, if the condition is satisfied, the method comprises performing a downstream processing step on the composition, wherein optionally the downstream processing step is chosen from: dividing the composition into portions, packaging the composition, labeling the composition, shipping the composition, distributing the composition, storing the composition, or releasing the composition into commerce. In some examples, the method comprises evaluating a sample of a composition disclosed herein. In some examples, the method comprises evaluating a sample of a composition made by a method disclosed herein.

In some examples, the ssDNA comprises a chemical modification, e.g., a chemical modification to a sugar, a chemical modification to a base, or a chemical modification to a nucleic acid backbone. In some examples, the ssDNA is not a bacteriophage genome. In some examples, the ssDNA lacks a bacteriophage packaging site. In some examples, the ssDNA lacks a bacteriophage origin of replication. In some examples, the ssDNA does not encode a bacteriophage capsid gene. In some examples, the ssDNA was not produced by rolling circle amplification. In some examples, the ssDNA was not produced by strand displacement amplification. In some examples, the ssDNA does not comprise a protelomerase target sequence. In some examples, the ssDNA does not comprise a hairpin structure. In some examples, the ssDNA does not comprise a first sequence that hybridizes with a second sequence, wherein the first sequence and the second sequence are at least 5 nt long, and wherein the first sequence and the second sequence are positioned less than 6 nucleotides apart from each other. In some examples, the ssDNA does not comprise a double strand origin (DSO).

In some examples, the chemical modification is a covalent modification selected from 5-formylcytosine; phosphorothioate; 7-methylguanine; and 5-glucosylmethylcytosine.

Definitions

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

CLAIMS

1. A pharmaceutical formulation comprising a lipid nanoparticle (LNP) that comprises a single stranded DNA (ssDNA), wherein the ssDNA:

(a) encodes a therapeutic protein,

(b) is covalently closed,

(c) does not comprise a region of intramolecular complementarity longer than 100 base pairs or does not form a double stranded structure longer than 100 base pairs,

(d) is at least 200 nucleotides in length, and

(e) does not comprise a protelomerase target sequence;

wherein the pharmaceutical formulation is substantially free of linear DNA and protein.

2. The pharmaceutical formulation of claim 1, wherein the ssDNA does not form a double stranded structure longer than 40 base pairs.

3. The pharmaceutical formulation of claim 1 or 2, wherein the ssDNA comprises a promoter sequence operably linked to a sequence encoding the therapeutic protein.

4. The pharmaceutical formulation of any of claims 1-3, wherein the therapeutic protein is selected from the group consisting of: a transcription factor, a chromatin remodeling factor, an antigen, a peptide, a hormone, an enzyme, an antibody, a receptor ligand, a receptor, a clotting factor, and a membrane protein.

5. The pharmaceutical formulation of claim 4, wherein the antibody is a monoclonal antibody, Fab fragment, or single-chain Fv.

6. The pharmaceutical formulation of any of claims 1-5, wherein the ssDNA lacks one or both of a bacteriophage packaging site and a bacteriophage origin of replication, or wherein the ssDNA does not encode a bacteriophage capsid gene.

7. The pharmaceutical formulation of any of claims 1-6, wherein the ssDNA was not produced by rolling circle amplification and/or was not produced by strand displacement amplification.

8. The pharmaceutical formulation of any of claims 1-7, wherein:
(i) the ssDNA has a GC content of 30-40%, 40-50%, 50-60%, or 60-70%;
(ii) the ssDNA further comprises a nuclear targeting sequence (NTS);
(iii) the ssDNA further comprises a maintenance sequence; and/or
(iv) the ssDNA further comprises a second strand motif (SSM).

9. The pharmaceutical formulation of any of claims 1-8, wherein the ssDNA comprises between 200 and 3,000 nucleotides.

10. The pharmaceutical formulation of any of claims 1-8, wherein the ssDNA comprises between 500 and 2,000 nucleotides.

11. The pharmaceutical formulation of any of claims 1-10, wherein the ssDNA is a sense ssDNA strand.

12. The pharmaceutical formulation of any of claims 1-10, wherein the ssDNA is an antisense ssDNA strand.

13. The pharmaceutical formulation of any of claims 1-12, wherein the ssDNA comprises at least one nucleotide modification.

14. The pharmaceutical formulation of claim 13, wherein the nucleotide modification is 5-formylcytosine.

15. The pharmaceutical formulation of any of claims 1-14, wherein the pharmaceutical formulation is formulated for parenteral administration or topical administration.

16. The pharmaceutical formulation of any of claims 1-15, being substantially free of one or more of: endotoxin, mononucleotides, modified mononucleotides, or double stranded DNA.

17. The pharmaceutical formulation of any one of claims 1-16, wherein the ssDNA comprises an EBV oriP site.

18. The pharmaceutical formulation of any of claims 1-17 for use in therapy.

19. The pharmaceutical formulation for use of claim 18, wherein the administration of the pharmaceutical formulation to a subject does not result in substantial integration of the ssDNA into the genome of the subject.