



(51) International Patent Classification:

A61K 31/165 (2006.01) C12Q 1/6886 (2018.01)

(21) International Application Number:

PCT/US2018/039179

(22) International Filing Date:

23 June 2018 (23.06.2018)

(25) Filing Language:

English

(26) Publication Language:

English

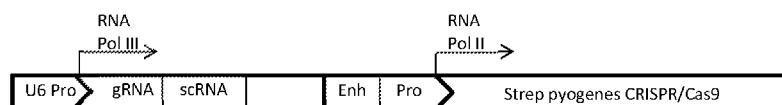
(30) Priority Data:

62/524,389 23 June 2017 (23.06.2017) US

(71) Applicant: **VICAL INCORPORATED** [US/US]; 10390
Pacific Center Court, San Diego, California 92121 (US).(72) Inventors: **SULLIVAN, Sean M.**; 2151 Lundy Lake Drive,
Escondido, California 92029 (US). **SMITH, Larry**;
12757 Larchmont Street, Poway, California 92064 (US).
CLEMENTE, Brenda; 3214 Camarillo Avenue, Ocean-
side, California 92056 (US). **WEI, Qun**; 11036 Corte Mar
De Delfinas, San Diego, California 92130 (US). **YE, Ming**;
6106 Lakewood Street, San Diego, California 92122 (US).
HAYASHI, Yasuhiro; 4F, Saito Bio-Incubator, 7-7-15,
Saito-asagi, Ibaraki, Osaka 567-0085 (JP).(74) Agent: **NOVOM, Antony M.** et al.; Wagenknecht IP Law
Group PC, 12396 World Trade Drive, Suite 312, San Diego,
California 92128 (US).(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: LIPID NANOPARTICLE (LNP)-MEDIATED DELIVERY OF A CRISPR-EXPRESSING PLASMID DNA FOR
TREATING CHRONIC HEPATITIS B VIRUS INFECTION**FIG. 1B**

(57) **Abstract:** Provided herein is a CRISPR-expressing plasmid DNA and a gene delivery system targeted to hepatocytes infected with hepatitis B virus. Also provided are methods of directing CRISPR system formation in such cells to ensure enhanced specificity for target recognition to alter, improve, or treat chronic HBV infection. In addition the CpG content of the expressing plasmid DNA has been minimized to reduce inflammation and maximize gene expression.



**LIPID NANOPARTICLE (LNP)-MEDIATED DELIVERY OF
A CRISPR-EXPRESSING PLASMID DNA FOR TREATING
CHRONIC HEPATITIS B VIRUS INFECTION**

CROSS REFERENCE TO RELATED APPLICATION(S)

[0001] This application claims the benefit of priority under 35 U.S.C. § 119(e) of US Serial No. 62/524,389, filed June 23, 2017, the entire content of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0002] The invention relates generally to hepatitis B infection and more specifically to delivery, engineering, optimization and therapeutic applications of a polynucleotide capable of gene expression and gene editing.

BACKGROUND INFORMATION

[0003] Hepatitis B is one of the world's most prevalent diseases. Although most individuals seem to resolve acute infection, approximately 30% of cases become chronic. According to current estimates, 350-400 million people worldwide have chronic hepatitis B, leading to 500,000-1,000,000 deaths per year due largely to the development of hepatocellular carcinoma, cirrhosis, and other complications. Despite the availability of an effective vaccine, immunoglobulin therapy, interferon, and antiviral drugs, hepatitis B remains a major global health problem.

[0004] The hepatitis B virus (HBV) is a double-stranded hepatotropic virus that infects only humans and non-human primates. Viral replication takes place predominantly in the liver and, to a lesser extent, in the kidneys, pancreas, bone marrow and spleen (Hepatitis B virus biology. *Microbiol Mol Biol Rev.* 64: 2000; 51-68.). Viral and immune markers are detectable in blood and characteristic antigen-antibody patterns evolve over time. The first detectable viral marker in blood is hepatitis B surface antigen (HBsAg), followed by hepatitis B e-antigen (HBeAg) and HBV DNA. Titers may be high during the incubation period, but HBV DNA and HBeAg levels begin to fall at the onset of illness and may be undetectable at the time of peak clinical illness (Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med.* 350: 2004; 1118-1129). HBeAg is a viral marker detectable in blood and correlates with active viral replication, and therefore high viral load

and infectivity (Hepatitis B e antigen--the dangerous end game of hepatitis B. N Engl J Med. 347: 2002; 208-210). The presence of anti-HBsAb and anti-hepatitis B core antibody (HBcAb) IgG indicates recovery and immunity in a previously infected individual.

[0005] Despite the availability of a prophylactic HBV vaccine, the burden of chronic HBV infection continues to be a significant unmet worldwide medical problem, due to suboptimal treatment options and sustained rates of new infections in most parts of the developing world. Current treatments do not provide a cure and are limited to only two classes of agents (interferon and nucleoside/nucleotide analogue inhibitors of the viral polymerase); drug resistance, low efficacy, and tolerability issues limit their impact. The low cure rates of HBV are attributed at least in part to the presence and persistence of covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes. However, persistent suppression of HBV DNA slows liver disease progression and helps to prevent hepatocellular carcinoma. Current therapy goals for HBV-infected patients are directed to reducing serum HBV DNA to low or undetectable levels, and to ultimately reducing or preventing the development of cirrhosis and hepatocellular carcinoma. Hence, there is a need in the art to discover and develop new HBV therapies to cure chronic HBV infection.

SUMMARY OF THE INVENTION

[0006] The present invention is based on the finding that a polynucleotide encoding a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) CRISPR-Cas system can be delivered using a lipid envelope engineered to target hepatocytes infected with HBV, resulting in reduction of viral nucleic acid. Accordingly, in one aspect, the invention provides a method of inactivating hepatitis B virus (HBV) nucleic acid in a host cell. The method includes transfecting a host cell containing HBV viral nucleic acid with a polycistronic expression cassette, wherein the expression cassette comprises: a RNA polymerase III promoter sequence, a 5'-untranslated region comprising two or more complexes, each complex consisting of a guide RNA (gRNA) sequence and a scaffold RNA (scRNA) sequence, and a 3'-untranslated region sequence, wherein CpG content of all sequences has been minimized to reduce immunostimulatory response but maintain maximal gRNA and CRISPR nuclease expression and wherein each gRNA sequence is capable of hybridizing to a target sequence of hepatitis B viral nucleic acid. In various embodiments, once the host cell has been transfected with the polycistronic

expression cassette, the method includes incubating the cell to promote expression of the polycistronic expression cassette, thereby inactivating HBV nucleic acid in the host cell. The method may be performed *in vitro* or *in vivo*, and the host cell may be a human cell, such as a hepatocyte. When performed *in vivo*, the vector may be administered in a lipid envelope encapsulating the vector, such as a lipid nanoparticle (LNP). In various embodiments, the LNP includes one or more cationic lipids of the ssPalm class, such as, but not limited to, ssPalmM, ssPalmE-P4C2, ssPalmE-Paz4-C2, and any combination thereof. In various embodiments, the LNP has a ratio of cationic lipid to plasmid DNA of 4:1 to 16:1, such as 8:1 or 12:1.

[0007] In various embodiments, the LNP may also include one or more lipids selected from the group consisting of cholesterol, phospholipids such as 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1,2-dielaiaoyl-sn-glycero-3-phosphoethanolamine (DEPE), 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (DPyPE), 1,2-dioleoyl-sn-glycero-phosphatidylserine (DOPS), 1-palmitoyl-2-oleoyl-sn-glycero-phosphatidylserine (POPS), 1,2-dimyristoyl-sn-glycero-phosphatidylserine (DMPS), 1,2 dipalmitoyl-sn-glycero-phosphatidylserine (DPPS), 1,2-distearoyl-sn-glycero-phosphatidylserine (DSPS), 1,2-dimyristoyl-sn-3-phosphatidylglycerol (DMPG), 1,2-dipalmitoyl-sn-3-phosphatidylglycerol (DPPG), 1,2-distearoyl-sn-3-phosphatidylglycerol (DSPG), 1-palmitoyl-2-oleoyl-sn-3-phosphatidylglycerol (POPG), 1,2-dimyristoyl-sn-glycero-3-phosphatidic acid (DMPA), 1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA), 1,2-distearoyl-sn-glycero-3-phosphatidic acid (DSPA), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidic acid (POPA); polyethyleneglycol (PEG) derivatized diacylglycerols such as 1,2-dimyristoyl-rac-glycerol-methylpolyoxyethylene (DMG-PEG₂₀₀₀), 1,2-dimyristoyl-rac-glycerol-methylpolyoxyethylene (DMG-PEG₅₀₀₀), 1,2-dipalmitoyl-rac-glycerol-methylpolyoxyethylene (DPG-PEG₂₀₀₀), 1,2-dipalmitoyl-rac-glycerol-methylpolyoxyethylene (DPG-PEG₅₀₀₀), 1,2-distearoyl-rac-glycerol-methylpolyoxyethylene

(DSG-PEG₂₀₀₀), 1,2-distearoyl-rac-glycerol-methylpolyoxyethylene (DSG-PEG₅₀₀₀); and gangliosides, such as GM1, GD1, globoside, lactosyl(β)ceramide, and lactosyl(β)sphingosine. In various embodiments, when present the DOPE can be substituted for other lipids, such as, but not limited to, dielidoylphosphatidylethanolamine (DEPE) and lipids with different head groups, such as phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, phosphatidic acid. The acyl chains can vary from myristoyl, palmitoyl, steryl, oleoyl. The acyl chains can be the same or mixed, such as oleoyl-palmitoyl-phosphatidylethanolamine. Other lipids useful in the LNP include diacyl glycerols.

[0008] In various embodiments, the LNP may also include dimyristoyl glycerol-polyethylene glycerol 2000 da (DMG-PEG), dipalmitoylglycerol-PEG, distearoylglycerol-PEG. In various embodiments, the LNP includes DMG-PEG at a concentration of about 2 mol% to 3.4 mol%. The polyethylene glycol portion of the DMG-PEG can vary in length from about 1,500 daltons to about 5,000 daltons. In various embodiments, the LNP includes cholesterol at about 20 mol% to about 33 mol%. In various embodiments, the LNP includes dexamethasone palmitate (DP) at a concentration of about 1.65 mol% to 5 mol%, such as 1.65 mol%, 3.3 mol%, or 5 mol%. In various embodiments, the LNP includes ssPalme-P4C2, DOPE, and cholesterol at mole ratios of about 60:10:30, 40:30:30, or 50:20:30.

[0009] In various embodiments, the method further includes detecting a decrease in hepatitis B surface antigen (HBsAg) production, a decrease in hepatitis B e-antigen (HBeAg) production, or a decrease in both HBsAg and HBeAg production in the cell. Thus, in embodiments performed *in vivo*, the step of transfecting comprises administering a vector comprising the polycistronic expression cassette to a subject suffering from chronic HBV infection.

[0010] In various embodiments, the gRNA sequence of each complex is selected from the group consisting of SEQ ID NOs: 15-38 and 39. Thus, in various embodiments, the polycistronic expression cassette is a bicistronic expression cassette, and the gRNA sequences may, for example, be SEQ ID NOs: 16 and 25, SEQ ID NOs: 27 and 28, or SEQ ID NOs: 28 and 39. In various embodiments, the bicistronic expression cassette may further include a plurality of transfer RNA (tRNA) sequences, each tRNA sequence

flanking one of the two or more complexes. In various embodiments, the plurality of tRNA sequences are independently selected from the group consisting of SEQ ID NOs: 1 and 2. In various embodiments, the scRNA sequence of each complex is SEQ ID NO: 3.

[0011] In various embodiments, the bicistronic expression cassette may further include a microRNA (miRNA) sequence located between each of the two or more complexes, wherein the miRNA sequence encodes a miRNA molecule that targets HBV nucleic acid, such as a sequence encoding precursor miRNA (pre-miRNA) molecule. In embodiments that include one or more miRNA sequences, the gRNA sequences are SEQ ID NOs: 28 and 39 or SEQ ID NOs: 16 and 25.

[0012] In various embodiments, the polycistronic expression cassette may be a tetracistronic expression cassette, and may further include a plurality of transfer RNA (tRNA) sequences, each tRNA sequence flanking one of the two or more complexes. In various embodiments, the tRNA sequences are independently selected from the group consisting of SEQ ID NOs: 1, 2, and 3, and the gRNA sequences may be SEQ ID NOs: 16, 25, 27, and 28.

[0013] In various embodiments, the expression cassette further includes a RNA polymerase II promoter sequence selectively active in hepatocytes located upstream of the CRISPR nuclease sequence. In various embodiments, the expression cassette further includes one or more additional promoters and/or enhancers such as, but not limited to, human alpha1 antitrypsin, human phenylalanine hydroxylase, apolipoprotein E/C-1 hepatic control region, α_1 microglobulin/bikunin enhancer, human thyroxine binding globulin (TBG), serum albumin, multimerized hepatocyte nuclear factor-3 (HNF-3) binding site, and an enhancer that can bind an HBV-specific transcription activator. In various embodiments, the 3'-untranslated region sequence comprises a poly(A) tail.

[0014] In another aspect, the invention provides a vector. In various embodiments, the vector includes a polynucleotide that includes: a RNA polymerase III promoter sequence, a 5'-untranslated region comprising at least one intron, wherein the intron comprises two or more guides, each being flanked by a transfer RNA (tRNA) sequence, wherein each guide comprises a guide RNA (gRNA) sequence and a scaffold RNA (scRNA) sequence, a CRISPR nuclease sequence, and a 3'-untranslated region sequence, wherein CpG content of

all sequences has been minimized to reduce immunostimulatory response but maintain maximal gRNA and CRISPR nuclease expression.

[0015] In various embodiments, the RNA polymerase III promoter is selected from the group consisting of SEQ ID NOs: 12, 13, and 14. In various embodiments, the flanking tRNA sequences are independently selected from the group consisting of SEQ ID NOs: 1 and 2. In various embodiments, the gRNA sequences are independently selected from the group consisting of SEQ ID NOs: 15-38 and 39. In various embodiments, the vector may also include a RNA polymerase II promoter sequence selectively active in hepatocytes located upstream of the CRISPR nuclease sequence. In various embodiments, the vector may also include one or more of human alpha1 antitrypsin, human phenylalanine hydroxylase, apolipoprotein E/C-1 hepatic control region, α_1 microglobulin/bikunin enhancer, human thyroxine binding globulin (TBG), serum albumin, multimerized hepatocyte nuclear factor-3 (HNF-3) binding site, and an enhancer that can bind an HBV-specific transcription activator. In various embodiments, the vector may also include a hepatocyte-specific enhancer sequence operably linked to the RNA polymerase II promoter.

[0016] In various embodiments, the intron comprises two guides, wherein the gRNA sequences are SEQ ID NOs: 16 and 25, SEQ ID NOs: 27 and 28, or SEQ ID NOs: 28 and 39. In various embodiments, the intron comprises four guides, wherein the gRNA sequences are SEQ ID NOs: 16, 25, 27, and 28.

[0017] In another aspect, the vector may include a polynucleotide which comprises: a RNA polymerase III promoter sequence, a 5'-untranslated region comprising at least one intron, wherein the intron comprises a microRNA (miRNA) molecule flanked by two guides, wherein each guide comprises a guide RNA (gRNA) sequence and a scaffold RNA (scRNA) sequence, an RNA polymerase II enhancer sequence, RNA polymerase II promoter sequence, a CRISPR nuclease sequence, and a 3'-untranslated region sequence, wherein CpG content of all sequences has been minimized to reduce immunostimulatory response but maintain maximal gRNA and CRISPR nuclease expression and wherein each gRNA sequence is capable of hybridizing to a target sequence of hepatitis B viral nucleic acid.

[0018] In various embodiments, the RNA polymerase III promoter is selected from the group consisting of SEQ ID NOs: 12, 13, and 14. In various embodiments, the miRNA

sequence encodes a precursor miRNA molecule (e.g., pre-HBV-miRNA). In various embodiments, the gRNA sequences are independently selected from the group consisting of SEQ ID NOs: 15-38 and 39. In various embodiments, the gRNA sequences are SEQ ID NOs: 28 and 39 or SEQ ID NOs: 16 and 25. In various embodiments, the vector also includes a RNA polymerase II promoter sequence selectively active in hepatocytes located upstream of the CRISPR nuclease sequence. In various embodiments, the vector may also include one or more of human alpha1 antitrypsin, human phenylalanine hydroxylase, apolipoprotein E/C-1 hepatic control region, α_1 microglobulin/bikunin enhancer, human thyroxine binding globulin (TBG), serum albumin, multimerized hepatocyte nuclear factor-3 (HNF-3) binding site, and an enhancer that can bind an HBV-specific transcription activator. In various embodiments, the vector also includes a hepatocyte-specific enhancer sequence operably linked to the RNA polymerase II promoter.

[0019] In any embodiments, the expression plasmid may contain introns at other locations besides the 5'UTR, such as the open reading frame (ORF). In various embodiments, the target hepatitis B viral nucleic acid is an episomal nucleic acid molecule, such as a cccDNA, and integrated into the genome of the organism. As such, the CRISPR nuclease may be capable of reducing the amount of episomal viral nucleic acid molecule in a cell of the organism compared to the amount of episomal viral nucleic acid molecule in a cell of the organism in the absence of providing the vector. Hepatitis B infected hepatocytes can have viral genomes integrated into the hepatocyte genome. Thus, the target may also be the HBV integrated genome as well as the episomal HBV cccDNA.

[0020] As described herein, the CRISPR nuclease sequence encodes a CRISPR nuclease selected from the group consisting of *Streptococcus pyogenes* Cas9 (SpCas9) and variants of SpCas9, such as VRER, VQR, and EQR, high-fidelity SpCas9 (SpCas9-HF1), *Staphylococcus aureus* Cas9 (SaCas9), *Streptococcus thermophilus* Cas9 (StCas9), a nuclease derived from *Prevotella* and *Francisella* bacteria (Cpf1), *Neisseria meningitidis* (NM), *Streptococcus thermophilus* (ST), *Treponema denticola* and a biologically active fragment or derivative thereof. In various embodiments, the CRISPR nuclease is CpG-free and human codon-optimized, and may be encoded by the sequence as set forth in SEQ ID NO: 10 or SEQ ID NO: 11. In various embodiments, the nuclease gene also includes DNA sequence(s) encoding one or more nuclear localization sequences (NLS).

[0021] In another aspect, the present invention provides a gene delivery system. The gene delivery system includes a lipid envelope encapsulating the vector as described herein. In various embodiments, the vector is complexed with a condensing agent, such as protamine, spermine, spermidine, cadaverine, putrescine, histones, and virus capsid proteins. In various embodiments, the condensing agent is protamine sulfate. The lipid envelope may form an LNP, or may be a naturally occurring or synthetic exosome. In various embodiments, the LNP is formed from one or more cationic lipids of the ssPALM class, such as, but not limited to, ssPalm, ssPalmE-P4C2, ssPalmE-Paz4-C2, and any combination thereof. In various embodiments, the LNP also includes one or more lipids selected from the group consisting of cholesterol, DOPE, DMG-PEG, distearoyl glycerol, SOPC, DEPE, DMPC, DPPC, DSPC, DOPC, POPC, DOPS, DMPS, DOPA, DMPA, and DPPA. In various embodiments, the lipid envelope includes one or more ligands that bind to hepatocyte receptors displayed thereon. Such ligands may be adsorbed to the surface of the lipid envelope or are covalently derivatized to specific components of the lipid envelope, such as a functionalized DMG-PEG-maleimide or simply a DMG-PEG ending with a primary amine or thiol. Alternatively, a phospholipid can be functionalized, such as phosphatidylethanolamine with a terminal maleimide or thiol group.

[0022] In another aspect, the present invention provides a method of treating chronic HBV infection in a subject. The method includes administering an effective amount of the gene delivery system as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Figures 1A and 1B are pictorial diagrams showing exemplary HBV gene editing expression plasmids of the invention.

[0024] Figure 2 is a pictorial diagram showing an exemplary poly-gRNA/tRNA expression cassette for use in the expression plasmids of the invention.

[0025] Figure 3 is a pictorial diagram showing an exemplary gRNA-miRNA-gRNA expression cassette for use in the expression plasmids of the invention.

DETAILED DESCRIPTION OF THE INVENTION

[0026] The present invention is based on the finding that a polynucleotide encoding a CRISPR-Cas system can be delivered using a lipid envelope engineered to target

hepatocytes infected with HBV, resulting in reduction of viral nucleic acid. Such reduction in viral nucleic acid reduces the extent of infection, thereby treating HBV infection in the subject.

[0027] Before the present compositions and methods are described, it is to be understood that this invention is not limited to particular compositions, methods, and experimental conditions described, as such compositions, methods, and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only in the appended claims.

[0028] As used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise. Thus, for example, references to “the method” includes one or more methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0029] The term “comprising,” which is used interchangeably with “including,” “containing,” or “characterized by,” is inclusive of open-ended language and does not exclude additional, unrecited elements or method steps. The phrase “consisting of” excludes any element, step, or ingredient not specified in the claim. The phrase “consisting essentially of” limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristics of the claimed invention. The present disclosure contemplates embodiments of the invention compositions and methods corresponding to the scope of each of these phrases. Thus, a composition or method comprising recited elements or steps contemplates particular embodiments in which the composition or method consists essentially of or consists of those elements or steps.

[0030] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described.

[0031] The term “subject” or “host organism,” as used herein, refers to any individual or patient to which the subject methods are performed. Generally the subject is human, although as will be appreciated by those in the art, the subject may be an animal. Thus other animals, including mammals such as rodents (including mice, rats, hamsters and guinea pigs), cats, dogs, rabbits, farm animals including cows, horses, goats, sheep, pigs, etc., and primates (including monkeys, chimpanzees, orangutans and gorillas) are included within the definition of subject.

[0032] The term “therapeutically effective amount” or “effective amount” means the amount of a compound or pharmaceutical composition that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician. Thus, the term “therapeutically effective amount” is used herein to denote any amount of a formulation that causes a substantial improvement in a disease condition when applied to the affected areas after a administration or multiple administrations over a period of time. The amount will vary with the condition being treated, the stage of advancement of the condition, and the type and concentration of formulation applied. Appropriate amounts in any given instance will be readily apparent to those skilled in the art or capable of determination by routine experimentation.

[0033] A “therapeutic effect,” as used herein, encompasses a therapeutic benefit and/or a prophylactic benefit as described herein.

[0034] The terms “administration” or “administering” are defined to include an act of providing a compound or pharmaceutical composition of the invention to a subject in need of treatment. The phrases “parenteral administration” and “administered parenterally” as used herein means modes of administration other than enteral (usually orally) and topical administration, or by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and infrasternal injection and infusion. The phrases “systemic administration,” “administered systemically,” “peripheral administration” and “administered peripherally” as used herein mean the administration of a compound, drug or other material other than

directly into the central nervous system, such that it enters the subject's system and, thus, is subject to metabolism and other like processes, for example, intravenous administration.

[0035] As used herein, the terms “reduce” and “inhibit” are used together because it is recognized that, in some cases, a decrease can be reduced below the level of detection of a particular assay. As such, it may not always be clear whether the expression level or activity is “reduced” below a level of detection of an assay, or is completely “inhibited.”

Nevertheless, it will be clearly determinable, following a treatment according to the present methods.

[0036] As used herein, “treatment” or “treating” means to administer a composition to a subject or a system with an undesired condition. The condition can include a disease or disorder. “Prevention” or “preventing” means to administer a composition to a subject or a system at risk for the condition, and therefore includes preventing disease progression in symptomatic or asymptomatic subjects. The condition can include a predisposition to a disease or disorder. The effect of the administration of the composition to the subject (either treating and/or preventing) can be, but is not limited to, the cessation of one or more symptoms of the condition, a reduction or prevention of one or more symptoms of the condition, a reduction in the severity of the condition, the complete ablation of the condition, a stabilization or delay of the development or progression of a particular event or characteristic, or minimization of the chances that a particular event or characteristic will occur.

[0037] As used herein “pharmaceutically acceptable carrier” encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water and emulsions such as an oil/water or water/oil emulsion, cryopreservatives for lyophilization and various types of wetting agents.

[0038] “Acute hepatitis B infection” results when a person is exposed to the hepatitis B virus, but may or may not have begun to develop the signs and symptoms of viral hepatitis. This period of time from infection to the presentations of symptoms, called the incubation period, is an average of 90 days, but could be as short as 45 days or as long as 6 months. For most people this infection will cause mild to moderate discomfort, but will go away by itself because of the body's immune response succeeds in fighting the virus. However, some people, particularly those with compromised immune systems, such as persons

suffering from AIDS, undergoing chemotherapy, taking immunosuppressant drugs, or taking steroids, have very serious problems as a result of the acute HBV infection, and go on to more severe conditions such as fulminant liver failure.

[0039] “Chronic hepatitis B infection” occurs when a person infected with HBV is unable to eliminate the virus; this is clinically defined as having detectable HBsAg in blood for over 6 months. Whether the disease becomes chronic or completely resolves depends mostly on the age when the person becomes infected. About 90% of infants infected at birth will develop to chronic HBV infection. However, as a person ages, the risk of developing chronic infection decreases such that between 20%-50% of children and less than 10% of older children or adults will develop chronic infection.

[0040] The terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

[0041] The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, α -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0042] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical

Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0043] “Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are “silent variations,” which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0044] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a “conservatively modified variant” where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0045] As used herein, a “protein coding sequence” or a sequence that encodes a particular protein or polypeptide, is a nucleic acid sequence that is transcribed into mRNA (in the case of DNA) and is translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' terminus (N-terminus) and a

translation stop nonsense codon at the 3' terminus (C-terminus). A coding sequence can include, but is not limited to, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic DNA, viral DNA, and synthetic nucleic acids. A transcription termination sequence will usually be located 3' to the coding sequence.

[0046] As used herein, an “expression cassette” refers to a portion of vector DNA that includes one or more genes and one or more regulatory sequences controlling their expression. In each successful transformation, the expression cassette directs the cell’s machinery to make RNA and/or protein(s).

[0047] As used herein, the term “gene” means the deoxyribonucleotide sequences that codes for a molecule that has a function. A “structural gene” refers to a gene that codes for an RNA or protein other than a regulatory factor, but is nonetheless encompassed within the definition of “gene.” A “gene” may also include non-translated sequences located adjacent to the coding region on both the 5' and 3' ends such that the gene corresponds to the length of the full-length mRNA. The sequences which are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term “gene” encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed “introns” or “intervening regions” or “intervening sequences.” Introns are segments of a gene which are transcribed into heterogeneous nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or “spliced out” from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

[0048] An mRNA molecule is said to be “monocistronic” when it contains the genetic information to translate only a single protein chain (polypeptide). On the other hand, “polycistronic mRNA” carries several open reading frames (ORFs), each of which is translated into a polypeptide. These polypeptides usually have a related function (they often are the subunits composing a final complex protein) and their coding sequence is grouped and regulated together in a regulatory region, containing a promoter and an operator.

[0049] As used herein, “transfer RNA” or “tRNA” refers to an adaptor molecule composed of RNA, typically 76 to 90 nucleotides in length that serves as the physical link between the mRNA and the amino acid sequence of proteins. Without being bound by theory, the role of tRNA is to specify which sequence from the genetic code corresponds to which amino acid during protein synthesis. Exemplary tRNA sequences useful in the invention include, but are not limited to:

AACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACC
CGGGTTCGATTCCCGGCTGGTGCA (SEQ ID NO: 1; glycine)

AAC TTGAAAAAGTGGCACC GAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGG
TAGAATAGTACCCTGCCACGGTACAGACCCGGGTTTCGATTCCCGGCTGGTGCA
(SEQ ID NO: 2)

[0050] As used herein, “covalently closed circular DNA” or “cccDNA” refers to a partially double-stranded DNA that is ligated by means of DNA ligase to a covalently closed ring. cccDNA arises during propagation of some viruses, such as HBV, in the cell nucleus.

[0051] As used herein, “microRNA” or “miRNA” refers to a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals and some viruses that functions in RNA silencing and post-transcriptional regulation of gene expression. While the majority of miRNAs are located within the cell, some miRNAs, commonly known as circulating miRNAs or extracellular miRNAs, have also been found in extracellular environment, including various biological fluids and cell culture media. miRNAs resemble the small interfering RNAs (siRNAs) of the RNA interference (RNAi) pathway, except miRNAs derive from regions of RNA transcripts that fold back on themselves to form short hairpins, whereas siRNAs derive from longer regions of double-stranded RNA.

[0052] The HBV capsid protein plays essential functions during the viral life cycle. HBV capsid/core proteins form metastable viral particles or protein shells that protect the viral genome during intercellular passage, and also play a central role in viral replication processes, including genome encapsidation, genome replication, and virion morphogenesis and egress. Capsid structures also respond to environmental cues to allow uncoating after

viral entry. Proper capsid assembly has consistently been found to be critical for viral infectivity.

[0053] The crucial function of HBV capsid proteins imposes stringent evolutionary constraints on the viral capsid protein sequence, leading to the observed low sequence variability and high conservation. Consistently, mutations in HBV capsid that disrupt its assembly are lethal with regard to negatively impacting virus replication, and mutations that perturb capsid stability severely attenuate viral replication.

[0054] HBV replication centers on the establishment of a cccDNA form of its genome in the host cell nucleus. This episomal form is established from conversion of the partially double stranded circular DNA (relaxed circular, or rcDNA) genome upon initial infection, and functions as the template for transcribing all HBV mRNAs. As indicated above, HBV DNA synthesis is coupled to assembly of its capsid, and most copies of the encapsidated genome then efficiently associate with the envelope proteins for virion assembly and secretion; a minority of these genomes are shunted to the nucleus where they are converted to cccDNA, thus amplifying levels of the episome. As such, inactivation of HBV cccDNA should impair these processes within the infected subject. The optimal outcome of cccDNA inactivation would be to cure a subject of chronic HBV infection and consequently mitigate the risk of developing cirrhosis and hepatocellular carcinoma.

[0055] As used herein, the term “genetic modification” is used to refer to any manipulation of an organism’s genetic material in a way that does not occur under natural conditions. Methods of performing such manipulations are known to those of ordinary skill in the art and include, but are not limited to, techniques that make use of vectors for transforming cells with a nucleic acid sequence of interest. Included in the definition are various forms of gene editing in which DNA is inserted, deleted or replaced in the genome of a living organism using engineered nucleases, or “molecular scissors.” These nucleases create site-specific double-strand breaks (DSBs) at desired locations in the genome. The induced double-strand breaks are repaired through nonhomologous end-joining (NHEJ) or homologous recombination (HR), resulting in targeted mutations (*i.e.*, edits).

[0056] There are several families of engineered nucleases used in gene editing, for example, but not limited to, meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALEN), and the CRISPR-Cas system.

[0057] CRISPR is an acronym for DNA loci that contain multiple, short, direct repetitions of base sequences. The prokaryotic CRISPR/Cas system has been adapted for use as gene editing (*i.e.*, silencing, enhancing or changing specific genes) in eukaryotes (see, for example, Cong, *Science*, 15:339(6121):819-823 (2013) and Jinek, *et al.*, *Science*, 337(6096):816-21 (2012)). By transfecting a cell with elements including a Cas gene, nucleic acid sequences can be cut and modified at any desired location. Methods of preparing compositions for use in genome editing using the CRISPR/Cas systems are described in detail in US Pub. No. 2016/0340661, US Pub. No. 20160340662, US Pub. No. 2016/0354487, US Pub. No. 2016/0355796, US Pub. No. 20160355797, and WO 2014/018423, which are specifically incorporated by reference herein in their entireties.

[0058] Thus, as used herein, “CRISPR system” refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated (“Cas”) genes, including sequences encoding a Cas gene, a *tracr* (trans-activating CRISPR) sequence (*e.g.*, *tracr*RNA or an active partial *tracr*RNA), a *tracr*-mate sequence (encompassing a “direct repeat” and a *tracr*RNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (“guide RNA” or “gRNA” in the context of an endogenous CRISPR system), or other sequences and transcripts from a CRISPR locus. One or more *tracr*-mate sequences operably linked to a guide sequence (*e.g.*, direct repeat-spacer-direct repeat) can also be referred to as “pre-crRNA” (pre-CRISPR RNA) before processing or crRNA after processing by a nuclease.

[0059] In some embodiments, a *tracr*RNA and crRNA are linked and form a chimeric crRNA-*tracr*RNA hybrid where a mature crRNA is fused to a partial *tracr*RNA via a synthetic stem loop to mimic the natural crRNA:*tracr*RNA duplex as described in Cong, *Science*, 15:339(6121):819-823 (2013) and Jinek, *et al.*, *Science*, 337(6096):816-21 (2012)). A single fused crRNA-*tracr*RNA construct can also be referred to as a guide RNA or gRNA (or single-guide RNA (sgRNA)). Within a sgRNA, the crRNA portion can be identified as the ‘target sequence’ and the *tracr*RNA is often referred to as the ‘scaffold’ RNA (scRNA).

[0060] There are many resources available for helping practitioners determine suitable target sites once a desired DNA target sequence is identified. For example, numerous public resources, including a bioinformatically generated list of about 190,000 potential sgRNAs, targeting more than 40% of human exons, are available to aid practitioners in

selecting target sites and designing the associated sgRNA to affect a nick or double strand break at the site. See also, crispr.u-psud.fr, a tool designed to help scientists find CRISPR targeting sites in a wide range of species and generate the appropriate crRNA sequences.

[0061] While the specifics can be varied in different engineered CRISPR systems, the overall methodology is similar. For example, a practitioner interested in using CRISPR technology to target a DNA sequence can insert a short DNA fragment containing the target sequence into a guide RNA expression plasmid. The sgRNA expression plasmid therefore contains the target sequence (about 20 nucleotides), a form of the tracrRNA sequence (*i.e.*, the scRNA) as well as a suitable promoter and necessary elements for proper processing in eukaryotic cells. Such vectors are commercially available (see, for example, Addgene). Many of the systems rely on custom, complementary oligos that are annealed to form a double stranded DNA and then cloned into the sgRNA expression plasmid. Co-expression of the sgRNA and the appropriate Cas enzyme from the same or separate plasmids in transfected cells results in a single or double strand break (depending of the activity of the Cas enzyme) at the desired target site.

[0062] Typically, the CRISPR complex of the invention, when introduced into a cell, creates a break (*e.g.*, a single or a double strand break) in the target DNA sequence. For example, the method can be used to cleave a disease gene in a cell. The break created by the CRISPR complex can be repaired by repair processes such as the error prone non-homologous end joining (NHEJ) pathway or the high fidelity homology-directed repair (HDR). During these repair processes, an exogenous polynucleotide template can be introduced into the genome sequence. In some methods, the HDR process is used to modify a genome sequence. For example, an exogenous polynucleotide template comprising a sequence to be integrated flanked by an upstream sequence and a downstream sequence is introduced into a cell. The upstream and downstream sequences share sequence similarity with either side of the site of integration in the chromosome. Where desired, a donor polynucleotide can be DNA, *e.g.*, a plasmid DNA (pDNA), a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), a viral vector, a linear piece of DNA, a PCR fragment, a naked nucleic acid, or a nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. Thus, the modifications of the target DNA due to NHEJ and/or homology-directed repair can be used to induce gene correction, gene

replacement, gene tagging, transgene insertion, nucleotide deletion, gene disruption, gene mutation, etc.

[0063] Accordingly, the present invention provides an expression system for delivering a CRISPR system to cells (*e.g.*, hepatocytes) harboring HBV cccDNA, such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at the target site, which leads to inactivation of the HBV cccDNA.

[0064] As used herein, a “vector” is a tool that allows or facilitates the transfer of an entity from one environment to another. It is a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment in the appropriate prokaryotic or eukaryotic cell. Generally, a vector is capable of replication when associated with the proper control elements. In general, the term “vector” refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (*e.g.* circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a “plasmid,” which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. The vector can be made synthetically using appropriate primers and a high fidelity proof reading DNA polymerase. Another type of vector is a “viral vector,” wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (*e.g.*, retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses, AAVs). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as “expression vectors.” Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

[0065] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, “operably linked” is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression (*e.g.*, transcription and translation) of the nucleotide sequence in a host cell when the vector is introduced into the host cell.

[0066] The term “regulatory element” is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (*e.g.*, transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences).

[0067] Accordingly, in one aspect, the invention provides a therapeutic expression plasmid for inactivating HBV cccDNA, thereby treating or preventing chronic HBV infection in a subject and mitigating the risk of developing cirrhosis and/or hepatocellular carcinoma. As shown in Figures 1A and 1B, the expression system is a HBV gene editing expression plasmid containing that includes at least one a promoter, at least one enhancer, a 5' untranslated region (5'-UTR), a nuclease spaced apart from the 5'-UTR by a spacer or intron, and a 3' untranslated region (3'-UTR), all of which will be explained in detail below.

[0068] Promoter

[0069] As used herein, a “promoter” is defined as a regulatory DNA sequence generally located upstream of a gene that mediates the initiation of transcription by directing RNA polymerase to bind to DNA and initiating RNA synthesis. A promoter can be a constitutively active promoter (*i.e.*, a promoter that is constitutively in an active/"ON" state), it may be an inducible promoter (*i.e.*, a promoter whose state, active/"ON" or inactive/"OFF", is controlled by an external stimulus, *e.g.*, the presence of a particular

compound or protein), it may be a spatially restricted promoter (*i.e.*, transcriptional control element, enhancer, etc.) (*e.g.*, tissue specific promoter, cell type specific promoter, etc.), and it may be a temporally restricted promoter (*i.e.*, the promoter is in the "ON" state or "OFF" state during specific stages of embryonic development or during specific stages of a biological process).

[0070] A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as a specific organ (*e.g.*, liver), or particular cell types (*e.g.*, hepatocytes). Thus, the plasmid of the invention includes a promoter that is selectively active in hepatocytes that will transcribe CRISPR RNA only in liver cells where HBV replication occurs. In various embodiments, the plasmid may express the gRNAs under a RNA polymerase II (pol II) promoter along with the nuclease. An exemplary promoter useful in the plasmid of the invention includes, but is not limited to, the elongation factor-1 alpha (EF-1 α) promoter. Alternatively, the gRNAs can be expressed using a RNA polymerase III (pol III) promoter, such as the U6 promoter that is commonly used for driving small hairpin RNA (shRNA) expression. Exemplary RNA pol III promoters useful in the invention include, but are not limited to, U6 promoter, 7SK promoter, and H1 promoter.

[0071] However, the pol III promoter will not restrict gRNA expression to hepatocytes whereas the pol II promoter will not only restrict expression to hepatocytes but can be engineered to restrict expression to hepatocytes infected with HBV. Exemplary hepatocyte-specific promoters useful in the plasmid of the invention include, but are not limited to, human alpha-1 antitrypsin (Hafenrichter, *et al.* 1994 *Blood* 84:3394-3404) and human phenylalanine hydroxylase (Chatterjee, *et al.* 1996 *PNAS* 93:728-733).

[0072] Enhancer

[0073] As used herein, an "enhancer" is a short (50-1500 bp) region of DNA that can be bound by proteins (activators) to increase the likelihood that transcription of a particular gene will occur. Thus, an enhancer may be used to increase promoter strength with regard to expression of the open reading frame for gene expression. In various embodiments, the enhancer may be paired with an endogenous hepatocyte promoter or it can be a non-specific enhancer that increases the promoter strength of an endogenous hepatocyte promoter.

[0074] Exemplary liver-specific enhancers useful in the plasmid of the invention include, but are not limited to, albumin or hepatitis B enhancers (Kramer, *et al.*, 2003 *Mol Ther* 7: 375-385), multimerized HNF-3 binding site (Hafenrichter, *et al.*, 1994 *J Surgical Research* 56: 510-717), multimerized α_1 -microglobulin enhancer + two copies of hepatocyte control region 1 (Jacobs, *et al.*, 2008 *Gene Therapy* 15: 594-603) and the cytomegalovirus (CMV) enhancer. Another aspect of the enhancer is that it can bind an HBV-specific transcription activator that is expressed during HBV replication, namely the X protein. Finally, the enhancer element can bind a transcription activator that is part of the hepatocyte genome but is preferentially upregulated during expression of hepatitis virus proteins.

[0075] As such, in various embodiments, the expression cassette further includes one or more additional promoters and/or enhancers such as, but not limited to, human alpha1 antitrypsin, human phenylalanine hydroxylase, apolipoprotein E/C-1 hepatic control region, α_1 microglobulin/bikunin enhancer, human thyroxine binding globulin (TBG), serum albumin, multimerized hepatocyte nuclear factor-3 (HNF-3) binding site, CMV enhancer, and an enhancer that can bind an HBV-specific transcription activator.

[0076] 5' Untranslated Region (UTR)

[0077] As used herein, “5' untranslated region” or “5'-UTR” (also known as a leader sequence or leader RNA) refers to a region of mRNA that is directly upstream from the initiation codon and important for the regulation of translation of a transcript. While called untranslated, the 5' UTR or a portion of it is sometimes translated into a protein product. This product can then regulate the translation of the main coding sequence of the mRNA.

[0078] In various embodiments, the 5' UTR contains introns that are spliced out prior to translocation of the mRNA from the nucleus into the cytoplasm. As such, the intron RNAs remain in the nucleus. However, it is important for the gRNAs to be in the nucleus. Hence, the gRNA can be incorporated into the intron and flanked on the 5' and 3' ends with processing ribozymes (*i.e.*, catalytic RNAs) to remove excess bases. As used herein, a “ribozyme” is an RNA molecule capable of catalyzing specific biochemical reactions. In various embodiments, the intron includes one or more (*i.e.*, 1, 2, 3, 4, 5, or more) gRNAs, each flanked with 5' and 3' processing ribozymes. For example, the intron may include three gRNAs, each being flanked by 5' and 3' processing ribozymes. Alternatively, multiple

gRNAs can be processed from the intron using tRNA or miRNA endogenous processing enzymes as in the polycistronic and ternary gRNAs expressed under the Pol III promoter, discussed in further detail below.

[0079] Since a single gRNA might be insufficient to completely inactivate the production of HBV, the expression system may include two or more gRNA sequences to maximize HBV inactivation. As is known in the art, expression of more than one gRNA can be accomplished in several ways, including, for example, administering multiple expression plasmids, each including a RNA Pol III promoter driving the respective gRNA. While inactivation of HBV may be accomplished utilizing a smaller dose of each plasmid, this strategy requires that all administered plasmids express the gRNA in the same cell at the same time.

[0080] Alternatively, a single plasmid may include two or more gRNA sequences, along with a RNA Pol III promoter for each gRNA sequence. However, this strategy can result in competition between the various RNA Pol III promoters resulting in expression of different amounts of each of the gRNA sequences. In addition, propagating the pDNAs in bacteria can be challenging because bacteria are known to sometimes delete DNA segments from pDNAs that contain repetitive sequences.

[0081] Accordingly, the expression system described herein expresses all gRNA sequences in a single transcript and utilizes the mammalian cell machinery to process the individual guides. In one embodiment, the expression system includes a polycistronic tRNA expression cassette that places a tRNA between each gRNA/scRNA and utilizes endogenous ribonuclease P (RNase P) and endogenous ribonuclease Z (RNase Z) to specifically cleave the RNA sequences on either side of the tRNA yielding individual gRNA/scRNAs. Figure 2 shows a diagram of an exemplary polycistronic tRNA expression cassette driven by the RNA Pol III promoter.

[0082] An exemplary scRNA sequence useful in the invention includes, but is not limited to:

GTTTGTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTGAAAAGTGGCACCAGTCGGTGC (SEQ ID NO: 3).

[0083] Thus, an exemplary polycistronic tRNA expression cassette may have the following structure:

Pol III promoter-tRNA-gRNA₁/scRNA-tRNA-gRNA₂/scRNA-tRNA-gRNA₃/scRNA-tRNA-gRNA₄/scRNA-tRNA-TTTTTT (SEQ ID NO: 4),

where each of gRNA₁, gRNA₂, gRNA₃, and gRNA₄ are independently selected from those shown in Table 2. Following this structure, the polycistronic tRNA expression cassette may, for example, be set forth as follows:

Pol III promoter-(SEQ ID NO: 1)-gRNA₁-(SEQ ID NO: 3)-(SEQ ID NO: 2)-gRNA₂-(SEQ ID NO: 3)-(SEQ ID NO: 1)-gRNA₃-(SEQ ID NO: 3)-(SEQ ID NO: 2)-gRNA₄-(SEQ ID NO: 3)-(SEQ ID NO: 4).

In yet another non-limiting example, the polycistronic tRNA expression cassette may be set forth as follows:

Pol III promoter-(SEQ ID NO: 1)-gRNA₁-(SEQ ID NO: 3)-(SEQ ID NO: 1)-gRNA₂-(SEQ ID NO: 3)-(SEQ ID NO: 1)-gRNA₃-(SEQ ID NO: 3)-(SEQ ID NO: 1)-gRNA₄-(SEQ ID NO: 3)-(SEQ ID NO: 4).

[0084] In another embodiment, the expression system includes a gRNA-miRNA-gRNA expression cassette, where the miRNA, Pre-HBV-miRNA, is positioned between two gRNA/scRNAs, as described by Wang, et al. (Wang, et al. The gRNA-miRNA-gRNA ternary cassette combining CRISPR/Cas9 with RNAi approach strongly inhibits hepatitis B virus Replication Theranostics (2017) 7: 3090-3105, incorporated herein by reference). A nuclear endonuclease, Drosha, specifically cleaves the RNA transcript on either side of the short hair pin RNA to release the short hair pin RNA. The stem-loop precursor is thereafter exported from the nucleus and subsequently processed by Dicer into microRNA, which is released in the cytoplasm. The miRNA is further processed for the siRNA strand to enter the RNA-induced silencing complex (RISC) (see, Petersen, et al. Short RNAs repress translation after initiation in mammalian cells Mol Cell (2006) 21:533-542, incorporated herein by reference). This provides an additional advantage in that the miRNA can be targeted to a key mRNA transcript and inhibit translation of an HBV protein. A diagram of an exemplary bicistronic miRNA processing cassette is shown in Figure 3.

[0085] Thus, an exemplary polycistronic miRNA expression cassette may have the following structure:

Pol III promoter-gRNA₁/scRNA-miRNA-gRNA₂/scRNA-TTTTTT (SEQ ID NO: 4),

where each of gRNA₁ and gRNA₂ are independently selected from those shown in Table 2.

In various embodiments, the Pre-HBV-miRNA molecule will include one or more of:

AGGGATGGTATTGCTCCTGTAAC TCGGAACTGGAGAGG (SEQ ID NO: 5; spacer RNA);

GGTGAAGCGAAGTGCACACGG (SEQ ID NO: 6; miRNA (minus strand));

GTTGAACTGGGAACG (SEQ ID NO: 7; loop);

ACGTGTGCACATCGATTACGGC (SEQ ID NO: 8; miRNA (plus strand)); and

TTTCCTGTCTGACAGCAGCTTGGCTACCTCCGTCCTGTTCGAG (SEQ ID NO: 9; spacer RNA).

Following this structure, the polycistronic miRNA expression cassette may, for example, be set forth as follows:

Pol III promoter-gRNA₁-(SEQ ID NO: 3)-(SEQ ID NO: 5)-(SEQ ID NO: 6)-(SEQ ID NO: 7)-(SEQ ID NO: 8)-(SEQ ID NO: 9)-gRNA₂-(SEQ ID NO: 3)-(SEQ ID NO: 4).

[0086] As described in greater detail below, a plurality of both types of poly-gRNA expression cassettes were constructed and tested for inhibition of HBeAg and HBsAg. gRNA selection was based on >20% inhibition of HBeAg production in HepAD38 cells and where the gRNA had 100% homology HBV genotypes A, B, C and D. Accordingly, exemplary poly-gRNA expression cassettes useful in the expression system are set forth in Table 1.

Table 1: Exemplary poly-gRNA Expression Cassettes

Plasmid Name	Number of gRNA	Guide ID numbers	HBeAg Inhibition (%)	HBsAg Inhibition (%)
VR7661 (PTG1)	Tetracistronic	g2-g10-g15-LUC	21-37	19-48

VR7662 (PTG2)	Tetracistronic	g2-g11-g13-g14	41-43	28-36
VR7663 (PTG3)	Tricistronic	g2-g11-g14	36-42	37-44
VR7664 (PTG4)	Bicistronic	g2-g11	37-51	40-52
VR7665 (PTG5)	Bicistronic	g13-g14	35-39	-23 to -22
VR7666 (PTG6)	Bicistronic	g14-g25	31-36	29-40
VR7667 (ternary 1)	Bicistronic+miRNA	g14-g25	47-69	71-72
VR7668 (ternary 2)	Bicistronic+miRNA	g2-g11	48-61	76

[0087] As shown in Table 1, each of the eight poly-gRNA (polycistronic gRNA) expression cassettes demonstrated >20% inhibition of HBeAg production in HepAD38 cells. The polycistronic gRNAs ranged from two gRNAs (bicistronic) to four gRNAs (tetracistronic), with guide numbers corresponding to those listed in Table 2, below. The first listed plasmid, PTG1, was a tetracistronic guide with a gRNA to luciferase (LUC). The last two plasmids (ternary 1 and ternary 2) incorporate a miRNA into bicistronic guides using the gRNA sequences from PTG4 and PTG6, to form gRNA-miRNA-gRNA expression cassettes.

[0088] Based on the data obtained for ternary 1 and ternary 2, it was concluded that additional inhibition of both HBeAg and HBsAg can be obtained by incorporating miRNA into the gRNA expression cassettes. While the miRNA selected was targeted to the HBV X protein for demonstrative purposes only, it should be understood that other sequences may be incorporated into the ternary cassettes for increased inhibition of the antigens. Unlike siRNA, which results in cleavage of the mRNA transcript, the RISC complex formed with the miRNA inhibits translation. In various embodiments, the miRNA may be targeted to the n-terminus of any of the structural HBV proteins eliminating the 7-methyl guanosine cap, which is required for mRNA export from the nucleus into the cytoplasm and for efficient translation. In various embodiments, the miRNA may be targeted to the AUG start site sequence of any of the HBV structural proteins or the common poly(A) tail sequence.

[0089] Nuclease

[0090] As used herein, “nuclease” refers to an enzyme capable of cleaving the phosphodiester bonds between monomers of nucleic acids. As described above, the CRISPR system includes a nuclease that is guided to the target DNA for cleavage. Thus, the terms “Cas”, “CRISPR enzyme”, and “nuclease” are generally used herein interchangeably, unless otherwise apparent. In various embodiments, the CRISPR enzyme is a type I, II, or III CRISPR enzyme from any species of microbe. A preferred Cas enzyme may be identified as “Cas9,” as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR system.

[0091] An exemplary Cas9 enzyme useful in the CRISPR system may be from the type II CRISPR locus in *Streptococcus pyogenes* (SpCas9). However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as but not limited to, *Staphylococcus aureus* (SaCas9), *Streptococcus thermophilus* (StCas9), and so forth. In various embodiments, the Cas9 enzyme may be codon-optimized, for example optimized for humans (*i.e.*, being optimized for expression in humans), such as human codon-optimized CpG-free *Streptococcus pyogenes* (Sp) Cas9 as set forth in SEQ ID NOs: 10 and 11. In various embodiments, the Cas9 enzyme may include one or more alterations designed to reduce non-specific DNA contacts, such as high-fidelity SpCas9 (SpCas9-HF1), as described in Kleinstiver, *et al.* 2016, *Nature*, 529,490-495, incorporated herein by reference. In various embodiments, the nuclease may be derived from *Prevotella* and *Francisella* bacteria, such as Cpf1, which is a smaller and simpler endonuclease than Cas9, and nucleases derived from *Neisseria meningitidis*, *Treponema denticola*, and any biologically active fragments or derivatives of the nucleases.

[0092] The plasmid may further include gene sequence(s) encoding one or more nuclear localization sequences (NLS). As used herein, the term “nuclear localization sequence” refers to an amino acid sequence that 'tags' a protein for import into the cell nucleus by nuclear transport. Typically, this signal consists of one or more short sequences of positively charged lysines or arginines exposed on the protein surface. In various embodiments, the NLS may be located at the 3' or 5' end or both, depending on the nuclease being used.

[0093] **3' Untranslated Region (UTR)**

[0094] As used herein, “3’ untranslated region” or “3’-UTR” refers to the section of messenger RNA (mRNA) that immediately follows the translation termination codon. An mRNA molecule is transcribed from the DNA sequence and is later translated into protein. Several regions of the mRNA molecule are not translated into protein including the 5' cap, 5' untranslated region, 3' untranslated region, and the poly(A) tail. The 3'-UTR often contains regulatory regions that post-transcriptionally influence gene expression. In various embodiments, the 3’-UTR may be synthetic or derived from naturally occurring genes. In various embodiments, the 3’-UTR may or may not include short hairpin RNA (shRNA) target sequences.

[0095] The 3’-UTR may also contain a sequence that directs addition of multiple adenine residues called the poly(A) tail to the end of the mRNA transcript. Poly(A) binding protein (PABP) binds to this tail, contributing to regulation of mRNA translation, stability, and export. For example, poly(A) tail bound PABP interacts with proteins associated with the 5' end of the transcript, causing a circularization of the mRNA that promotes translation. While the presence of a poly(A) tail usually aids in triggering translation, the absence or removal of one often leads to exonuclease-mediated degradation of the mRNA. Thus, the poly(A) tail protects the mRNA molecule from enzymatic degradation in the cytoplasm and aids in transcription termination, export of the mRNA from the nucleus, and translation.

[0096] Polyadenylation itself is regulated by sequences within the 3'-UTR of the transcript. These sequences include cytoplasmic polyadenylation elements (CPEs), which are uridine-rich sequences that contribute to both polyadenylation activation and repression. CPE-binding protein (CPEB) binds to CPEs in conjunction with a variety of other proteins in order to elicit different responses. Without being bound by theory, polyadenylate polymerase builds the poly(A) tail by adding adenosine monophosphate units from adenosine triphosphate to the RNA, cleaving off pyrophosphate. Another protein, PAB2, binds to the new, short poly(A) tail and increases the affinity of polyadenylate polymerase for the RNA. When the poly(A) tail is approximately 250 nucleotides long the enzyme can no longer bind to CPSF and polyadenylation stops, thus determining the length of the poly(A) tail. In eukaryotic cells, the poly(A) tail of most mRNAs in the cytoplasm gradually get shorter, and mRNAs with shorter poly(A) tail are translated less and degraded sooner, thereby influencing expression levels. This deadenylation and degradation process can be accelerated by microRNAs complementary to the 3' untranslated region of an

mRNA. In various embodiments, the 3'-UTR includes a rabbit beta globin poly(A) sequence.

Delivery System

[0097] In another aspect, the invention provides a gene delivery system that includes the plasmid DNA of the invention complexed with a condensing agent and encapsulated by a lipid envelope. Thus, the invention provides methods comprising delivering one or more polynucleotides, such as one or more plasmids of the invention and/or one or more transcripts thereof to a host cell, such as a hepatocyte. In various embodiments, a CRISPR enzyme in combination with (and optionally complexed with) one or more guide sequences is delivered to a cell. Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids in mammalian cells or target tissues. Such methods can be used to administer nucleic acids encoding components of a CRISPR system to cells in culture, or in a host organism. Non-viral vector delivery systems include plasmid DNA, RNA (*e.g.*, a transcript of a vector described herein), naked nucleic acid, and nucleic acid complexed with a delivery vehicle, such as a liposome. See, *e.g.*, US Pub. No. 2016/0317677, incorporated herein by reference).

[0098] DNA Condensation Agent with NLS

[0099] As used herein, "DNA condensation" refers to the process of compacting DNA molecules *in vitro* or *in vivo*. DNA diameter is about 2 nm, while the length of a stretched single molecule may be up to several dozens of centimeters depending on the organism. However, the physical properties of the DNA double helix, such as the sugar-phosphate backbone, electrostatic repulsion between phosphates, stacking interactions between the bases of each individual strand, and strand-strand interactions contribute to the overall stiffness of the molecule. To cope with volume constraints, DNA can pack itself in the appropriate solution conditions with the help of ions and other molecules (*i.e.*, DNA condensation agents).

[0100] DNA condensation can be induced *in vitro* either by applying external force to bring the double helices together, or by inducing attractive interactions between the DNA segments. The former can be achieved with the help of the osmotic pressure exerted by crowding neutral polymers in the presence of monovalent salts. In this case, the forces

pushing the double helices together are coming from entropic random collisions with the crowding polymers surrounding DNA condensates, and salt is required to neutralize DNA charges and decrease DNA-DNA repulsion. DNA condensation may also be realized by inducing attractive interactions between the DNA segments by multivalent cationic charged ligands (multivalent metal ions, inorganic cations, polyamines, protamines, peptides, lipids, liposomes and proteins).

[0101] As such, a condensing agent useful in the delivery system of the invention can be naturally occurring, such as a DNA binding protein or molecules capable of binding to DNA either through ionic interactions, hydrogen bonding or combination of both types of bonding. In various embodiments, the DNA condensing agent may have an endogenous domain or may be derivatized from an exogenous domain that traffics the plasmid DNA to the nucleus. Exemplary plasmid DNA condensing agents include, but are not limited to, protamine (*e.g.*, protamine sulfate), spermine, spermidine, cadaverine, putrescine, histones, and virus capsid proteins.

[0102] Lipid Envelope

[0103] The gene delivery system incorporates an encapsulating envelope that may be composed of lipids, such as cationic lipids, that are both naturally occurring and synthetic. While the encapsulating lipids can protect the plasmid DNA from degradation prior to target cell entry, the lipids may also function as condensing agents themselves, either alone or in combination with other condensing agents, as described above. As such, upon binding to the DNA, the lipids compact the DNA structure to facilitate packaging into lipid particles of less than about 100 nm in diameter. In various embodiments, the lipid composition can be made up of naturally occurring lipids, synthetic lipids or synthetic amphiphiles that trigger intracellular release into the cytoplasm of the target cell.

[0104] Thus, encapsulating lipids may be formulated to include the expression cassette and the CRISPR Cas system of the present invention to form lipid nanoparticles (LNPs). LNPs are typically spherical with an average diameter of between 10 nanometers to less than 1 μm . Various lipid nanoparticles useful in the gene delivery system may be formed from, for example, triglycerides (*e.g.*, tristearin), diglycerides (*e.g.*, glycerol behenate), monoglycerides (*e.g.*, glycerol monostearate), fatty acids (*e.g.*, stearic acid), steroids (*e.g.*,

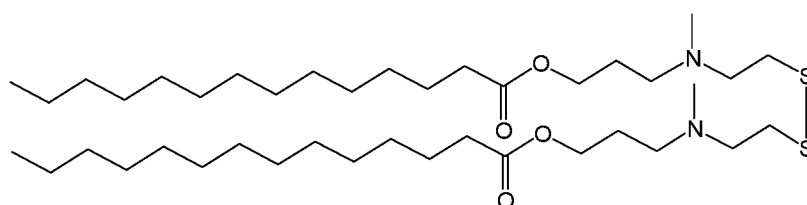
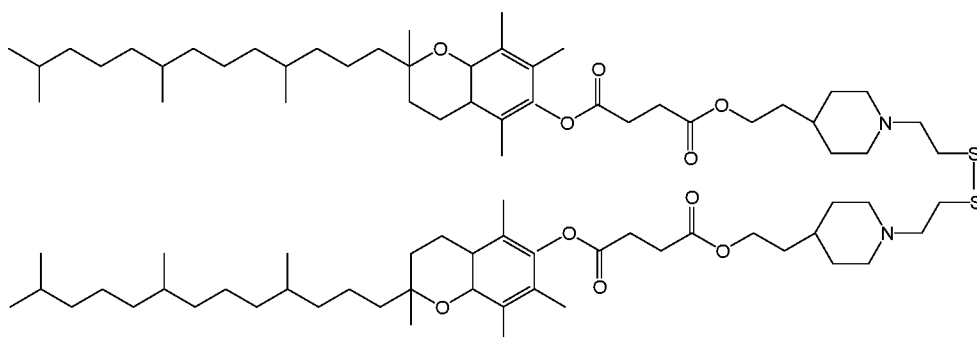
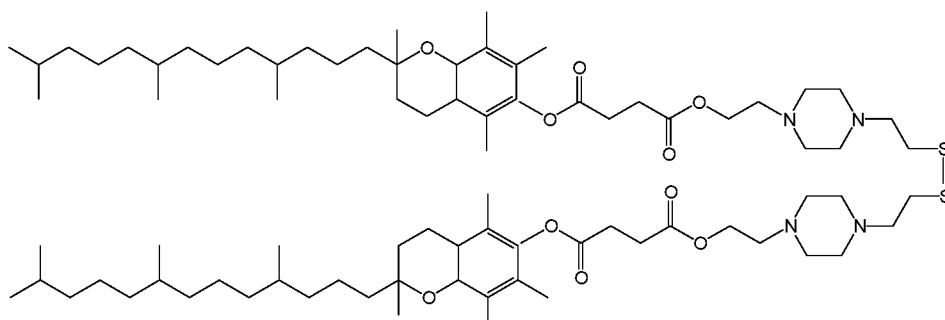
cholesterol), and waxes (*e.g.*, cetyl palmitate), and may further include emulsifiers to stabilize the lipid dispersion. Lipid formulations useful in the gene delivery system are described in US Pub. No. 2017/0143631, US Pat. No. 9,682,139, US Pat. No. 9,675,710, US Pat. No. 9,670,487, US Pat. No. 9,668,980, US Pat. No. 9,636,302, US Pat. No. 9,629,804, US Pat. No. 9,617,562, and US Pat. No. 9,579,338, each of which is incorporated herein by reference.

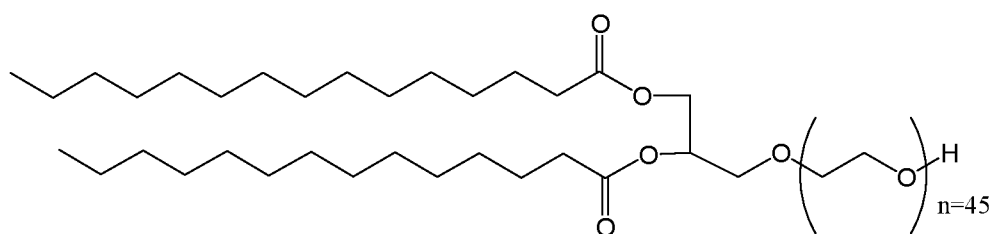
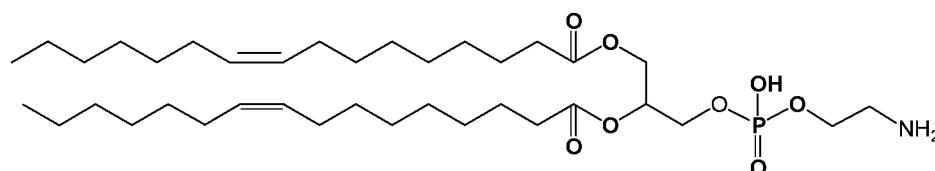
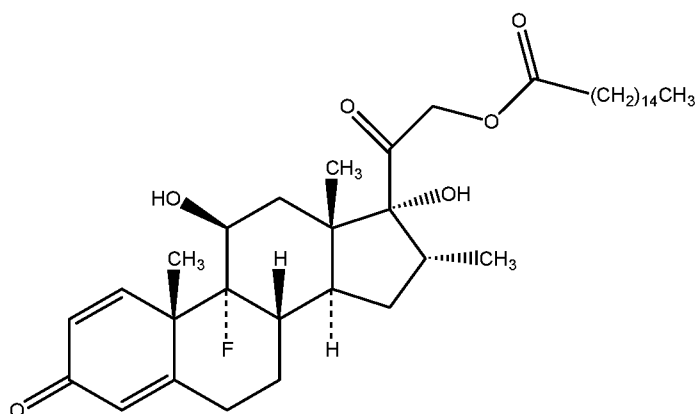
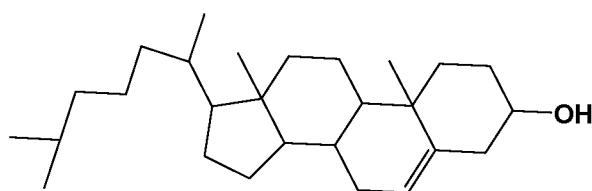
[0105] In various embodiments, the LNPs may be formed from one or more cationic lipids of the ssPalm class. The ssPalm class of cationic lipids were selected for evaluation based on the following parameters: (1) all are biodegradable in that the polar head groups can be hydrolyzed from the hydrophobic domain by acid labile ester bond and the dual amphiphile can be dissociated into single amphiphiles by reduction of the disulfide bond; (2) formulation of these lipids with helper lipids (*e.g.*, cholesterol, phosphatidylethanolamine or phosphatidylcholine) create a surface to which ApoE, an endogenous apolipoprotein can bind. This association results in liver uptake of the nanoparticles through the low density lipoprotein (LDL) receptor, which is highly expressed on hepatocytes; and (3) the cationic lipids have a head group that when formulated with the other helper lipids has a pK at 6.0 ± 0.5 (Ukawa, et al. Neutralized nanoparticle composed of SS-Cleavable and pH-activated lipid-like material as a long lasting and liver -specific gene delivery system *Adv. Healthcare Materials* (2014) 3:1222-1229, incorporated herein by reference).

[0106] Thus, formulation studies were conducted to develop plasmid DNA encapsulated in a LNP for delivery to hepatocytes following systemic administration. In various embodiments, the lipid formulation includes disulfide-cleavable, pH-responsive amphiphiles, dioleoylphosphatidylethanolamine (*e.g.*, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOPE), cholesterol and polyethylene glycol (~MW2,000)-dimyristoyl glycerol (*e.g.*, 1,2-Dimyristoyl-rac-glycero-3-methylpolyoxyethylene; DMG-PEG 2000) with an actual molar ratio of 58.3:9.71:29.1:2.91, respectively.

[0107] Exemplary cationic lipids from which the LNPs may be formed, include, but are not limited to, ssPalm, ssPalmE-P4C2, ssPalmE-Paz4-C2. In various embodiments, the LNP may further include one or more lipids selected from the group consisting of cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-Dimyristoyl-rac-

glycero-3-methylpolyoxyethylene (DMG-PEG), distearoyl glycerol, stearyl-oleoylphosphatidylcholine (SOPC), dipalmitoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), dipalmitoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-L-serine (DOPS), dimyristoylphosphatidylserine (DMPS), dioleoylphosphatidic acid (DOPA), 1,2-Dimyristoyl-sn-glycero-3-phosphate (DMPA), and 1,2-Dipalmitoyl-sn-glycero-3-phosphate (DPPA). In various embodiments, the disulfide cleavable, pH-responsive amphiphile includes a vitamin E (α -tocopherol) hydrophobic chain covalently bonded to a piperidine. Chemical structures of exemplary cationic lipids useful in the invention are as follows:

**ssPalmM****ssPalmE-P4C2****ssPalmE-Paz4-C2**

**DMG-PEG 2000****DOPE****Dexamethasone Palmitate****Cholesterol**

[0108] The lipid composition of the gene delivery system may further include a mechanism for releasing the pDNA into the cytoplasm. In various embodiments, disulfide-linked dimyristoyl amphiphiles containing a quaternary amine with a $pK_a \sim 6$ are formulated with phospholipid, cholesterol and a diglycerol-polyethylene glycol. Without being bound

by theory, the release mechanism takes advantages of a pH gradient as intracellular vesicles, primary and secondary endosomes, bud off from the plasma membrane and migrate to the lysosome. The pH of each endosomal membrane transitions from about a pH of 6.0 at the plasma membrane to about a pH of 4.0 in the lysosome. The pDNA thereby gets released from the endosome into the cytoplasm and is then trafficked to the nucleus where it exists episomally for expression.

[0109] In various embodiments, the lipid envelope may be in the form of naturally occurring secreted vesicles, such as exosomes, or may be synthetic versions thereof (*e.g.*, synthetic exosomes). Exosomes are endogenous nano-vesicles that transport RNAs and proteins that can deliver short interfering (si)RNA to target organs. Composed of cellular membranes with multiple adhesive proteins on their surface, exosomes are known to specialize in cell-cell communications and provide the ability to deliver various therapeutic agents to target cells.

[0110] Hepatocyte Targeting Ligands

[0111] In various embodiments, the lipid envelope of the gene delivery system may further include one or more ligands that bind to hepatocyte receptors disposed thereon. In various embodiments, the ligands may be either adsorbed to the surface of the lipid envelope or may be covalently derivatized to specific components of the lipid envelope. Depending on the composition of the lipid envelope, the surface chemistry thereof may be engineered to adsorb a protein ligand on the surface while maintaining exposure of the hepatocyte receptor binding domain, thereby facilitating docking of the gene delivery system specifically to a hepatocyte. Exemplary protein ligands useful in the gene delivery system include, but are not limited to, ligands that bind to the LDL receptor, *i.e.*, ApoE, viral glycoproteins either inserted into the membrane or derivatized to lipid. Peptides derived from viral glycoproteins may also either be adsorbed to the surface or derivatized to lipid components of the lipid envelope. Likewise, peptides that bind to hepatocyte receptors, such as those derived from malaria sporozoite proteins, may also be covalently derivatized to the phospholipids or to the ends of the polyethylene glycol derivatized diacylglycerols of the lipid envelope.

[0112] Finally, other non-protein ligands, such as carbohydrates or vitamins, may be incorporated into the lipid envelope for hepatocyte targeting. Such non-protein ligands may be covalently attached to various lipid components of the lipid envelope. In the case of carbohydrates, incorporation of a specific glycolipid may contain the appropriate carbohydrate configuration to trigger endocytosis of the delivery system into the hepatocyte. In various embodiments, synthetic peptides useful for hepatocyte targeting may either be adsorbed to the surface or chemically derivatized to one of the components of the lipid envelope.

CpG Free CRISPR/Cas9 Sequence (SEQ ID NO: 10):

GCCTGTACAGCCACCATGGATAAGAAATACTCAATAGGACTGGATATTGGCAC
AAATTCTGTGGGATGGGCTGTGATCACTGATGAATATAAGGTTCTTCTAAAAA
GTTCAAGGTTCTGGGAAATACAGACAGACACAGTATCAAAAAAATCTTATAG
GGGCTCTTCTGTTTGACAGTGGAGAGACAGCTGAAGCTACTAGACTCAAAGG
ACAGCTAGGAGAAGGTATACAAGAAGGAAGAATAGGATTTGTTATCTCCAGGA
GATTTTTTCAAATGAGATGGCCAAAGTGGATGATAGCTTCTTCCATAGACTTGA
AGAATCTTTTTTGGTGGAAAGAAGACAAGAAGCATGAAAGACATCCTATTTTTGG
AAATATAGTGGATGAAGTTGCTTATCATGAGAAATATCCAACCTATCTATCATCT
GAGAAAAAATTGGTGGATTCTACTGATAAAGCTGATTTGAGACTGATCTATTT
GGCCCTGGCCCACATGATTAAGTTTAGAGGTCATTTTTTGATTGAGGGGGATCT
GAATCCTGATAATAGTGATGTGGACAACTGTTTATCCAGTTGGTGCAAACCTA
CAATCAACTGTTTGAAGAAAACCCTATTAATGCAAGTGGAGTGGATGCTAAAG
CCATTCTTTCTGCAAGATTGAGTAAATCAAGAAGACTGGAAAATCTCATTGCTC
AGCTCCCTGGAGAGAAGAAAAATGGCCTGTTTGGGAATCTCATTGCTTTGTCAT
TGGGTTTGACCCCTAATTTTAAATCAAATTTTGATTTGGCAGAAGATGCTAAAC
TCCAGCTTTCAAAGATACTTATGATGATGATCTGGATAATCTGTTGGCTCAA
TTGGAGATCAATATGCTGATTTGTTTTTGGCTGCTAAGAATCTGTCAGATGCTAT
TCTGCTTTCTGACATCCTGAGAGTGAATACTGAAATAACTAAGGCTCCCCTGTC
AGCTTCAATGATTAAAAGATATGATGAACATCATCAAGACTTGACTCTTCTGAA
AGCCCTGGTTAGACAACAACCTCCAGAAAAGTATAAAGAAATCTTTTTTGATCA
ATCAAAAAATGGATATGCAGGTTATATTGATGGAGGAGCAAGCCAAGAAGAAT
TTTATAAATTTATCAAACCAATTCTGGAAAAAATGGATGGTACTGAGGAAGTGT
TGGTGAAACTGAATAGAGAAGATTTGCTGAGAAAGCAAAGGACCTTTGACAAT

GGCTCTATTCCCATCAAATTCACCTTGGGAGAGCTGCATGCTATTTTGAGAAGA
CAAGAAGACTTTTATCCATTTCTGAAAGACAATAGAGAGAAGATTGAAAAAAT
CTTGACTTTTAGGATTCCTTATTATGTTGGTCCATTGGCCAGAGGCAATAGTAGG
TTTGCATGGATGACTAGGAAGTCTGAAGAAACAATTACCCCATGGAATTTTGAA
GAAGTTGTGGATAAAGGAGCTTCAGCTCAATCATTATTGAAAGAATGACAAA
CTTTGATAAAAAATCTTCCAAATGAAAAAGTGCTGCCAAAACATAGTTTGCTTTA
TGAGTATTTTACAGTTTATAATGAATTGACAAAGGTCAAATATGTTACTGAAGG
AATGAGAAAACCAGCATTCTTTCTGGAGAACAGAAGAAAGCCATTGTTGATCT
GCTCTTCAAAACAAATAGGAAAGTGACAGTTAAGCAACTGAAAGAAGATTATT
TCAAAAAAATAGAATGTTTTGATAGTGTTGAAATTTTCAGGAGTTGAAGATAGAT
TTAATGCTTCACTGGGCACATACCATGATTTGCTGAAAATTATTAAGATAAAG
ATTTTTTGGATAATGAAGAAAATGAAGACATCCTGGAGGATATTGTTCTGACAT
TGACCCTGTTTGAAGATAGAGAGATGATTGAGGAAAGACTTAAACATATGCT
CACCTCTTTGATGATAAGGTGATGAAACAGCTTAAAAGAAGAAGATATACTGG
TTGGGGAAGGTTGTCCAGAAAATTGATTAATGGTATTAGGGATAAGCAATCTGG
CAAAACAATACTGGATTTTTTTGAAATCAGATGGTTTTTGCCAATAGAAATTTTAT
GCAGCTCATCCATGATGATAGTTTGACATTTAAAGAAGACATCCAAAAAGCAC
AAGTGTCTGGACAAGGAGATAGTCTGCATGAACATATTGCAAATCTGGCTGGTA
GCCCTGCTATTAAAAAAGGCATTCTCCAGACTGTGAAAGTTGTTGATGAATTGG
TCAAAGTGATGGGGAGGCATAAGCCAGAAAATATTGTTATTGAAATGGCAAGA
GAAAATCAGACAACTCAAAAGGGCCAGAAAAATTCCAGAGAGAGGATGAAAA
GAATTGAAGAAGGCATCAAAGAAGTGGGAAGTCAGATTCTTAAAGAGCATCCT
GTTGAAAATACTCAATTGCAAAATGAAAAGCTCTATCTCTATTATCTCCAAAT
GGAAGAGATATGTATGTGGACCAAGAACTGGATATTAATAGGCTGAGTGATTA
TGATGTGGATCACATTGTTCCACAAAGTTTCCTTAAAGATGATTCAATAGACAA
TAAGGTCCTGACCAGATCTGATAAAAAATAGAGGCCAAATCTGATAATGTTCCAA
GTGAAGAAGTGGTCAAAAAGATGAAAAACTATTGGAGACAACTTCTGAATGCC
AAGCTGATCACTCAAAGGAAGTTTGATAATCTGACCAAAGCTGAAAGAGGAGG
TTTGAGTGAACCTTGATAAAGCTGGTTTTATCAAAAGACAATTGGTTGAAACTAG
ACAAATCACTAAGCATGTGGCACAAATTTTGGATAGTAGAATGAATACTAAAT
ATGATGAAAATGATAAACTTATTAGAGAGGTTAAAGTGATTACCCTGAAATCTA
AACTGGTTTCTGACTTCAGAAAAGATTTCCAATTCTATAAAGTGAGAGAGATTA
ACAATTACCATCATGCCCATGATGCCTATCTGAATGCTGTGGTTGGAAGTGCCTT

GATTAAGAAATATCCAAAACCTTGAATCTGAGTTTGTCTATGGTGATTATAAAGT
TTATGATGTTAGGAAAATGATTGCTAAGTCTGAGCAAGAAATAGGCAAAGCAA
CAGCAAAGTATTTCTTTTACTCTAATATCATGAACTTCTTCAAAACAGAAATTAC
ACTTGCAAATGGAGAGATTAGAAAAAGACCTCTGATTGAACTAATGGGGAAA
CTGGAGAAATTGTCTGGGATAAAGGGAGAGATTTTGCCACAGTGAGAAAAGTG
TTGTCCATGCCCCAAGTCAATATTGTCAAGAAAACAGAAGTGCAGACAGGAGG
ATTCTCTAAGGAGTCAATTCTGCCAAAAAGAAATTCTGACAAGCTGATTGCTAG
GAAAAAAGACTGGGACCCAAAAAAATATGGTGGTTTTGATAGTCCAACAGTGG
CTTATTCAGTCCTGGTGGTTGCTAAGGTGGAAAAAGGGAAATCCAAGAAGCTG
AAATCTGTAAAGAGCTGCTGGGGATCACAATTATGGAAAGAAGTTCCTTTGAA
AAAAATCCCATTGACTTTCTGGAAGCTAAAGGATATAAGGAAGTTAAAAAAGA
CCTGATCATTAACTGCCTAAATATAGTCTTTTTTGAGCTGGAAAATGGCAGGAA
AAGGATGCTGGCTAGTGCTGGGAGAACTGCAAAAAGGAAATGAGCTGGCTCTGC
CAAGCAAATATGTGAATTTTCTGTATCTGGCTAGTCATTATGAAAAGTTGAAGG
GTAGTCCAGAAGATAATGAACAAAAACAATTGTTTGTGGAGCAGCATAAGCAT
TATCTGGATGAGATTATTGAGCAAATCAGTGAATTTTCTAAGAGAGTTATTCTG
GCAGATGCCAATCTGGATAAAGTTCTTAGTGATATAACAAACATAGAGACAA
ACCAATAAGAGAACAAGCAGAAAATATCATTTCATCTGTTTACCTTGACCAATCT
TGGAGCACCTGCTGCTTTTAAATACTTTGATACAACAATTGATAGGAAAAGATA
TACCTCTACAAAAGAAGTTCTGGATGCCACTCTTATCCATCAATCCATCACTGG
TCTTTATGAAACAAGAATTGATTTGAGTCAGCTGGGAGGTGACCCCAAGAAAA
AAAGAAAGGTGGAAGATCCTAAGAAAAAGAGGAAAGTGTAATCAGCTAGCAC
C

CpG Free CRISPR/Cas9 Codon Optimized Sequence (SEQ ID NO: 11):

ATGGATAAGAAATACTCAATAGGACTGGATATTGGCACAAATTCTGTGGGATG
GGCTGTGATCACTGATGAATATAAGGTTCTTCTAAAAAGTTCAAGGTTCTGGG
AAATACAGACAGACACAGTATCAAAAAAATCTTATAGGGGCTCTTCTGTTTGA
CAGTGGAGAGACAGCTGAAGCTACTAGACTCAAAGGACAGCTAGGAGAAGGT
ATACAAGAAGGAAGAATAGGATTTGTTATCTCCAGGAGATTTTTTCAAATGAGA
TGGCCAAAGTGGATGATAGCTTCTTCCATAGACTTGAAGAATCTTTTTTGGTGG
AAGAAGACAAGAAGCATGAAAGACATCCTATTTTTGGAAATATAGTGGATGAA
GTTGCTTATCATGAGAAATATCCAACATCTATCATCTGAGAAAAAAATTGGTG

GATTCTACTGATAAAGCTGATTTGAGACTGATCTATTTGGCCCTGGCCCACATG
ATTAAGTTTAGAGGTCATTTTTTTGATTGAGGGGGATCTGAATCCTGATAATAGT
GATGTGGACAACTGTTTATCCAGTTGGTGCAAACCTACAATCAACTGTTTGAA
GAAAACCCTATTAATGCAAGTGGAGTGGATGCTAAAGCCATTCTTTCTGCAAGA
TTGAGTAAATCAAGAAGACTGGAAAATCTCATTGCTCAGCTCCCTGGAGAGAA
GAAAAATGGCCTGTTTGGGAATCTCATTGCTTTGTCATTGGGTTTGACCCCTAAT
TTTAAATCAAATTTTTGATTTGGCAGAAGATGCTAAACTCCAGCTTTCAAAAGAT
ACTTATGATGATGATCTGGATAATCTGTTGGCTCAAATTGGAGATCAATATGCT
GATTTGTTTTTTGGCTGCTAAGAATCTGTCAGATGCTATTCTGCTTTCTGACATCC
TGAGAGTGAATACTGAAATAACTAAGGCTCCCCTGTCAGCTTCAATGATTAAAA
GATATGATGAACATCATCAAGACTTGACTCTTCTGAAAGCCCTGGTTAGACAAC
AACTTCCAGAAAAGTATAAAGAAATCTTTTTTGATCAATCAAAAAATGGATATG
CAGGTTATATTGATGGAGGAGCAAGCCAAGAAGAATTTTATAAATTTATCAAAC
CAATTCTGGAAAAAATGGATGGTACTGAGGAACTGTTGGTGAAACTGAATAGA
GAAGATTTGCTGAGAAAGCAAAGGACCTTTGACAATGGCTCTATTCCCATCAA
ATTCACTTGGGAGAGCTGCATGCTATTTTGAGAAGACAAGAAGACTTTTATCCA
TTTCTGAAAGACAATAGAGAGAAGATTGAAAAAATCTTGACTTTTAGGATTCCT
TATTATGTTGGTCCATTGGCCAGAGGCAATAGTAGGTTTGCATGGATGACTAGG
AAGTCTGAAGAAACAATTACCCCATGGAATTTTGAAGAAGTTGTGGATAAAGG
AGCTTCAGCTCAATCATTTATTGAAAGAATGACAACTTTGATAAAAAATCTTCC
AAATGAAAAAGTGCTGCCAAAACATAGTTTGCTTTATGAGTATTTTACAGTTTA
TAATGAATTGACAAAGGTCAAATATGTTACTGAAGGAATGAGAAAACCAGCAT
TTCTTTCTGGAGAACAGAAGAAAGCCATTGTTGATCTGCTCTTCAAAACAAATA
GGAAAGTGACAGTTAAGCAACTGAAAGAAGATTATTTCAAAAAAATAGAATGT
TTTGATAGTGTTGAAATTTTCAGGAGTTGAAGATAGATTTAATGCTTCACTGGGC
ACATACCATGATTTGCTGAAAATTATTAAAGATAAAGATTTTTTTGGATAATGAA
GAAAATGAAGACATCCTGGAGGATATTGTTCTGACATTGACCCTGTTTGAAGAT
AGAGAGATGATTGAGGAAAGACTTAAACATATGCTCACCTCTTTGATGATAA
GGTGATGAAACAGCTTAAAGAAGAAGATATACTGGTTGGGGAAGGTTGTCCA
GAAAATTGATTAATGGTATTAGGGATAAGCAATCTGGCAAAACAATACTGGAT
TTTTTGAAATCAGATGGTTTTTGCCAATAGAAATTTTATGCAGCTCATCCATGATG
ATAGTTTGACATTTAAAGAAGACATCCAAAAAGCACAAAGTGTCTGGACAAGGA
GATAGTCTGCATGAACATATTGCAAATCTGGCTGGTAGCCCTGCTATTAAAAAA

GGCATTCTCCAGACTGTGAAAGTTGTTGATGAATTGGTCAAAGTGATGGGGAGG
CATAAGCCAGAAAATATTGTTATTGAAATGGCAAGAGAAAAATCAGACAACTCA
AAAGGGCCAGAAAAATTCCAGAGAGAGGATGAAAAGAATTGAAGAAGGCATC
AAAGAACTGGGAAGTCAGATTCTTAAAGAGCATCCTGTTGAAAATACTCAATTG
CAAAATGAAAAGCTCTATCTCTATTATCTCCAAAATGGAAGAGATATGTATGTG
GACCAAGAACTGGATATTAATAGGCTGAGTGATTATGATGTGGATCACATTGTT
CCACAAAGTTTCCTTAAAGATGATTCAATAGACAATAAGGTCCTGACCAGATCT
GATAAAAATAGAGGCCAAATCTGATAATGTTCCAAGTGAAGAAGTGGTCAAAAA
GATGAAAACTATTGGAGACAACCTTCTGAATGCCAAGCTGATCACTCAAAGGA
AGTTTGATAATCTGACCAAAGCTGAAAGAGGAGGTTTGAGTGAACCTGATAAA
GCTGGTTTTATCAAAAGACAATTGGTTGAACTAGACAAATCACTAAGCATGTG
GCACAAATTTTGGATAGTAGAATGAATACTAAATATGATGAAAATGATAAACTT
ATTAGAGAGGTTAAAGTGATTACCCTGAAATCTAACTGGTTTCTGACTTCAGA
AAAGATTTCCAATTCTATAAAGTGAGAGAGATTAACAATTACCATCATGCCCAT
GATGCCTATCTGAATGCTGTGGTTGGAAGCTGCTTTGATTAAGAAATATCCAAAA
CTTGAATCTGAGTTTGTCTATGGTGATTATAAAGTTTATGATGTTAGGAAAATG
ATTGCTAAGTCTGAGCAAGAAATAGGCCAAAGCAACAGCAAAGTATTTCTTTTAC
TCTAATATCATGAACTTCTTCAAAACAGAAATTACACTTGCAAATGGAGAGATT
AGAAAAAGACCTCTGATTGAACTAATGGGGAACTGGAGAAATTGTCTGGGA
TAAAGGGAGAGATTTTGCCACAGTGAGAAAAGTGTTGTCCATGCCCCAAGTCA
ATATTGTCAAGAAAACAGAAGTGCAGACAGGAGGATTCTCTAAGGAGTCAATT
CTGCCAAAAAGAAATTCTGACAAGCTGATTGCTAGGAAAAAAGACTGGGACCC
AAAAAAATATGGTGGTTTTTGATAGTCCAACAGTGGCTTATTCAGTCCTGGTGGT
TGCTAAGGTGGAAAAAGGGAAATCCAAGAAGCTGAAATCTGTAAAGAGCTGC
TGGGGATCACAAATTATGGAAAGAAGTTCCTTTGAAAAAAATCCCATTGACTTTC
TGGAAGCTAAAGGATATAAGGAAGTTAAAAAAGACCTGATCATTAACTGCCT
AAATATAGTCTTTTTTGAGCTGGAAAAATGGCAGGAAAAGGATGCTGGCTAGTGCT
GGAGAACTGCAAAAAGGAAATGAGCTGGCTCTGCCAAGCAAATATGTGAATTT
TCTGTATCTGGCTAGTCATTATGAAAAGTTGAAGGGTAGTCCAGAAGATAATGA
ACAAAAACAATTGTTTGTGGAGCAGCATAAGCATTATCTGGATGAGATTATTGA
GCAAATCAGTGAATTTTCTAAGAGAGTTATTCTGGCAGATGCCAATCTGGATAA
AGTTCTTAGTGCATATAACAAACATAGAGACAAACCAATAAGAGAACAAGCAG
AAAATATCATTATCTGTTTACCTTGACCAATCTTGGAGCACCTGCTGCTTTTAA

ATACTTTGATACAACAATTGATAGGAAAAGATATACCTCTACAAAAGAAGTTCT
 GGATGCCACTCTTATCCATCAATCCATCACTGGTCTTTATGAAACAAGAATTGA
 TTTGAGTCAGCTGGGAGGTGACCCCAAGAAAAAAGAAAGGTGGAAGATCCTA
 AGAAAAAGAGGAAAGTGTA

RNA Polymerase III Promoter 7SK (SEQ ID NO: 12):

CTGCAGTATTTAGCATGCCCCACCCATCTGCAAGGCATTCTGGATAGTGTCAAA
 ACAGCCGGAAATCAAGTCCGTTTATCTCAAACCTTTAGCATTTTGGGAATAAATG
 ATATTTGCTATGCTGGTTAAATTAGATTTTAGTTAAATTCCTGCTGAAGCTCTA
 GTACGATAAGTAACTTGACCTAAGTGTAAGTTGAGATTTCTTCAGGTTTATA
 TAGCTTGTGCGCCGCCTGGGTACCTC

RNA Polymerase III Promoter U6 (SEQ ID NO: 13):

AAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATA
 CGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAA
 GATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGC
 AGTTTTAAAATTATGTTTTAAATGGACTATCATATGCTTACCGTAACTTGAAA
 GTATTTTCGATTTCTTGGGTTTATATATCTTGTGGAAAGGACGCGGGATC

RNA Polymerase III Promoter H1 (SEQ ID NO: 14):

GGAATTCGAACGCTGACGTCATCAACCCGCTCCAAGGAATCGCGGGCCCAGTG
 TCACTAGGCGGGAACACCCAGCGCGCGTGCGCCCTGGCAGGAAGATGGCTGTG
 AGGGACAGGGGAGTGGCGCCCTGCAATATTTGCATGTCGCTATGTGTTCTGGGA
 AATCACCATAAACGTGAAATGTCTTTGGATTTGGGAATCTTATAAGTTCTGTAT
 GAGACCACAGATCCCC

[0113] Accordingly, in another aspect, the invention provides a method of treating chronic hepatitis B viral infection in a subject by administering the plasmid of the invention using the gene delivery system described herein. As described above, such treatment may be useful in subjects having viral infection wherein target viral sequences are comprised in an episomal nucleic acid molecule which is not integrated into the genome of the organism, such as a cccDNA.

[0114] The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1

Screening for Anti-HBV gRNAs

[0115] The HepAD38 cell line is derived from a HepG2 cell line in which a 1.3X hepatitis B virus genotype D is integrated into the HepG2 genome (Ladner, et al. Inducible expression of human hepatitis B virus in stable transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication *Antimicrob Agents Chemother* (1997) 41:1715-1720, incorporated herein by reference). The human HBV genome is under the control of the tetracycline operator modified to be activatable by the tetR/VP16 transactivator by inclusion of the cytomegalovirus early promoter. Thus, in the presence of tetracycline (tet), viral production is suppressed and in the absence of tet, the cells produce a large number of HBV Dane particles and other HBV replicative intermediates (*i.e.*, cccDNA, single stranded DNA, relaxed circular DNA, and other forms). Moreover, since the HBV surface antigen contains its own promoter sequences, the HepAD38 cell line is seropositive for the HBV surface antigen in either the repressed or unrepressed states (see, *e.g.*, US Pat. No. 5,723,319, incorporated herein by reference in its entirety).

[0116] HepAD38 cells were obtained and grown in the absence of tet for 6 days to produce virus. The cells were then plated in a 24-well plate on Day 6 and transfected with CRISPR/Cas9/gRNA expression plasmids using a commercially available transfection reagent (Viafect, Promega Corporation). Media was removed and replaced after 48 hrs, and after an additional 48 hrs the media was removed. The media was then assayed for HBeAg and HBsAg. Reduction in viral antigens was compared to transfection of cells with plasmid DNA expressing CRISPR/Cas9 in the absence of gRNA.

EXAMPLE 2

Screening of gRNAs for Inhibition of HBV Virus Production

[0117] 25 gRNAs were screened for in vitro inhibition of HBV production using the HepAD38 cell line. Table 2 provides the list of gRNAs tested.

Table 2: gRNAs Tested for Inhibition of HBV Genotype D Production

Guide ID#	HBV Location (bp) (Accession No. U95551)	gRNA Sequence	HBV Gene Product
g1	1523-1542	GGGGCGCACCTCTCTTTACG (SEQ ID NO: 15)	DNA polymerase/ X protein
g2	1256-1275	TCCTCTGCCGATCCATACTG (SEQ ID NO: 16)	DNA polymerase
g3	239-258	TACCGCAGAGTCTAGACTCG (SEQ ID NO: 17)	DNA polymerase/ S protein
g4	346-3655	TCCTGTCCTCCAACTTGTCC (SEQ ID NO: 18)	DNA polymerase/ S protein
g5	356-375	CAACTTGTCTGGTTATCGC (SEQ ID NO: 19)	DNA polymerase/ S protein
g6	731-750	TTTCAGTTATATGGATGATG (SEQ ID NO: 20)	DNA polymerase/ S protein
g7	1504-1523	TGCCGTTCCGACCGACCACG (SEQ ID NO: 21)	DNA polymerase/ X Protein
g8	1591-1610	CTTCACCTCTGCACGTCGCA (SEQ ID NO: 22)	DNA polymerase/ X protein /DR2
g9	1599-1618	GGTCTCCATGCGACGTGCAG (SEQ ID NO: 23)	DNA polymerase/ X protein /DR2
g10	1620-1639	CATTCGGTGGGCGTTCACGG (SEQ ID NO: 24)	X protein
g11	1681-1700	AATGTCAACGACCGACCTTG (SEQ ID NO: 25)	X protein
g12	1695-1714	TTTGAAGTATGCCTCAAGGT (SEQ ID NO: 26)	X protein
g13	1817-1836	GCAGAGGTGAAAAAGTTGCA (SEQ ID NO: 27)	X protein/Core protein/DR1
g14	1867-1886	CAAGCCTCCAAGCTGTGCCT (SEQ ID NO: 28)	Core protein
g15	1920-1939	TAAAGAATTTGGAGCTACTG (SEQ ID NO: 29)	Core protein
g16	1997-2016	ATACCGCCTCAGCTCTGTAT (SEQ ID NO: 30)	Core protein
g17	2386-2405	GACCTTCGTCTGCGAGGCGA (SEQ ID NO: 31)	Precore protein /DNA polymerase
g18	1918-1937	GTAGCTCCAAATTCTTTATA (SEQ ID NO: 32)	Core protein/Poly A Sequence
g19	2681-2700	GGTTTAATACCCTTATCCAA (SEQ ID NO: 33)	DNA polymerase L2 Loop of Terminal Protein Domain
g20	2749-2768	TTTACACACTCTATGGAAGG (SEQ ID NO: 34)	DNA polymerase

g21	2861-2880	TCTTTCCACCAGCAATCCTC (SEQ ID NO: 35)	DNA polymerase/PreS1 protein-neutralizing epitope
g22	2900-2919	TGAAGGCTGGATCCAACCTGG (SEQ ID NO: 36)	PreS1 protein-neutralizing epitope/ DNA polymerase
g23	2068-2087	AGGCAAGCAATTCTTTGCTG (SEQ ID NO: 37)	Targeting core C63 responsible for disulfide core dimerization
g24	2069-2088	GGCAAGCAATTCTTTGCTGG (SEQ ID NO: 38)	Targeting core C63 responsible for disulfide core dimerization
g25	1181-1199	GGTTGCGTCAGCAAACACT (SEQ ID NO: 39)	DNA polymerase

[0118] Of the gRNAs listed in Table 2, the 14 gRNAs shown in Table 3 were selected based on a showing of greater than 20% inhibition of HBeAg and HBsAg production in HepAD38 cells *in vitro*. Also shown is the HBV genotype to which the gRNA sequence is 100% identical.

Table 3: gRNAs showing >20% Reduction in Antigen Production

Guide ID#	gRNA Sequence (Bold is complementary to negative strand)	HBV Genotype
g2	TCCTCTGCCGATCCATACTG (SEQ ID NO: 16)	A, B, C, D
g10	CATTCGGTGGGCGTTCACGG (SEQ ID NO: 24)	Some D strains
g11	AATGTCAACGACCGACCTTG (SEQ ID NO: 25)	A, B, C, D
g13	GCAGAGGTGAAAAAGTTGCA (SEQ ID NO: 27)	A, B, C, D
g14	CAAGCCTCCAAGCTGTGCCT (SEQ ID NO: 28)	A, B, C, D
g15	TAAAGAATTTGGAGCTACTG (SEQ ID NO: 29)	A, D
g16	ATACCGCCTCAGCTCTGTAT (SEQ ID NO: 30)	D
g17	GACCTTCGTCTGCGAGGCCGA (SEQ ID NO: 31)	B, C, D
g18	GTAGCTCCAAATTCTTTATA (SEQ ID NO: 32)	A, D

g21	TCTTTCCACCAGCAATCCTC (SEQ ID NO: 35)	D
g22	TGAAGGCTGGATCCA ACTGG (SEQ ID NO: 36)	D
g23	AGGCAAGCAATTCTTTGCTG (SEQ ID NO: 37)	D
g24	GGCAAGCAATTCTTTGCTGG (SEQ ID NO: 38)	D
g25	GGTTGCGTCAGCAA AACT (SEQ ID NO: 39)	A, B, C, D

EXAMPLE 3

Poly-gRNAs Expression Cassettes

[0119] In first demonstrating functionality of the expression cassette shown in Figure 1B, a plasmid DNA containing a single gRNA and CRISPR/Cas9 expression cassette was cotransfected with a luciferase expression pDNA into HepG2 cells. The gRNA was targeted to the luciferase open reading frame. Increasing the ratio of the gRNA/CRISPR/Cas9 pDNA to the luciferase pDNA resulted in inhibition of luciferase expression to >95% inhibition at a plasmid wt/wt ratio of 15:1.

[0120] Therefore, this same luciferase gRNA was placed at the end of a tetracistronic gRNA and the same cotransfection experiment was used to demonstrate that the multiple gRNA expression cassette was being processed to generate individual gRNAs. Cotransfection of the PTG1 pDNA (see Table 1) with the luciferase pDNA into HepG2 cells showed inhibition of luciferase expression, thereby demonstrating correct gRNA processing.

[0121] Referring back to Table 1, all subsequently listed PTGs (*i.e.*, PTG2-PTG6) and ternary pDNA (*i.e.*, ternary 1 and ternary 2) were cotransfected into the HepAD38 cells. The cells were subsequently assayed for HBeAg and HBsAg production at 48 and 98 hrs after transfection. Comparison of inhibition of HBeAg production for PTG4, PTG3 and PTG2 showed approximately the same degree of inhibition. However, comparison of inhibition of HBsAg production demonstrated a reverse in the anticipated trend. As the number of guides increased from two (bicistronic) to four (tetracistronic), the degree of inhibition of HBsAg surprisingly decreased from an average inhibition of 47% to 32%.

[0122] Building upon the findings of Wang, et al., 2017 (*Id.*), a ternary cassette (ternary1) was constructed for comparison to a PTG (PTG6) expressing identical guide sequences (g14 and g25, Table 3). A secondary ternary cassette-containing construct (ternary2) was constructed for comparison to its PTG counterpart (PTG4) expressing identical guide sequences (g2 and g11, Table 3). Comparison of the inhibition of HBeAg and HBsAg production for the bicistronic PTGs (PTG4 and PTG6) vs the ternaries with the same gRNAs (ternary2 and ternary1, respectively) showed that additional inhibition of both antigens was obtained by incorporating the miRNA (see Table 1). Furthermore, it was observed that ternary2 strongly inhibited both HBeAg and HBsAg to levels comparable to ternary1, thereby indicating that guides g2 and g11 function effectively in a ternary cassette.

[0123] The miRNA sequence was targeted to the HBV X protein and was modeled after a naturally occurring pri-miR-31 (Ely, et al. Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles. *Nuc Acids Res* (2009) 37:e91, incorporated herein by reference). Alternative naturally occurring short hairpin RNAs (shRNA) include miR-31 (Ely A, Naidoo T, Mufamadi S, et al. Expressed anti-HBV primary microRNA shuttles inhibit viral replication efficiently in vitro and in vivo. *Mol Ther.* 2008; Ely A, Naidoo T, Arbuthnot P. Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles. *Nucleic Acids Res.* 2009; 37: e91.), and miR-155 (Chung, et al. Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155 *Nuc. Acids Res* 34:e53, incorporated herein by reference).

EXAMPLE 4

Formulation of Lipid Nanoparticles

[0124] Reagents:

DL-Malic Acid

NaCl

Amicon Ultra 15, MWCO 100K

5 mL tube

HEPES

Dexamethasone palmitate (CAS number, 14899-36-6)

ssPalmE-P4C2

PicoGreen

Triton X100

[0125] Solutions and Buffers:

5 mM ssPalmE (in 100% EtOH)
10mM Cholesterol (in 100% EtOH)
1mM PEG2000-DMG (in 100% EtOH)
100mM Malic acid (pH=4.0)
5M NaCl
1 mg/mL pDNA
20mM Malic acid (pH=4.0)
10mM Hepes Buffer (pH=7.4)
Phosphate Buffered Saline (PBS), pH 7.4

[0126] 300 μ L of pDNA solution was prepared in a 1.5 mL tube using 1 mg/mL of pDNA, 100mM Malic acid (pH=4.0), and 5M NaCl. Final concentrations of pDNA, Malic acid, NaCl were 0.1 mg/mL, 20mM, 40mM, respectively.

[0127] In a 5 mL tube, a total of 600nmol lipids per 200 μ L, including ssPalm lipid and cholesterol were prepared. PEG2000-DMG lipids were added at about 3 mol% of total lipids (18nmol). For production of dexamethasone palmitate modified LNPs, dexamethasone palmitate solution was added into the lipid solution at a final concentration of about 0.5mM / 200 μ L.

[0128] 300 μ L of the pDNA solution was rapidly added into the lipid solution with vigorous stirring, followed by 500 μ L of 20mM Malic acid (pH=4.0) and 1000 μ L of PBS solution to form a pDNA-lipid mixture. This procedure was repeated a total of 4 times (total volume: 2 mL x 4=8 mL, pDNA amount: 30 μ g x 4=120 μ g). The resulting solution was transferred to an Amicon Ultra-15 centrifugal filter (100K), and 8 mL of PBS solution was added (final EtOH conc. 5%). The solution was centrifuged (1000g for 25 min. at room temp.) to a final volume of 200 μ L. The concentrated solution was thereafter diluted with 14mL of PBS and centrifuged again to a final volume of 200 μ L.

[0129] The formulation process was optimized to yield 80 ± 10 nm average diameter particles with a polydispersity index of ≤ 0.2 and a plasmid DNA trapping efficiency of $\geq 80\%$.

[0130] For production of LNPs with ssPalmM, the ssPalmM LNPs were prepared with the following formulation: ssPalmM:cholesterol:DOPE:DMG-PEG, 30:40:30:3 (mol:mol:mol:mol). Stearoyl-Oleoylphosphatidylcholine (SOPC) and distearoyl-glycerol-polyethylene glycol (avg MW 5KDa) were substituted for DOPE and DMG-PEG. The

pDNA was precondensed with protamine sulfate at a 1:1.25 (wt/wt) ratio and then complexed with the lipid formulations forming the LNPs.

EXAMPLE 5

Optimizing *In Vivo* Gene Transfer in Mice

[0131] This example discusses a series of assays performed on different LNP formulations to obtain maximal gene expression and maximal tolerability in mice.

[0132] CpG free luciferase was expressed from a CpG free plasmid DNA backbone (Invivogen, San Diego, CA) and expression was monitored by bioluminescence imaging. The expression plasmids were formulated using different cationic lipids and helper lipids to form LNPs that were characterized based on average particle size diameters, polydispersity index and plasmid DNA trapping efficiency.

[0133] A 10 µg dose of trapped plasmid DNA in an injection volume of 0.2 mL was administered into the tail veins of mice. The mice were then imaged for luciferase expression at 18 to 24 hrs after administration.

[0134] Imaging was accomplished by first anesthetizing the mice with a 0.2 mL ketamine cocktail administered by intraperitoneal (i.p.) injection, and then given 0.1 mL of 28.5 mg/mL D-luciferin i.p. Animals were then imaged for a duration of 0.5 sec to 5 min using the Xenogen IVIS imaging system. Assessed luciferase activity levels were expressed in photons emitted per second per square centimeter, denoted as p/sec/cm². The results shown in Table 4 summarize the gene expression obtained with LNPs composed of different cationic lipids, where the amounts of cationic lipids are listed as mol%, and the amount of DMG-PEG is shown as the mol% of the total amount of cationic lipid, DOPE and cholesterol.

Table 4: Comparison of Gene Expression from various LNPs

LNP	ssPalmM	ssPalm E-P4C2	ssPalm E-PazC4	DOPE	Chol	DMG- PEG	Trapping Efficiency (%)	Particle Size (nm)	Bioluminesce nce (p/sec/cm ²)
1	60			10	30	3.4	92	139	4.1x10 ⁴
2			70	0	30	3.4	69	141	3.2x10 ⁵
3		60		10	30	3.4	56	103	4.5x10 ⁶

[0135] It was observed that the ssPalmM LNP (LNP1) had expression levels close to background (10^4 p/sec/cm²) (data not shown). It was reported in the literature that changing the helper lipid from DOPE to SOPC increased gene expression. However, this was independently tested *in vivo* and was found not to improve expression. LNPs prepared with ssPalmE-PazC4 (LNP2) yielded 1 log higher expression than the ssPalm M LNPs. Surprisingly, the ssPalmE-P4C2 LNP (LNP3) yielded 2 log higher expression and was further developed to increase gene expression.

[0136] A dose response assay was performed using LNP3 (*i.e.*, the 60:10 formulation) in which the injection volume was maintained at 0.2 mL, while the amount of trapped DNA was increased. The LNP average diameter was 91 nm with a polydispersity index of 0.15 and a pDNA trapping efficiency of 92%. The average bioluminescence from three mice as a function of pDNA dose is shown in Table 5. As demonstrated herein, maximum expression was obtained with the 10 μ g dose of trapped pDNA.

Table 5: Luciferase Expression as a Function of Trapped pDNA Dose Response

Trapped pDNA Dose (μ g)	Bioluminescence (p/sec/cm ²)
5	5.1×10^6
10	6.1×10^7
15	4.1×10^7

[0137] The 10 μ g trapped pDNA dose was thereafter tested in a multidose study, but because some of the mice did not recover after anesthesia after luciferase bioluminescence imaging, a series of studies were conducted in which the formulation parameters were varied to minimize any potential lack of tolerability while maintaining high levels of gene expression. The varied formulation parameters included (a) removal of residual tert-butyl alcohol; (b) holding the ratio of the lipid components constant while varying the pDNA nucleotide to cationic lipid ratio; (c) varying the cationic lipid to DOPE ratio; and (d) adding dexamethasone palmitate to the formulation. The luciferase expression results summarized in Table 6 show the effect of conditions (a) and (b).

Table 6: Effects of Decreasing the Cationic Lipid to pDNA Molar Ratio

LNP Formulation	ssPalmE-P4C2/pDNA	Particle Size (nm)	Polydispersity Index	pDNA Trapping Efficiency (%)	Bioluminescence (p/sec/cm ²)
1 (undialyzed)	16:1	97	0.18	90	1.8x10 ⁷
2 (dialyzed)	16:1	97	0.18	94	2.4x10 ⁷
3 (dialyzed)	12:1	85	0.10	96	5.6x10 ⁶
4 (dialyzed)	8:1	93	0.13	90	3.7x10 ⁷

[0138] As shown in Table 6, decreasing the cationic lipid to pDNA nucleotide ratio did not impact gene expression in that the 8:1 ratio yielded slightly higher expression than the 16:1 ratio (compare Formulation 4 to Formulation 2). Thus, by decreasing the cationic lipid to pDNA nucleotide ratio and maintaining the mole% of each of DOPE, Chol, and DMG-PEG, there is a total reduction of lipid by 25% and 50%, which does not alter any of the characterization parameters (*i.e.*, particle size or pDNA trapping efficiency).

[0139] The ratio was further reduced to 4:1, which significantly reduced the pDNA trapping efficiency to 60% while increasing average particle size diameter to >100 nm. Physical observations from this study showed that the livers from mice administered Formulations #1 and #2 were pale. It should also be noted that increasing the ratio to 32 and 64 to 1 resulted in all animals dying (results not shown) within 24 hrs after administration. The importance of this finding is that the amount of cationic lipid determines the tolerability of the LNPs.

[0140] An additional formulation was tested in the above study where the total lipid to pDNA ratio was equivalent to the 16:1 cationic lipid to pDNA ratio formulation (Formulation 2), but the ratio of ssPalmE-P4C2 to DOPE was changed from 60:10 to 40:30. This resulted in a smaller average particle size diameter (66 nm), and a 98% pDNA trapping efficiency. However, the decrease in cationic lipid also decreased expression by approximately 1 log (8.0x10⁵ p/sec/cm²).

[0141] A pilot study was thereafter conducted to determine the impact of holding the total lipid to pDNA ratio constant, while varying the ratio of cationic lipid to helper lipid in the formulation. In addition, the mol% of DMG-PEG was varied since a lower mol% DMG-PEG has been previously reported to interfere with intracellular release of pDNA,

which would decrease gene expression. The *in vivo* luciferase expression results from this study are summarized in Table 7.

Table 7: Effect of Varying LNP Formulation Components on *In Vivo* Expression

LNP	ssPalmE-P4C2	DOPE	Chol	DMG-PEG	Trapping Efficiency (%)	Particle Size (nm)	Bioluminescence (p/sec/cm ²)
4	40	30	30	3.4	99	65	8.7x10 ⁵
5	40	30	30	2.0	100	80	3.5x10 ⁶
6	50	20	30	3.4	99	66	1.6x10 ⁶
7	50	20	30	2.0	100	79	3.1x10 ⁶

[0142] As shown in Table 7, decreasing the mol% of DMG-PEG in the 40:30 formulation (LNP4 and LNP5), increased luciferase expression 4 fold. It was also observed that increasing the ratio of ssPalmE-P4C2 from 40:30 (LNP4) to 50:20 (LNP6) also increased gene expression slightly (*i.e.*, by approximately 2 fold). Finally, it was observed that expression was further increased by approximately 2 fold, when the mol% of DMG-PEG was decreased from 3.4 (LNP6) to 2.0 (LNP7).

[0143] A study was thereafter conducted to optimize LNP formulation parameters to increase formulation tolerance while maintaining high levels of gene expression. The 60:10 LNP formulation with 3.4 mol% DMG-PEG (LNP1) and the 40:30 LNP formulation with 2.0 mol% DMG-PEG (LNP5) were tested.

[0144] In this study, the cationic lipid to pDNA nucleotide ratio was fixed at 8:1 and dexamethasone palmitate (DP) was added to both LNP formulations. Mice were bled 4 hrs after administration of the LNP formulation and assayed for interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α) and interferon-gamma (INF- γ). Mice were imaged for luciferase expression 24 hrs after LNP administration. Once mice recovered from anesthesia, blood was collected and analyzed for liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The characterization and luciferase expression results are reported in Table 8A and the cytokine and liver enzyme results are reported in Table 8B.

Table 8A: Effect of DP on LNP Characterization Parameters and Luciferase Expression

LNP Formulation	Mol% Dexamethasone Palmitate	Particle Size (nm)	pDNA Trapping Efficiency (%)	Bioluminescence p/sec/cm ²
40:30 with 2.0 mol% DMG-PEG (LNP5)	0	81	99	2.0x10 ⁷
	1.65	78	99	4.5x10 ⁷
	3.3	79	99	4.0x10 ⁷
60:10 (LNP1)	0	87	92	1.1x10 ⁷
	3.3	86	92	1.8x10 ⁸

Table 8B: Effect of LNP Dexamethasone Palmitate on Cytokine Secretion and Release of Liver Enzymes into the Blood

LNP Formulation	Mol % Dexamethasone Palmitate	IL-6 (pg/mL)	INF- γ (pg/mL)	TNF- α (pg/mL)	ALT (U/L)	AST (U/L)
40:30 with 2.0 mol% DMG-PEG (LNP5)	0	15631	523	14	125	197
	1.65	10440	266	9	135	326
	3.3	4899	182	8	240	403
60:10 (LNP1)	0	20388	1276	29	547	674
	3.3	6169	235	16	260	417

[0145] As shown in Table 8A, incorporating DP into the 40:30 ssPalmE-P4C2:DOPE formulation had no effect on luciferase expression, whereas incorporation of DP into the 60:10 ssPalmE-P4C2:DOPE formulation increased luciferase expression by 1 log.

[0146] As shown in Table 8B, incorporation of 1.65 mol% and 3.3 mol% of DP into the 40:30 LNP formulation decreased IL-6 by 33% and 69%, respectively; decreased INF- γ by 49% and 65%, respectively; and decreased TNF- α by 36% and 43%, respectively. Incorporation of 3.3 mol% DP into the 60:10 LNP formulation decreased IL-6 by 69%, decreased INF- γ by 82%, and decreased TNF- α by 45%. These results demonstrate that incorporation of DP into the formulation dramatically reduced inflammatory cytokine production, and in the case of the 60:10 LNP formulation, increased gene expression. With regard to the liver enzymes, increasing the mol% of DP for the 40:30 LNP formulation increased the liver enzymes, whereas for the 60:10 formulation, the ALT levels and AST levels were decreased by 52% and 38%, respectively.

[0147] As demonstrated herein, the 60:10:30 ssPalmE-P4C2:DOPE:Chol formulation with 3.4 mol% DMG-PEG and 3.3 mol% DP yielded the highest *in vivo* gene expression

with reduced inflammatory cytokine levels. Increasing the mol% of DP from 3.3 mol% to 6.6 mol% increased particle size to >100 nm and also reduced trapping efficiency. It is therefore conceivable that any mol% of DP equivalent to 3.3 mol%, or between 3.3 mol% and 6.6 mol%, may be used in the formulations disclosed herein to further reduce inflammatory cytokines and liver enzymes.

[0148] Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A method of inactivating hepatitis B virus (HBV) nucleic acid in a host cell comprising:
 - (a) transfecting a host cell containing HBV viral nucleic acid with a polycistronic expression cassette, wherein the expression cassette comprises:
 - (i) a RNA polymerase III promoter sequence;
 - (ii) a 5'-untranslated region comprising two or more complexes, each complex consisting of a guide RNA (gRNA) sequence and a scaffold RNA (scRNA) sequence;
 - (iii) a CRISPR nuclease sequence; and
 - (iv) a 3'-untranslated region sequence,wherein CpG content of all sequences has been minimized to reduce immunostimulatory response but maintain maximal gRNA and CRISPR nuclease expression and wherein each gRNA sequence is capable of hybridizing to a target sequence of hepatitis B viral nucleic acid; and
 - (b) incubating the cell to promote expression of the polycistronic expression cassette, thereby inactivating HBV nucleic acid in the host cell.
2. The method of claim 1, further comprising detecting a decrease in hepatitis B surface antigen (HBsAg) production, a decrease in hepatitis B e-antigen (HBeAg) production, or a decrease in both HBsAg and HBeAg production in the cell.
3. The method of claim 1, wherein the host cell is human.
4. The method of claim 3, wherein the host cell is a hepatocyte.
5. The method of claim 1, wherein the method is performed *in vitro*.
6. The method of claim 1, wherein the step of transfecting comprises administering a vector comprising the polycistronic expression cassette to a subject suffering from chronic HBV infection.

7. The method of claim 6, wherein the vector is administered in a lipid envelope encapsulating the vector.
8. The method of claim 7, wherein the lipid envelope forms a lipid nanoparticle (LNP).
9. The method of claim 8, wherein the LNP is formed from one or more cationic lipids of the ssPALM class.
10. The method of claim 9, wherein the one or more cationic lipids are independently selected from the group consisting of ssPalm, ssPalmE-P4C2, ssPalmE-Paz4-C2, and any combination thereof.
11. The method of claim 10, wherein the LNP has a ratio of cationic lipid to plasmid DNA of 4:1 to 16:1.
12. The method of claim 11, wherein the ratio of cationic lipid to plasmid DNA is 8:1 or 12:1.
13. The method of claim 10, wherein the LNP further comprises one or more lipids selected from the group consisting of cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-Dimyristoyl-rac-glycero-3-methylpolyoxyethylene (DMG-PEG), distearoyl glycerol, stearyl-oleoylphosphatidylcholine (SOPC), dielidoylphosphatidylethanolamine (DEPE), dipalmitoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), dipalmitoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-L-serine (DOPS), dimyristoylphosphatidylserine (DMPS), dioleoylphosphatidic acid (DOPA), 1,2-Dimyristoyl-sn-glycero-3-phosphate (DMPA), and 1,2-Dipalmitoyl-sn-glycero-3-phosphate (DPPA).
14. The method of claim 13, wherein the LNP comprises DMG-PEG at a concentration of about 2 mol% to 3.4 mol%.

15. The method of claim 14, wherein the DMG-PEG has a polyethylene glycol portion having a length of about 1500 daltons to 5000 daltons.
16. The method of claim 14, wherein the DMG-PEG is present at about 3.4 mol%.
17. The method of claim 13, wherein the LNP further comprises cholesterol at about 20 mol% to 33 mol%.
18. The method of claim 13, wherein the LNP further comprises dexamethasone palmitate (DP).
19. The method of claim 18, wherein the DP is present at a concentration of about 1.65 mol% to 5 mol%.
20. The method of claim 19, wherein the DP is present at about 1.65 mol%.
21. The method of claim 19, wherein the DP is present at about 3.3 mol%.
22. The method of claim 19, wherein the DP is present at about 5 mol%.
23. The method of claim 13, wherein the LNP comprises ssPalmE-P4C2, DOPE, and cholesterol.
24. The method of claim 23, wherein the ssPalmE-P4C2, DOPE, and cholesterol are present in a mol% ratio of 60:10:30, 40:30:30, or 50:20:30.
25. The method of claim 24, wherein the LNP further comprises DMG-PEG at a concentration of about 2 mol% to 3.4 mol%.
26. The method of claim 1, wherein the gRNA sequence of each complex is selected from the group consisting of SEQ ID NOs: 15-38 and 39.

27. The method of claim 26, wherein the polycistronic expression cassette is a bicistronic expression cassette.
28. The method of claim 27, wherein the bicistronic expression cassette further comprises a plurality of transfer RNA (tRNA) sequences, each tRNA sequence flanking one of the two or more complexes.
29. The method of claim 28, wherein the plurality of tRNA sequences are independently selected from the group consisting of SEQ ID NOs: 1 and 2.
30. The method of claim 28, wherein the gRNA sequences are SEQ ID NOs: 16 and 25, SEQ ID NOs: 27 and 28, or SEQ ID NOs: 28 and 39.
31. The method of claim 28, wherein the scRNA sequence of each complex is SEQ ID NO: 3.
32. The method of claim 4, wherein the expression cassette further comprises a RNA polymerase II promoter sequence selectively active in hepatocytes located upstream of the CRISPR nuclease sequence.
33. The method of claim 32, wherein the expression cassette further comprises one or more of human alpha1 antitrypsin, human phenylalanine hydroxylase, apolipoprotein E/C-1 hepatic control region, α_1 microglobulin/bikunin enhancer, human thyroxine binding globulin (TBG), serum albumin, multimerized hepatocyte nuclear factor-3 (HNF-3) binding site, and an enhancer that can bind an HBV-specific transcription activator.
34. The method of claim 33, wherein the expression cassette further comprises a hepatocyte-specific enhancer sequence operably linked to the RNA polymerase II promoter.
35. The method of claim 1, wherein the 3'-untranslated region sequence comprises a poly(A) tail.

36. The method of claim 27, wherein the bicistronic expression cassette further comprises a microRNA (miRNA) sequence located between each of the two or more complexes, wherein the miRNA sequence encodes a miRNA molecule that targets HBV nucleic acid.
37. The method of claim 36, wherein the miRNA sequence encodes a precursor miRNA (pre-miRNA) molecule.
38. The method of claim 37, wherein the gRNA sequences are SEQ ID NOs: 28 and 39 or SEQ ID NOs: 16 and 25.
39. The method of claim 38, wherein the scRNA sequence of each complex is SEQ ID NO: 3.
40. The method of claim 26, wherein the polycistronic expression cassette is a tetracistronic expression cassette.
41. The method of claim 40, wherein the tetracistronic expression cassette further comprises a plurality of transfer RNA (tRNA) sequences, each tRNA sequence flanking one of the two or more complexes.
42. The method of claim 41, wherein the plurality of tRNA sequences are independently selected from the group consisting of SEQ ID NOs: 1 and 2.
43. The method of claim 40, wherein the gRNA sequences are SEQ ID NOs: 16, 25, 27, and 28.
44. The method of claim 40, wherein the scRNA sequence of each complex is SEQ ID NO: 3.

45. The method of claim 40, wherein the expression cassette further comprises a RNA polymerase II promoter sequence selectively active in hepatocytes located upstream of the CRISPR nuclease sequence.
46. The method of claim 45, wherein the expression cassette further comprises one or more of human α_1 antitrypsin, human phenylalanine hydroxylase, apolipoprotein E/C-1 hepatic control region, α_1 microglobulin/bikunin enhancer, human thyroxine binding globulin (TBG), serum albumin, multimerized hepatocyte nuclear factor-3 (HNF-3) binding site, and an enhancer that can bind an HBV-specific transcription activator.
47. The method of claim 46, wherein the expression cassette further comprises a hepatocyte-specific enhancer sequence operably linked to the RNA polymerase II promoter.
48. The method of claim 40, wherein the tetracistronic expression cassette further comprises a microRNA (miRNA) sequence located between each of the two or more complexes, wherein the miRNA sequence encodes a miRNA molecule that targets HBV nucleic acid.
49. The method of claim 48, wherein the miRNA sequence encodes pre-miRNA molecule.
50. A vector comprising a polynucleotide which comprises in 5' to 3' orientation:
- (a) a RNA polymerase III promoter sequence;
 - (b) a 5'-untranslated region comprising at least one intron, wherein the intron comprises two or more guides, each being flanked by a transfer RNA (tRNA) sequence, wherein each guide comprises a guide RNA (gRNA) sequence and a scaffold RNA (scRNA) sequence;
 - (c) a CRISPR nuclease sequence; and
 - (d) a 3'-untranslated region sequence,
- wherein CpG content of all sequences has been minimized to reduce immunostimulatory response but maintain maximal gRNA and CRISPR nuclease

expression and wherein each gRNA sequence is capable of hybridizing to a target sequence of hepatitis B viral nucleic acid.

51. The vector of claim 50, wherein the RNA polymerase III promoter is selected from the group consisting of SEQ ID NOs: 12, 13, and 14.
52. The vector of claim 50, wherein the flanking tRNA sequences are independently selected from the group consisting of SEQ ID NOs: 1 and 2.
53. The vector of claim 50, wherein the gRNA sequences are independently selected from the group consisting of SEQ ID NOs: 15-38 and 39.
54. The vector of claim 50, wherein the vector further comprises a RNA polymerase II promoter sequence selectively active in hepatocytes located upstream of the CRISPR nuclease sequence.
55. The vector of claim 54, wherein the vector further comprises one or more of human alpha1 antitrypsin, human phenylalanine hydroxylase, apolipoprotein E/C-1 hepatic control region, α_1 microglobulin/bikunin enhancer, human thyroxine binding globulin (TBG), serum albumin, multimerized hepatocyte nuclear factor-3 (HNF-3) binding site, and an enhancer that can bind an HBV-specific transcription activator.
56. The vector of claim 55, further comprising a hepatocyte-specific enhancer sequence operably linked to the RNA polymerase II promoter.
57. The vector of claim 50, wherein the 3'-untranslated region sequence comprises a poly(A) tail.
58. The vector of claim 50, wherein the intron comprises two guides, wherein the gRNA sequences are SEQ ID NOs: 16 and 25, SEQ ID NOs: 27 and 28, or SEQ ID NOs: 28 and 39.

59. The vector of claim 50, wherein the intron comprises four guides, wherein the gRNA sequences are SEQ ID NOs: 16, 25, 27, and 28.
60. The vector of claim 50, wherein the CRISPR nuclease sequence encodes a CRISPR nuclease is capable of reducing the amount of episomal viral nucleic acid molecule in a cell of the organism compared to the amount of episomal viral nucleic acid molecule in a cell of the organism in the absence of providing the vector.
61. The vector of claim 60, wherein the CRISPR nuclease sequence encodes a CRISPR nuclease selected from the group consisting of *Streptococcus pyogenes* Cas9 (SpCas9), VRER, EQR, VQR, high-fidelity SpCas9 (SpCas9-HF1), *Staphylococcus aureus* Cas9 (SaCas9), *Streptococcus thermophilus* Cas9 (StCas9), a nuclease derived from *Prevotella* and *Francisella* bacteria (Cpf1), a nuclease derived from *Neisseria meningitidis*, a nuclease derived from *Treponema denticola*, and biologically active fragments or derivatives thereof.
62. The vector of claim 53, wherein the CRISPR nuclease sequence is CpG-free and human codon-optimized.
63. The vector of claim 62, wherein the CRISPR nuclease sequence consists of the sequence set forth in SEQ ID NO: 10 or SEQ ID NO: 11.
64. The vector of claim 50, wherein the vector further comprises nuclease DNA sequence(s) encoding at least one or more nuclear localization sequences (NLS).
65. A vector comprising a polynucleotide which comprises in 5' to 3' orientation:
- (a) a RNA polymerase III promoter sequence selectively active in hepatocytes;
 - (b) a 5'-untranslated region comprising at least one intron, wherein the intron comprises a microRNA (miRNA) molecule flanked by two guides, wherein each guide comprises a guide RNA (gRNA) sequence and a scaffold RNA (scRNA) sequence;
 - (c) a CRISPR nuclease sequence; and
 - (d) a 3'-untranslated region sequence,

wherein CpG content of all sequences has been minimized to reduce immunostimulatory response but maintain maximal gRNA and CRISPR nuclease expression and wherein each gRNA sequence is capable of hybridizing to a target sequence of hepatitis B viral nucleic acid.

66. The vector of claim 65, wherein the RNA polymerase III promoter is selected from the group consisting of SEQ ID NOs: 12, 13, and 154.
67. The vector of claim 65, wherein the miRNA sequence encodes pre-miRNA molecule.
68. The vector of claim 65, wherein the gRNA sequences are independently selected from the group consisting of SEQ ID NOs: 15-38 and 39.
69. The vector of claim 68, wherein the gRNA sequences are SEQ ID NOs: 28 and 39 or SEQ ID NOs: 16 and 25.
70. The vector of claim 65, wherein the vector further comprises a RNA polymerase II promoter sequence selectively active in hepatocytes located upstream of the CRISPR nuclease sequence.
71. The vector of claim 70, wherein the vector further comprises one or more sequences encoding human alpha1 antitrypsin, human phenylalanine hydroxylase, apolipoprotein E/C-1 hepatic control region, α_1 microglobulin/bikunin enhancer, human thyroxine binding globulin (TBG), serum albumin, multimerized hepatocyte nuclear factor-3 (HNF-3) binding site, and an enhancer that can bind an HBV-specific transcription activator.
72. The vector of claim 71, wherein the vector further comprises a hepatocyte-specific enhancer sequence operably linked to the RNA polymerase II promoter.
73. The vector of claim 65, wherein the 3'-untranslated region sequence comprises a poly(A) tail.

74. The vector of claim 65, wherein the CRISPR nuclease is capable of reducing the amount of episomal viral nucleic acid molecule in a cell of the organism compared to the amount of episomal viral nucleic acid molecule in a cell of the organism in the absence of providing the vector.
75. The vector of claim 74, wherein the CRISPR nuclease sequence encodes a CRISPR nuclease selected from the group consisting of *Streptococcus pyogenes* Cas9 (SpCas9), VRER, EQR, VQR, high-fidelity SpCas9 (SpCas9-HF1), *Staphylococcus aureus* Cas9 (SaCas9), *Streptococcus thermophilus* Cas9 (StCas9), a nuclease derived from *Prevotella* and *Francisella* bacteria (Cpf1), a nuclease derived from *Neisseria meningitidis*, a nuclease derived from *Treponema denticola*, and biologically active fragments or derivatives thereof.
76. The vector of claim 68, wherein the CRISPR nuclease sequence is CpG-free and human codon-optimized.
77. The vector of claim 76, wherein the CRISPR nuclease sequence consists of the sequence set forth in SEQ ID NO: 10 or SEQ ID NO: 11.
78. The vector of claim 65, further comprising nuclease DNA sequence(s) encoding at least one or more nuclear localization sequences (NLS).
79. A gene delivery system comprising a lipid envelope encapsulating the vector of any one of claims 50-78.
80. The gene delivery system of claim 74, wherein the vector is complexed with a condensing agent.
81. The gene delivery system of claim 80, wherein the condensing agent is selected from the group consisting of protamine, spermine, spermidine, cadaverine, putrescine, histones, and viral DNA binding proteins.

82. The gene delivery system of claim 79, wherein the lipid envelope forms an LNP.
83. The gene delivery system of claim 82, wherein the LNP is formed from one or more cationic lipids of the ssPALM class.
84. The gene delivery system of claim 83, wherein the one or more cationic lipids are independently selected from the group consisting of ssPalm, ssPalmE-P4C2, ssPalmE-Paz4-C2, and any combination thereof.
85. The gene delivery system of claim 84, wherein the LNP further comprises one or more lipids selected from the group consisting of cholesterol, DOPE, DMG-PEG, distearoyl glycerol, SOPC, DEPE, DMPC, DPPC, DSPC, DOPC, POPC, DOPS, DMPS, DOPA, DMPA, and DPPA.
86. The gene delivery system of claim 79, wherein the lipid envelope comprises one or more ligands that bind to hepatocyte receptors disposed thereon.
87. A method of treating chronic HBV infection in a subject in need thereof comprising administering an effective amount of the gene delivery system of any one of claims 79-86.

FIG. 1A

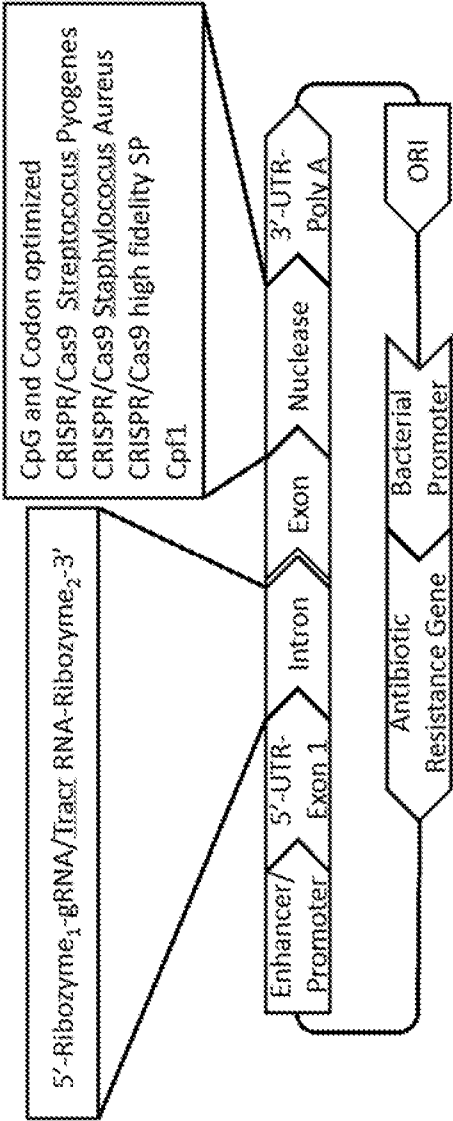
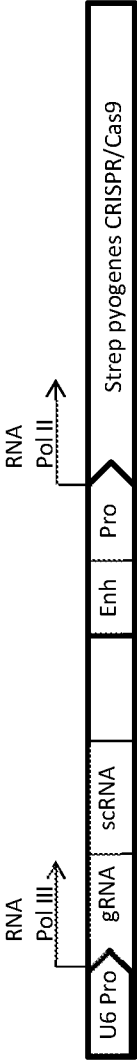


FIG. 1B



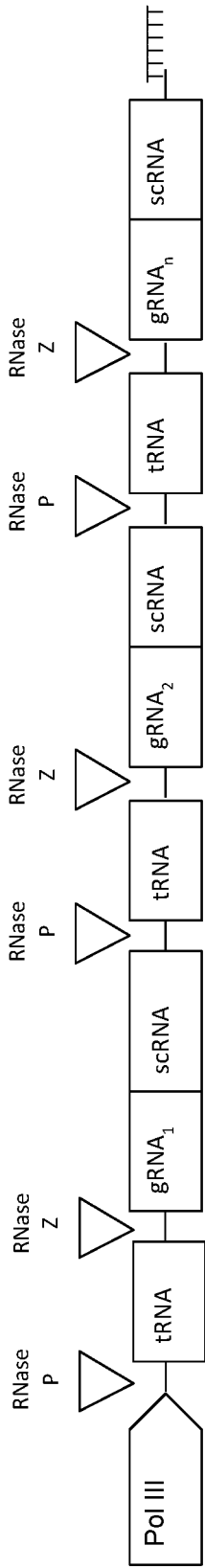


FIG. 2

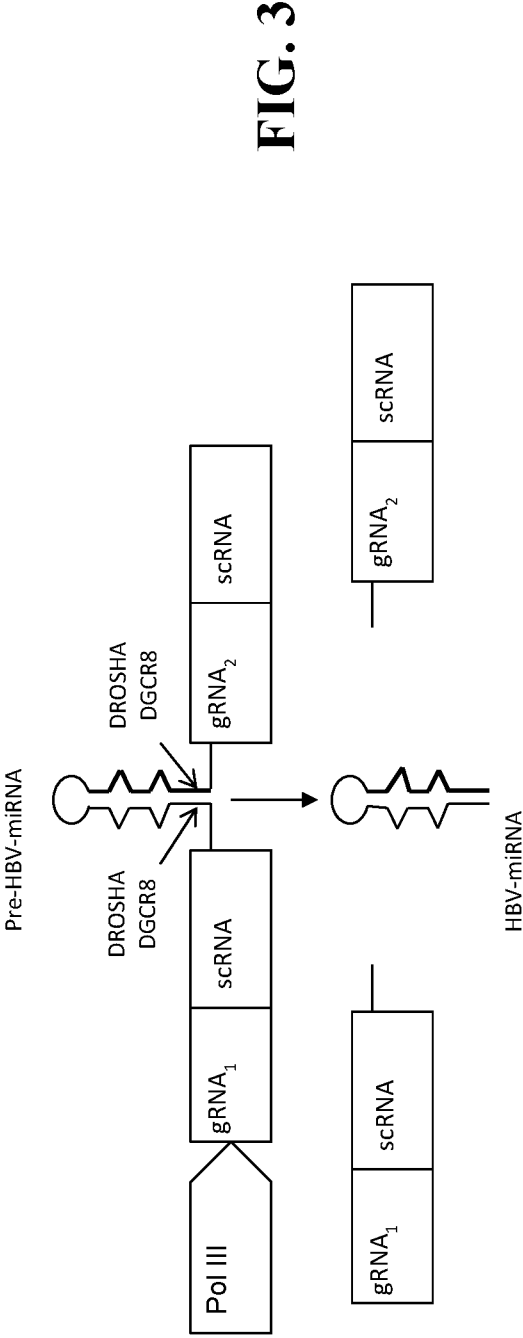


FIG. 3