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(54) **LIPID COMPOSITION FOR THE DELIVERY OF THERAPEUTIC CARGOS**

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(21) Appl. No.: **17/874,818**

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Publication Classification

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A61K 47/69	(2006.01)
B82Y 5/00	(2006.01)
C12N 9/22	(2006.01)

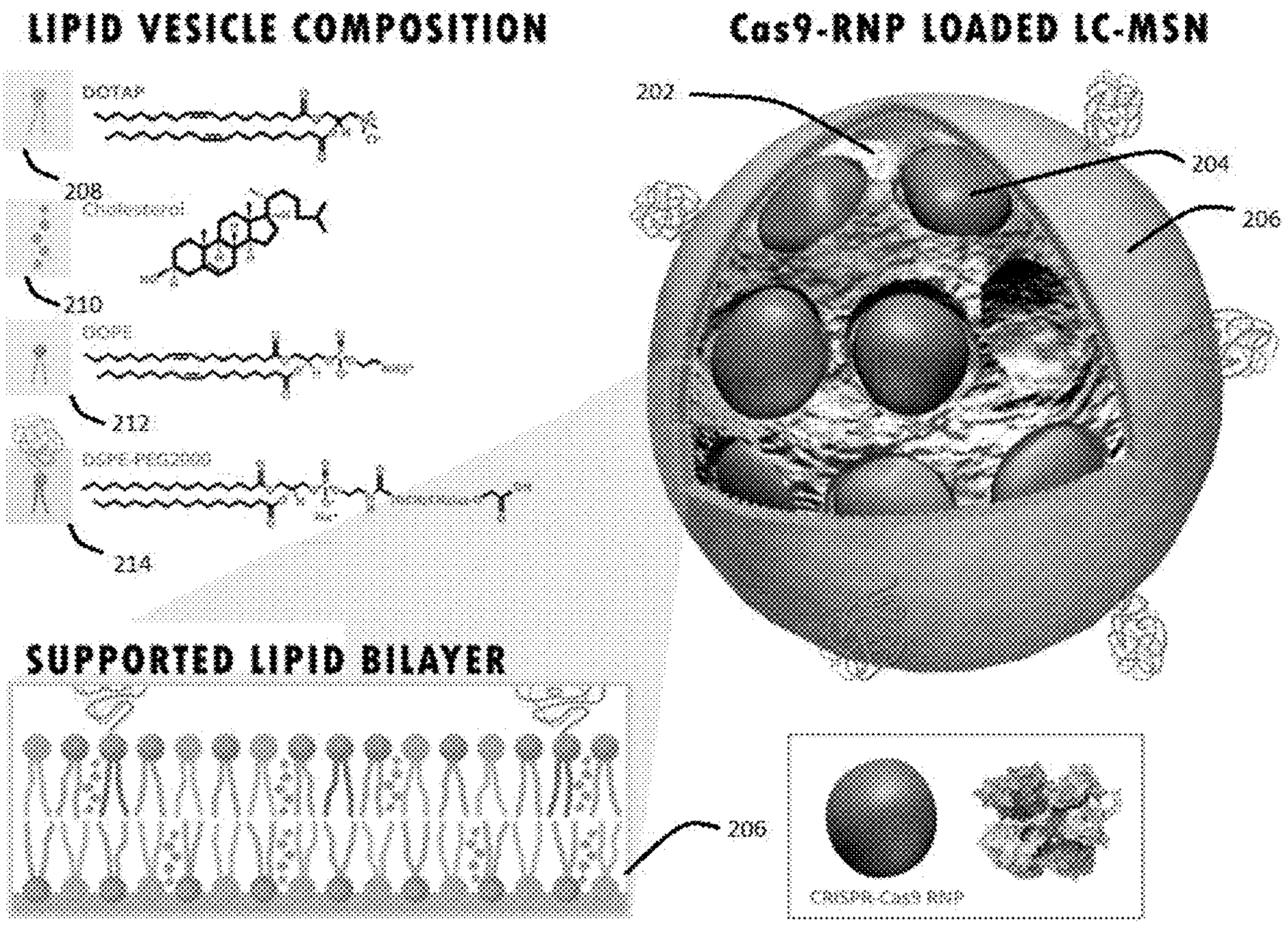
(52) **U.S. Cl.**

CPC ***A61K 47/543*** (2017.08); ***A61K 47/10*** (2013.01); ***A61K 47/6923*** (2017.08); ***B82Y 5/00*** (2013.01); ***C12N 9/22*** (2013.01); *C12N 2310/20* (2017.05)

(57) **ABSTRACT**

A construct includes a core comprising an external surface and a plurality of pores, a cargo disposed in a pore of the plurality of pores, the cargo comprising a CRISPR Cas9 component or a nucleic acid sequence encoding a CRISPR Cas9 component; and a coating coupled to the core, wherein the coating comprises a cationic lipid, a pegylated lipid, a zwitterionic lipid, and a sterol. The coating may comprise a molar ratio of about 1 cationic lipid to 1 zwitterionic lipid to 0.9 sterol to 0.15 PEGylated lipid, wherein each molar ratio optionally varies by about plus or minus 10%. A method of treatment is also provided.

Specification includes a Sequence Listing.



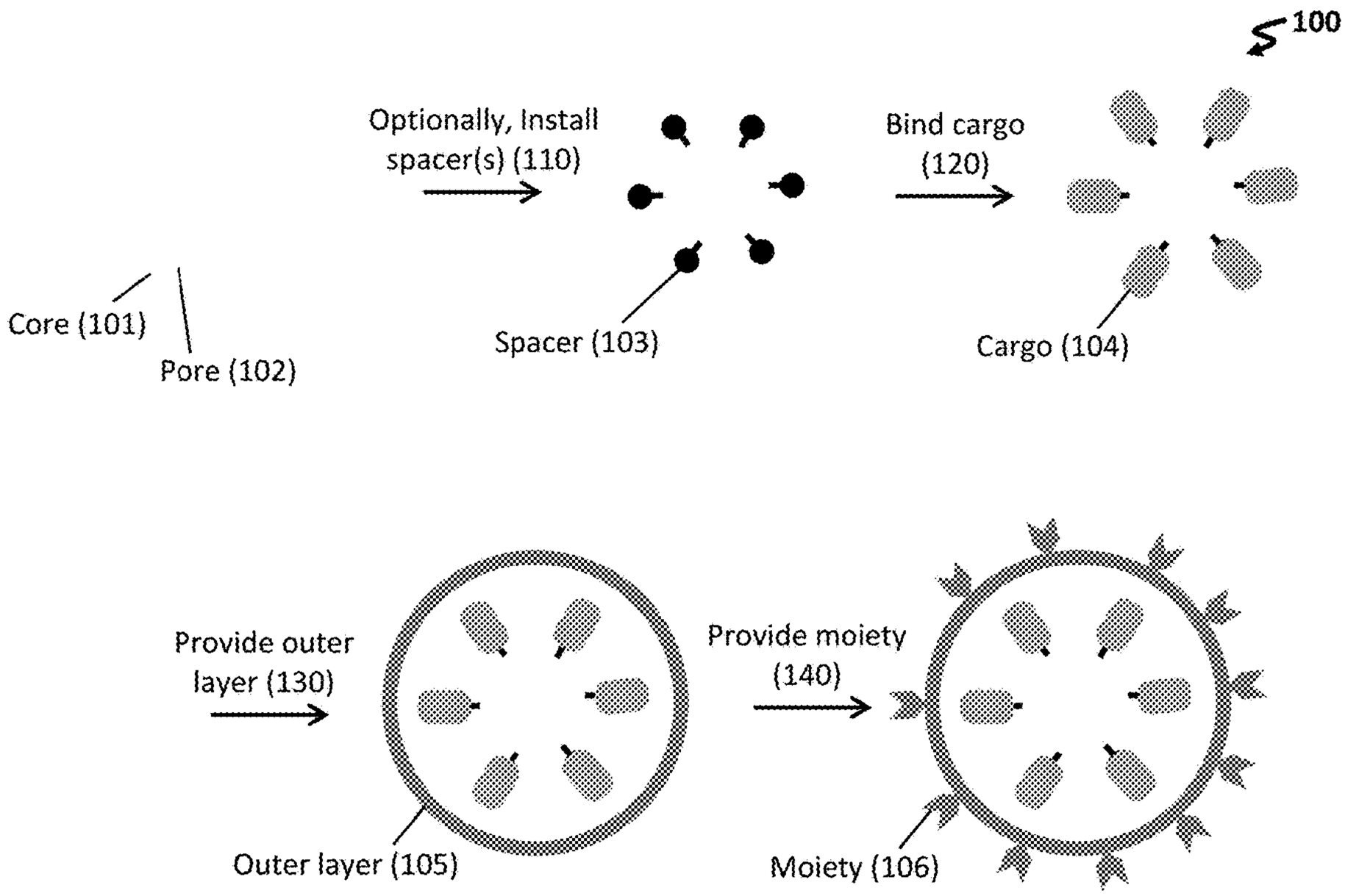


FIG. 1A

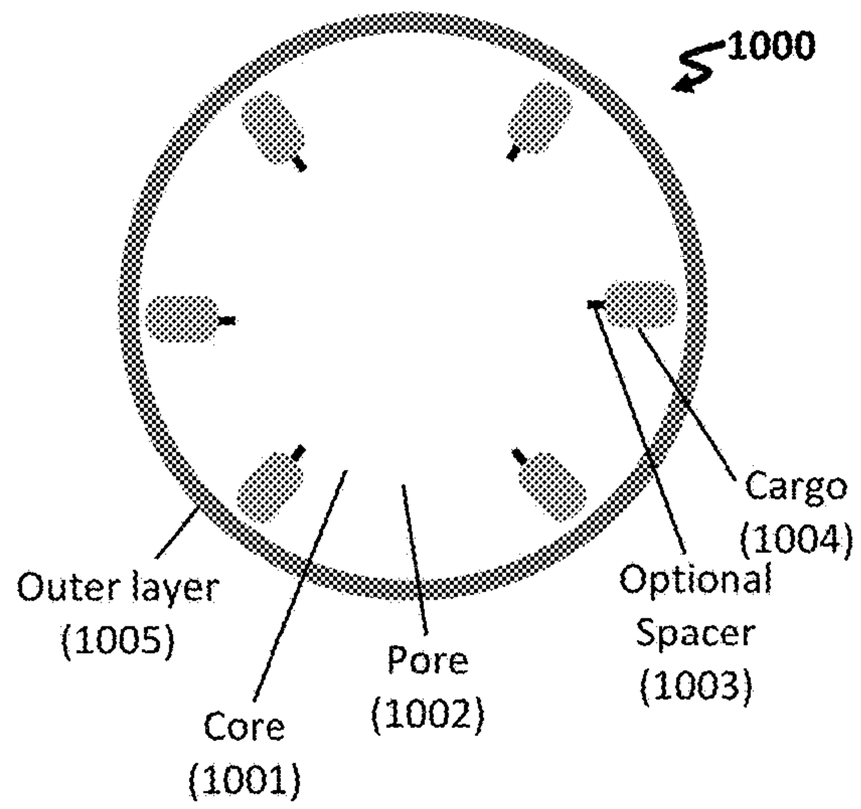


FIG. 1B

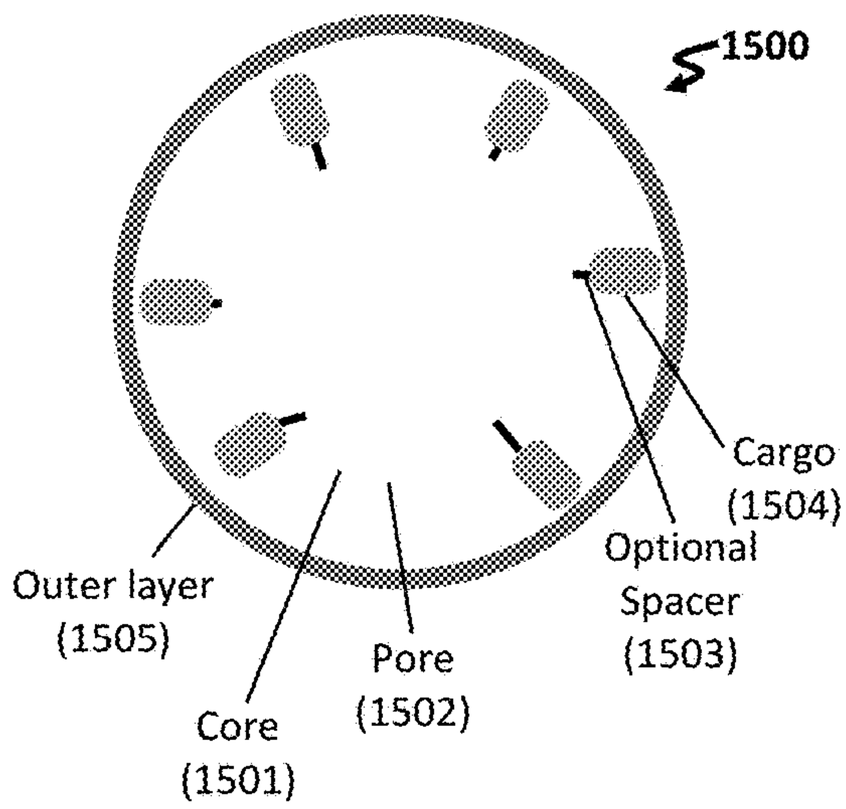


FIG. 1C

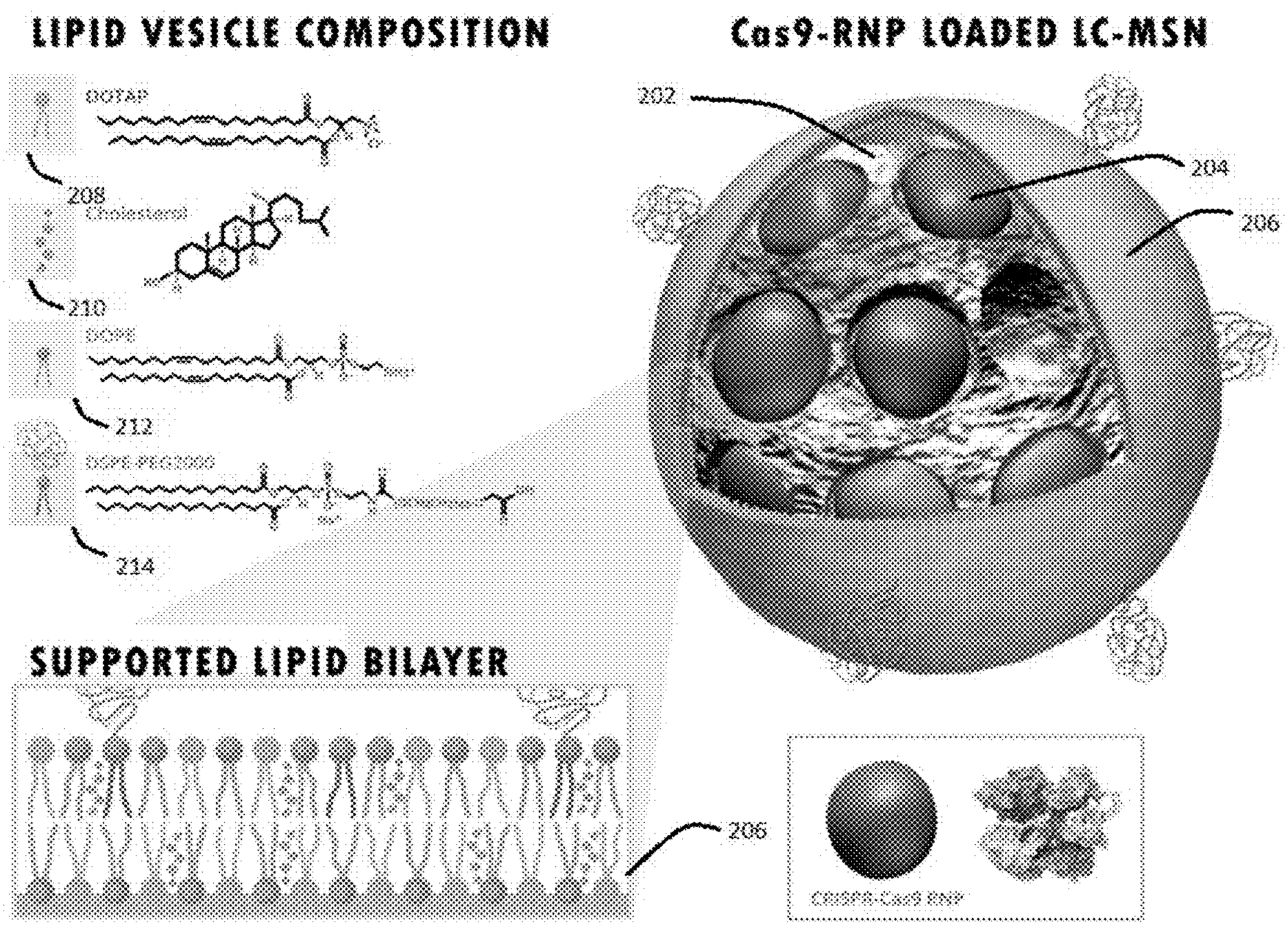

LIPID VESICLE COMPOSITION
Cas9-RNP LOADED LC-MSN
DOTAP
208
Cholesterol
210
DOPE
212
DOPE-PEG2000
214
202
204
206
SUPPORTED LIPID BILAYER
206
Cas9-Cas9 RNP

Fig. 2

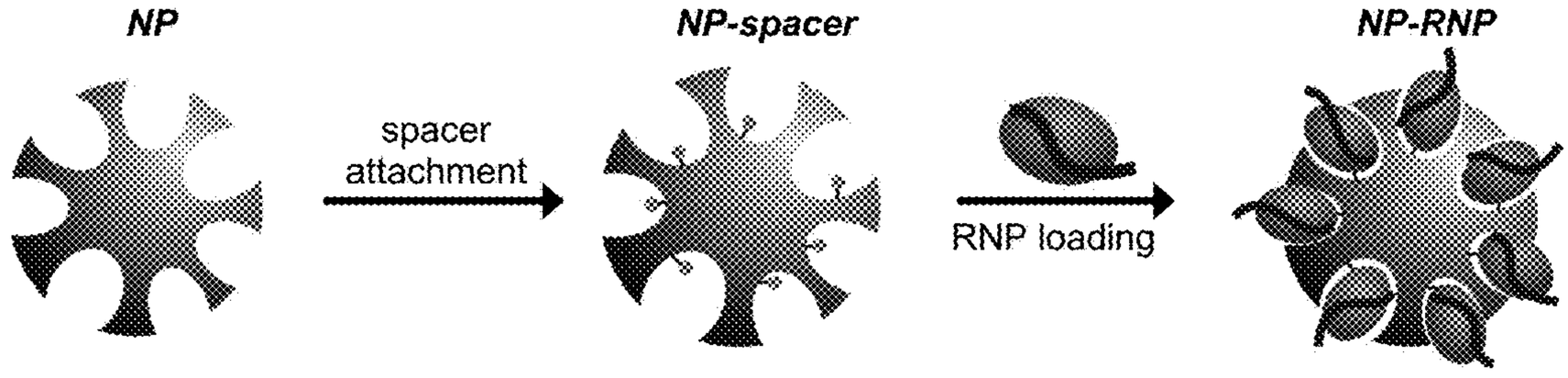


FIG. 3A

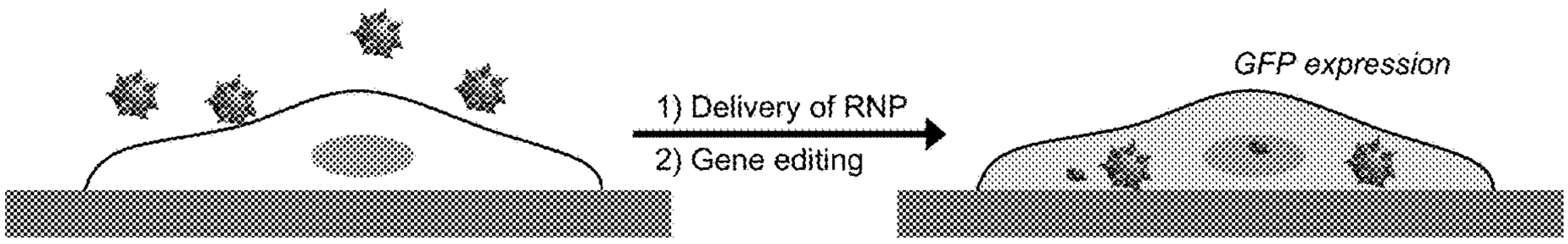


FIG. 3B

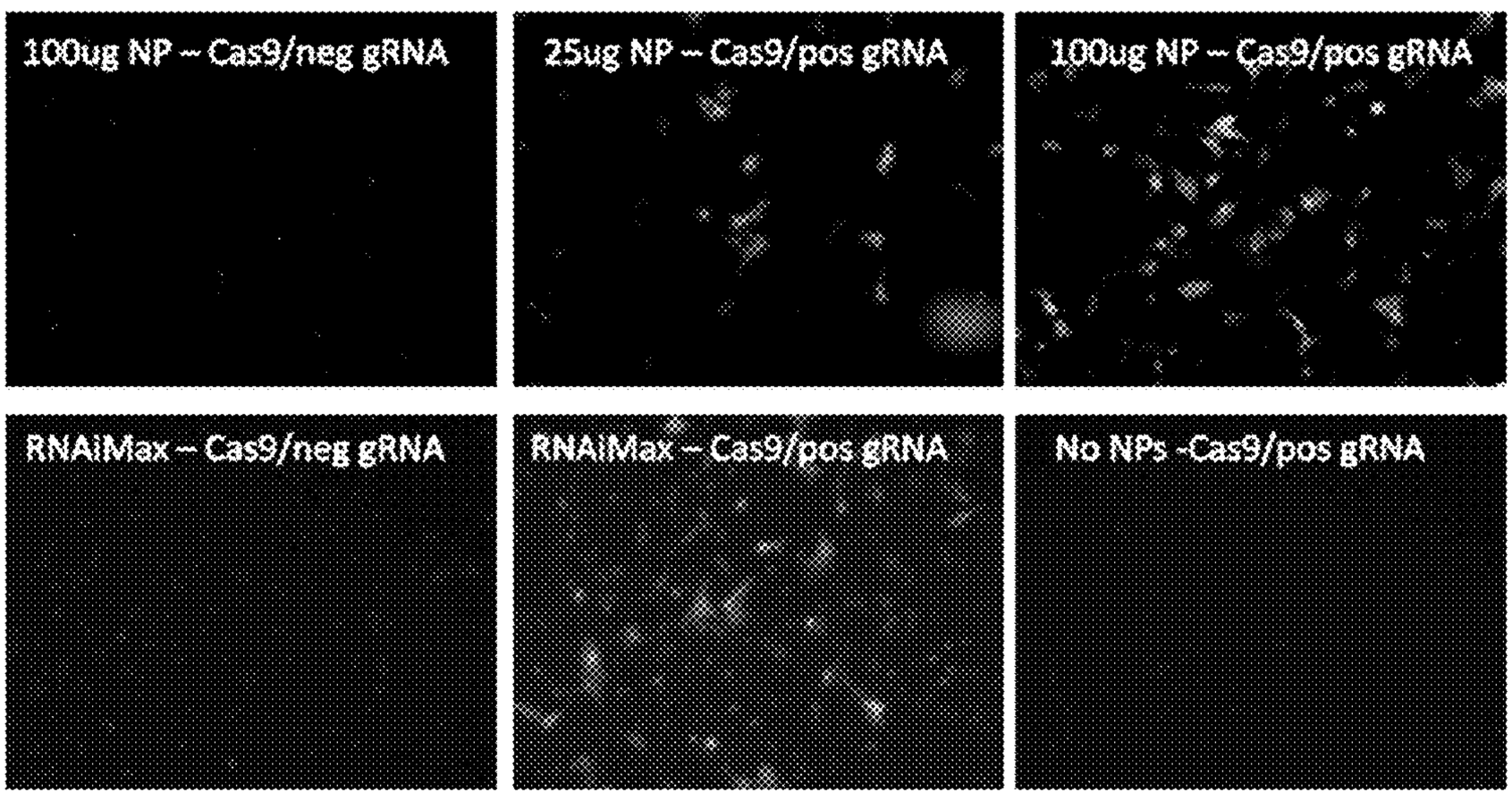


FIG. 3C

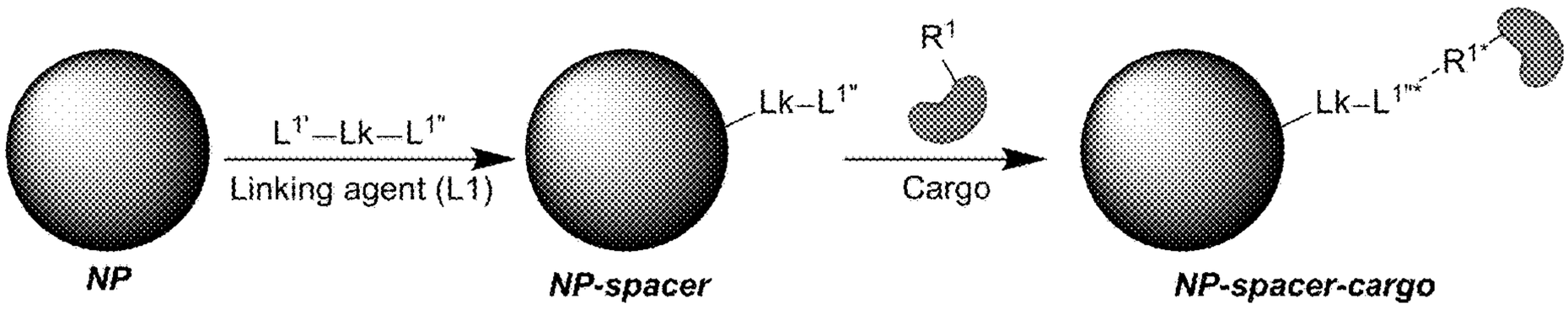


FIG. 4A

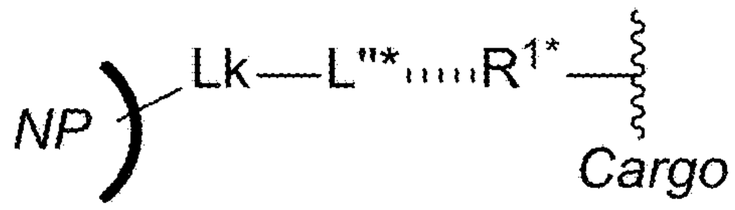


FIG. 4B

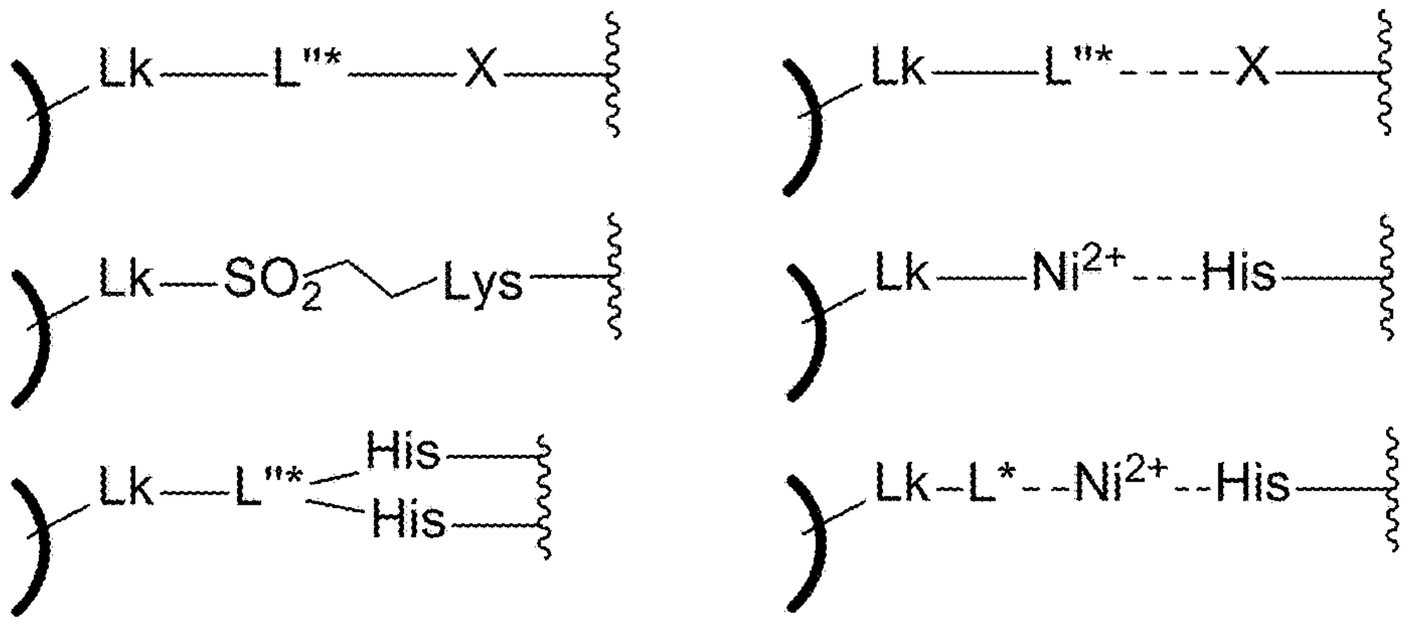


FIG. 4C

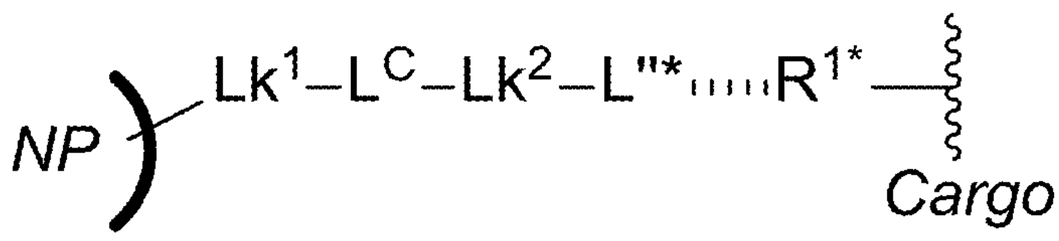

NP
Lk1–LC–Lk2–L"*
R1*
Cargo

FIG. 4D

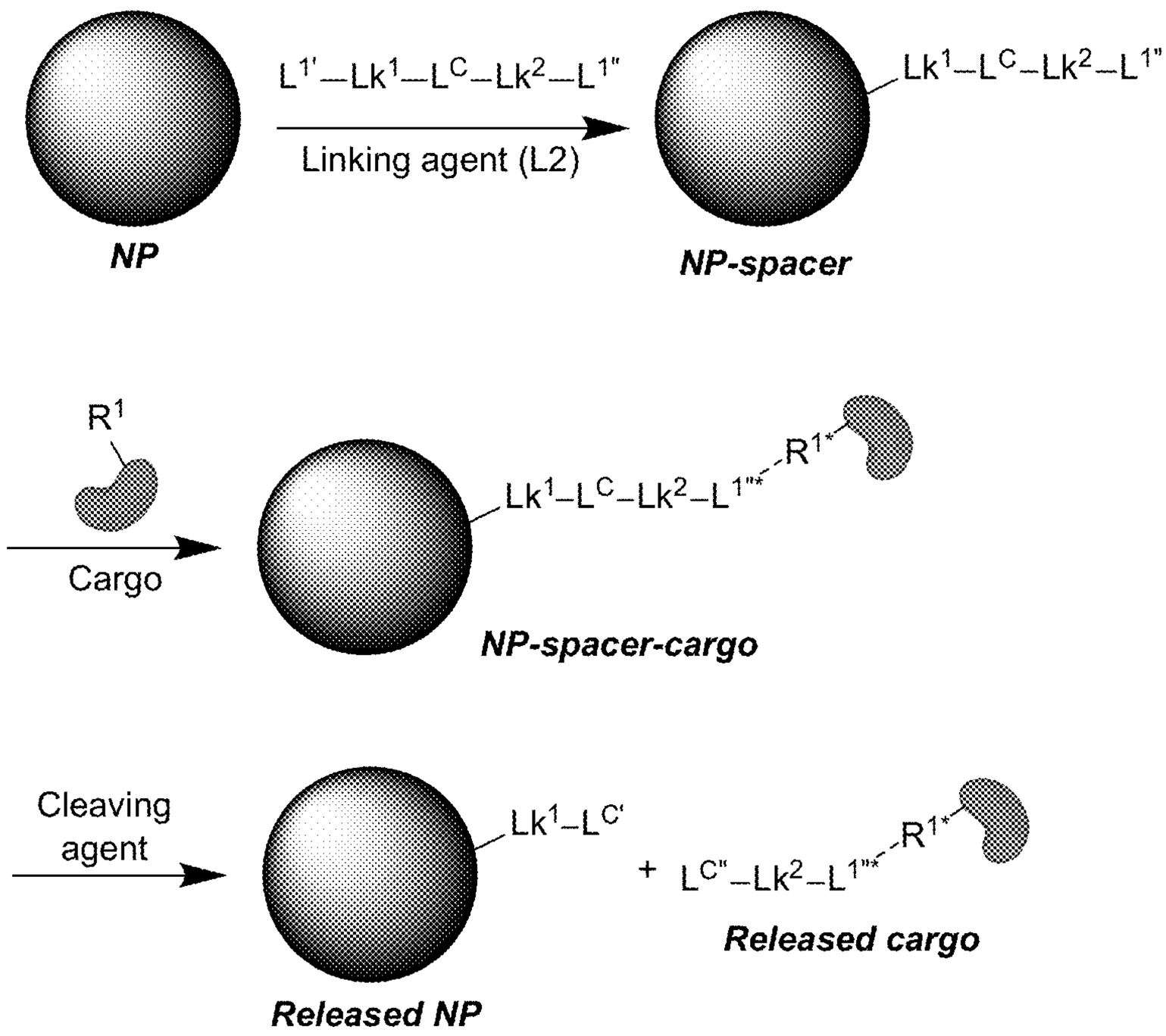

L1'–Lk1–LC–Lk2–L1"
Linking agent (L2)
Lk1–LC–Lk2–L1"
NP
NP-spacer
R1
Cargo
Lk1–LC–Lk2–L1"*
R1*
NP-spacer-cargo
Cleaving agent
Lk1–LC'
+ LC"–Lk2–L1"*
R1*
Released cargo
Released NP

FIG. 4E

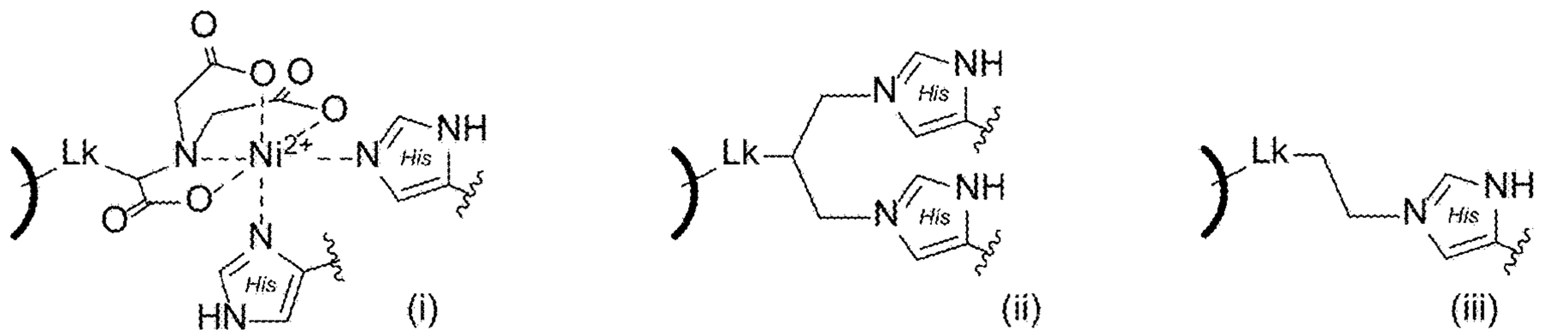


FIG. 5A

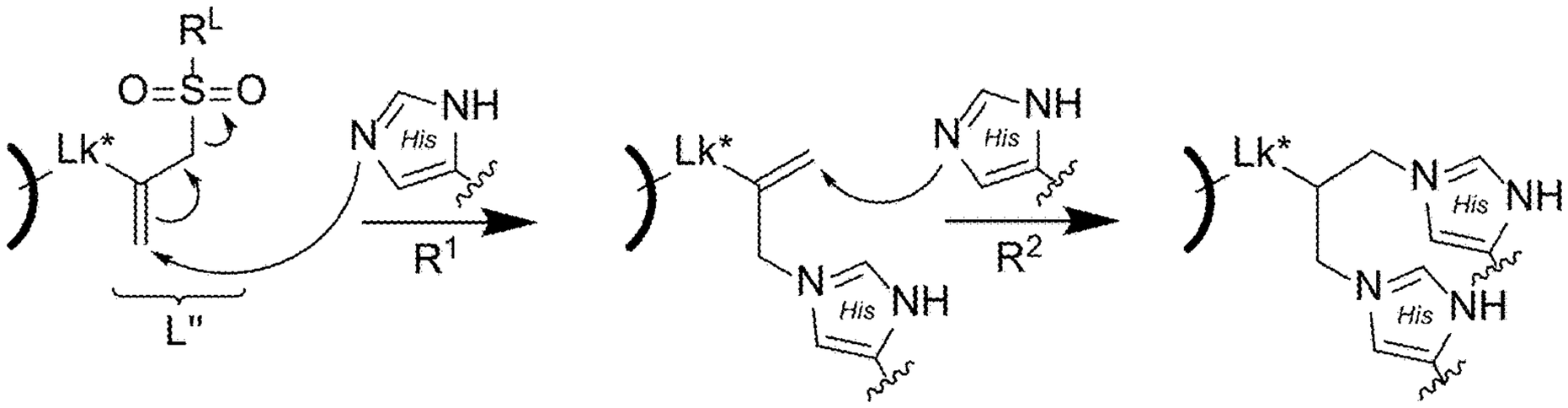


FIG. 5B

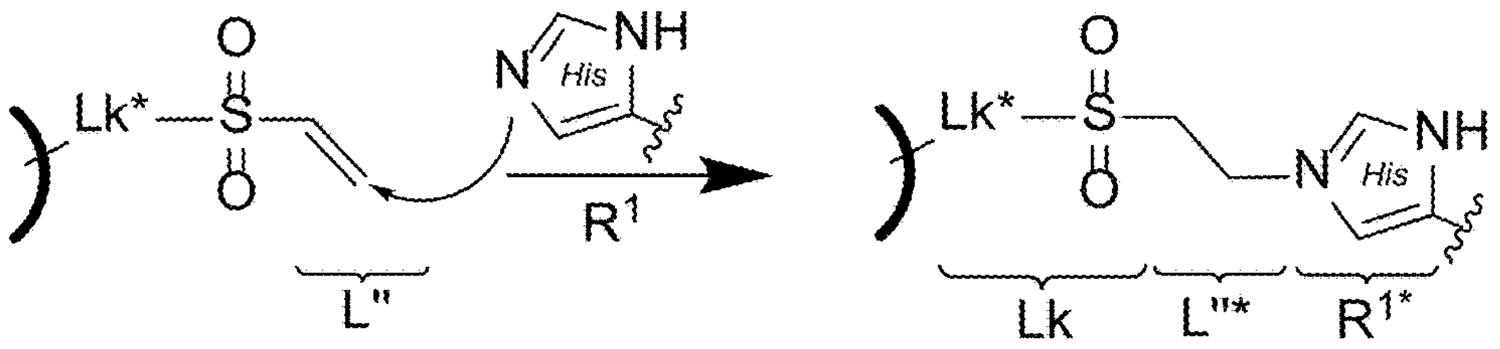


FIG. 5C

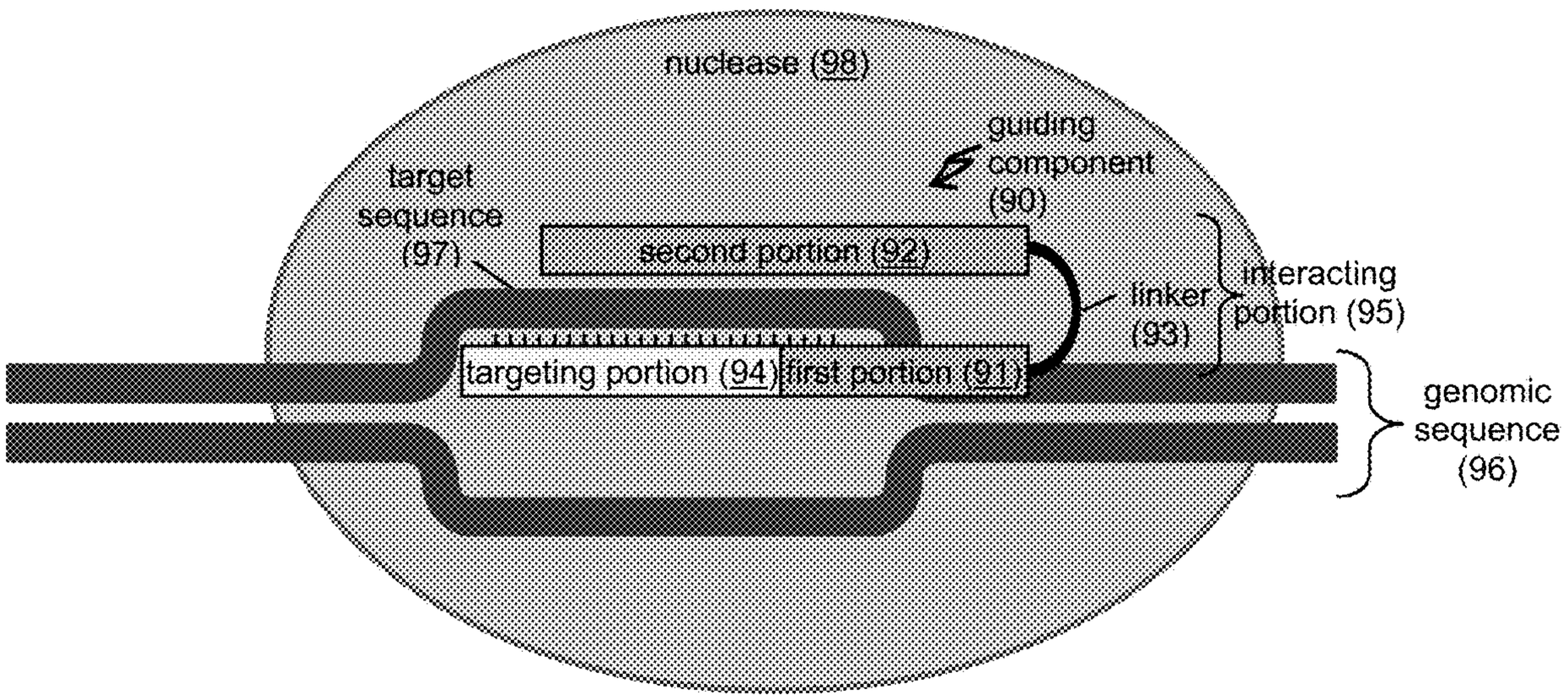


FIG. 6

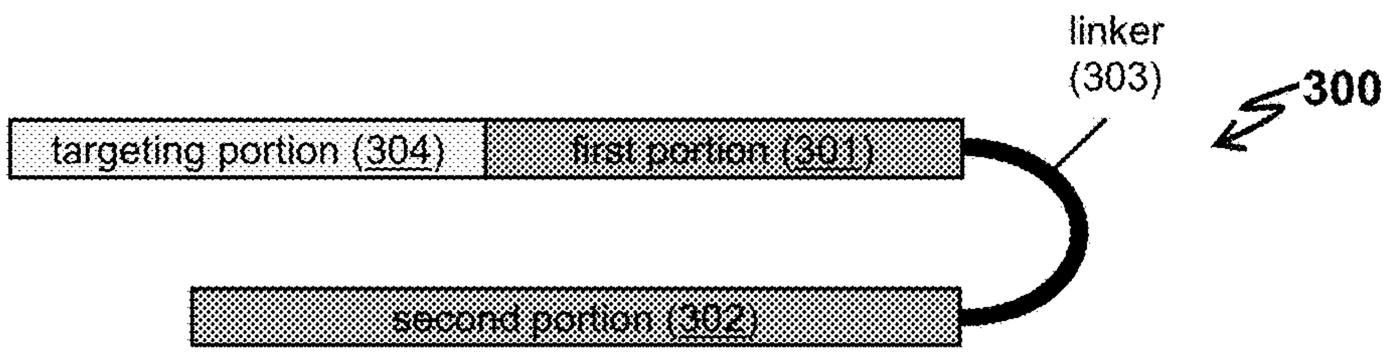


FIG. 7A

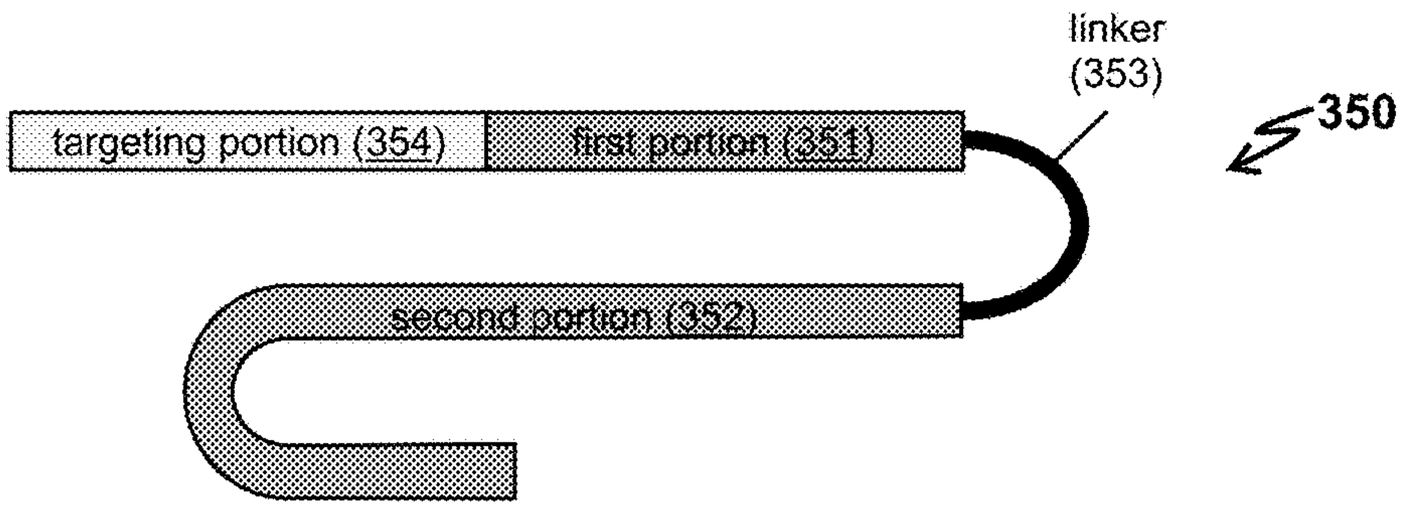


FIG. 7B

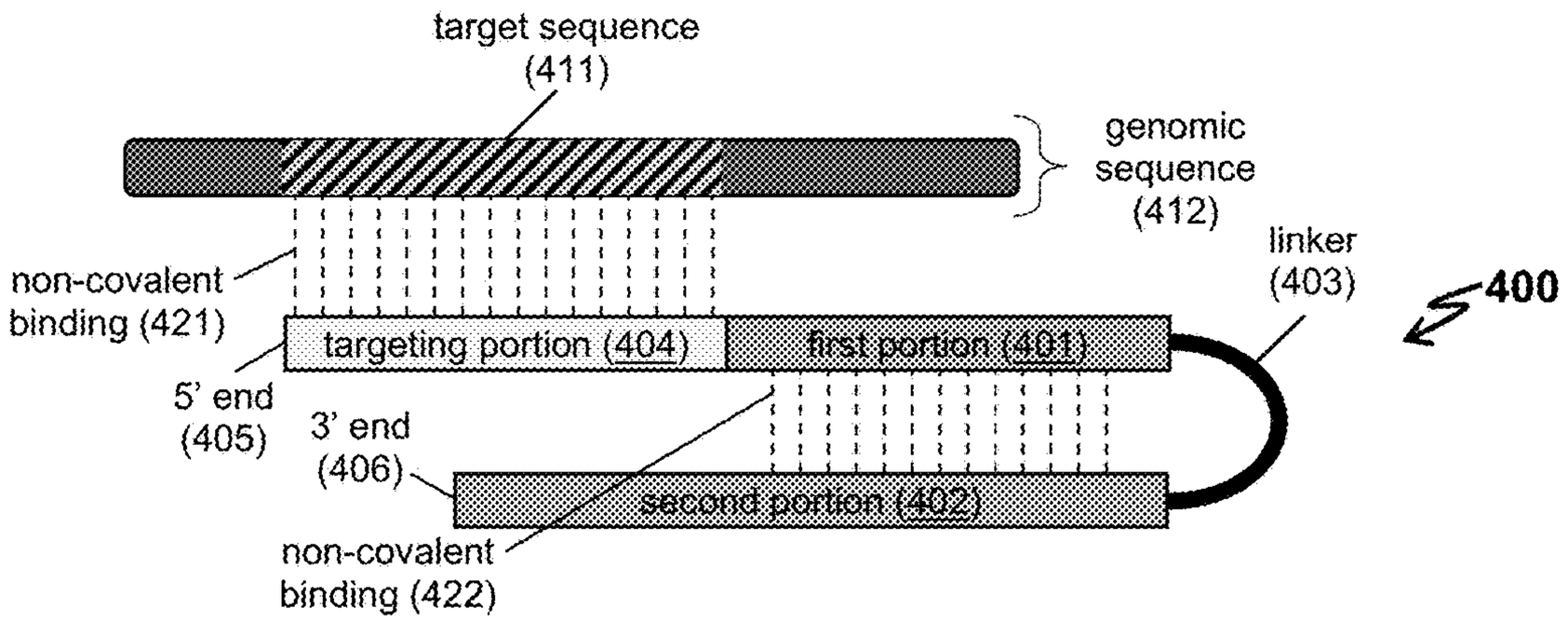


FIG. 7C

SEQ ID NO:110
UniProtKB/Swiss-Prot: Q99ZW2.1
RecName: Full=CRISPR-associated endonuclease Cas9/Csn1; AltName: Full=SpyCas9
[Streptococcus pyogenes serotype M1]

```
      1 MDKKYSIGLD IGTNSVGWAV ITDEYKVPSK KFKVLGNTDR HSIKKNLIGA LLFDSGETAE
     61 ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG
    121 NIVDEVAYHE KYPTIYHLRK KLVDSTDKAD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD
    181 VDKLFIQLVQ TYNQLFEENP INASGVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN
    241 LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAI
    301 LLSDILRVNT EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA
    361 GYIDGGASQE EFYKFIKPIL EKMDGTEELL VKLNREDLLR KQRTFDNGSI PHQIHLGELH
    421 AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE
    481 VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL
    541 SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI
    601 IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG
    661 RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL
    721 HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER
    781 MKRIEEGIKE LGSQILKEHP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDH
    841 IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL
    901 TKAERGGLSE LDKAGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVKVITLKS
    961 KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK
   1021 MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF
   1081 ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA
   1141 YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKNPID FLEAKGYKEV KKDLIIKLPK
   1201 YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE
   1261 QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA
   1321 PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ SITGLYETRI DLSQLGGD
```

FIG. 8A

SEQ ID NO:111

dCas9 (D10A,H840A)

```
   1 MDKKYSIGLA IGTNSVGWAV ITDEYKVPSK KFKVLGNTDR HSIKKNLIGA LLFDSGETAE
  61 ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG
 121 NIVDEVAYHE KYPTIYHLRK KLVDSTDKAD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD
 181 VDKLFIQLVQ TYNQLFEENP INASGVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN
 241 LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAI
 301 LLSDILRVNT EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA
 361 GYIDGGASQE EFYKFIKPIL EKMDGTEELL VKLNREDLLR KQRTFDNGSI PHQIHLGELH
 421 AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE
 481 VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL
 541 SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI
 601 IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG
 661 RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL
 721 HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER
 781 MKRIEEGIKE LGSQILKEHP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDA
 841 IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL
 901 TKAERGGLSE LDKAGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVKVITLKS
 961 KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK
1021 MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF
1081 ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA
1141 YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKNPID FLEAKGYKEV KKDLIIKLPK
1201 YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE
1261 QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA
1321 PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ SITGLYETRI DLSQLGGD
```

FIG. 8B

SEQ ID NO:112

NCBI Reference Sequence: WP_011054416.1

CRISPR-associated protein Csn1

[Streptococcus pyogenes]

```
   1 MDKKYSIGLD IGTNSVGWAV ITDDYKVPSK KLKGLGNTDR HGIKKNLIGA LLFDSGETAE
  61 ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG
 121 NIVDEVAYHE KYPTIYHLRK KLADSTDKVD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD
 181 VDKLFIQLVQ TYNQLFEENP INASRVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN
 241 LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAT
 301 LLSDILRVNS EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA
 361 GYIDGGASQE EFYKFIKPIL EKMDGTEELL AKLNREDLLR KQRTFDNGSI PYQIHLGELH
 421 AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE
 481 VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL
 541 SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI
 601 IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG
 661 RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL
 721 HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER
 781 MKRIEEGIKE LGSDILKEYP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDH
 841 IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL
 901 TKAERGGLSE LDKVGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVRVITLKS
 961 KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK
1021 MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF
1081 ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA
1141 YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKDPID FLEAKGYKEV RKDLIIKLPK
1201 YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE
1261 QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA
1321 PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ SITGLYETRI DLSQLGGD
```

FIG. 8C

SEQ ID NO:113

UniProtKB/Swiss-Prot: A0Q5Y3.1
RecName: Full=CRISPR-associated endonuclease Cas9
[Francisella tularensis subsp. novicida U112]

```
   1 MNFKILPIAI DLGVKNTGVF SAFYQKGTSL ERLDNKNGKV YELSKDSYTL LMNNRTARRH
  61 QRRGIDRKQL VKRLFKLIWT EQLNLEWDKD TQQAISFLFN RRGFSFITDG YSPEYLNIVP
 121 EQVKAILMDI FDDYNGEDDL DSYLKLATEQ ESKISEIYNK LMQKILEFKL MKLCTDIKDD
 181 KVSTKTLKEI TSYEFELLAD YLANYSESLK TQKFSYTDKQ GNLKELSYYH HDKYNIQEFL
 241 KRHATINDRI LDTLLTDDLD IWNFNFEKFD FDKNEEKLQN QEDKDHIQAH LHHFVFAVNK
 301 IKSEMASGGR HRSQYFQEIT NVLDENNHQE GYLKNFCENL HNKKYSNLSV KNLVNLIGNL
 361 SNLELKPLRK YFNDKIHAKA DHWDEQKFTE TYCHWILGEW RVGVKDQDKK DGAKYSYKDL
 421 CNELKQKVTK AGLVDFLLEL DPCRTIPPYL DNNNRKPPKC QSLILNPKFL DNQYPNWQQY
 481 LQELKKLQSI QNYLDSFETD LKVLKSSKDQ PYFVEYKSSN QQIASGQRDY KDLDARILQF
 541 IFDRVKASDE LLLNEIYFQA KKLKQKASSE LEKLESSKKL DEVIANSQLS QILKSQHTNG
 601 IFEQGTFLHL VCKYYKQRQR ARDSRLYIMP EYRYDKKLHK YNNTGRFDDD NQLLTYCNHK
 661 PRQKRYQLLN DLAGVLQVSP NFLKDKIGSD DDLFISKWLV EHIRGFKKAC EDSLKIQKDN
 721 RGLLNHKINI ARNTKGKCEK EIFNLICKIE GSEDKKGNYK HGLAYELGVL LFGEPNEASK
 781 PEFDRKIKKF NSIYSFAQIQ QIAFAERKGN ANTCAVCSAD NAHRMQQIKI TEPVEDNKDK
 841 IILSAKAQRL PAIPTRIVDG AVKKMATILA KNIVDDNWQN IKQVLSAKHQ LHIPIITESN
 901 AFEFEPALAD VKGKSLKDRR KKALERISPE NIFKDKNNRI KEFAKGISAY SGANLTDGDF
 961 DGAKEELDHI IPRSHKKYGT LNDEANLICV TRGDNKNKGN RIFCLRDLAD NYKLKQFETT
1021 DDLEIEKKIA DTIWDANKKD FKFGNYRSFI NLTPQEQKAF RHALFLADEN PIKQAVIRAI
1081 NNRNRTFVNG TQRYFAEVLA NNIYLRAKKE NLNTDKISFD YFGIPTIGNG RGIAEIRQLY
1141 EKVDSDIQAY AKGDKPQASY SHLIDAMLAF CIAADEHRND GSIGLEIDKN YSLYPLDKNT
1201 GEVFTKDIFS QIKITDNEFS DKKLVRKKAI EGFNTHRQMT RDGIYAENYL PILIHKELNE
1261 VRKGYTWKNS EEIKIFKGKK YDIQQLNNLV YCLKFVDKPI SIDIQISTLE ELRNILTTNN
1321 IAATAEYYYI NLKTQKLHEY YIENYNTALG YKKYSKEMEF LRSLAYRSER VKIKSIDDVK
1381 QVLDKDSNFI IGKITLPFKK EWQRLYREWQ NTTIKDDYEF LKSFFNVKSI TKLHKKVRKD
1441 FSLPISTNEG KFLVKRKTWD NNFIYQILND SDSRADGTKP FIPAFDISKN EIVEAIIDSF
1501 TSKNIFWLPK NIELQKVDNK NIFAIDTSKW FEVETPSDLR DIGIATIQYK IDNNSRPKVR
1561 VKLDYVIDDD SKINYFMNHS LLKSRYPDKV LEILKQSTII EFESSGFNKT IKEMLGMKLA
1621 GIYNETSNN
```

FIG. 8D

SEQ ID NO:114

UniProtKB/Swiss-Prot: G3ECR1.2
RecName: Full=CRISPR-associated endonuclease Cas9
[Streptococcus thermophilus]

```
   1 MLFNKCIIIS INLDFSNKEK CMTKPYSIGL DIGTNSVGWA VITDNYKVPS KKMKVLGNTS
  61 KKYIKKNLLG VLLFDSGITA EGRRLKRTAR RRYTRRRNRI LYLQEIFSTE MATLDDAFFQ
 121 RLDDSFLVPD DKRDSKYPIF GNLVEEKVYH DEFPTIYHLR KYLADSTKKA DLRLVYLALA
 181 HMIKYRGHFL IEGEFNSKNN DIQKNFQDFL DTYNAIFESD LSLENSKQLE EIVKDKISKL
 241 EKKDRILKLF PGEKNSGIFS EFLKLIVGNQ ADFRKCFNLD EKASLHFSKE SYDEDLETLL
 301 GYIGDDYSDV FLKAKKLYDA ILLSGFLTVT DNETEAPLSS AMIKRYNEHK EDLALLKEYI
 361 RNISLKTYNE VFKDDTKNGY AGYIDGKTNQ EDFYVYLKNL LAEFEGADYF LEKIDREDFL
 421 RKQRTFDNGS IPYQIHLQEM RAILDKQAKF YPFLAKNKER IEKILTFRIP YYVGPLARGN
 481 SDFAWSIRKR NEKITPWNFE DVIDKESSAE AFINRMTSFD LYLPEEKVLP KHSLLYETFN
 541 VYNELTKVRF IAESMRDYQF LDSKQKKDIV RLYFKDKRKV TDKDIIEYLH AIYGYDGIEL
 601 KGIEKQFNSS LSTYHDLLNI INDKEFLDDS SNEAIIEEII HTLTIFEDRE MIKQRLSKFE
 661 NIFDKSVLKK LSRRHYTGWG KLSAKLINGI RDEKSGNTIL DYLIDDGISN RNFMQLIHDD
 721 ALSFKKKIQK AQIIGDEDKG NIKEVVKSLP GSPAIKKGIL QSIKIVDELV KVMGGRKPES
 781 IVVEMARENQ YTNQGKSNSQ QRLKRLEKSL KELGSKILKE NIPAKLSKID NNALQNDRLY
 841 LYYLQNGKDM YTGDDLDIDR LSNYDIDHII PQAFLKDNSI DNKVLVSSAS NRGKSDDFPS
 901 LEVVKKRKTF WYQLLKSKLI SQRKFDNLTK AERGGLLPED KAGFIQRQLV ETRQITKHVA
 961 RLLDEKFNNK KDENNRAVRT VKIITLKSTL VSQFRKDFEL YKVREINDFH HAHDAYLNAV
1021 IASALLKKYP KLEPEFVYGD YPKYNSFRER KSATEKVYFY SNIMNIFKKS ISLADGRVIE
1081 RPLIEVNEET GESVWNKESD LATVRRVLSY PQVNVVKKVE EQNHGLDRGK PKGLFNANLS
1141 SKPKPNSNEN LVGAKEYLDP KKYGGYAGIS NSFAVLVKGT IEKGAKKKIT NVLEFQGISI
1201 LDRINYRKDK LNFLLEKGYK DIELIIELPK YSLFELSDGS RRMLASILST NNKRGEIHKG
1261 NQIFLSQKFV KLLYHAKRIS NTINENHRKY VENHKKEFEE LFYYILEFNE NYVGAKKNGK
1321 LLNSAFQSWQ NHSIDELCSS FIGPTGSERK GLFELTSRGS AADFEFLGVK IPRYRDYTPS
1381 SLLKDATLIH QSVTGLYETR IDLAKLGEG
```

FIG. 8E

SEQ ID NO:115
NCBI Reference Sequence: WP_011681470.1
CRISPR-associated endonuclease Cas9 2
[Streptococcus thermophilus]

```
   1 MTKPYSIGLD IGTNSVGWAV TTDNYKVPSK KMKVLGNTSK KYIKKNLLGV LLFDSGITAE
  61 GRRLKRTARR RYTRRRNRIL YLQEIFSTEM ATLDDAFFQR LDDSFLVPDD KRDSKYPIFG
 121 NLVEEKAYHD EFPTIYHLRK YLADSTKKAD LRLVYLALAH MIKYRGHFLI EGEFNSKNND
 181 IQKNFQDFLD TYNAIFESDL SLENSKQLEE IVKDKISKLE KKDRILKLFP GEKNSGIFSE
 241 FLKLIVGNQA DFRKCFNLDE KASLHFSKES YDEDLETLLG YIGDDYSDVF LKAKKLYDAI
 301 LLSGFLTVTD NETEAPLSSA MIKRYNEHKE DLALLKEYIR NISLKTYNEV FKDDTKNGYA
 361 GYIDGKTNQE DFYVYLKKLL AEFEGADYFL EKIDREDFLR KQRTFDNGSI PYQIHLQEMR
 421 AILDKQAKFY PFLAKNKERI EKILTFRIPY YVGPLARGNS DFAWSIRKRN EKITPWNFED
 481 VIDKESSAEA FINRMTSFDL YLPEEKVLPK HSLLYETFNV YNELTKVRFI AESMRDYQFL
 541 DSKQKKDIVR LYFKDKRKVT DKDIIEYLHA IYGYDGIELK GIEKQFNSSL STYHDLLNII
 601 NDKEFLDDSS NEAIIEEIIH TLTIFEDREM IKQRLSKFEN IFDKSVLKKL SRRHYTGWGK
 661 LSAKLINGIR DEKSGNTILD YLIDDGISNR NFMQLIHDDA LSFKKKIQKA QIIGDEDKGN
 721 IKEVVKSLPG SPAIKKGILQ SIKIVDELVK VMGGRKPESI VVEMARENQY TNQGKSNSQQ
 781 RLKRLEKSLK ELGSKILKEN IPAKLSKIDN NALQNDRLYL YYLQNGKDMY TGDDLDIDRL
 841 SNYDIDHIIP QAFLKDNSID NKVLVSSASN RGKSDDVPSL EVVKKRKTFW YQLLKSKLIS
 901 QRKFDNLTKA ERGGLSPEDK AGFIQRQLVE TRQITKHVAR LLDEKFNNKK DENNRAVRTV
 961 KIITLKSTLV SQFRKDFELY KVREINDFHH AHDAYLNAVV ASALLKKYPK LEPEFVYGDY
1021 PKYNSFRERK SATEKVYFYS NIMNIFKKSI SLADGRVIER PLIEVNEETG ESVWNKESDL
1081 ATVRRVLSYP QVNVVKKVEE QNHGLDRGKP KGLFNANLSS KPKPNSNENL VGAKEYLDPK
1141 KYGGYAGISN SFTVLVKGTI EKGAKKKITN VLEFQGISIL DRINYRKDKL NFLLEKGYKD
1201 IELIIELPKY SLFELSDGSR RMLASILSTN NKRGEIHKGN QIFLSQKFVK LLYHAKRISN
1261 TINENHRKYV ENHKKEFEEL FYYILEFNEN YVGAKKNGKL LNSAFQSWQN HSIDELCSSF
1321 IGPTGSERKG LFELTSRGSA ADFEFLGVKI PRYRDYTPSS LLKDATLIHQ SVTGLYETRI
1381 DLAKLGEG
```

FIG. 8F

SEQ ID NO:116
UniProtKB/Swiss-Prot: Q927P4.1
RecName: Full=CRISPR-associated endonuclease Cas9
Listeria innocua Clip11262

```
   1 MKKPYTIGLD IGTNSVGWAV LTDQYDLVKR KMKIAGDSEK KQIKKNFWGV RLFDEGQTAA
  61 DRRMARTARR RIERRRNRIS YLQGIFAEEM SKTDANFFCR LSDSFYVDNE KRNSRHPFFA
 121 TIEEEVEYHK NYPTIYHLRE ELVNSSEKAD LRLVYLALAH IIKYRGNFLI EGALDTQNTS
 181 VDGIYKQFIQ TYNQVFASGI EDGSLKKLED NKDVAKILVE KVTRKEKLER ILKLYPGEKS
 241 AGMFAQFISL IVGSKGNFQK PFDLIEKSDI ECAKDSYEED LESLLALIGD EYAELFVAAK
 301 NAYSAVVLSS IITVAETETN AKLSASMIER FDTHEEDLGE LKAFIKLHLP KHYEEIFSNT
 361 EKHGYAGYID GKTKQADFYK YMKMTLENIE GADYFIAKIE KENFLRKQRT FDNGAIPHQL
 421 HLEELEAILH QQAKYYPFLK ENYDKIKSLV TFRIPYFVGP LANGQSEFAW LTRKADGEIR
 481 PWNIEEKVDF GKSAVDFIEK MTNKDTYLPK ENVLPKHSLC YQKYLVYNEL TKVRYINDQG
 541 KTSYFSGQEK EQIFNDLFKQ KRKVKKKDLE LFLRNMSHVE SPTIEGLEDS FNSSYSTYHD
 601 LLKVGIKQEI LDNPVNTEML ENIVKILTVF EDKRMIKEQL QQFSDVLDGV VLKKLERRHY
 661 TGWGRLSAKL LMGIRDKQSH LTILDYLMND DGLNRNLMQL INDSNLSFKS IIEKEQVTTA
 721 DKDIQSIVAD LAGSPAIKKG ILQSLKIVDE LVSVMGYPPQ TIVVEMAREN QTTGKGKNNS
 781 RPRYKSLEKA IKEFGSQILK EHPTDNQELR NNRLYLYYLQ NGKDMYTGQD LDIHNLSNYD
 841 IDHIVPQSFI TDNSIDNLVL TSSAGNREKG DDVPPLEIVR KRKVFWEKLY QGNLMSKRKF
 901 DYLTKAERGG LTEADKARFI HRQLVETRQI TKNVANILHQ RFNYEKDDHG NTMKQVRIVT
 961 LKSALVSQFR KQFQLYKVRD VNDYHHAHDA YLNGVVANTL LKVYPQLEPE FVYGDYHQFD
1021 WFKANKATAK KQFYTNIMLF FAQKDRIIDE NGEILWDKKY LDTVKKVMSY RQMNIVKKTE
1081 IQKGEFSKAT IKPKGNSSKL IPRKTNWDPM KYGGLDSPNM AYAVVIEYAK GKNKLVFEKK
1141 IIRVTIMERK AFEKDEKAFL EEQGYRQPKV LAKLPKYTLY ECEEGRRRML ASANEAQKGN
1201 QQVLPNHLVT LLHHAANCEV SDGKSLDYIE SNREMFAELL AHVSEFAKRY TLAEANLNKI
1261 NQLFEQNKEG DIKAIAQSFV DLMAFNAMGA PASFKFFETT IERKRYNNLK ELLNSTIIYQ
1321 SITGLYESRK RLDD
```

FIG. 8G

SEQ ID NO:117
UniProtKB/Swiss-Prot: Q7MRD3
CRISPR-associated endonuclease Cas9
Wolinella succinogenes

```
   1 MIERILGVDL GISSLGWAIV EYDKDDEAAN RIIDCGVRLF TAAETPKKKE SPNKARREAR
  61 GIRRVLNRRR VRMNMIKKLF LRAGLIQDVD LDGEGGMFYS KANRADVWEL RHDGLYRLLK
 121 GDELARVLIH IAKHRGYKFI GDDEADEESG KVKKAGVVLR QNFEAAGCRT VGEWLWRERG
 181 ANGKKRNKHG DYEISIHRDL LVEEVEAIFV AQQEMRSTIA TDALKAAYRE IAFFVRPMQR
 241 IEKMVGHCTY FPEERRAPKS APTAEKFIAI SKFFSTVIID NEGWEQKIIE RKTLEELLDF
 301 AVSREKVEFR HLRKFLDLSD NEIFKGLHYK GKPKTAKKRE ATLFDPNEPT ELEFDKVEAE
 361 KKAWISLRGA AKLREALGNE FYGRFVALGK HADEATKILT YYKDEGQKRR ELTKLPLEAE
 421 MVERLVKIGF SDFLKLSLKA IRDILPAMES GARYDEAVLM LGVPHKEKSA ILPPLNKTDI
 481 DILNPTVIRA FAQFRKVANA LVRKYGAFDR VHFELAREIN TKGEIEDIKE SQRKNEKERK
 541 EAADWIAETS FQVPLTRKNI LKKRLYIQQD GRCAYTGDVI ELERLFDEGY CEIDHILPRS
 601 RSADDSFANK VLCLARANQQ KTDRTPYEWF GHDAARWNAF ETRTSAPSNR VRTGKGKIDR
 661 LLKKNFDENS EMAFKDRNLN DTRYMARAIK TYCEQYWVFK NSHTKAPVQV RSGKLTSVLR
 721 YQWGLESKDR ESHTHHAVDA IIIAFSTQGM VQKLSEYYRF KETHREKERP KLAVPLANFR
 781 DAVEEATRIE NTETVKEGVE VKRLLISRPP RARVTGQAHE QTAKPYPRIK QVKNKKKWRL
 841 APIDEEKFES FKADRVASAN QKNFYETSTI PRVDVYHKKG KFHLVPIYLH EMVLNELPNL
 901 SLGTNPEAMD ENFFKFSIFK DDLISIQTQG TPKKPAKIIM GYFKNMHGAN MVLSSINNSP
 961 CEGFTCTPVS MDKKHKDKCK LCPEENRIAG RCLQGFLDYW SQEGLRPPRK EFECDQGVKF
1021 ALDVKKYQID PLGYYYEVKQ EKRLGTIPQM RSAKKLVKK
```

FIG. 8H

crRNA (first portion)

S. pyogenes	GUUUUAGAGCUAUG-CUGUUUUGAAU-GGUCCCAAAAC	**(SEQ ID NO:20)**
L. innocua	GUUUUAGAGCUAUG-UUAUUUUGAAU-GCUAACAAAAC	**(SEQ ID NO:21)**
S. thermophilus 1	GUUUUAGAGCUGUG-UUGUUUCGAAU-GGUUCCAAAAC	**(SEQ ID NO:22)**
S. thermophilus 2	GUUUUUGUACUCUC-AAGAUUUAAGU-AACUGUACAAC	**(SEQ ID NO:23)**
F. novicida	CUAACAGUAGUUUA-CCAAAUAAUUCAGCAACUGAAAC	**(SEQ ID NO:24)**
W. succinogenes	GCAACACUU-UAUAGCAAAUCCGCUUAGCCUGUGAAAC	**(SEQ ID NO:25)**
Consen. 1st seq. A	XXXXXXXXXXUXUXXXXXXXXXXXXXXXXXXXXXXXAAC	**(SEQ ID NO:26)**
Consen. 1st seq. B	XXXXXXXXXXUX	**(SEQ ID NO:27)**
Consen. 1st seq. C	XXXXXXXXXXUXUXXX	**(SEQ ID NO:28)**

S. pyogenes	GUUUUAGAGCUAUGCUGUUUUGAAUGGUCCCAAAAC	(SEQ ID NO:20)
L. innocua	GUUUUAGAGCUAUGUUAUUUUGAAUGCUAACAAAAC	(SEQ ID NO:21)
S. thermophilus 1	GUUUUAGAGCUGUGUUGUUUCGAAUGGUUCCAAAAC	(SEQ ID NO:22)
S. thermophilus 2	GUUUUUGUACUCUCAAGAUUUAAGUAACUGUACAAC	(SEQ ID NO:23)
Consens. 1st seq. D	GUUUUXGXXCUXUXXXXXUUXXAXUXXXXXXAXAAC	**(SEQ ID NO:29)**
Consens. 1st seq. E	GUUUUXGXXCUX	**(SEQ ID NO:30)**

F. novicida	CUAACAGUAGUUUACCAAAU-AAUUCAGCAACUGAAAC	(SEQ ID NO:24)
W. succinogenes	GCAACACUU-UAUAGCAAAUCCGCUUAGCCUGUGAAAC	(SEQ ID NO:25)
Consens. 1st seq. F	XXAACAXUXXUXUAXCAAAUXXXXUXAXCXXXUGAAAC	**(SEQ ID NO:31)**
Consens. 1st seq. G	XXAACAXUXXUXUAXC	**(SEQ ID NO:32)**

FIG. 9

tracrRNA (second portion)

S. pyogenes	UUGUUGGAAC-CAUUCAA--AAC--AGCAUAGCAAGUUAAA	(SEQ ID NO:40)
L. innocua	AUAUUGUUAG-UAUUCAA--AAU--AACAUAGCAAGUUAAA	(SEQ ID NO:41)
S. thermophilus 1	GGUUUGAAAC-CAUUCGA--AAC--AACACAGCGAGUUAAA	(SEQ ID NO:42)
S. thermophilus 2	CUU-ACACAGUUACUUA---AAUCUUGCAGAAGCUA-CAAA	(SEQ ID NO:43)
F. novicida 1	GUU-UCAGU--UGUUAGA-UUAUUUGGUAUGUACUUGUGUU	(SEQ ID NO:44)
F. novicida 2	AUU-ACAGAG-CAUU-AA-UUAUUUGGUACAUUUAUAAUUU	(SEQ ID NO:45)
W. succinogenes 1	UUU--CAAGG-CAUCGAACGGAUUUGCUAUAAAGUG-UUGC	(SEQ ID NO:46)
W. succinogenes 2	UUUGUUAAAG-C-UGGAUGGGAUU-AUUAUAGAGUG-UUGC	(SEQ ID NO:47)
Consen. 2nd seq. A	ZZZZZZZZZZZZZZZZZZZZAZZZZZZAZZZZZZZZZZZZ	(SEQ ID NO:48)
Consen. 2nd seq. B	ZAZZZZZZZZZZZ	(SEQ ID NO:49)
Consen. 2nd seq. C	ZZZZZZZZZZZZ	(SEQ ID NO:50)

FIG. 10A

S. pyogenes	UUGUUGGAACCAUUCAAAACAGCAUAGCAAGU-UAAA	(SEQ ID NO:40)
L. innocua	AUAUUGUUAGUAUUCAAAUAACAUAGCAAGU-UAAA	(SEQ ID NO:41)
S. thermophilus 1	GGUUUGAAACCAUUCGAAACAACACAGCGAGU-UAAA	(SEQ ID NO:42)
S. thermophilus 2	CUUACACAGUUACUUAAAUC-UUGCAGAAGCUACAAA	(SEQ ID NO:43)
Consen. 2nd seq. D	ZZZZZZZZZZZAZUZZAAZZZZZZZAGZZZZUZZAAA	(SEQ ID NO:51)
Consen. 2nd seq. E	ZAGZZZZUZZAAA	(SEQ ID NO:52)

FIG. 10B

F. novicida 1	GUUUCAGUUGUUAG-AU-UAUUUGGUAUGUACUUGUGUU	(SEQ ID NO:44)
F. novicida 2	AUUACAGAGCAUUA-AU-UAUUUGGUACAUUUAUAAUUU	(SEQ ID NO:45)
W. succinogenes 1	U-UUCAAGGCAUCGAACGGAUUUGCUAUAAAG-UGUUGC	(SEQ ID NO:46)
W. succinogenes 2	UUUGUUAAAGCUGG-AUGGGAUUAUUAUAGAG-UGUUGC	(SEQ ID NO:47)
Consen. 2nd seq. F	ZZUZZZZZZZUZZZAZZZZZUUZZUAZZZZZZUZZZZZ	(SEQ ID NO:53)
Consen. 2nd seq. G	ZZUAZZZZZZUZZZZZ	(SEQ ID NO:54)

FIG. 10C

Long tracrRNA (second portion)

S. pyogenes	-----G**U**----**UG**GA**A**------CCA**U**U**C**AAA**A**CAG**C**AU------A**GC**--	
L. innocua	AU---A**U**----**UG**UU**A**------GUA**U**U**C**AAA**A**UAA**C**AU------A**GC**--	
S. thermophilus 1	UU---G**U**GGUU**UG**AA**A**------CCA**U**U**C**GA**A**ACAA**C**AC------A**GC**--	
S. thermophilus 2	UAAUAA**U**AG--**UG**UA**A**GGGACGCCU**U**A**C**AC**A**GUUA**C**UUAAAUCUU**GC**AG	
Consen. tracRNA seq. A	ZZZZZZUZZZUGZZAZZZZZZZZUZCZZAZZZZCZZZZZZZZGCZZ	
Consen. tracRNA seq. B	ZZZZZZZGCZZ	

S. pyogenes	A**AG**U**UA**---**A**AA**U**AA**GGCU**-AG**U**-**CCG**UUA**UCAAC**---UU**G**AA**A**--AAG	
L. innocua	A**AG**U**UA**---**A**AA**U**AA**GGCU**UUG**U**-**CCG**UUA**UCAAC**---U**U**UUA**A**UUAAG	
S. thermophilus 1	G**AG**U**UA**---**A**AA**U**AA**GGCU**UAG**U**-**CCG**UAC**UCAAC**---UU**G**AA**A**--AGG	
S. thermophilus 2	A**AG**C**UA**CAA**A**G**AU**AA**GGCU**UCAU**G**C**CG**AAA**UCAAC**ACCC**U**GUC**A**UUUUA	
Consen. tracRNA seq. A	ZAGZUAZZZAZAUAAGGCUZZZUZCCGZZZUCAACZZZZUZZZAZZZZZ	
Consen. tracRNA seq. B	ZAGZUAZZZAZAUAAGGCUZZZUZCCG	

S. pyogenes	**U**GG**C**ACC**G**AG**U**CGGU**G**C**UUUUUU**	**(SEQ ID NO:60)**
L. innocua	**UAGC**GCU**G**UU**U**CGGC**G**C**UUUUUU**	**(SEQ ID NO:61)**
S. thermophilus 1	**UGGC**ACC**G**AU**U**CGGU**G**U**UUUUUU**	**(SEQ ID NO:62)**
S. thermophilus 2	**UGGC**AGG**G**UG**U**UUUC**G-UUAUUU**	**(SEQ ID NO:63)**
Consen. tracRNA seq. A	UZGCZZZGZZUZZZZGZUUZUUU	**(SEQ ID NO:64)**
Consen. tracRNA seq. B		**(SEQ ID NO:65)**

FIG. 11

Genus		**A -L- B**	
Sp var. 1		GUUUUAGAGCUA -L- UAGCAAGUUAAAAUAAGGCUAGUCCG	SEQ ID NO:80
Cons. var. 1	XXXXXXXXXXUXUXXXXXXXXXXXXXXXXXXXXXXXAAC	-L- ZZZZZZZZZZZZZZZZZZZZZAZZZZZZAZZZZZZZZZZZZ	SEQ ID NO:81
Cons. var. 2A		XXXXXXXXXXUX -L- ZAZZZZZZZZZZZ	SEQ ID NO:82
Cons. var. 2B		XXXXXXXXXXUXUXXX -L- ZAZZZZZZZZZZZ	SEQ ID NO:83
Cons. var. 3A		XXXXXXXXXXUX -L- ZZZZZZZZGCZZZAGZUAZZZAZAUAAGGCUZZZUXCCG	SEQ ID NO:84
Cons. var. 3A		XXXXXXXXXXUXUXXX -L- ZZZZZZZZGCZZZAGZUAZZZAZAUAAGGCUZZZUXCCG	SEQ ID NO:85
Sp var. 2	GUUUUXGXXCUXUXXXXXUUXXAXUXXXXXXAXAAC	-L- ZZZZZZZZZZZAZUZZAAZZZZZZZAGZZZZUZZAAA	SEQ ID NO:86
Sp var. 3		GUUUUXGXXCUX -L- ZZZZZZZZZZAZUZZAAZZZZZZZAGZZZZUZZAAA	SEQ ID NO:87
Sp var. 4		GUUUUXGXXCUX -L- ZAGZZZZUZZAAA	SEQ ID NO:88
Sp var. 5		GUUUUXGXXCUX -L- ZZZZZZZZGCZZZAGZUAZZZAZAUAAGGCUZZZUXCCG	SEQ ID NO:89
Fn var. 1	XXAACAXUXXUXUAXCAAAUXXXXUXAXCXXXUGAAAC	-L- ZZZZZZZZZZZAZUZZAAZZZZZZZAGZZZZUZZAAA	SEQ ID NO:90
Fn var. 2		XXAACAXUXXUXUAXC -L- ZZUZZZZZZZZUZZZAZZZZZUUZZUAZZZZZZUZZZZZ	SEQ ID NO:91
Fn var. 3		XXAACAXUXXUXUAXC -L- ZZUAZZZZZZUZZZZZ	SEQ ID NO:92
Fn var. 4		XXAACAXUXXUXUAXC -L- ZZZZZZZZGCZZZAGZUAZZZAZAUAAGGCUZZZUXCCG	SEQ ID NO:93

FIG. 12

SEQ ID NO:100

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 60
nnnnnnnnnn nnnnnnnnnn guuuuagagc uannnnnnnn nnnnnnnnnn nnnnnnnnnn 120
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 180
nnnnnnnnnn nnuagcaagu uaaaaauaagg cuaguccg 218

where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and n is a, c, t, g, u, or modified forms thereof; and where n at each of positions 93-192 can be present or absent such that this region can contain anywhere from 3 to 100 nucleotides and n is a, c, t, g, u, or modified forms thereof

SEQ ID NO:101

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 60
nnnnnnnnnn nnnnnnnnnn guuuuagagc uannnnnnnn nnnnnnnnnn nnnnnnnnnn 120
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 180
nnnnnnnnnn nnuagcaagu uaaaaauaagg cuuuguccg 219

where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and n is a, c, t, g, u, or modified forms thereof; and where n at each of positions 93-192 can be present or absent such that this region can contain anywhere from 3 to 100 nucleotides and n is a, c, t, g, u, or modified forms thereof

SEQ ID NO:102

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 60
nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaaauagc aaguuaaaau aaggcuaguc 120
cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu uuu 163

where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and n is a, c, t, g, u, or modified forms thereof

SEQ ID NO:103

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 60
nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaaauagc aaguuaaaau aaggcuaguc 120
cguuaucaac uugaaaaagu ggcaccgagu cggugcuuuu uuu 163
where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and n is a, c, t, g, u, or modified forms thereof

FIG. 13

FIG. 14A

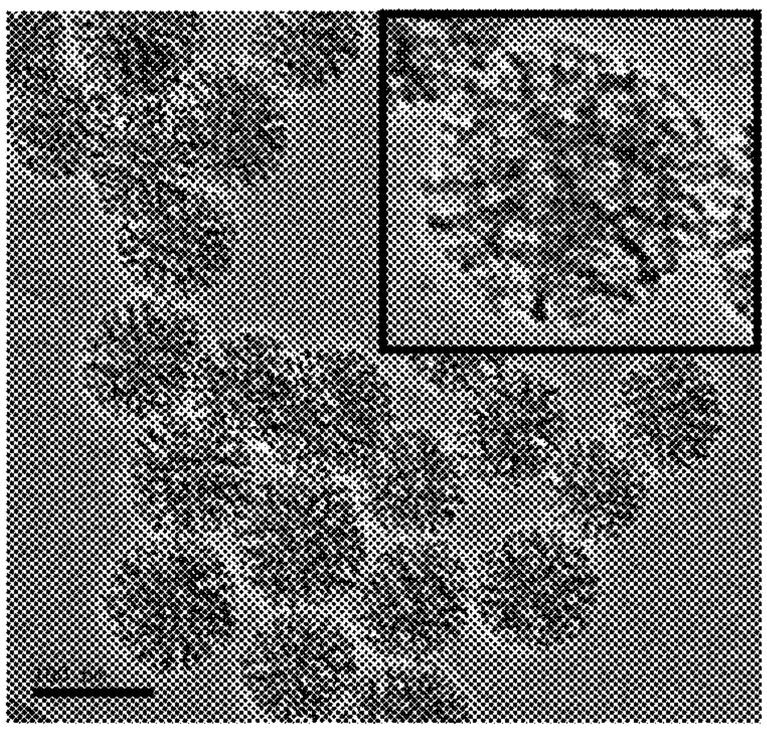


FIG. 14B

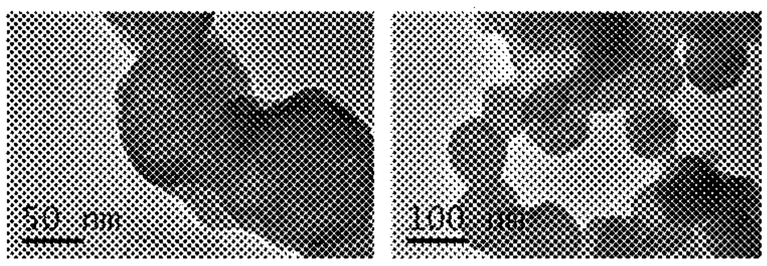
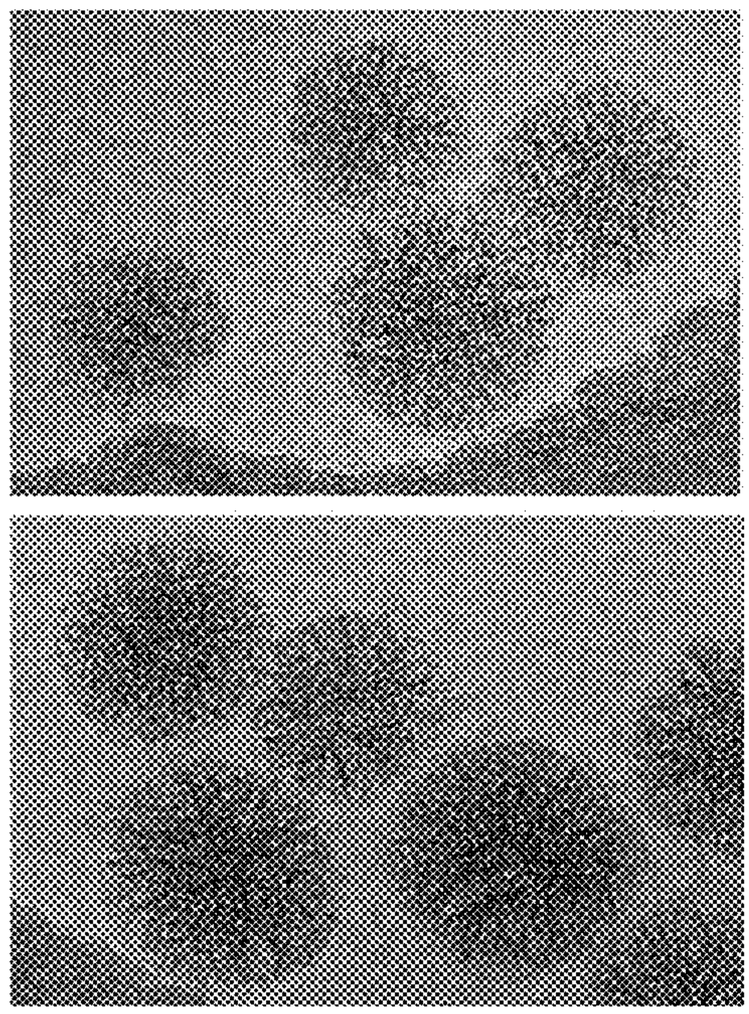


FIG. 14C



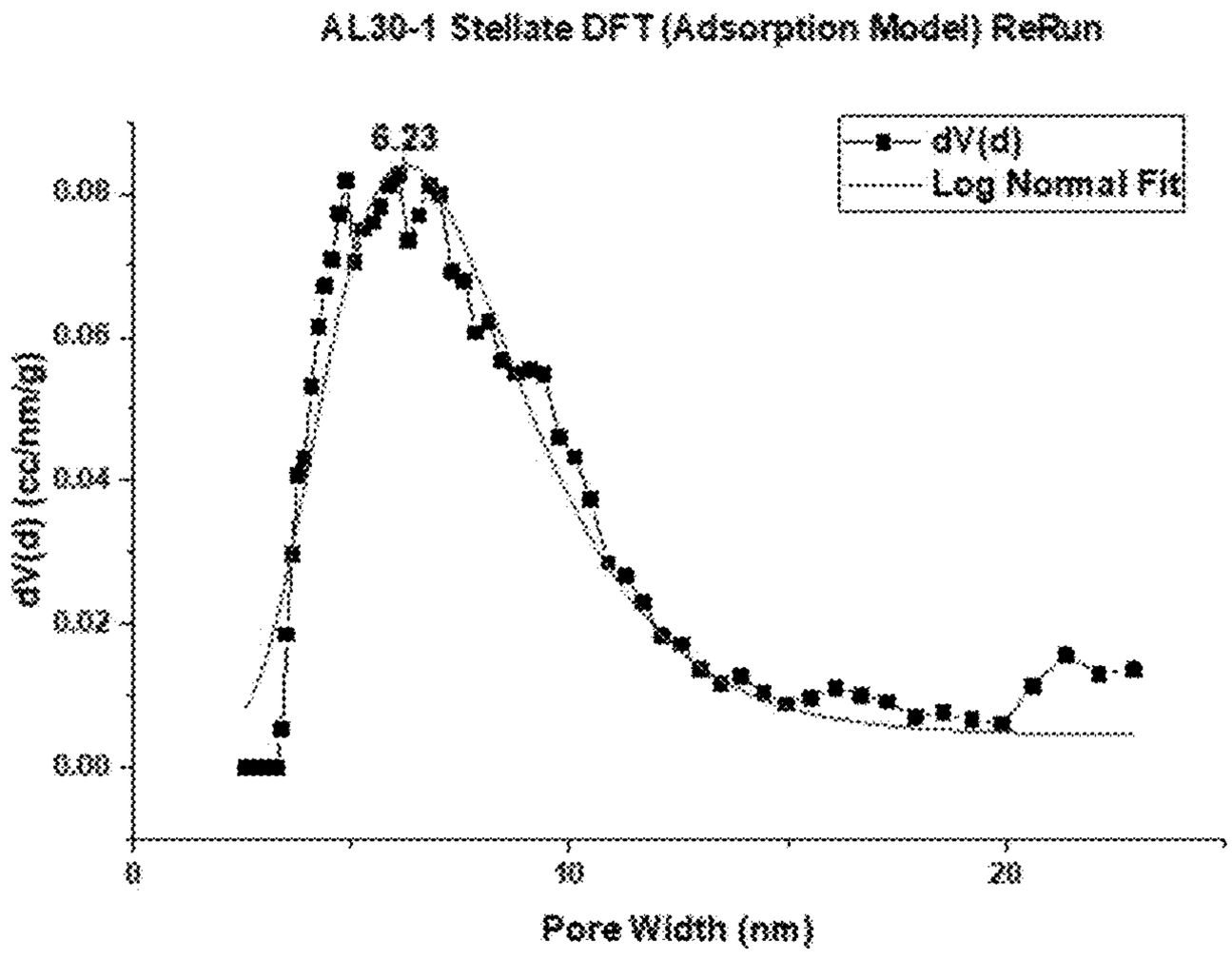
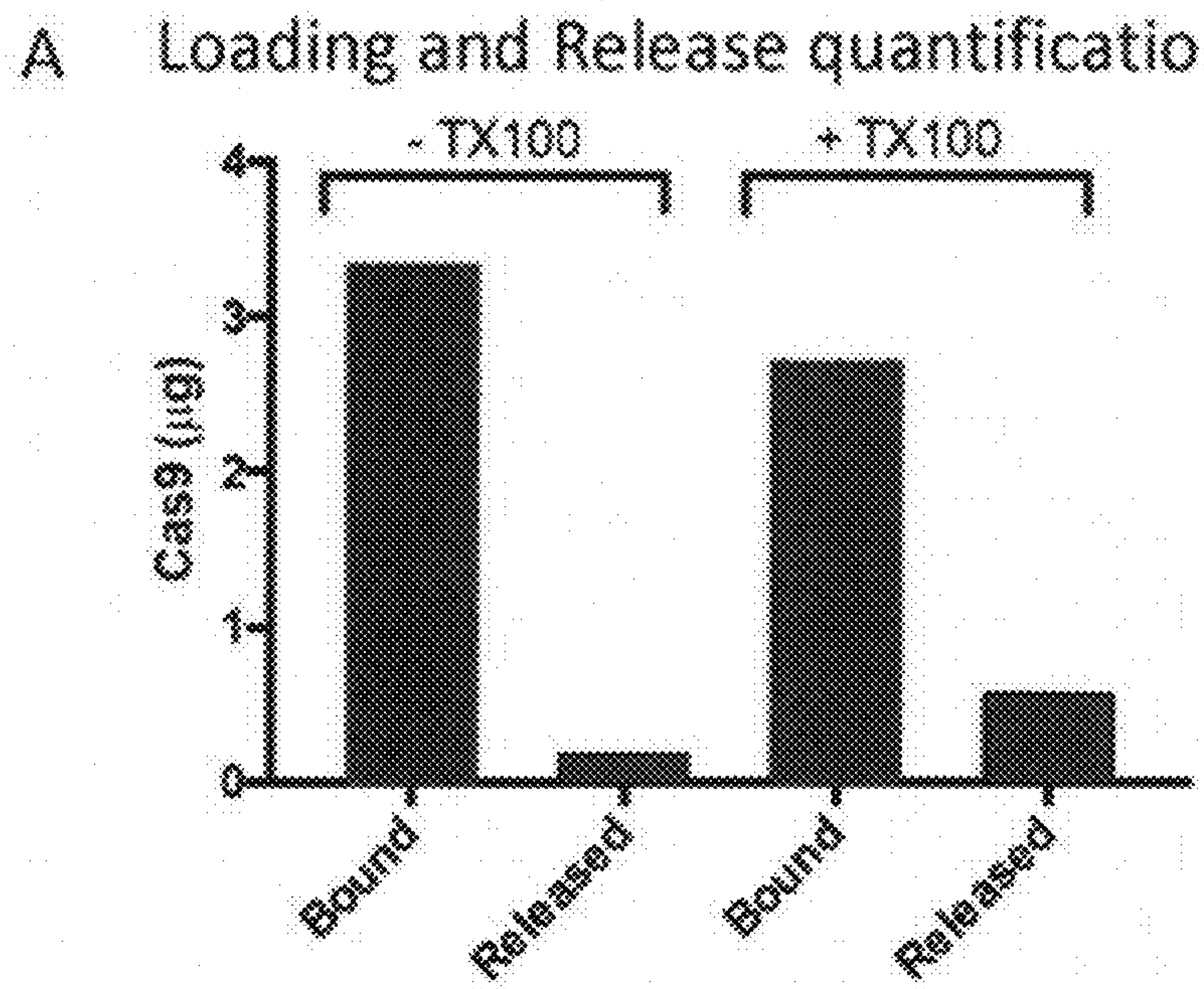

AL30-1 Stellate DFT (Adsorption Model) ReRun
6.23
dV(d)
Log Normal Fit
dV(d) (cc/nm/g)
0.08
0.06
0.04
0.02
0.00
0
10
20
Pore Width (nm)

FIG. 15


A
Loading and Release quantification
- TX100
+ TX100
Cas9 (µg)
0
1
2
3
Bound
Released
Bound
Released

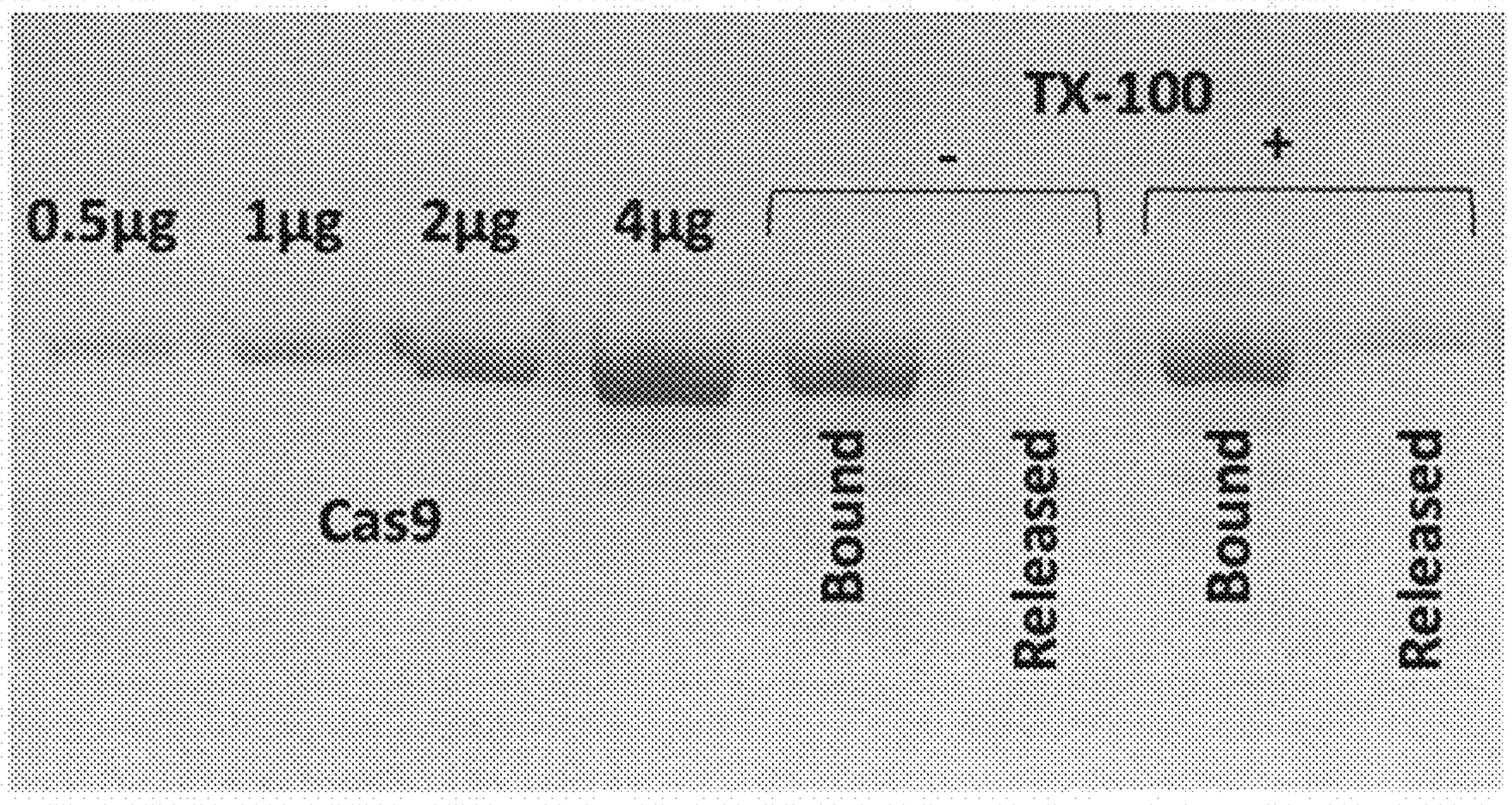

TX-100
-
+
0.5µg
1µg
2µg
4µg
Cas9
Bound
Released
Bound
Released

FIG. 16

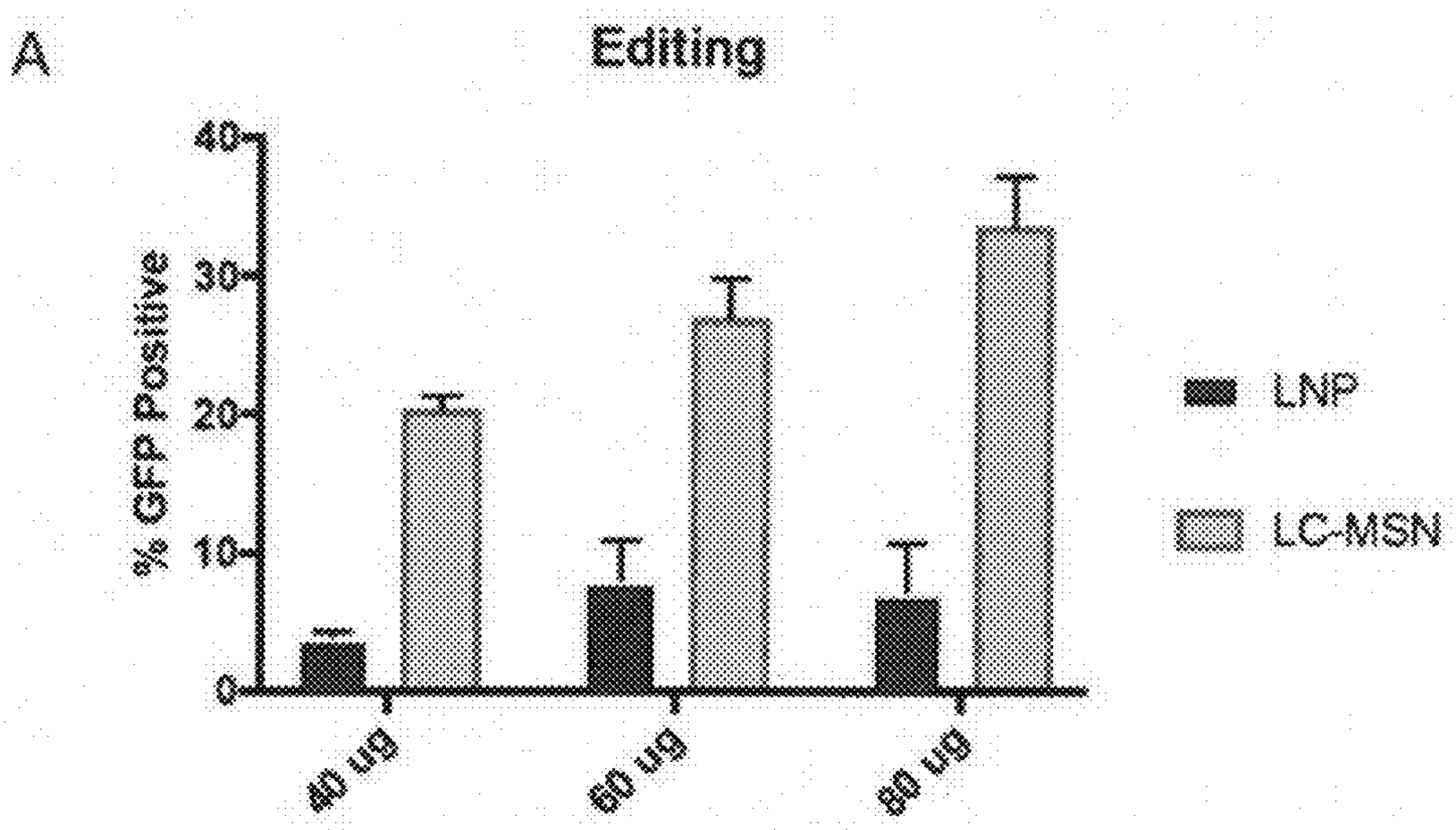


FIG. 17A

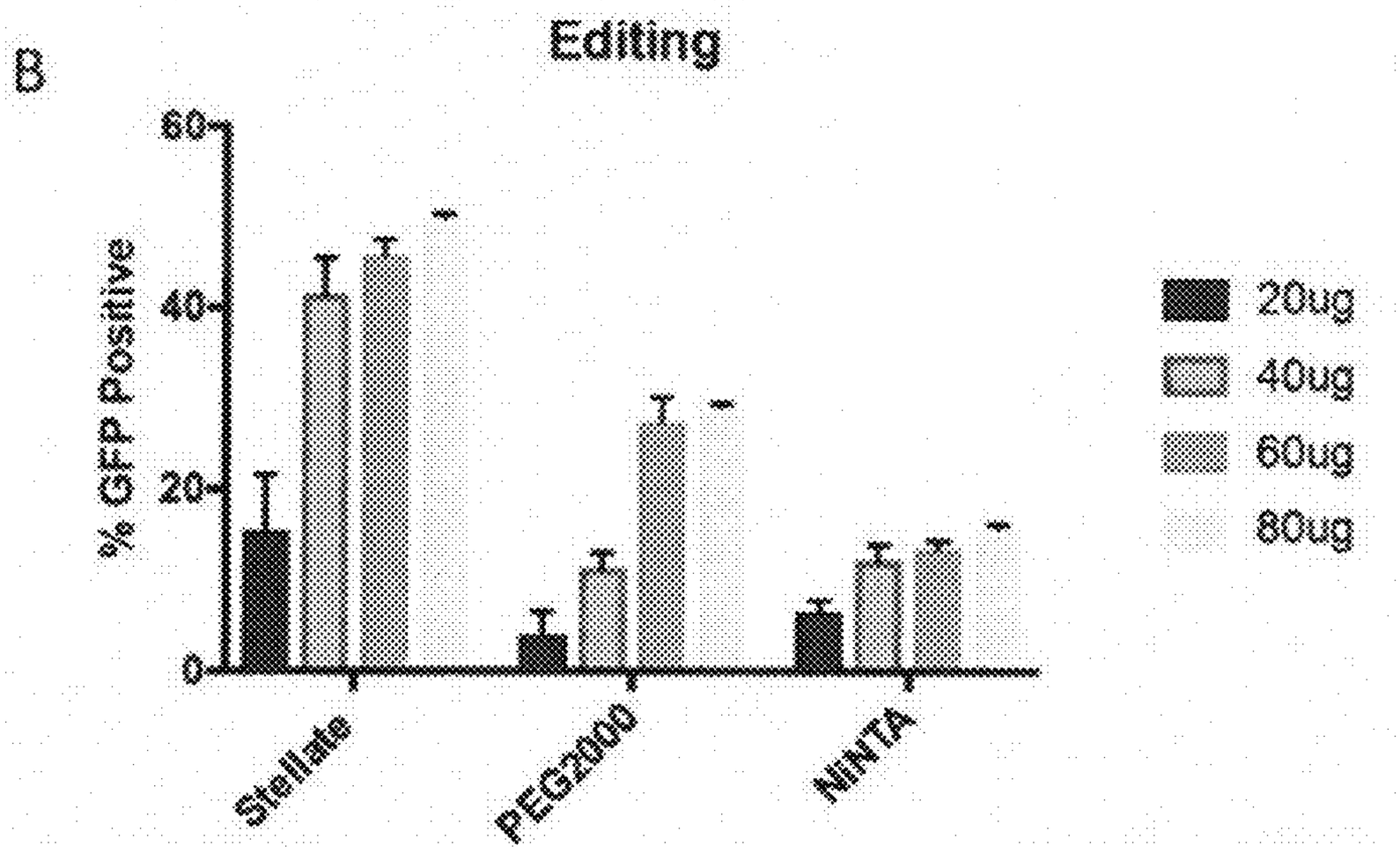


FIG. 17B

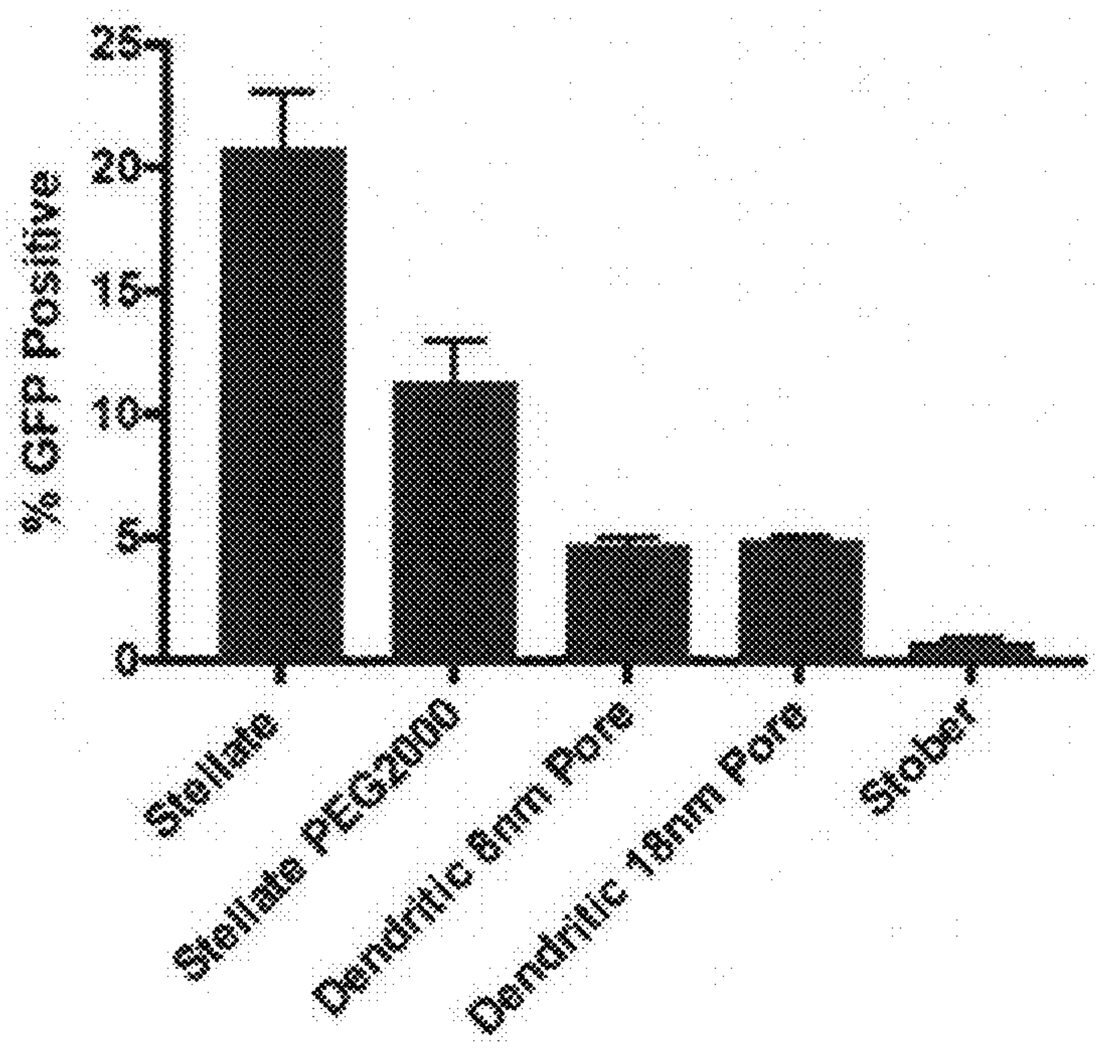

% GFP Positive
25
20
15
10
5
0
Stellate
Stellate PEG2000
Dendritic 8nm Pore
Dendritic 18nm Pore
Stober

FIG. 17C

FIG. 18A

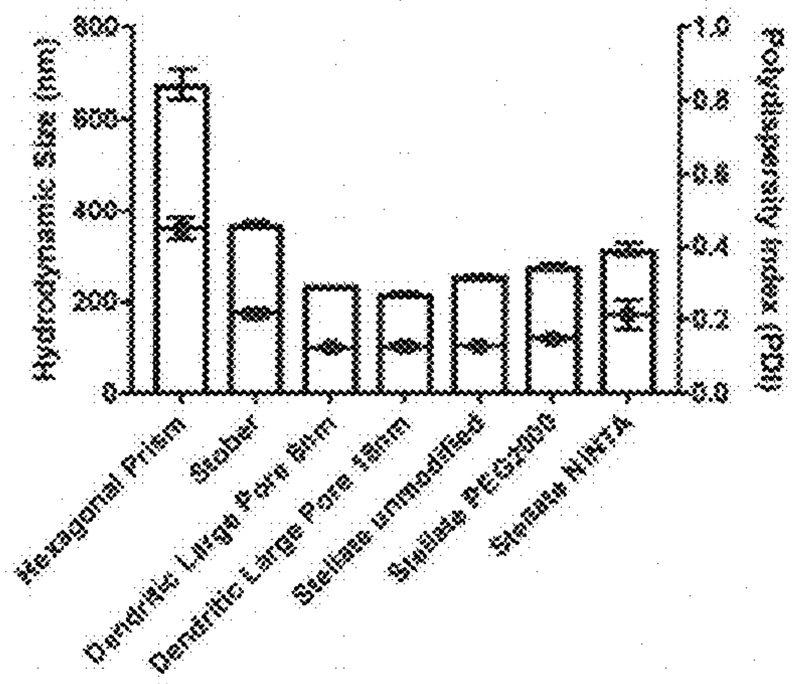


FIG. 18B

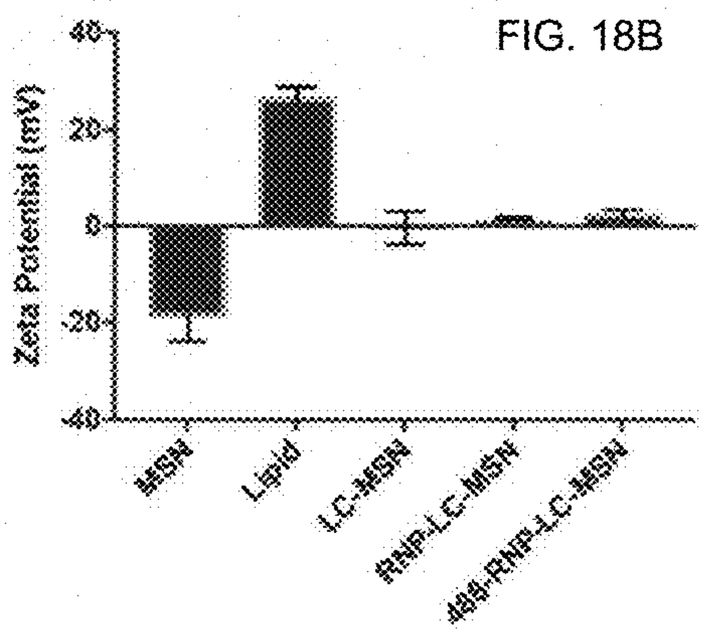


FIG. 18C

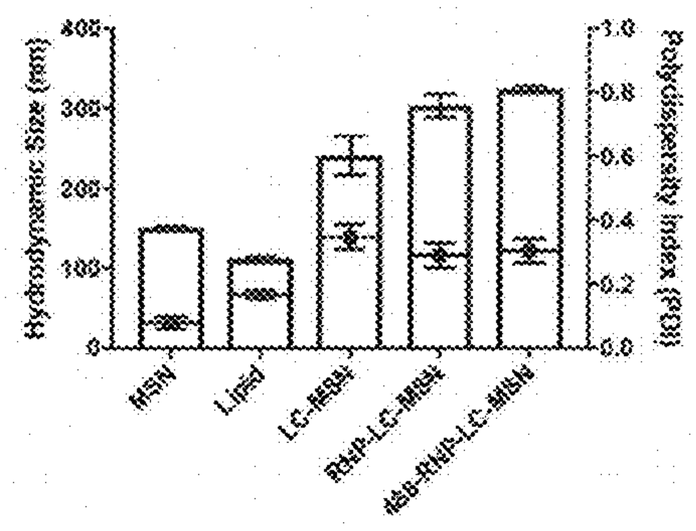
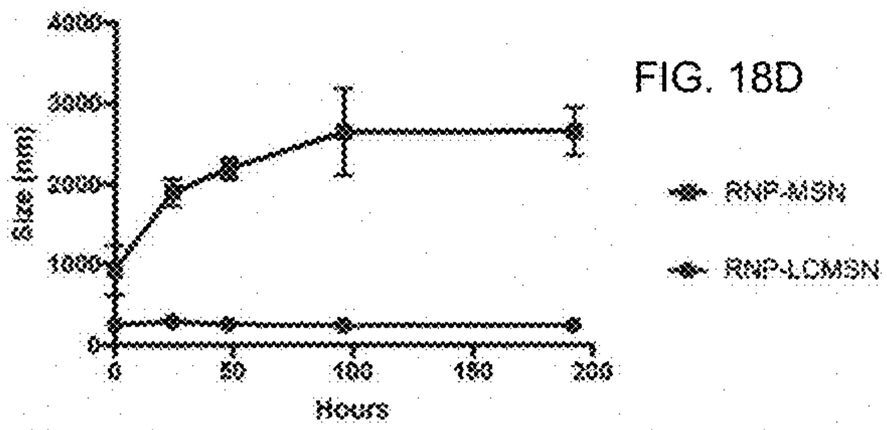


FIG. 18D



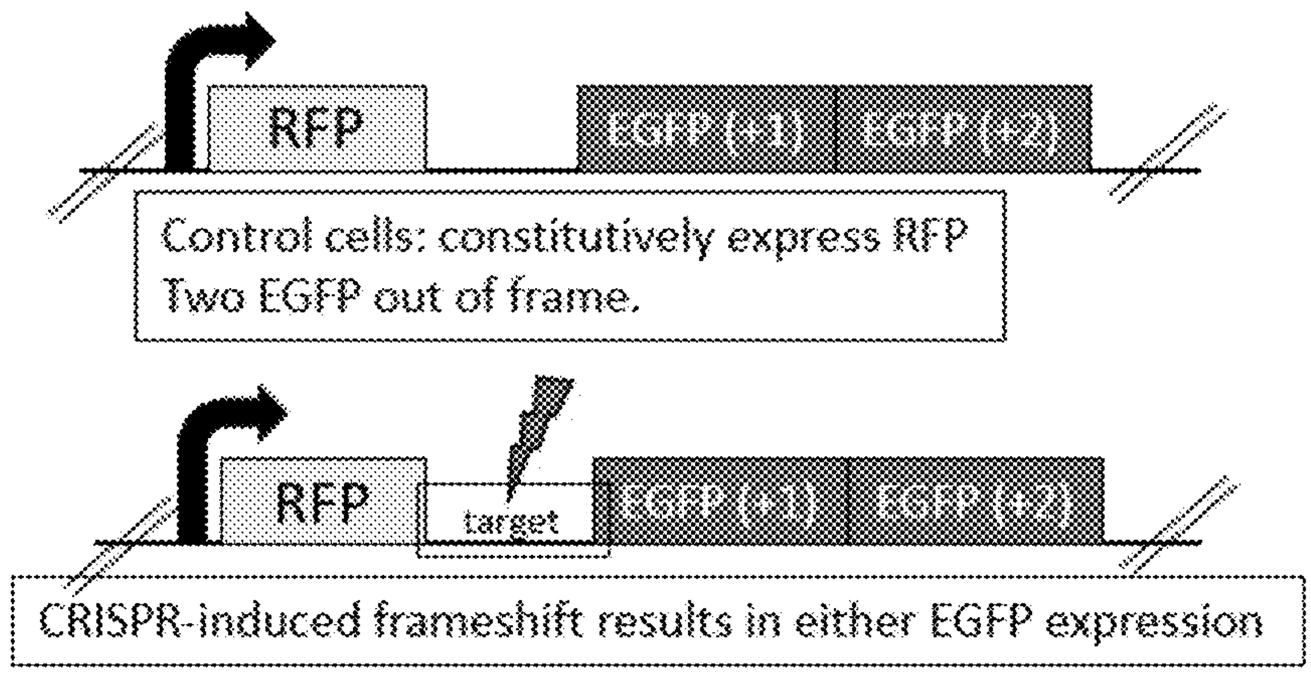

RFP
EGFP (+1)
EGFP (+2)
Control cells: constitutively express RFP
Two EGFP out of frame.
RFP
target
EGFP (+1)
EGFP (+2)
CRISPR-induced frameshift results in either EGFP expression

FIG. 19

LIPID COMPOSITION FOR THE DELIVERY OF THERAPEUTIC CARGOS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a divisional of U.S. application Ser. No. 16/839,194, filed on Apr. 3, 2020. That prior application is herein incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT INTEREST

[0002] This invention was made with Government support under Contract No. DE-NA0003525 awarded by the United States Department of Energy/National Nuclear Security Administration. The Government has certain rights in the invention.

REFERENCE TO A SEQUENCE LISTING APPENDIX

[0003] A sequence listing appendix including an XML formatted file accompanies this application. The appendix includes a file named "150131-1.xml," created on Feb. 3, 2023 (size of 231 kilobytes), which is hereby incorporated by reference in its entirety.

FIELD

[0004] This disclosure relates to a blend of coating materials for a nanoparticle with a gene-editing agent payload.

BACKGROUND

[0005] CRISPR (clustered, regularly interspaced, short 37 palindromic repeats)/Cas9 is a bacterial derived gene editing system that has been repurposed to edit specifically targeted sequences opening a new avenue with enormous unrealized implications in health, disease prevention, diagnosis, and treatment. Efficient intracellular delivery of CRISPR-Cas9 remains a hurdle in the advancement of this technology. Porous nanoparticles are an attractive delivery vehicle for a variety of cargos including small molecule drugs and proteins due to the low toxicity, high biocompatibility, facile synthesis and amenability to chemical functionalization.

[0006] However, efficacious delivery of therapeutic agents such as CRISPR-Cas9 still remains a challenge for certain classes of agents. For instance, due to delivery efficiency, certain coatings and gene-editing agents display effectiveness in cellular assays but show reduced efficacy in vivo. In addition, different coatings will interact differently with different types of particles and gene-editing agent payloads. Thus, there is a need for additional delivery constructs that can be configured to accommodate such agents.

SUMMARY

[0007] The present disclosure relates, in part, to a biocompatible, non-toxic construct including a core, a cargo, and a coating. The present disclosure demonstrates a tailored coating layer resulting in and improved and efficient delivery of CRISPR/Cas9 using a lipid coated mesoporous silica nanoparticle resulting in editing in over 30% of target cells in vitro.

[0008] In some embodiments, the core is a mesoporous nanoparticle (e.g., a mesoporous silica nanoparticle). In particular embodiments, the core has a dimension (e.g., a diameter, a width, or a length, or an effective average particle size) greater than about 50 nm (e.g., from about 50 nm to 300 nm, 50 nm to 100 nm, 50 nm to 150 nm, 50 nm to 200 nm, 50 nm to 250 nm, 75 nm to 100 nm, 75 nm to 150 nm, 75 nm to 200 nm, 75 nm to 250 nm, 75 nm to 300 nm, 100 nm to 150 nm, 100 nm to 200 nm, 100 nm to 250 nm, 100 nm to 300 nm, 125 nm to 150 nm, 125 nm to 200 nm, 125 nm to 250 nm, 125 nm to 300 nm, 150 nm to 200 nm, 150 nm to 250 nm, 150 nm to 300 nm, 175 nm to 200 nm, 175 nm to 250 nm, 175 nm to 300 nm, 200 nm to 250 nm, 200 nm to 300 nm, 225 nm to 250 nm, 225 nm to 300 nm, 250 nm to 300 nm, or 275 nm to 300 nm).

[0009] In particular embodiments, the core includes a plurality of pores, in which an average dimension of the pores is sufficiently large enough to accommodate a large cargo. In some embodiments, the average dimension is greater than about 2 nm, (e.g., of from about 5 nm to 35 nm, including from 5 nm to 10 nm, 5 nm to 15 nm, 5 nm to 20 nm, 5 nm to 25 nm, 5 nm to 30 nm, 8 nm to 10 nm, 8 nm to 15 nm, 8 nm to 20 nm, 8 nm to 25 nm, 8 nm to 30 nm, 8 nm to 35 nm, 10 nm to 15 nm, 10 nm to 20 nm, 10 nm to 25 nm, 10 nm to 30 nm, 10 nm to 35 nm, 12 nm to 15 nm, 12 nm to 20 nm, 12 nm to 25 nm, 12 nm to 30 nm, 12 nm to 35 nm, 15 nm to 20 nm, 15 nm to 25 nm, 15 nm to 30 nm, 15 nm to 35 nm, 18 nm to 20 nm, 18 nm to 25 nm, 18 nm to 30 nm, 18 nm to 35 nm, 20 nm to 25 nm, 20 nm to 30 nm, 20 nm to 35 nm, 25 nm to 30 nm, 25 nm to 35 nm, or 30 nm to 35 nm).

[0010] The cargo is, for example, an mRNA or a CRISPR component, such as any described herein. Exemplary CRISPR components include a Cas protein, a guide nucleic acid, a plasmid, as well as combinations thereof (e.g., a ribonucleoprotein complex). In some embodiments, an average dimension of the pores is greater than a dimension of the cargo. In other embodiments, an average dimension of the pores is smaller than a dimension of the cargo.

[0011] The construct further includes an outer layer or coating. An exemplary coating includes a cationic lipid, a zwitterionic lipid, a PEGylated lipid, and a sterol.

[0012] In a second aspect, a formulation including a plurality of constructs (e.g., any described herein) is disclosed along with a pharmaceutically acceptable excipient.

[0013] In a third aspect, a construct includes: a core comprising an external surface and a plurality of pores, wherein an average dimension of the plurality of pores is greater than about 2 nm. It also includes a cargo disposed in a pore of the plurality of pores, the cargo comprising one or more selected from the group consisting of: peptides, proteins, nucleic acids, mRNA, aptamers, antibodies, pharmaceuticals, carbohydrates, dyes, and markers. It also includes a coating coupled to the core, wherein the coating comprises a cationic lipid, a pegylated lipid, a zwitterionic lipid, and a sterol. The coating comprises a molar ratio of about 1 cationic lipid to 1 zwitterionic lipid to 0.9 sterol to 0.15 PEGylated lipid, wherein each molar ratio optionally varies by about plus or minus 10%. The the cationic lipid is 1,2-dioleoyl-3-trimethylammonium-propane, the zwitterionic lipid is 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, the sterol is cholesterol, and the PEGylated lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000].

[0014] In a fourth aspect, the present disclosure features a method of treating a subject, the method including: administering to a subject in need thereof, an effective amount of a construct, the construct including: a core comprising an

external surface and a plurality of pores, wherein an average dimension of the plurality of pores is greater than about 2 nm; a cargo disposed in a pore of the plurality of pores, the cargo comprising a CRISPR Cas9 component or a nucleic acid sequence encoding a CRISPR Cas9 component; and a coating coupled to the core. The coating comprises a cationic lipid, a pegylated lipid, a zwitterionic lipid, and a sterol.

[0015] In a fifth aspect, the disclosure features a method of forming the construct.

Definitions

[0016] As used herein, the term "about" means+/−10% of any recited value. As used herein, this term modifies any recited value, range of values, or endpoints of one or more ranges.

[0017] By "micro" is meant having at least one dimension that is less than 1 mm but equal to or larger than 1 μm. For instance, a microstructure (e.g., any structure described herein, such as a microparticle) can have a length, width, height, cross-sectional dimension, circumference, radius (e.g., external or internal radius), or diameter that is less than 1 mm but equal to or larger than 1 μm. In another instance, the microstructure has a dimension that is of from about 1 μm to 1 mm.

[0018] By "nano" is meant having at least one dimension that is less than 1 m but equal to or larger than 1 nm. For instance, a nanostructure (e.g., any structure described herein, such as a nanoparticle) can have a length, width, height, cross-sectional dimension, circumference, radius (e.g., external or internal radius), or diameter that is less than 1 m but equal to or larger than 1 nm. In another instance, the nanostructure has a dimension that is of from about 1 nm to about 1 μm.

[0019] The term "cargo" is used herein to describe a molecule or compound, whether a small molecule or macromolecule having an activity relevant to its use in particles (e.g., a construct, a nanoparticle, or a mesoporous silica nanoparticle), especially including biological activity, that can be included in or with particles according to the present disclosure. In principal embodiments of the present disclosure, the cargo is a nucleic acid sequence, such as double stranded (ds) plasmid DNA. The cargo may be included within the pores, associated with the pore (e.g., by way of a spacer), and/or on the surface of the core (e.g., by way of a spacer) according to the present disclosure. Additional representative cargo may include, for example, a small molecule bioactive agent, a nucleic acid (e.g., RNA or DNA), a polypeptide, including a protein or a carbohydrate. Particular examples of such cargo include RNA, such as mRNA, siRNA, shRNA micro RNA, a polypeptide or protein, including a protein toxin (e.g., ricin toxin A-chain or diphtheria toxin A-chain), and/or DNA (including double stranded or linear DNA, complementary DNA (cDNA), minicircle DNA, naked DNA and plasmid DNA, which optionally may be supercoiled and/or packaged (e.g., with histones) and which may be optionally modified with a nuclear localization sequence). Cargo may also include a reporter as described herein.

[0020] The phrase "effective average particle size" as used herein to describe a multiparticulate (e.g., a porous nanoparticulate) means that at least 50% of the particles therein are of a specified size. Accordingly, "effective average particle size of less than about 2,000 nm in diameter" means that at least 50% of the particles therein are less than about 2,000 nm in diameter. In certain embodiments, nanoparticulates have an effective average particle size of less than about 2,000 nm (i.e., 2 microns), less than about 1,900 nm, less than about 1,800 nm, less than about 1,700 nm, less than about 1,600 nm, less than about 1,500 nm, less than about 1,400 nm, less than about 1,300 nm, less than about 1,200 nm, less than about 1,100 nm, less than about 1,000 nm, less than about 900 nm, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 250 nm, less than about 200 nm, less than about 150 nm, less than about 100 nm, less than about 75 nm, or less than about 50 nm, as measured by light-scattering methods. In certain aspects of the present disclosure, the particles are monodisperse and generally no greater than about 50 nm in average diameter, often less than about 30 nm in average diameter, as otherwise described herein. The term "D_{50}" refers to the particle size below which 50% of the particles in a multiparticulate fall. Similarly, the term "D_{90}" refers to the particle size below which 90% of the particles in a multiparticulate fall.

[0021] The term "monodisperse" is used as a standard definition established by the National Institute of Standards and Technology (NIST) (Particle Size Characterization, Special Publication 960-1, January 2001) to describe a distribution of particle size (diameter) within a population of particles, in this case nanoparticles, which particle distribution may be considered monodisperse if at least 90% of the distribution lies within 5% of the median size, measured by dynamic light scattering (DLS) that calculates particles hydrodynamic diameter and polydispersity index (PDI) using a Malvern Zetasizer. See, e.g., LaBauve, et al, *Lipid-Coated Mesoporous Silica Nanoparticles for the Delivery of the ML336Antiviral to Inhibit Encephalitic Alphavirus Infection*, Sci Rep. 2018; 8: 13990, 2018 Sep. 18. doi: 10.1038/s41598-018-32033-w, incorporated herein by reference for more information on measurement technique.

[0022] The term "lipid" is used to describe the components which are used to form lipid mono-, bi-, or multilayers on the surface of the particles (e.g., a core of the particle), that are used in the present disclosure (e.g., as constructs) and may include a PEGylated lipid. Various embodiments provide nanostructures, that are constructed from nanoparticles, which support one or more lipid layers (e.g., bilayer (s) or multilayer(s)).

[0023] The terms "targeting ligand" and "targeting active species" are used to describe a compound or moiety (e.g., an antigen), which is complexed or covalently bonded to the surface of a particle (e.g., either directly on an outer surface of a delivery platform or on an outer layer). The targeting ligand, in turn, binds to a moiety on the surface of a cell to be targeted so that the constructs may bind to the surface of the targeted cell, enter the cell or an organelle thereof, and/or deposit their contents into the cell. The targeting active species for use in the present disclosure may be a targeting peptide (e.g., a receptor ligand, a cell penetration peptide, a fusogenic peptide, or an endosomolytic peptide, as otherwise described herein), a polypeptide including an antibody or antibody fragment, an aptamer, or a carbohydrate, among other species that bind (e.g., selectively bind) to a targeted cell.

[0024] The term "reporter" is used to describe an imaging agent or moiety that is incorporated into the outer layer or

cargo of particles according to an embodiment of the present disclosure and provides a signal that can be measured. The moiety may provide a fluorescent signal or may be a radioisotope which allows radiation detection, among others. Exemplary fluorescent labels for use in particles (e.g., via conjugation or adsorption to the outer layer or the core, via integration into the matrix of the core, and/or via incorporation into cargo elements such as DNA, RNA-sn-glycero-3-phosphoethanolamine (Texas Red DHPE, 583/608), Alexa Fluor® 647 hydrazide (649/666), Alexa Fluor® 647 carboxylic acid, succinimidyl ester (650/668), Ulysis™ Alexa Fluor® 647 Nucleic Acid Labeling Kit (650/670), Alexa Fluor® 647 conjugate of annexin V (650/665), other fluorescent labels, colorimetric labels, quantum dots, nanoparticles, microparticles, barcodes, radio labels (e.g., RF labels or barcodes), avidin, biotin, tags, dyes, an enzyme that can optionally include one or more linking agents and/or one or more dyes, as well as combinations thereof etc. Additional reporters can include a detection agent (e.g., a dye, such as an electroactive detection agent, a fluorescent dye, a luminescent dye, a chemiluminescent dye, a colorimetric dye, a radioactive agent, a contrast agent, etc.), a particle (e.g., such as a microparticle, a nanoparticle, a latex bead, a colloidal particle, a magnetic particle, a fluorescent particle, etc.), and/or a label (e.g., an electroactive label, an electrocatalytic label, a fluorescent label, a colorimetric label, a quantum dot, a nanoparticle, a microparticle, a barcode, a radio label (e.g., an RF label or barcode), avidin, biotin, a tag, a dye, a marker, an enzyme that can optionally include one or more linking agents and/or one or more dyes). Moieties that enhance the fluorescent signal or slow the fluorescent fading may also be incorporated and include SlowFade® Gold antifade reagent (with and without DAPI) and Image-iT® FX signal enhancer. All of these are well known in the art.

[0025] The terms "polynucleotide" and "nucleic acid," used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-stranded (e.g., sense or antisense), double-stranded, or multi-stranded ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs), or hybrids thereof, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. Polynucleotides can have any useful two-dimensional or three-dimensional structure or motif, such as regions including one or more duplex, triplex, quadruplex, hairpin, and/or pseudoknot structures or motifs. In any nucleic acid described herein, U may be replaced with T and vice versa.

[0026] The term "modified," as used in reference to nucleic acids, means a nucleic acid sequence including one or more modifications to the nucleobase, nucleoside, nucleotide, phosphate group, sugar group, and/or internucleoside linkage (e.g., phosphodiester backbone, linking phosphate, or a phosphodiester linkage).

[0027] The nucleoside modification may include, but is not limited to, pyridin-4-one ribonucleoside, 5-aza-uridine, 2-thio-5-aza-uridine, 2-thiouridine, 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxyuridine, 3-methyluridine, 5-carboxymethyl-uridine, 1-carboxymethyl-pseudouridine, 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-taurinomethyluridine, 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uridine, 1-taurinomethyl-4-thio-uridine, 5-methyl-uridine, 1-methyl-pseudouridine, 4-thio-1-methyl-pseudouridine, 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine, dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxyuridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, 5-aza-cytidine, pseudoisocytidine, 3-methyl-cytidine, N4-acetylcytidine, 5-formylcytidine, N4-methylcytidine, 5-hydroxymethylcytidine, 1-methyl-pseudoisocytidine, pyrrolo-cytidine, pyrrolo-pseudoisocytidine, 2-thio-cytidine, 2-thio-5-methyl-cytidine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 1-methyl-1-deaza-pseudoisocytidine, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, 2-aminopurine, 2,6-diaminopurine, 7-deaza-adenine, 7-deaza-8-aza-adenine, 7-deaza-2-aminopurine, 7-deaza-8-aza-2-aminopurine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 1-methyladenosine, N6-methyladenosine, N6-isopentenyladenosine, N6-(cis-hydroxyisopentenyl)adenosine, 2-methylthio-N6-(cis-hydroxyisopentenyl) adenosine, N6-glycinylcarbamoyladenosine, N6-threonylcarbamoyladenosine, 2-methylthio-N6-threonyl carbamoyladenosine, N6,N6-dimethyladenosine, 7-methyl-adenine, 2-methylthio-adenine, and 2-methoxy-adenine, inosine, 1-methyl-inosine, wyosine, wybutosine, 7-deaza-guanosine, 7-deaza-8-aza-guanosine, 6-thio-guanosine, 6-thio-7-deaza-guanosine, 6-thio-7-deaza-8-aza-guanosine, 7-methyl-guanosine, 6-thio-7-methyl-guanosine, 7-methyl-inosine, 6-methoxy-guanosine, 1-methylguanosine, N2-methylguanosine, N2,N2-dimethylguanosine, 8-oxo-guanosine, 7-methyl-8-oxo-guanosine, 1-methyl-6-thio-guanosine, N2-methyl-6-thio-guanosine, and N2,N2-dimethyl-6-thio-guanosine, and combinations thereof.

[0028] A sugar modification may include, but is not limited to, a locked nucleic acid (LNA, in which the 2'-hydroxyl is connected by a C_{1-6} alkylene or C_{1-6} heteroalkylene bridge to the 4'-carbon of the same ribose sugar), replacement of the oxygen in ribose (e.g., with S, Se, or alkylene, such as methylene or ethylene), addition of a double bond (e.g., to replace ribose with cyclopentenyl or cyclohexenyl), ring contraction of ribose (e.g., to form a 4-membered ring of cyclobutane or oxetane), ring expansion of ribose (e.g., to form a 6- or 7-membered ring having an additional carbon or heteroatom, such as for anhydrohexitol, altritol, mannitol, cyclohexanyl, cyclohexenyl, and morpholino that also has a phosphoramidate backbone), multicyclic forms (e.g., tricyclic), and "unlocked" forms, such as glycol nucleic acid (GNA) (e.g., R-GNA or S-GNA, where ribose is replaced by glycol units attached to phosphodiester bonds), threose nucleic acid (TNA, where ribose is replace with a-L-threofuranosyl-(3'→2')), and peptide nucleic acid (PNA, where 2-amino-ethyl-glycine linkages replace the ribose and phosphodiester backbone). The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a polynucleotide molecule can include nucleotides containing, e.g., arabinose, as the sugar.

[0029] A backbone modification may include, but is not limited to, 2'-deoxy- or 2'-O-methyl modifications. A phosphate group modification may include, but is not limited to, phosphorothioate, phosphoroselenates, boranophosphates, boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, phosphotriesters, phosphorodithioates, bridged phosphoramidates, bridged phosphorothioates, or bridged methylene-phosphonates.

[0030] "Complementarity" or "complementary" refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types, e.g., form Watson-Crick base pairs and/or G/U base pairs, "anneal", or "hybridize," to another nucleic acid in a sequence-specific, antiparallel, manner (i.e., a nucleic acid specifically binds to a complementary nucleic acid) under the appropriate in vitro and/or in vivo conditions of temperature and solution ionic strength. As is known in the art, standard Watson-Crick base-pairing includes: adenine (A) pairing with thymidine (T), adenine (A) pairing with uracil (U), and guanine (G) pairing with cytosine (C). In addition, it is also known in the art that for hybridization between two RNA molecules (e.g., dsRNA), guanine (G) base pairs with uracil (U). A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. "Substantially complementary" or "sufficient complementarity" as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%. 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions.

[0031] As used herein, "stringent conditions" for hybridization refer to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent, and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions are described in detail in Tijssen (1993), Laboratory Techniques In Biochemistry And Molecular Biology-Hybridization With Nucleic Acid Probes Part 1, Second Chapter "Overview of principles of hybridization and the strategy of nucleic acid probe assay", Elsevier, N.Y.

[0032] "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogstein binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PCR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the "complement" of the given sequence.

[0033] Hybridization and washing conditions are well known and exemplified in Sambrook J, Fritsch E F, and Maniatis T, "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), particularly Chapter 11 and Table 11.1 therein; and Sambrook J and Russell W, "Molecular Cloning: A Laboratory Manual," Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2001). The conditions of temperature and ionic strength determine the "stringency" of the hybridization.

[0034] Hybridization requires that the two nucleic acids contain complementary sequences, although mismatches between bases are possible. The conditions appropriate for hybridization between two nucleic acids depend on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of complementation between two nucleotide sequences, the greater the value of the melting temperature (Tm) for hybrids of nucleic acids having those sequences. For hybridizations between nucleic acids with short stretches of complementarity (e.g., complementarity over 35 or less, 30 or less, 25 or less, 22 or less, 20 or less, or 18 or less nucleotides) the position of mismatches becomes important (see Sambrook et al., supra, 11.7-11.8). Typically, the length for a hybridizable nucleic acid is at least about 10 nucleotides. Illustrative minimum lengths for a hybridizable nucleic acid are: at least about 15 nucleotides; at least about 20 nucleotides; at least about 22 nucleotides; at least about 25 nucleotides; and at least about 30 nucleotides). Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary, according to factors such as length of the region of complementation and the degree of complementation.

[0035] It is understood in the art that the sequence of polynucleotide need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable or hybridizable. Moreover, a polynucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). A polynucleotide can comprise at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, or 100% sequence complementarity to a target region within the target nucleic acid sequence to which they are targeted. For example, an antisense nucleic acid in which 18 of 20 nucleotides of the antisense compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining non-complementary nucleotides may be clustered or interspersed with complementary nucleotides and need not be contiguous to each other or to complementary nucleotides. Percent complementarity between particular stretches of nucleic acid sequences within nucleic acids can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul S F et al., *J. Mol. Biol.* 1990; 215:403-10; Zhang J et al., Genome Res. 1997; 7:649-56) or by using the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research

Park, Madison Wis.), using default settings, which uses the algorithm of Smith T F et al., *Adv. Appl. Math.* 1981; 2(4):482-9).

[0036] By "protein," "peptide," or "polypeptide," as used interchangeably, is meant any chain of more than two amino acids, regardless of post-translational modification (e.g., glycosylation or phosphorylation), constituting all or part of a naturally occurring polypeptide or peptide, or constituting a non-naturally occurring polypeptide or peptide, which can include coded amino acids, non-coded amino acids, modified amino acids (e.g., chemically and/or biologically modified amino acids), and/or modified backbones.

[0037] The term "fragment" is meant a portion of a nucleic acid or a polypeptide that is at least one nucleotide or one amino acid shorter than the reference sequence. This portion contains, at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 1800 or more nucleotides; or 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 640 amino acids or more. In another example, any polypeptide fragment can include a stretch of at least about 5 (e.g., about 10, about 20, about 30, about 40, about 50, or about 100) amino acids that are at least about 40% (e.g., about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 87%, about 98%, about 99%, or about 100%) identical to any of the sequences described herein can be utilized in accordance with this disclosure. In certain embodiments, a polypeptide to be utilized in accordance with the disclosed technology includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations (e.g., one or more conservative amino acid substitutions, as described herein). In yet another example, any nucleic acid fragment can include a stretch of at least about 5 (e.g., about 7, about 8, about 10, about 12, about 14, about 18, about 20, about 24, about 28, about 30, or more) nucleotides that are at least about 40% (about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 87%, about 98%, about 99%, or about 100%) identical to any of the sequences described herein can be utilized in accordance with the disclosed technology.

[0038] The term "conservative amino acid substitution" refers to the interchangeability in proteins of amino acid residues having similar side chains (e.g., of similar size, charge, and/or polarity). For example, a group of amino acids having aliphatic side chains consists of glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains consists of serine and threonine; a group of amino acids having amide containing side chains consisting of asparagine and glutamine; a group of amino acids having aromatic side chains consists of phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains consists of lysine, arginine, and histidine; a group of amino acids having acidic side chains consists of glutamic acid and aspartic acid; and a group of amino acids having sulfur containing side chains consists of cysteine and methionine. Exemplary conservative amino acid substitution groups are valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glycine-serine, glutamate-aspartate, and asparagine-glutamine.

[0039] As used herein, when a polypeptide or nucleic acid sequence is referred to as having "at least X % sequence identity" to a reference sequence, it is meant that at least X percent of the amino acids or nucleotides in the polypeptide or nucleic acid are identical to those of the reference sequence when the sequences are optimally aligned. An optimal alignment of sequences can be determined in various ways that are within the skill in the art, for instance, the Smith Waterman alignment algorithm (Smith T F et al., *J. Mol. Biol.* 1981; 147:195-7) and BLAST (Basic Local Alignment Search Tool; Altschul S F et al., *J. Mol. Biol.* 1990; 215:403-10). These and other alignment algorithms are accessible using publicly available computer software such as "Best Fit" (Smith T F et al., *Adv. Appl. Math.* 1981; 2(4):482-9) as incorporated into GeneMatcher Plus™ (Schwarz and Dayhof, "Atlas of Protein Sequence and Structure," ed. Dayhoff, M. O., pp. 353-358, 1979), BLAST, BLAST-2, BLAST-P, BLAST-N, BLAST-X, WU-BLAST-2, ALIGN, ALIGN-2, CLUSTAL, T-COFFEE, MUSCLE, MAFFT, or Megalign (DNASTAR). In addition, those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve optimal alignment over the length of the sequences being compared. In general, for polypeptides, the length of comparison sequences can be at least five amino acids, such as, for example, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, or more amino acids, up to the entire length of the polypeptide. For nucleic acids, the length of comparison sequences can generally be at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, or more nucleotides, up to the entire length of the nucleic acid molecule. It is understood that for the purposes of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymine nucleotide is equivalent to an uracil nucleotide.

[0040] By "substantial identity" or "substantially identical" is meant a polypeptide or nucleic acid sequence that has the same polypeptide or nucleic acid sequence, respectively, as a reference sequence, or has a specified percentage of amino acid residues or nucleotides, respectively, that are the same at the corresponding location within a reference sequence when the two sequences are optimally aligned. For example, an amino acid sequence that is "substantially identical" to a reference sequence has at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the reference amino acid sequence. For polypeptides, the length of comparison sequences will generally be at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 50, 75, 90, 100, 150, 200, 250, 300, or 350 contiguous amino acids (e.g., a full-length sequence). For nucleic acids, the length of comparison sequences will generally be at least 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 contiguous nucleotides (e.g., the full-length nucleotide sequence). Sequence identity may be measured using sequence analysis software on the default setting (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI, 53705). Such software may match similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

[0041] The term "chimeric" as used herein as applied to a nucleic acid or polypeptide refers to two components that are defined by structures derived from different sources. For

example, where "chimeric" is used in the context of a chimeric polypeptide (e.g., a chimeric Cas9/Csn1 protein), the chimeric polypeptide includes amino acid sequences that are derived from different polypeptides. A chimeric polypeptide may comprise either modified or naturally-occurring polypeptide sequences (e.g., a first amino acid sequence from a modified or unmodified Cas9/Csn1 protein; and a second amino acid sequence other than the Cas9/Csn1 protein). Similarly, "chimeric" in the context of a polynucleotide encoding a chimeric polypeptide includes nucleotide sequences derived from different coding regions (e.g., a first nucleotide sequence encoding a modified or unmodified Cas9/Csn1 protein; and a second nucleotide sequence encoding a polypeptide other than a Cas9/Csn1 protein).

[0042] The term "chimeric polypeptide" refers to a polypeptide that is made by the combination (i.e., "fusion") of two otherwise separated segments of amino sequence, usually through human intervention. A polypeptide that comprises a chimeric amino acid sequence is a chimeric polypeptide.

[0043] Some chimeric polypeptides can be referred to as "fusion variants." "Heterologous," as used herein, means a nucleotide or polypeptide sequence that is not found in the native nucleic acid or protein, respectively. For example, in a chimeric Cas9/Csn1 protein, the RNA-binding domain of a naturally-occurring bacterial Cas9/Csn1 polypeptide (or a variant thereof) may be fused to a heterologous polypeptide sequence (i.e., a polypeptide sequence from a protein other than Cas9/Csn1 or a polypeptide sequence from another organism). The heterologous polypeptide sequence may exhibit an activity (e.g., enzymatic activity) that will also be exhibited by the chimeric Cas9/Csn1 protein (e.g., methyltransferase activity, acetyltransferase activity, kinase activity, ubiquitinating activity, etc.). A heterologous nucleic acid sequence may be linked to a naturally-occurring nucleic acid sequence (or a variant thereof) (e.g., by genetic engineering) to generate a chimeric nucleotide sequence encoding a chimeric polypeptide. As another example, in a fusion variant Cas9 site-directed polypeptide, a variant Cas9 site-directed polypeptide may be fused to a heterologous polypeptide (i.e., a polypeptide other than Cas9), which exhibits an activity that will also be exhibited by the fusion variant Cas9 site-directed polypeptide. A heterologous nucleic acid sequence may be linked to a variant Cas9 site-directed polypeptide (e.g., by genetic engineering) to generate a nucleotide sequence encoding a fusion variant Cas9 site-directed polypeptide.

[0044] "Recombinant," as used herein, means that a particular nucleic acid, as defined herein, is the product of various combinations of cloning, restriction, polymerase chain reaction (PCR) and/or ligation steps resulting in a construct having a structural coding or non-coding sequence distinguishable from endogenous nucleic acids found in natural systems. DNA sequences encoding polypeptides can be assembled from cDNA fragments or from a series of synthetic oligonucleotides, to provide a synthetic nucleic acid which is capable of being expressed from a recombinant transcriptional unit contained in a cell or in a cell-free transcription and translation system. Genomic DNA comprising the relevant sequences can also be used in the formation of a recombinant gene or transcriptional unit. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where such sequences do not interfere with manipulation or expression of the coding regions, and may indeed act to modulate production of a desired product by various mechanisms. Alternatively, DNA sequences encoding RNA (e.g., DNA-targeting RNA) that is not translated may also be considered recombinant. Thus, e.g., the term "recombinant" nucleic acid refers to one which is not naturally occurring, e.g., is made by the artificial combination of two otherwise separated segments of sequence through human intervention. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a codon encoding the same amino acid, a conservative amino acid, or a non-conservative amino acid. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a desired combination of functions. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. When a recombinant polynucleotide encodes a polypeptide, the sequence of the encoded polypeptide can be naturally occurring ("wild type") or can be a variant (e.g., a mutant) of the naturally occurring sequence. Thus, the term "recombinant" polypeptide does not necessarily refer to a polypeptide whose sequence does not naturally occur. Instead, a "recombinant" polypeptide is encoded by a recombinant DNA sequence, but the sequence of the polypeptide can be naturally occurring ("wild type") or non-naturally occurring (e.g., a variant, a mutant, etc.). Thus, a "recombinant" polypeptide is the result of human intervention, but may be a naturally occurring amino acid sequence.

[0045] A "target sequence" as used herein is a polynucleotide (e.g., as defined herein, including a DNA, RNA, or DNA/RNA hybrid, as well as modified forms thereof) that includes a "target site." The terms "target site" or "target protospacer DNA" are used interchangeably herein to refer to a nucleic acid sequence present in a target genomic sequence (e.g., DNA or RNA in a host cell) to which a targeting portion of the guiding component will bind provided sufficient conditions (e.g., sufficient complementarity) for binding exist. Suitable DNA/RNA binding conditions include physiological conditions normally present in a cell. Other suitable DNA/RNA binding conditions (e.g., conditions in a cell-free system) are known in the art; see, e.g., Sambrook, supra.

[0046] By "cleavage" it is meant the breakage of the covalent backbone of a target sequence (e.g., a nucleic acid molecule). Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, a complex comprising a guiding component and a nuclease is used for targeted double-stranded DNA cleavage. In other embodiments, a complex comprising a guiding component and a nuclease is used for targeted single-stranded RNA cleavage.

[0047] "Nuclease" and "endonuclease" are used interchangeably herein to mean an enzyme which possesses catalytic activity for DNA cleavage and/or RNA cleavage.

[0048] By "cleavage domain" or "active domain" or "nuclease domain" of a nuclease it is meant the polypeptide

sequence or domain within the nuclease which possesses the catalytic activity for nucleic acid cleavage. A cleavage domain can be contained in a single polypeptide chain or cleavage activity can result from the association of two (or more) polypeptides. A single nuclease domain may consist of more than one isolated stretch of amino acids within a given polypeptide.

[0049] A "host cell," as used herein, denotes an in vivo or in vitro eukaryotic cell, a prokaryotic cell (e.g., bacterial or archaeal cell), or a cell from a multicellular organism (e.g., a cell line) cultured as a unicellular entity, which eukaryotic or prokaryotic cells can be, or have been, used as recipients for a nucleic acid, and include the progeny of the original cell which has been transformed by the nucleic acid. It is understood that the progeny of a single cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. A "recombinant host cell" (also referred to as a "genetically modified host cell") is a host cell into which has been introduced a heterologous nucleic acid, e.g., an expression vector. For example, a subject bacterial host cell is a genetically modified bacterial host cell by virtue of introduction into a suitable bacterial host cell of an exogenous nucleic acid (e.g., a plasmid or recombinant expression vector) and a subject eukaryotic host cell is a genetically modified eukaryotic host cell (e.g., a mammalian germ cell), by virtue of introduction into a suitable eukaryotic host cell of an exogenous nucleic acid.

[0050] By "linker" or "spacer", unless otherwise indicated, is meant any useful multivalent (e.g., bivalent) component useful for joining to different portions or segments. Exemplary linkers and spacers include a nucleic acid sequence, a chemical linker, etc. In one instance, the linker of the guiding component (e.g., linker L in the interacting portion of the guiding component) can have a length of from about 3 nucleotides to about 100 nucleotides. For example, the linker can have a length of from about 3 nucleotides (nt) to about 90 nt, from about 3 nucleotides (nt) to about 80 nt, from about 3 nucleotides (nt) to about 70 nt, from about 3 nucleotides (nt) to about 60 nt, from about 3 nucleotides (nt) to about 50 nt, from about 3 nucleotides (nt) to about 40 nt, from about 3 nucleotides (nt) to about 30 nt, from about 3 nucleotides (nt) to about 20 nt or from about 3 nucleotides (nt) to about 10 nt. For example, the linker can have a length of from about 3 nt to about 5 nt, from about 5 nt to about 10 nt, from about 10 nt to about 15 nt, from about 15 nt to about 20 nt, from about 20 nt to about 25 nt, from about 25 nt to about 30 nt, from about 30 nt to about 35 nt, from about 35 nt to about 40 nt, from about 40 nt to about 50 nt, from about 50 nt to about 60 nt, from about 60 nt to about 70 nt, from about 70 nt to about 80 nt, from about 80 nt to about 90 nt, or from about 90 nt to about 100 nt. In some embodiments, the linker of a single-molecule guiding component is 4 nt.

[0051] The term "histone-packaged supercoiled plasmid DNA" is used to describe a component of particles according to the present disclosure that employ a plasmid DNA that has been "supercoiled" (i.e., folded in on itself using a supersaturated salt solution or other ionic solution which causes the plasmid to fold in on itself and "supercoil" in order to become more dense for efficient packaging into the particles). The plasmid may be virtually any plasmid that expresses any number of polypeptides or encode RNA, including small hairpin RNA/shRNA or small interfering RNA/siRNA, as otherwise described herein. Once supercoiled (using the concentrated salt or other anionic solution), the supercoiled plasmid DNA is then complexed with histone proteins to produce a histone-packaged "complexed" supercoiled plasmid DNA.

[0052] "Packaged" DNA herein refers to DNA that is loaded into particles (e.g., adsorbed into the pores, confined directly within the core itself, or confined partially within a pore). To minimize the DNA spatially, it is often packaged, which can be accomplished in several different ways, from adjusting the charge of the surrounding medium to creation of small complexes of the DNA with, for example, lipids, proteins, or other nanoparticles (usually, although not exclusively cationic). Packaged DNA is often achieved via lipoplexes (i.e., complexing DNA with cationic lipid mixtures). In addition, DNA has also been packaged with cationic proteins (including proteins other than histones), as well as gold nanoparticles (e.g., NanoFlares—an engineered DNA and metal complex in which the core of the nanoparticle is gold).

[0053] One ore more histone proteins, as well as other means to package the DNA into a smaller volume such as normally cationic nanoparticles, lipids, or proteins, may be used to package the supercoiled plasmid DNA "histone-packaged supercoiled plasmid DNA." In certain aspects of the disclosed technology, a combination of histone proteins H1, H2A, H2B, H3, and H4 in a preferred ratio of 1:2:2:2:2, although other histone proteins may be used in other, similar ratios, as is known in the art or may be readily practiced pursuant to the teachings of the present disclosure. The DNA may also be double stranded linear DNA, instead of plasmid DNA, which also may be optionally supercoiled and/or packaged with histones or other packaging components.

[0054] Other histone proteins which may be used in this aspect of the disclosed technology include, for example, H1F, H1A, H1B, H2A, H2B, H1F0, H1FNT, H1FOO, H1FX, H1H1, HIST1H1A, HIST1H1B, HIST1H1C, HIST1H1D, HIST1H1E, HIST1H1T, H2AF, H2AFB1, H2AFB2, H2AFB3, H2AFJ, H2AFV, H2AFX, H2AFY, H2AFY2, H2AFZ, H2A1, HIST1H2AA, HIST1H2AB, HIST1H2AC, HIST1H2AD, HIST1H2AE, HIST1H2AG, HIST1H2AI, HIST1H2AJ, HIST1H2AK, HIST1H2AL, HIST1H2AM, H2A2, HIST2H2AA3, HIST2H2AC, H2BF, H2BFM, HSBFS, HSBFWT, H2B1, HIST1H2BA, HIST1HSBB, HIST1HSBC, HIST1HSBD, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2BH, HIST1H2B1, HIST1H2BJ, HIST1H2BK, HIST1H2BL, HIST1H2BM, HIST1H2BN, HIST1H2BO, H2B2, HIST2H2BE, H3A1, HIST1H3A, HIST1H3B, HIST1H3C, HIST1H3D, HIST1H3E, HIST1H3F, HIST1H3G, HIST1H3H, HIST1H3I, HIST1H3J, H3A2, HIST2H3C, H3A3, HIST3H3, H41, HIST1H4A, HIST1H4B, HIST1H4C, HIST1H4D, HIST1H4E, HIST1H4F, HIST1H4G, HIST1H4H, HIST1H4I, HIST1H4J, HIST1H4K, HIST1H4L, H44, and HIST4H4.

[0055] The term "nuclear localization sequence" refers to a peptide sequence incorporated or otherwise crosslinked into histone proteins, which comprise the histone-packaged supercoiled plasmid DNA. In certain embodiments, particles according to the present disclosure may further comprise a plasmid (often a histone-packaged supercoiled plasmid DNA) which is modified (crosslinked) with a nuclear localization sequence (note that the histone proteins may be crosslinked with the nuclear localization sequence or the

plasmid itself can be modified to express a nuclear localization sequence), which enhances the ability of the histone-packaged plasmid to penetrate the nucleus of a cell and deposit its contents there (to facilitate expression and ultimately cell death. These peptide sequences assist in carrying the histone-packaged plasmid DNA and the associated histones into the nucleus of a targeted cell, whereupon the plasmid will express peptides and/or nucleotides as desired to deliver therapeutic and/or diagnostic molecules (polypeptide and/or nucleotide) into the nucleus of the targeted cell. One or more crosslinking agents, known in the art, may be used to covalently link a nuclear localization sequence to a histone protein (often at a lysine group or other group which has a nucleophilic or electrophilic group in the side chain of the amino acid exposed pendant to the polypeptide), which can be used to introduce the histone packaged plasmid into the nucleus of a cell. Alternatively, a nucleotide sequence that expresses the nuclear localization sequence can be positioned in a plasmid in proximity to that which expresses histone protein, such that the expression of the histone protein conjugated to the nuclear localization sequence will occur thus facilitating transfer of a plasmid into the nucleus of a targeted cell.

[0056] The terms "nucleic acid regulatory sequences," "control elements," and "regulatory elements," used interchangeably herein, refer to transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, internal ribosomal entry sites (IRES), terminators, and protein degradation signals, that provide for and/or regulate transcription of a non-coding sequence (e.g., DNA-targeting RNA) or a coding sequence (e.g., site-directed modifying polypeptide, or Cas9/Csn1 polypeptide) and/or regulate translation of an encoded polypeptide.

[0057] A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present disclosed technology, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the −10 and −35 consensus sequences.

[0058] An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence. Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, and terminators, that provide for the expression of a coding sequence in a host cell.

[0059] A "vector" or "expression vector" is a replicon, such as a plasmid, phage, virus, or cosmid, to which another nucleic acid segment, i.e., an "insert", may be attached so as to bring about the replication of the attached segment in a cell.

[0060] An "expression cassette" comprises a nucleic acid coding sequence operably linked, as defined herein, to a promoter sequence, as defined herein.

[0061] A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

[0062] "Operably linked" or "operatively linked" or "operatively associated with," as used interchangeably, refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For instance, a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression. A nucleic acid molecule is operatively linked or operably linked to, or operably associated with, an expression control sequence when the expression control sequence controls and regulates the transcription and translation of nucleic acid sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the nucleic acid sequence to be expressed and maintaining the correct reading frame to permit expression of the nucleic acid sequence under the control of the expression control sequence and production of the desired product encoded by the nucleic acid sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

[0063] In accordance with the present disclosure there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, 2001, "Molecular Cloning: A Laboratory Manual"; Ausubel, ed., 1994, "Current Protocols in Molecular Biology" Volumes I-III; Celis, ed., 1994, "Cell Biology: A Laboratory Handbook" Volumes I-III; Coligan, ed., 1994, "Current Protocols in Immunology" Volumes I-III; Gait ed., 1984, "Oligonucleotide Synthesis"; Hames & Higgins eds., 1985, "Nucleic Acid Hybridization"; Hames & Higgins, eds., 1984, "Transcription And Translation"; Freshney, ed., 1986, "Animal Cell Culture"; IRL.

[0064] By an "effective amount" or a "sufficient amount" of an agent (e.g., a cargo), as used herein, is that amount sufficient to effect beneficial or desired results, such as clinical results, and, as such, an "effective amount" depends upon the context in which it is being applied. For example, in the context of administering an agent that employs a CRISPR component to genetically modify a gene, an effective amount of an agent is, for example, an amount sufficient to achieve increased or decreased expression of that gene, as compared to the response obtained without administration of the agent.

[0065] By "subject" is meant a human or non-human animal (e.g., a mammal).

[0066] By "treating" a disease, disorder, or condition in a subject is meant reducing at least one symptom of the disease, disorder, or condition by administrating a therapeutic agent to the subject. By "treating prophylactically" a

disease, disorder, or condition in a subject is meant reducing the frequency of occurrence of or reducing the severity of a disease, disorder or condition by administering a therapeutic agent to the subject prior to the onset of disease symptoms. Beneficial or desired results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions; diminishment of extent of disease, disorder, or condition; stabilized (i.e., not worsening) state of disease, disorder, or condition; preventing spread of disease, disorder, or condition; delay or slowing the progress of the disease, disorder, or condition; amelioration or palliation of the disease, disorder, or condition; and remission (whether partial or total), whether detectable or undetectable.

[0067] By "salt" is meant an ionic form of a compound or structure (e.g., any formulas, compounds, or compositions described herein), which includes a cation or anion compound to form an electrically neutral compound or structure. For example, non-toxic salts are described in Berge S M et al., "Pharmaceutical salts," *J. Pharm. Sci.* 1977 January; 66(1):1-19; and in "Handbook of Pharmaceutical Salts: Properties, Selection, and Use," Wiley-VCH, April 2011 (2nd rev. ed., eds. P. H. Stahl and C. G. Wermuth). The salts can be prepared in situ during the final isolation and purification of the compounds of the disclosed technology or separately by reacting the free base group with a suitable organic acid (thereby producing an anionic salt) or by reacting the acid group with a suitable metal or organic salt (thereby producing a cationic salt). Representative anionic salts include acetate, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bicarbonate, bisulfate, bitartrate, borate, bromide, butyrate, camphorate, camphorsulfonate, chloride, citrate, cyclopentanepropionate, digluconate, dihydrochloride, diphosphate, dodecylsulfate, edetate, ethanesulfonate, fumarate, glucoheptonate, glucomate, glutamate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, hydroxyethanesulfonate, hydroxynaphthoate, iodide, lactate, lactobionate, laurate, lauryl sulfate, malate, maleate, malonate, mandelate, mesylate, methanesulfonate, methylbromide, methylnitrate, methylsulfate, mucate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, polygalacturonate, propionate, salicylate, stearate, subacetate, succinate, sulfate, tannate, tartrate, theophyllinate, thiocyanate, triethiodide, toluenesulfonate, undecanoate, and valerate salts. Representative cationic salts include metal salts, such as alkali or alkaline earth salts, e.g., barium, calcium (e.g., calcium edetate), lithium, magnesium, potassium, and sodium; other metal salts, such as aluminum, bismuth, iron, and zinc; as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and pyridinium. Other cationic salts include organic salts, such as chloroprocaine, choline, dibenzylethylenediamine, diethanolamine, ethylenediamine, methylglucamine, and procaine. Exemplary salts include pharmaceutically acceptable salts.

[0068] By "pharmaceutically acceptable salt" is meant a salt that is, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and animals without for example, undue toxicity, irritation, or allergic response, and are commensurate with a reasonable benefit/risk ratio.

[0069] By "pharmaceutically acceptable excipient" is meant any ingredient other than a compound or structure (e.g., any formulas, compounds, or compositions described herein) and having the properties of being nontoxic and non-inflammatory in a subject. Exemplary, non-limiting excipients include adjuvants, antiadherents, antioxidants, binders, carriers, coatings, compression aids, diluents, disintegrants, dispersing agents, dyes (colors), emollients, emulsifiers, fillers (diluents), film formers or coatings, flavors, fragrances, glidants (flow enhancers), isotonic carriers, lubricants, preservatives, printing inks, solvents, sorbents, stabilizers, suspensing or dispersing agents, surfactants, sweeteners, waters of hydration, or wetting agents. Any of the excipients can be selected from those approved, for example, by the United States Food and Drug Administration or other governmental agency as being acceptable for use in humans or domestic animals. Exemplary excipients include, but are not limited to alcohol, butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, cross-linked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, glycerol, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactated Ringer's solution, lactose, magnesium stearate, maltitol, maltose, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, Ringer's solution, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium chloride injection, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, stearic acid, sucrose, talc, titanium dioxide, vegetable oil, vitamin A, vitamin E, vitamin C, water, and xylitol.

[0070] As used herein, the terms "top," "bottom," "upper," "lower," "above," and "below" are used to provide a relative relationship between structures. The use of these terms does not indicate or require that a particular structure must be located at a particular location in the apparatus.

[0071] By "alkenyl" is meant an optionally substituted C_{2-24} alkyl group having one or more double bonds. The alkenyl group can be cyclic (e.g., C_{3-24} cycloalkenyl) or acyclic. The alkenyl group can also be substituted or unsubstituted. For example, the alkenyl group can be substituted with one or more substitution groups, as described herein for alkyl.

[0072] By "alkyl" and the prefix "alk" is meant a branched or unbranched saturated hydrocarbon group of 1 to 24 carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, s-butyl, t-butyl, n-pentyl, isopentyl, s-pentyl, neopentyl, hexyl, heptyl, octyl, nonyl, decyl, dodecyl, tetradecyl, hexadecyl, eicosyl, and tetracosyl. The alkyl group can be cyclic (e.g., C_{3-24} cycloalkyl) or acyclic.

[0073] The alkyl group can be branched or unbranched. The alkyl group can also be substituted or unsubstituted. For example, the alkyl group can be substituted with one, two, three or, in the case of alkyl groups of two carbons or more, four substituents independently selected from the group consisting of: (1) C_{1-6} alkoxy (e.g., —OAk, in which Ak is an alkyl group, as defined herein); (2) C_{1-6} alkylsulfinyl (e.g., —S(O)Ak, in which Ak is an alkyl group, as defined herein); (3) C_{1-6} alkylsulfonyl (e.g., —SO_2Ak, in which Ak

is an alkyl group, as defined herein); (4) amino (e.g., —$NR^{N1}R^{N2}$, where each of R^{N1} and R^{N2} is, independently, H or optionally substituted alkyl, or R^{N1} and R^{N2}, taken together with the nitrogen atom to which each are attached, form a heterocyclyl group); (5) aryl; (6) arylalkoxy (e.g., —OA^LAr, in which A^L is an alkylene group and Ar is an aryl group, as defined herein); (7) aryloyl (e.g., —C(O)Ar, in which Ar is an aryl group, as defined herein); (8) azido (e.g., an —N_3 group); (9) cyano (e.g., a —CN group); (10) carboxyaldehyde (e.g., a —C(O)H group); (11) C_{3-8} cycloalkyl; (12) halo; (13) heterocyclyl (e.g., a 5-, 6- or 7-membered ring, unless otherwise specified, containing one, two, three, or four non-carbon heteroatoms (e.g., independently selected from the group consisting of nitrogen, oxygen, phosphorous, sulfur, or halo)); (14) heterocyclyloxy (e.g., —OHet, in which Het is a heterocyclyl group); (15) heterocyclyloyl (e.g., —C(O)Het, in which Het is a heterocyclyl group); (16) hydroxyl (e.g., a —OH group); (17) N-protected amino; (18) nitro (e.g., an —NO_2 group); (19) oxo (e.g., an =O group); (20) C_{3-8} spirocyclyl (e.g., an alkylene diradical, both ends of which are bonded to the same carbon atom of the parent group to form a spirocyclyl group); (21) C_{1-6} thioalkoxy (e.g., —SAk, in which Ak is an alkyl group, as defined herein); (22) thiol (e.g., an —SH group); (23) —CO_2R^A, where R^A is selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{4-18} aryl, and (d) C_{1-6} alk-C_{4-18} aryl; (24) —$C(O)NR^BR^C$, where each of R^B and R^C is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{4-18} aryl, and (d) C_{1-6} alk-C_{4-18} aryl; (25) —SO_2R^D, where R^D is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{4-18} aryl, and (c) C_{1-6} alk-C_{4-18} aryl; (26) —$SO_2NR^ER^F$, where each of R^E and R^F is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{4-18} aryl, and (d) C_{1-6} alk-C_{4-18} aryl; and (27) —NR^GR^H, where each of R^G and R^H is, independently, selected from the group consisting of (a) hydrogen, (b) an N-protecting group, (c) C_{1-6} alkyl, (d) C_{2-6} alkenyl, (e) C_{2-6} alkynyl, (f) C_{4-18} aryl, (g) C_{1-6} alk-C_{4-18} aryl, (h) C_{3-8} cycloalkyl, and (i) C_{1-6} alk-C_{3-8} cycloalkyl, wherein in one embodiment no two groups are bound to the nitrogen atom through a carbonyl group or a sulfonyl group. The alkyl group can be a primary, secondary, or tertiary alkyl group substituted with one or more substituents (e.g., one or more halo or alkoxy). In some embodiments, the unsubstituted alkyl group is a C_{1-3}, C_{1-6}, C_{1-12}, C_{1-16}, C_{1-18}, C_{1-20}, or C_{1-24} alkyl group.

[0074] By "alkylene" is meant a multivalent (e.g., bivalent, trivalent, tetravalent, etc.) form of an alkyl group, as described herein. Exemplary alkylene groups include methylene, ethylene, propylene, butylene, etc. In some embodiments, the alkylene group is a C_{1-3}, C_{1-6}, C_{1-12}, C_{1-16}, C_{1-18}, C_{1-20}, C_{1-24}, C_{2-3}, C_{2-6}, C_{2-12}, C_{2-16}, C_{2-18}, C_{2-20}, or C_{2-24} alkylene group. The alkylene group can be branched or unbranched. The alkylene group can also be substituted or unsubstituted. For example, the alkylene group can be substituted with one or more substitution groups, as described herein for alkyl.

[0075] By "alkynyl" is meant an optionally substituted C_{2-24} alkyl group having one or more triple bonds. The alkynyl group can be cyclic or acyclic such as ethynyl or 1-propynyl. The alkynyl group can also be substituted or unsubstituted. For example, the alkynyl group can be substituted with one or more substitution groups, as described herein for alkyl.

[0076] By "amido" is meant —$C(O)NR^{N1}R^{N2}$, where each of R^{N1} and R^{N2} is, independently, H, optionally substituted alkyl, or optionally substituted aryl; or where a combination of R^{N1} and R^{N2}, taken together with the nitrogen atom to which each are attached, form a heterocyclyl group, as defined herein.

[0077] By "amino" is meant —$NR^{N1}R^{N2}$, where each of R^{N1} and R^{N2} is, independently, H or optionally substituted alkyl, or R^{N1} and R^{N2}, taken together with the nitrogen atom to which each are attached, form a heterocyclyl group, as defined herein.

[0078] By "aryl" is meant a group that contains any carbon-based aromatic group including, but not limited to, benzyl, naphthalene, phenyl, biphenyl, and phenoxybenzene. The term "aryl" also includes "heteroaryl," which is defined as a group that contains an aromatic group that has at least one heteroatom incorporated within the ring of the aromatic group. Examples of heteroatoms include, but are not limited to, nitrogen, oxygen, sulfur, and phosphorus. Likewise, the term "non-heteroaryl," which is also included in the term "aryl," defines a group that contains an aromatic group that does not contain a heteroatom. The aryl group can be substituted or unsubstituted. The aryl group can be substituted with one, two, three, four, or five substituents independently selected from the group consisting of: (1) C_{1-6} alkanoyl (e.g., —C(O)Ak, in which Ak is an alkyl group, as defined herein); (2) C_{1-6} alkyl; (3) C_{1-6} alkoxy (e.g., —OAk, in which Ak is an alkyl group, as defined herein); (4) C_{1-6} alkoxy-C_{1-6} alkyl (e.g., an alkyl group, which is substituted with an alkoxy group —OAk, in which Ak is an alkyl group, as defined herein); (5) C_{1-6} alkylsulfinyl (e.g., —S(O)Ak, in which Ak is an alkyl group, as defined herein); (6) C_{1-6} alkylsulfinyl-C_{1-6} alkyl (e.g., an alkyl group, which is substituted by an alkylsulfinyl group —S(O)Ak, in which Ak is an alkyl group, as defined herein); (7) C_{1-6} alkylsulfonyl (e.g., —SO_2Ak, in which Ak is an alkyl group, as defined herein); (8) C_{1-6} alkylsulfonyl-C_{1-6} alkyl (e.g., an alkyl group, which is substituted by an alkylsulfonyl group —SO_2Ak, in which Ak is an alkyl group, as defined herein); (9) aryl; (10) amino (e.g., —$NR^{N1}R^{N2}$, where each of R^{N1} and R^{N2} is, independently, H or optionally substituted alkyl, or R^{N1} and R^{N2}, taken together with the nitrogen atom to which each are attached, form a heterocyclyl group); (11) C_{1-6} aminoalkyl (e.g., meant an alkyl group, as defined herein, substituted by an amino group); (12) heteroaryl; (13) C_{1-6} alk-C_{4-18} aryl (e.g., -A^LAr, in which A^L is an alkylene group and Ar is an aryl group, as defined herein); (14) aryloyl (e.g., —C(O)Ar, in which Ar is an aryl group, as defined herein); (15) azido (e.g., an —N3 group); (16) cyano (e.g., a —CN group); (17) C_{1-6} azidoalkyl (e.g., a —N3 azido group attached to the parent molecular group through an alkyl group, as defined herein); (18) carboxyaldehyde (e.g., a —C(O)H group); (19) carboxyaldehyde-C_{1-6} alkyl (e.g., -A^LC(O)H, in which A^L is an alkylene group, as defined herein); (20) C_{3-8} cycloalkyl; (21) C_{1-6} alk-C_{3-8} cycloalkyl (e.g., -A^LCy, in which A^L is an alkylene group and Cy is a cycloalkyl group, as defined herein); (22) halo (e.g., F, Cl, Br, or I); (23) C_{1-6} haloalkyl (e.g., an alkyl group, as defined herein, substituted with one or more halo); (24) heterocyclyl; (25) heterocyclyloxy (e.g., —OHet, in which Het is a heterocyclyl group); (26) heterocyclyloyl (e.g., —C(O)Het, in which Het is a heterocyclyl group); (16) hydroxyl (e.g., a —OH group); (27) hydroxyl (e.g., a —OH group); (28) C_{1-6} hydroxyalkyl (e.g., an alkyl

group, as defined herein, substituted by one to three hydroxyl groups, with the proviso that no more than one hydroxyl group may be attached to a single carbon atom of the alkyl group); (29) nitro (e.g., an —NO_2 group); (30) C_{1-6} nitroalkyl (e.g., an alkyl group, as defined herein, substituted by one to three nitro groups); (31)N-protected amino; (32) N-protected amino-C_{1-6} alkyl; (33) oxo (e.g., an =O group); (34) C_{1-6} thioalkoxy (e.g., —SAk, in which Ak is an alkyl group, as defined herein); (35) thio-C_{1-6} alkoxy-C_{1-6} alkyl (e.g., an alkyl group, which is substituted by an thioalkoxy group —SAk, in which Ak is an alkyl group, as defined herein); (36) —$(CH_2)_rCO_2R^A$, where r is an integer of from zero to four, and R^A is selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{4-18} aryl, and (d) C_{1-6} alk-C_{4-18} aryl; (37) —$(CH_2)_rCONR^BR^C$, where r is an integer of from zero to four and where each R^B and R^C is independently selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{4-18} aryl, and (d) C_{1-6} alk-C_{4-18} aryl; (38) —$(CH_2)_rSO_2R^D$, where r is an integer of from zero to four and where R^D is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{4-18} aryl, and (c) C_{1-6} alk-C_{4-18} aryl; (39) —$(CH_2)_rSO_2NR^ER^F$, where r is an integer of from zero to four and where each of R^E and R^F is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{4-18} aryl, and (d) C_{1-6} alk-C_{4-18} aryl; (40) —$(CH_2)_rNR^GR^H$, where r is an integer of from zero to four and where each of R^G and R^H is, independently, selected from the group consisting of (a) hydrogen, (b) an N-protecting group, (c) C_{1-6} alkyl, (d) C_{2-6} alkenyl, (e) C_{2-6} alkynyl, (f) C_{4-18} aryl, (g) C_{1-6} alk-C_{4-18} aryl, (h) C_{3-8} cycloalkyl, and (i) C_{1-6} alk-C_{3-8} cycloalkyl, wherein in one embodiment no two groups are bound to the nitrogen atom through a carbonyl group or a sulfonyl group; (41) thiol; (42) perfluoroalkyl (e.g., an alkyl group, as defined herein, having each hydrogen atom substituted with a fluorine atom); (43) perfluoroalkoxy (e.g., —ORf, in which Rf is an alkyl group, as defined herein, having each hydrogen atom substituted with a fluorine atom); (44) aryloxy (e.g., —OAr, where Ar is an optionally substituted aryl group, as described herein); (45) cycloalkoxy (e.g., —OCy, in which Cy is a cycloalkyl group, as defined herein); (46) cycloalkylalkoxy (e.g., —OA^LCy, in which A^L is an alkylene group and Cy is a cycloalkyl group, as defined herein); and (47) arylalkoxy (e.g., —OA^LAr, in which A^L is an alkylene group and Ar is an aryl group, as defined herein). In particular embodiments, an unsubstituted aryl group is a C_{4-18}, C_{4-14}, C_{4-12}, C_{4-10}, C_{6-18}, C_{6-14}, C_{6-12}, or C_{6-10} aryl group.

[0079] By "arylene" is meant a multivalent (e.g., bivalent, trivalent, tetravalent, etc.) form of an aryl group, as described herein. Exemplary arylene groups include phenylene, naphthylene, biphenylene, triphenylene, diphenyl ether, acenaphthenylene, anthrylene, or phenanthrylene. In some embodiments, the arylene group is a C_{4-18}, C_{4-14}, C_{4-12}, C_{4-10}, C_{6-18}, C_{6-14}, C_{6-12}, or C_{6-10} arylene group. The arylene group can be branched or unbranched. The arylene group can also be substituted or unsubstituted. For example, the arylene group can be substituted with one or more substitution groups, as described herein for aryl.

[0080] By "azido" is meant an —N_3 group.

[0081] By "carbamido" is meant —$NR^{N1}C(O)NR^{N2}R^{N3}$, where each of R^{N1} and R^{N2} and R^{N3} is, independently, H, optionally substituted alkyl, or optionally substituted aryl; or where a combination of R^{N2} and R^{N3}, taken together with the nitrogen atom to which each are attached, form a heterocyclyl group, as defined herein.

[0082] By "carbonyl" is meant a —C(O)— group, which can also be represented as >C=O.

[0083] By "carboxyl" is meant a —CO_2H group.

[0084] By "halo" is meant F, Cl, Br, or I.

[0085] By "heteroalkyl" is meant an alkyl group, as defined herein, containing one, two, three, or four non-carbon heteroatoms (e.g., independently selected from the group consisting of nitrogen, oxygen, phosphorous, sulfur, or halo).

[0086] By "heteroalkylene" is meant a divalent form of an alkylene group, as defined herein, containing one, two, three, or four non-carbon heteroatoms (e.g., independently selected from the group consisting of nitrogen, oxygen, phosphorous, sulfur, or halo).

[0087] By "heteroaryl" is meant a subset of heterocyclyl groups, as defined herein, which are aromatic, i.e., they contain 4n+2 pi electrons within the mono- or multicyclic ring system.

[0088] By "heterocyclyl" is meant a 5-, 6- or 7-membered ring, unless otherwise specified, containing one, two, three, or four non-carbon heteroatoms (e.g., independently selected from the group consisting of nitrogen, oxygen, phosphorous, sulfur, or halo). The 5-membered ring has zero to two double bonds and the 6- and 7-membered rings have zero to three double bonds. The term "heterocyclyl" also includes bicyclic, tricyclic and tetracyclic groups in which any of the above heterocyclic rings is fused to one, two, or three rings independently selected from the group consisting of an aryl ring, a cyclohexane ring, a cyclohexene ring, a cyclopentane ring, a cyclopentene ring, and another monocyclic heterocyclic ring, such as indolyl, quinolyl, isoquinolyl, tetrahydroquinolyl, benzofuryl, and benzothieny. Heterocyclics include, for example, thiiranyl, thietanyl, tetrahydrothienyl, thianyl, thiepanyl, aziridinyl, azetidinyl, pyrrolidinyl, piperidinyl, azepanyl, pyrrolyl, pyrrolinyl, pyrazolyl, pyrazolinyl, pyrazolidinyl, imidazolyl, imidazolinyl, imidazolidinyl, pyridyl, homopiperidinyl, pyrazinyl, piperazinyl, pyrimidinyl, pyridazinyl, oxazolyl, oxazolidinyl, isoxazolyl, isoxazolidiniyl, morpholinyl, thiomorpholinyl, thiazolyl, thiazolidinyl, isothiazolyl, isothiazolidinyl, indolyl, quinolinyl, isoquinolinyl, benzimidazolyl, benzothiazolyl, benzoxazolyl, furyl, thienyl, thiazolidinyl, isothiazolyl, isoindazoyl, triazolyl, tetrazolyl, oxadiazolyl, uricyl, thiadiazolyl, pyrimidyl, tetrahydrofuranyl, dihydrofuranyl, tetrahydrothienyl, dihydrothienyl, dihydroindolyl, tetrahydroquinolyl, tetrahydroisoquinolyl, pyranyl, dihydropyranyl, dithiazolyl, benzofuranyl, and benzothienyl.

[0089] By "hydroxyl" is meant —OH.

[0090] By "imido" is meant —$C(O)N^{N1}C(O)$—, where R^{N1} is, independently, H, optionally substituted alkyl, or optionally substituted aryl.

[0091] By "protecting group" is meant any group intended to protect a reactive group against undesirable synthetic reactions. Commonly used protecting groups are disclosed in "Greene's Protective Groups in Organic Synthesis," John Wiley & Sons, New York, 2007 (4th ed., eds. P. G. M. Wuts and T. W. Greene), which is incorporated herein by reference. O-protecting groups include an optionally substituted alkyl group (e.g., forming an ether with reactive group O), such as methyl, methoxymethyl, methylthiomethyl, benzoyloxymethyl, t-butoxymethyl, etc.; an optionally substituted

alkanoyl group (e.g., forming an ester with the reactive group O), such as formyl, acetyl, chloroacetyl, fluoroacetyl (e.g., perfluoroacetyl), methoxyacetyl, pivaloyl, t-butylacetyl, phenoxyacetyl, etc.; an optionally substituted aryloyl group (e.g., forming an ester with the reactive group O), such as —C(O)—Ar, including benzoyl; an optionally substituted alkylsulfonyl group (e.g., forming an alkylsulfonate with reactive group O), such as —SO_2—R^{S1}, where R^{S1} is optionally substituted C_{1-12} alkyl, such as mesyl or benzylsulfonyl; an optionally substituted arylsulfonyl group (e.g., forming an arylsulfonate with reactive group O), such as —SO_2—R^{S4}, where R^{S4} is optionally substituted C_{4-18} aryl, such as tosyl or phenylsulfonyl; an optionally substituted alkoxycarbonyl or aryloxycarbonyl group (e.g., forming a carbonate with reactive group O), such as —C(O)—OR^{T1}, where R^{T1} is optionally substituted C_{1-12} alkyl or optionally substituted C_{4-18} aryl, such as methoxycarbonyl, methoxymethylcarbonyl, t-butyloxycarbonyl (Boc), or benzyloxycarbonyl (Cbz); or an optionally substituted silyl group (e.g., forming a silyl ether with reactive group O), such as —Si—$(R^{T2})_3$, where each R^{T2} is, independently, optionally substituted C_{1-12} alkyl or optionally substituted C_{4-18} aryl, such as trimethylsilyl, t-butyldimethylsilyl, or t-butyldiphenylsilyl. N-protecting groups include, e.g., formyl, acetyl, benzoyl, pivaloyl, t-butylacetyl, alanyl, phenylsulfonyl, benzyl, Boc, and Cbz. Such protecting groups can employ any useful agent to cleave the protecting group, thereby restoring the reactivity of the unprotected reactive group.

[0092] By "thio" is meant an —S— group.

[0093] By "thiol" is meant an —SH group.

[0094] By "attaching," "attachment," or related word forms is meant any covalent or non-covalent bonding interaction between two components. Non-covalent bonding interactions include, without limitation, hydrogen bonding, ionic interactions, halogen bonding, electrostatic interactions, π bond interactions, hydrophobic interactions, inclusion complexes, clathration, van der Waals interactions, and combinations thereof.

[0095] Other features and advantages of the disclosed technology will be apparent from the following description and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0096] FIG. **1**A-**1**C is a schematic showing exemplary constructs. Provided are (A) an exemplary method **100** for providing a non-limiting construct having a core **101**, a spacer **103**, a cargo **104**, and an outer layer **105**; (B) another exemplary construct **1000**; and (C) yet another exemplary construct **1500**.

[0097] FIG. **2** is a schematic showing an exemplary lipid coated mesoporous silica nanoparticles (LC-MSN) in accordance with present disclosure.

[0098] FIG. **3**A-**3**C is a schematic showing exemplary methods for providing a construct and its use for in vitro gene editing.

[0099] FIG. **4**A-**4**E is a schematic view showing (A) exemplary linking agents and methods employing linking agent L1 to provide a construct having a nanoparticle (NP) core, a spacer, and an attached cargo, (B) another exemplary spacer having a reactive group L" that interacts with a reactive group R^1 present on a cargo, and yet other exemplary spacers present between the NP and the cargo. Also provided are (D) another exemplary spacer and (B) another exemplary method employing linking agent L2 to provide a construct having a NP core, a spacer, and an attached cargo.

[0100] FIG. **5**A-**5**C is a schematic view showing further exemplary linking agents and spacers. Provided are schematics of (A) exemplary spacers (i)-(iii) present between a core and a cargo, (B) an exemplary reaction scheme between a linking agent and a reactive group present on a cargo, thereby forming a spacer between the core and the cargo, and (C) yet another reaction scheme between another linking agent and a reactive group present on a cargo.

[0101] FIG. **6** is a schematic view of an exemplary CRISPR component that includes a guiding component **90** to bind to the target sequence **97**, as well as a nuclease **98**.

[0102] FIG. **7**A-**7**C are schematics showing exemplary CRISPR components.

[0103] FIG. **8**A-**8**H shows non-limiting amino acid sequences for various nucleases. Provided are sequences for (A) a Cas9 endonuclease for *S. pyogenes* serotype M1 (SEQ ID NO:110), (B) a deactivated Cas9 having D10A and H840A mutations (SEQ ID NO:111), (C) a Cas protein Csn1 for *S. pyogenes* (SEQ ID NO:112), (D) a Cas9 endonuclease for *F. novicida* U112 (SEQ ID NO:113), (E) a Cas9 endonuclease for *S. thermophilus* 1 (SEQ ID NO:114), (F) a Cas9 endonuclease for *S. thermophilus* 2 (SEQ ID NO:115), (G) a Cas9 endonuclease for *L. innocua* (SEQ ID NO:116), and (H) a Cas9 endonuclease for W *succinogenes* (SEQ ID NO:117).

[0104] FIG. **9** shows non-limiting nucleic acid sequences of crRNA that can be employed as a first portion in any guiding component described herein. Provided are sequences for *S. pyogenes* (SEQ ID NO:20), *L. innocua* (SEQ ID NO:21), *S. thermophilus* 1 (SEQ ID NO:22), *S. thermophilus* 2 (SEQ ID NO:23), *F. novicida* (SEQ ID NO:24), and W. *succinogenes* (SEQ ID NO:25). Also provided are various consensus sequences (SEQ ID NOs:26-32), in which each X, independently, can be absent, A, C, T, G, or U, as well as modified forms thereof (e.g., as described herein). In another embodiment, for each consensus sequence (SEQ ID NOs:26-32), each X at each position is a nucleic acid (or a modified form thereof) that is provided in an aligned reference sequence. For instance, for consensus SEQ ID NO:26, the first position includes an X, and this X can be absent or any nucleic acid (e.g., A, C, T, G, or U, as well as modified forms thereof). Alternatively, this X can be any nucleic acid provided in an aligned reference sequence (e.g., aligned reference sequences SEQ ID NO:20-25 for the consensus sequence in SEQ ID NO:26). Thus, X at position 1 in SEQ ID NO:26 can also be G (as in SEQ ID NOs:20-23 and 25) or C (as in SEQ ID NO:24), in which this subset of substitutions is defined as a conservative subset. Similarly, for each X at each position for the consensus sequences (SEQ ID NOs:26-32), conservative subsets can be determined based on FIG. **9**, and these consensus sequences include nucleic acid sequences encompassed by such conservative subsets. Gray highlight indicates a conserved nucleic acid, and the dash indicates an absent nucleic acid.

[0105] FIG. **10**A-**10**C shows non-limiting nucleic acid sequences of tracrRNA that can be employed as a second portion and/or linker in any guiding component described herein. Provided are sequences for *S. pyogenes* (SEQ ID NO:40), *L. innocua* (SEQ ID NO:41), *S. thermophilus* 1 (SEQ ID NO:42), *S. thermophilus* 2 (SEQ ID NO:43), *F. novicida* 1 (SEQ ID NO:44), *F. novicida* 2 (SEQ ID NO:45), *W. succinogenes* 1 (SEQ ID NO:46), and W *succinogenes* 2

(SEQ ID NO:47). Also provided are various consensus sequences (SEQ ID NOs:48-54), in which each Z, independently, can be absent, A, C, T, G, or U, as well as modified forms thereof (e.g., as described herein). Consensus sequences are shown for (A) an alignment of all SEQ ID NOs:40-47, providing consensus sequences SEQ ID NOs: 48-50; (B) an alignment of SEQ ID NOs:40-43, providing consensus sequences SEQ ID NOs:51-52; and (C) an alignment of SEQ ID NOs:44-47, providing consensus sequences SEQ ID NOs:53-54. In another embodiment, for each consensus sequence (SEQ ID NOs:48-54), each Z at each position is a nucleic acid (or a modified form thereof) that is provided in an aligned reference sequence. For instance, for consensus SEQ ID NO:48, the first position includes a Z, and this Z can be absent or any nucleic acid (e.g., A, C, T, G, or U, as well as modified forms thereof). Alternatively, this Z can be any nucleic acid provided in an aligned reference sequence (e.g., aligned reference sequences SEQ ID NO:40-47 for the consensus sequence in SEQ ID NO:48). Thus, Z at position 2 in SEQ ID NO:48 can also be U (as in SEQ ID NOs:40, 41, and 43-47) or G (as in SEQ ID NO:42), in which this subset of substitutions is defined as a conservative subset. Similarly, for each Z at each position for the consensus sequences (SEQ ID NOs:48-54), conservative subsets can be determined based on FIG. **10**A-**10**C, and these consensus sequences include nucleic acid sequences encompassed by such conservative subsets. Gray highlight indicates a conserved nucleic acid, and the dash indicates an absent nucleic acid.

[0106] FIG. **17** shows non-limiting nucleic acid sequences of extended tracrRNA that can be employed as a second portion and/or linker in any guiding component described herein. Provided are sequences for *S. pyogenes* (SEQ ID NO:60), *L. innocua* (SEQ ID NO:61), *S. thermophilus* 1 (SEQ ID NO:62), and *S. thermophilus* 2 (SEQ ID NO:63). Also provided are various consensus sequences (SEQ ID NOs:64-65), in which each Z, independently, can be absent, A, C, T, G, or U, as well as modified forms thereof (e.g., as described herein). In another embodiment, for each consensus sequence (SEQ ID NOs:64-65), each Z at each position is a nucleic acid (or a modified form thereof) that is provided in an aligned reference sequence. For instance, for consensus SEQ ID NO:64, the first position includes a Z, and this Z can be absent or any nucleic acid (e.g., A, C, T, G, or U, as well as modified forms thereof). Alternatively, this Z can be any nucleic acid provided in an aligned reference sequence (e.g., aligned reference sequences SEQ ID NO:60-63 for the consensus sequence in SEQ ID NO:64). Thus, Z at position 1 in SEQ ID NO:64 can also be absent (as in SEQ ID NO:60), A (as in SEQ ID NO:61), or U (as in SEQ ID NOs:63-64), in which this subset of substitutions is defined as a conservative subset. Similarly, for each Z at each position for the consensus sequences (SEQ ID NOs:64-65), conservative subsets can be determined based on FIG. **17**, and these consensus sequences include nucleic acid sequences encompassed by such conservative subsets. Gray highlight indicates a conserved nucleic acid, and the dash indicates an absent nucleic acid.

[0107] FIG. **12** shows non-limiting nucleic acid sequences of a guiding component (e.g., a synthetic, non-naturally occurring guiding component) having a generic structure of A-L-B, in which A includes a first portion (e.g., any one of SEQ ID NOs:20-32, or a fragment thereof), L is a linker (e.g., a covalent bond, a nucleic acid sequence, a fragment of any one of SEQ ID NOs:40-54 and 60-65, or any other useful linker), and B is a second portion (e.g., any one of SEQ ID NOs:40-54 and 60-65, or a fragment thereof). Also provided are various embodiments of single-stranded guiding components (SEQ ID NOs:80-93). Exemplary non-limiting guiding components include SEQ ID NO:81, or a fragment thereof, where X at each position is defined as in SEQ ID NO:26 and Z at each position is as defined in SEQ ID NO:48; SEQ ID NO:82, or a fragment thereof, where X at each position is defined as in SEQ ID NO:27 and Z at each position is as defined in SEQ ID NO:49; SEQ ID NO:83, where X at each position is defined as in SEQ ID NO:28 and Z at each position is as defined in SEQ ID NO:49; SEQ ID NO:84, or a fragment thereof, where X at each position is defined as in SEQ ID NO:27 and Z at each position is as defined in SEQ ID NO:65; SEQ ID NO:85, or a fragment thereof, where X at each position is defined as in SEQ ID NO:28 and Z at each position is as defined in SEQ ID NO:65; SEQ ID NO:86, or a fragment thereof, where X at each position is defined as in SEQ ID NO:29 and Z at each position is defined as in SEQ ID NO:51; SEQ ID NO:87, or a fragment thereof, where X at each position is defined as in SEQ ID NO:30 and Z at each position is defined as in SEQ ID NO:51; SEQ ID NO:88, or a fragment thereof, where X at each position is defined as in SEQ ID NO:30 and Z at each position is defined as in SEQ ID NO:52; SEQ ID NO:89, or a fragment thereof, where X at each position is defined as in SEQ ID NO:30 and Z at each position is defined as in SEQ ID NO:65; SEQ ID NO:90, or a fragment thereof, where X at each position is defined as in SEQ ID NO:31 and Z at each position is defined as in SEQ ID NO:51; SEQ ID NO:91, or a fragment thereof, where X at each position is defined as in SEQ ID NO:32 and Z at each position is as defined in SEQ ID NO:53; SEQ ID NO:92, or a fragment thereof, where X at each position is defined as in SEQ ID NO:32 and Z at each position is as defined in SEQ ID NO:54; and SEQ ID NO:93, or a fragment thereof, where X at each position is defined as in SEQ ID NO:32 and Z at each position is defined as in SEQ ID NO:65. The fragment can include any useful number of nucleotides (e.g., one or more contiguous nucleotides, such as a fragment including about 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 20, or more contiguous nucleotides of any sequences described herein, such as a sequence for the first portion, e.g., any one of SEQ ID NOs:20-32; and also such as a fragment including about 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 20, 24, 26, 28, 30, 32, 34, 38, 36, 40, or more contiguous nucleotides of any sequences described herein, such as a sequence for the first portion, e.g., any one of SEQ ID NOs:40-54 and 60-65).

[0108] FIG. **13** shows additional non-limiting nucleic acid sequences of a guiding component (e.g., a synthetic, non-naturally occurring guiding component). Provided are various embodiments of single-stranded guiding components (SEQ ID NOs:100-103). Exemplary non-limiting guiding components include SEQ ID NO:100, or a fragment thereof, where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and n is A, C, T, G, U, or modified forms thereof; and where n at each of positions 93-192 can be present or absent such that this region can contain anywhere from 3 to 100 nucleotides and n is A, C, T, G, U, or modified forms thereof; SEQ ID NO:101, or a fragment thereof, where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and

n is A, C, T, G, U, or modified forms thereof; and where n at each of positions 93-192 can be present or absent such that this region can contain anywhere from 3 to 100 nucleotides and n is A, C, T, G, U, or modified forms thereof; SEQ ID NO:102, or a fragment thereof, where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and n is A, C, T, G, U, or modified forms thereof, and SEQ ID NO:103, or a fragment thereof, where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and n is A, C, T, G, U, or modified forms thereof.

[0109] FIG. **14**A-C are TEM micrographs showing exemplary stellate nanoparticles in various levels of magnification.

[0110] FIG. **15** is a graph showing porosimetry data.

[0111] FIG. **16** is a graph showing Cas9 loading and release in exemplary LC-MSNs by densitometry.

[0112] FIG. **17**A-C are graphs showing editing efficiency of exemplary loaded LC-MSNs and comparative examples.

[0113] FIG. **18**A-D are graphs showing size, charge, polydispersity, and colloidal stability information of exemplary particles and comparative examples.

[0114] FIG. **19** is a schematic showing the mechanism of Example 5.

DETAILED DESCRIPTION

[0115] The present disclosure relates to particle-based constructs configured to transport a CRISPR-based cargo in vivo and in vitro as an attempt to overcome a major hurdle in clinical translation of CRISPR-based countermeasures. The construct is directed to attacking viral infections by targeting critical host factor or viral genomes directly. In particular embodiments, the construct includes a porous nanoparticle core, in which the pores are employed to completely or partially confine the cargo. Nanoparticles of various pore sizes were screened to determine those most effective in vitro editing efficiency, stability, and monodispersity. The construct and cargo are encapsulated partially or completely by a coating comprising a cationic lipid, a pegylated lipid, a zwitterionic lipid, and a sterol.

[0116] The construct can include any useful component and can be assembled in any useful process. FIG. **1**A provides an exemplary method **100** for assembling a construct. As can be seen, the method can include providing a core **101** including a plurality of pores **102**. The pores are inside or part of the external surface. Furthermore, a first pore can optionally be connected to or coupled to a second pore. The pores can be characterized in any useful manner, such as, e.g., by an average dimension of the plurality of pores.

[0117] Optionally, the method can include expanding the pores present in the core. In this instance, the pores of the initial core can be characterized by a first dimension. After expansion, the initial core can include a plurality of expanded pores, where an average dimension of the plurality of expanded pores is characterized by a second dimension of the same type (e.g. length, width, or height) that is greater than the first dimension. Pore expansion can be accomplished in any useful manner, e.g., by use of a swelling agent to expand an initial pore size to a larger pore size.

[0118] Spacers can optionally be used to attach a cargo to the core. As seen in FIG. **1**A, the method can optionally include installing **110** a spacer **103** to be disposed within at least one pore and/or upon the external surface of the core. The spacer can be installed by use of a linking agent (e.g., L^1-R^L-L^2, in which R^L is a linking group such as any described herein; each of L^1 and L^2 is, independently, a reactive group such as any functional group described herein; and each of L^1 and L^2 can be the same or different). The linking agent can include a first reactive group to form a bond with the core, as well as a second reactive group to form a bond with the cargo. The linking agent may be divalent (having two reactive groups) or multivalent (having more than two reactive groups). If a linker is not used, certain cargo can be loaded through electrostatic interacations.

[0119] The cargo can be introduced to the core to provide a loaded core. As seen in FIG. **1**A, the method can include binding **120** a cargo **104** to a spacer. In one non-limiting instance, the installed spacer **103** can include a reactive group that interacts with a reactive group present on the cargo, thereby forming a bond (e.g., a covalent or non-covalent bond). In other embodiments, the cargo itself has a reactive group that interacts directly with the core surface (particle).

[0120] Then, an outer layer **105** is provided on the external surface of the core **130**. The outer layer includes a blend of lipids and a sterol. In one instance, the method includes providing an outer layer **130**, thereby forming an exemplary construct. The outer layer **105** can be formed, e.g., by exposing the loaded core to a lipid and sterol formulation to form the outer layer **105**.

[0121] The outer layer can include one or more moieties (e.g., targeting ligands). These moieties can be introduced before or after providing the outer layer. The cargo should be loaded before the outer layer. In yet other embodiments, the moieties can be introduced simultaneously with providing the outer layer. In forming the outer layer **105**, a lipid formulation including cationic and anionic lipids, the sterol components (e.g., cholesterol), and the targeting ligand moieties can be prepared; and the resulting formulation can be used to form the outer layer. As seen in FIG. **1**A, the method can includes providing **140** one or more moieties **106**, thereby forming an exemplary construct.

[0122] As seen in FIG. **1**A, one exemplary construct includes a core **101** having a plurality of pores **102**, a spacer **103** disposed within a pore, a cargo **104** attached to the spacer, and an outer layer **105** optionally including a moiety **106**. FIG. **1**B provides another exemplary construct **1000** having interconnected pores **1002** within the core **1001**, spacers **1003** disposed on an external surface of the core, a cargo **1004** attached to the spacer, and an outer layer **1005**. In another instance, FIG. **1**C provides yet another exemplary construct **1500** having interconnected pores **1502** within the core **1501**, spacers **1503** disposed on an external surface of the core or within a pore, a cargo **1504** attached to the spacer, and an outer layer **1505**.

[0123] A particular embodiment with a highly effective outer layer is shown in FIG. **2**. The mesoporous nanoparticle **202** has a CRISPR-Cas9 cargo **204** disposed in its pores, and the core is at least partially encapsulated by an outer coating **206** comprising four components, a cationic lipid, DOTAP **208**, a sterol, cholesterol **210**, a zwitterionic lipid DOPE **212**, and a Pegylated lipid, DSPE-PEG2000 **214**. A PEG portion **216** of the DSPE-PEG2000 component **214** extends outward from the encapsulating outer coating **206**. The Examples below shows the efficient delivery of CRISPR-56

Cas9 RNP components using lipid coated mesoporous silica nanoparticles (LC-MSN) modified from previous studies.

[0124] Core

[0125] In an embodiment, the core is a particle, providing a surface upon which an outer layer and a cargo can be supported. In other non-limiting embodiments, the core provides a charged surface that allows for electrostatic interactions with the cargo and/or the outer layer, or a portion thereof.

[0126] In one instance, the core can be characterized by a first dimension (e.g., core circumference, pore size of the core, core diameter, core length, or core width). Exemplary values for a core dimension (e.g., core circumference, core diameter, core length, or core width, as well as an average or mean value for any of these) include, without limitation, greater than about 1 nm (e.g., greater than about 5 nm, 10 nm, 20 nm, 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 125 nm, 150 nm, 200 nm, 300 nm, 500 nm, 750 nm, 1 m, 2 m, 5 m, 10 m, or 20 m), including from about 5 nm to about 300 nm (e.g., from 5 nm to 20 nm, 5 nm to 30 nm, 5 nm to 40 nm, 5 nm to 50 nm, 5 nm to 75 nm, 5 nm to 100 nm, 5 nm to 150 nm, 5 nm to 200 nm, 5 nm to 250 nm, 10 nm to 20 nm, 10 nm to 30 nm, 10 nm to 40 nm, 10 nm to 50 nm, 10 nm to 75 nm, 10 nm to 100 nm, 10 nm to 150 nm, 10 nm to 200 nm, 10 nm to 250 nm, 10 nm to 300 nm, 25 nm to 30 nm, 25 nm to 40 nm, 25 nm to 50 nm, 25 nm to 75 nm, 25 nm to 100 nm, 25 nm to 150 nm, 25 nm to 200 nm, 25 nm to 250 nm, 25 nm to 300 nm, 50 nm to 75 nm, 50 nm to 100 nm, 50 nm to 150 nm, 50 nm to 200 nm, 50 nm to 250 nm, 50 nm to 300 nm, 75 nm to 100 nm, 75 nm to 150 nm, 75 nm to 200 nm, 75 nm to 250 nm, 75 nm to 300 nm, 100 nm to 150 nm, 100 nm to 200 nm, 100 nm to 250 nm, 100 nm to 300 nm, 150 nm to 200 nm, 150 nm to 250 nm, 150 nm to 300 nm, 200 nm to 250 nm, 200 nm to 300 nm, 250 nm to 300 nm, or 275 nm to 300 nm).

[0127] In one instance, the particle includes a porous core (e.g., a silica core that is spherical and ranges in diameter from about 10 nm to about 250 nm (e.g., having a mean diameter of about 150 nm)). In particular embodiments, the silica core is monodisperse in size distribution.

[0128] The core can be further characterized by an electrostatic property. In some embodiments, the core has a negative charge (e.g., a net negative charge), such as a zeta potential of from about −10 mV to about −200 mV (e.g., from −10 mV to −100 mV, −10 mV to −75 mV, −10 mV to −50 mV, −10 mV to −30 mV, −15 mV to −100 mV, −15 mV to −75 mV, −15 mV to −50 mV, −15 mV to −30 mV, −20 mV to −100 mV, −20 mV to −75 mV, −20 mV to −50 mV, −20 mV to −30 mV, −30 mV to −100 mV, −30 mV to −75 mV, −30 mV to −50 mV, −40 mV to −100 mV, −40 mV to −75 mV, −40 mV to −50 mV, −50 mV to −100 mV, −50 mV to −75 mV, −60 mV to −100 mV, or −60 mV to −75 mV). Zeta potential measurements are obtained using a Malvern Zetasizer.

[0129] The core can be porous. In particular embodiments, the pore has a dimension (e.g., average pore size, pore diameter, pore radius, pore circumference, pore length, pore width, or pore depth) that is greater than about 0.5 nm (e.g., of from about 0.5 nm to about 30 nm, including from 0.5 nm to 10 nm, 0.5 nm to 20 nm, 0.5 nm to 25 nm, 1 nm to 10 nm, 1 nm to 15 nm, 1 nm to 20 nm, 1 nm to 25 nm, 1 nm to 30 nm, 2 nm to 5 nm, 2 nm to 10 nm, 2 nm to 20 nm, 2 nm to 25 nm, or 2 nm to 30 nm).

[0130] A particle or a portion thereof (e.g., a core) may have a variety of shapes and cross-sectional geometries that may depend, in part, upon the process used to produce the particles. The core or particle can be a nanoparticle (e.g., having a diameter less than about 1 m) or a microparticle (e.g., having a diameter greater than or equal to about 1 m). In one embodiment, a core or particle may have a shape that is a sphere, a donut (toroidal), a rod, a tube, a flake, a fiber, a plate, a wire, a cube, or a whisker. A collection of cores may have two or more of the aforementioned shapes. In one embodiment, a cross-sectional geometry of the core may be one or more of circular, ellipsoidal, triangular, rectangular, or polygonal. In one embodiment, a core may consist essentially of non-spherical cores. For example, such cores may have the form of ellipsoids, which may have all three principal axes of differing lengths, or may be oblate or prelate ellipsoids of revolution. Non-spherical cores alternatively may be laminar in form, wherein laminar refers to particles in which the maximum dimension along one axis is substantially less than the maximum dimension along each of the other two axes. Non-spherical cores may also have the shape of frusta of pyramids or cones, or of elongated rods. In one embodiment, the cores may be irregular in shape. In one embodiment, a plurality of cores may consist essentially of spherical cores. Particles and cores for use in the present disclosure may be PEGylated and/or aminated as otherwise described in Int. Pub. Nos. WO 2015/042268 and WO 2015/042279, which is incorporated herein by reference in their entirety.

[0131] The particle size distribution (e.g., size of the core for the protocell or a size of the silica carrier), according to the present disclosure, depends on the application, but is principally monodisperse (e.g., a uniform sized population varying no more than about 5-20% in diameter, as otherwise described herein). In certain embodiments, particles or cores can range, e.g., from around 1 nm to around 500 nm in size, including all integers and ranges there between. The size is measured as the longest axis of the core. In various embodiments, the cores are from around 5 nm to around 500 nm and from around 10 nm to around 100 nm in size. In certain alternative embodiments, the cores or particles are monodisperse and range in size from about 25 nm to about 300 nm. The sizes used include 50 nm (+/−10 nm) and 150 nm (+/−15 nm), within a narrow monodisperse range, but may be more narrow in range.

[0132] When the core is porous, the pores can be from around 0.5 nm to about 25 nm in diameter, often about 1 to around 20 nm in diameter, including all integers and ranges there between. In one embodiment, the pores are from around 1 to around 10 nm in diameter. In one embodiment, around 90% of the pores are from around 1 to around 20 nm in diameter. In another embodiment, around 95% of the pores are around 1 to around 20 nm in diameter.

[0133] In certain embodiments, cores or particles according to the disclosed technology: are monodisperse and range in size from about 25 nm to about 300 nm; are anionic, neutral or cationic for specific targeting (e.g, cationic); are optionally modified with agents such as PEI (polyethylene imine), NMe^{3+}, dye, crosslinker, ligands (ligands provide neutral charge); and optionally, are used in combination with a cargo to be delivered to the target.

[0134] In some embodiments, these cores or particles are often monodisperse and provide colloidally stable compositions. These compositions can be used to target host cells

because of enhanced biodistribution/bioavailability of these compositions, and optionally, specific cells, with a wide variety of therapeutic and/or diagnostic agents that exhibit varying release rates at the site of activity.

[0135] The cores can be produced, for example, by templating with a surfactant, a cross-linked micelle, or a detergent (see, e.g., Gao F et al., *J Phys. Chem. B.* 2009; 113:1796-804; Lin Y S et al., *Chem. Mater.* 2009; 21(17): 3979-86; Carroll N J et al., *Langmuir* 2009; 25(23):13540-4; and Zhang K et al., *J. Am. Chem. Soc.* 2013 Feb. 20; 135(7):2427-30). In yet another instance, cores are formed by dendritic growth (see, e.g., Shen D et al., *Nano Lett.* 2014; 14(2):923-32). In some instances, the cores are formed by expanding a pore, e.g., by use of a swelling agent, such as an alkylbenzene (e.g., 1,3,5-trimethylbenzene or triisopropylbenzene), an alkane (e.g., heptane, decane, or dodecane), a glycol (e.g., poly(propylene glycol)), or a tertiary amine (see, e.g., Kim M H et al., *ACS Nano* 2011; 5(5):3568-76; and Na H K et al., *Small* 2012; 8(11):1752-61). In other instances, cores are formed by an aerosol process, such as EISA (see, e.g., Lu Y et al., *Nature* 1999; 398:223-6; and Durfee P N et al., *ACS Nano* 2016; 10:8325-45).

[0136] Each batch of cores or particles can be characterized by, for example, assessment of size and polydispersity using dynamic light scattering (DLS) (NIST-NCL PCC-1), and surface charge or zeta potential measurements (e.g., with a Zetasizer instrument (Malvern Instruments, Ltd) (NIST-NCL PCC-2 (charge and zeta potential), and verification of low endotoxin contamination per health industry product standards (NCL STE-1.1). Resultant cores can be further processed, such as by modifying core condensation (e.g., by using acidified ethanol for silica) or modifying core surface charge (e.g., by use of amine-containing silanes, such as APTES).

[0137] The core can be formed of, for example, a metal oxide, alum, or silica, including mesoporous forms thereof). In particular embodiments, the core is composed of a mesoporous silica nanoparticle (MSN). Exemplary, non-limiting MSNs for use in the disclosed technology are described in Int. Pub. Nos. WO 2015/042268 and WO 2015/042279, each of which is incorporated herein in its entirety.

[0138] In an embodiment, a stellate particle is used as the core. A stellate particle has a radial pore morphology with a small particle diameter. It also has more uniform polydispersity (FIGS. **18**A and **18**C) and colloidal stability (FIG. **18**D) compared to other particle types such as small-pore hexagonal prism, dendritic MSN with 8 or 18 nm pores, and non-porous Stober MSN. FIG. **18**D shows comparison of the RNP-loaded MSN with and without lipids. FIG. **18**C shows size measurements during LC-MSN assembly. The FIG. **18**B illustrates the overall charge of the particle. Neutrally charged particles are more stable in vivo than charged particles. The combination of the negatively charged nanoparticle cores (MSN) with the positively charged lipid (cationic lipid), has a neutral charge if particles are unloaded (LC-MSN) or even when loaded with RNP (RNP-LC-MSN and 488-RNP-LC-MSN). The negatively charged core and positively charged (cationic) lipid promotes in vivo stability and delivery efficacy.

[0139] The synthesis of stellate MSN involves a base-catalyzed condensation reaction of tetraorthosilicate with a surfactant that acts as a substrate. A single synthesis reaction can yield between 500-800 milligrams of MSN that are stable when stored in ethanol for over a year.

[0140] DLS and TEM were used to assess the size and morphology of stellate MSN (FIG. **14**A-C). The unique radiating arm morphology of the stellate particles can be seen in FIGS. **14**A-C. The average size (diameter) of stellate particles may be, for example, about 75 to 400 nm, such as about 100 to 200 nm, or about 110 to 160 nm. Arm length for the stellate particle may range from about 30 to 200 nm, such as about 50 to 100 nm, or about 55 to 80 nm. The average diameter may be assessed by a Zetasizer instrument (Malvern Instruments, Ltd). Porosimetry was performed with nitrogen adsorption-desorption analysis to determine the pore size range, which may be, for example, 3 to 20 nm, such as 5 to 15 nm or 6.5 to 10 nm (FIG. **15**).

[0141] While MSNs are promising therapeutic carriers, MSNs can have low colloidal stability and are subject to aggregation in physiological solutions, reducing circulation time and preventing desirable cell uptake. Similarly, permanently charged cationic liposomes are successfully used as nucleic acid transfection reagents in cell culture, however, they have limited in vivo stability. The net neutral charge (FIG. **18**B) of the RNP loaded LC-MSNs overcomes challenges presented by these individual components to enable improvements in colloidal stability and subsequent circulation time, with biocompatibility and lower cytoxicity.

[0142] In an embodiment, a spacer can be employed to attach a core (e.g., an external surface and/or a pore of the core) to one or more cargos. A spacer can include, for example, a bond (e.g., a covalent bond or a coordination bond), an atom, a molecule, a nucleic acid, or a protein. A spacer can be provided as a linking agent, which in turn reacts with a reactive group (e.g., a functional group present on the core or the cargo) to form a bond. Thus, a reacted linking agent can result in a spacer present between the core and the cargo.

[0143] A spacer can include a coordination bond. In some instances, the coordination bond includes one or more functional groups that form a bond to a metal (e.g., a divalent metal). Exemplary functional groups include an amino, an amido, a carboxyl, a thiol, a heterocyclyl (e.g., a heteroaryl, imidazolyl, etc.), or an amino acid (e.g., histidine, cysteine, lysine, etc.), as well as chelate forms thereof (e.g., as in iminodiacetic acid or nitriloacetic acid). Exemplary metals include nickel, cobalt, copper, iron, or zinc, as well as cationic forms thereof.

[0144] A non-zero length spacer can include a linking group. In some instances, a linking agent (e.g., to form the non-zero length spacer) includes at least two reactive groups and a linking group disposed between the reactive groups. In some instances, a first reactive group forms a bond with the core, and a second reactive group forms a bond with the cargo. The linking group can be, for example, an optionally substituted alkylene, heteroalkylene, arylene, nucleic acid, or peptide, and can have a functionality, such as, for example, a cleavable moiety, thereby detaching the cargo from the core.

[0145] The spacer can optionally include a cleavable moiety. Exemplary cleavable moieties include a labile group, or a scissile group, including but not limited to a disulfide bond.

[0146] The spacer can be provided as a linking agent, which in turn reacts with a reactive group (e.g., a functional group present on the core or the cargo) to form a bond. In some instances, the linking agent is L^1-R^L-L^2, in which R^L

is a linking group (e.g., any useful chemical group, such as a covalent bond, a nucleic acid sequence, a monomer, etc.) and each of L^1 and L^2 is, independently, a reactive group (e.g., a functional group that is one of a cross-linker group, a binding group, or a click-chemistry group, such as any described herein), and in which each of L^1 and L^2 can be the same or different.

[0147] FIG. **4**A provides an exemplary linking agent $L^{1'}$-Lk-$L^{1''}$ (compound L1), where Lk is a linking group and where each of $L^{1'}$ and $L^{1''}$ is, independently, a reactive group (e.g., a functional group that is one of a cross-linker group, a binding group, or a click-chemistry group, such as any described herein). In some instances, a reactive group can include a protecting group (e.g., any described herein), which provides a reactive group upon exposure to particular chemical or biological conditions (e.g., an acidic condition, a basic condition, the presence of a protease, etc.).

[0148] As seen in FIG. **4**A, a first group of the linking agent can be used to react with a core (NP), thereby providing a NP-spacer. A second group of the linking agent can then react with a functional group R^1 of the cargo, thereby providing a NP-spacer-cargo construct. Any useful linking agent and spacer can be employed.

[0149] FIG. **4**B provided an exemplary spacer present between a core (NP) and the cargo. The spacer -Lk-L"*-R*, in which Lk is a linking group (e.g., any described herein), L"* is a reactive group of the linking agent (that underwent a reaction), and R^1 is a second reactive group present on the cargo (that underwent a reaction). The dashed line indicates that the bond can be covalent or non-covalent.

[0150] Further spacers are provided in FIG. **4**C. In some embodiments, the spacer can include a covalent bond between reacted L"*and reacted X. In one instance, L"*-X can be represented by a reacted sulfone group of the linking agent, a reacted Lys of the cargo, and an alkylene group between the sulfone and Lys. In another instance, two groups on the cargo react with the linking agent, thereby providing a multivalent spacer L"*<$(His)_2$ between the NP and the cargo. In other embodiments, the spacer can include a non-covalent bond between reacted L"* and reacted X. In one instance, L"*-X can be represented by a chelated Ni^{2+} of the linking agent, a chelated His of the cargo, and a coordination bond between the nickel and His. In another instance, the linking agent provides a reactive group L*, the cargo includes a reactive His, and an intermediate Ni^{2+} is provided to provide a chelating bridge between the linking agent and the cargo.

[0151] FIG. **4**D provided another exemplary spacer present between a core (NP) and the cargo. The spacer -Lk^1-L^C-Lk^2-L"*-R^1*, in which Lk^1 is a first linking group (e.g., any described herein), L^C is a cleavable moiety (e.g., any described herein), Lk^2 is a second linking group (e.g., any described herein), L"* is a reactive group of the linking agent (that underwent a reaction), and R^1* is a second reactive group present on the cargo (that underwent a reaction). The dashed line indicates that the bond can be covalent or non-covalent. Exemplary linking groups (e.g., for Lk^1 and/or Lk^2) includes an optionally substituted alkylene group, an optionally substituted heteroalkylene group, or a poly(ethylene glycol) group.

[0152] A cleavable moiety (L^C) can include any useful moiety capable of releasing a bound cargo upon exposure to a particular cleaving condition or cleaving agent. In one non-limiting embodiment, the cleavable moiety includes a disulfide group (e.g., —S—S—), in which the cleaving condition includes a reducing condition and the cleaving agent is a reducing agent (e.g., dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), or (2S)-2-amino-1,4-dimercaptobutane (DTBA)). In another non-limiting embodiment, the cleavable moiety includes a hydrazone group (e.g., >C═N—NH—), in which the cleaving condition includes an acidic condition and the cleaving agent is an acidic agent (e.g., an acid having a pH less than about 4.5).

[0153] A reactive linking group of the linking agent (L" or L"*) can include any useful moiety, such as one or more anionic moieties (e.g., a chelating anionic moiety, such as a polycarboxylic acid, a carboxylic acid, a carbonate, etc.) and one or more cationic moieties (e.g., a chelated cationic metal, such as a cationic transition metal, including Co^{2+}, Ni^{2+}, Fe^{2+}, Cu^{2+}, or Zn^{2+}). Further exemplary anionic moieties can include those having one or more carboxylic or carbonate moieties, such as iminodiacetic acid (IDA), nitrilotriacetic acid (NTA), ethylenediamine tetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) (EGTA), (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) (BAPTA), carboxylmethylaspartate (CMA), as well as acidic and basic forms thereof.

[0154] A second reactive group present on the cargo (R^1 or R^1*) can include any useful moiety capable of forming a bond with the reactive linking group of the linking agent. In one non-limiting embodiment, the reactive linking group includes a cationic moiety, and the second reactive group present on the cargo is a moiety capable of forming a bond with the cationic moiety. Exemplary second reactive groups include one or more histidine residues located at any useful position of the cargo (e.g., at the N-terminus or the C-terminus for a protein cargo).

[0155] FIG. **4**E provides an exemplary linking agent $L^{1'}$-Lk^1-L^C-Lk^2-$L^{1''}$ (compound L2), where Lk^1 and Lk^2 are linking groups, where L^C is a cleavable moiety, and where each of $L^{1'}$ and $L^{1''}$ is, independently, a reactive group (e.g., a functional group that is one of a cross-linker group, a binding group, or a click-chemistry group, such as any described herein). A first group of the linking agent L2 can be used to react with a core (NP), thereby providing a NP-spacer. A second group of the linking agent can then react with a functional group R^1 of the cargo, thereby providing a NP-spacer-cargo construct. Any useful linking agent and spacer can be employed. Next, as the linker as a cleavable moiety L^C, the construct can be exposed to a cleaving agent that reacts with L^C to provide a released particle (released NP) and a released cargo.

[0156] FIG. **5**A-**5**B provides schematics of exemplary reaction schemes for linking agents and spacers. As seen in FIG. **5**A, the spacer can include multiple coordination bonds (i), multiple covalent bonds (ii), or a single covalent bond (iii) between the core and the cargo. Such spacers can employ any useful linking agent and reaction schemes. FIG. **5**B provides an exemplary reaction scheme in which the linking agent includes a reactive group L" having an alkene and a sulfone leaving group. The reactive group R^1 of the cargo participates in an addition reaction with L", thereby providing a single covalent bond present in the spacer. Then, a second reactive group R^2 of the cargo participated in another addition reaction with the linking agent, thereby providing a second covalent bond present in the spacer. FIG. **5**C provides an exemplary reaction scheme in which the

linking agent includes a reactive group L" having an alkene and a sulfone leaving group, and the reactive group R^1 of the cargo participates in an addition reaction to provide a single covalent bond. Other exemplary spacers and linking agents are described in Cong Y et al., *Bioconjug. Chem.* 2012; 23(2):248-63; Liberatore F A et al., *Bioconjug. Chem.* 1990; 1(1):36-50; Han D H et al., *Nature Commun.* 2014; 5(5): 5633; and Shen D et al., *Nano Lett.* 2014; 14(2):923-32, each of which is incorporated herein by reference in its entirety.

[0157] Reactive groups can be present on any useful bonding components, such as spacers, linking agents, a surface of the core, and/or a cargo. Pairs of reactive groups can be chosen to facilitate any useful reaction between any bonding components. In one instance, the first bonding component includes a nucleophilic reactive group (e.g., an amino group, a thio group, a hydroxyl group, an anion, etc.), and the second bonding component includes an electrophilic reactive group (e.g., an alkenyl group, an alkynyl group, a carbonyl group, an ester group, an imido group, an epoxide group, an amido group, a carbamido group, a cation, etc.).

[0158] Exemplary reactive groups include any chemical group configured to form a bond. In general, a first chemical group reacts with a second chemical group to form a bond (e.g., a covalent bond), in which the first and second chemical groups form a reactive pair.

[0159] In one instance, the reactive group is a cross-linker group. In another non-limiting instance, the reactive pair is a cross-linker reaction pair, which includes a first cross-linker group and a second cross-linker group that reacts with that first cross-linker group. Exemplary cross-linker groups and cross-linker reaction pairs include those for forming a covalent bond between a carboxyl group (e.g., —CO_2H) and an amino group (e.g., —NH_2); or between an imido group (e.g., maleimido or succinimido) and a thiol group (e.g., —SH); or between an epoxide group and a thiol group (e.g., —SH); or between an epoxide group and an amino group (e.g., —NH_2); or between an ester group (e.g., —CO_2R, in which R is an organic moiety, such as optionally substituted alkyl, aryl, etc.) and an amino group (e.g., —NH_2); or between an carbamido group (e.g., —NHC(O)Het, where Het is a N-containing heterocyclyl) and an amino group (e.g., —NH_2); or between a phospho group (e.g., —P(O)$(OH)_2$) and an amino group (e.g., —NH_2), such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and dicyclohexylcarbodiimide (DCC), optionally used with N-hydroxysuccinimide (NHS) and/or N-hydroxysulfosuccinimide (sulfo-NHS). Other cross-linkers include those for forming a covalent bond between an amino group (e.g., —NH_2) and a thymine moiety, such as succinimidyl-[4-(psoralen-8-yloxy)]-butyrate (SPB); a hydroxyl group (e.g., —OH) and a sulfur-containing group (e.g., free thiol, —SH, sulfhydryl, cysteine moiety, or mercapto group), such as p-maleimidophenyl isocyanate (PMPI); between an amino group (e.g., —NH_2) and a sulfur-containing group (e.g., free thiol, —SH, sulfhydryl, cysteine moiety, or mercapto group), such as succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) and/or succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC); between a sulfur-containing group (e.g., free thiol, —SH, sulfhydryl, cysteine moiety, or mercapto group) and a carbonyl group (e.g., an aldehyde group, such as for an oxidized glycoprotein carbohydrate), such as N-beta-maleimidopropionic acid hydrazide-trifluoroacetic acid salt (BMPH), 3-(2-pyridyldithio)propionyl hydrazide (PDPH), and/or a 3-(2-pyridyldithio)propionyl group (PDP); and between a maleimide-containing group and a sulfur-containing group (e.g., free thiol, —SH, sulfhydryl, cysteine moiety, or mercapto group). Yet other cross-linkers include those for forming a covalent bond between two or more unsaturated hydrocarbon bonds, e.g., such as a reaction of forming a covalent bond between a first alkene group and a second alkene group.

[0160] In another instance, the reactive group is a binding group. In another non-limiting instance, the reactive pair is a binding reaction pair, which includes a first binding group and a second binding group that reacts with that first binding group. Exemplary binding groups and binding reaction pairs include those for forming a bond between biotin and avidin, biotin and streptavidin, biotin and neutravidin, desthiobiotin and avidin (or a derivative thereof, such as streptavidin or neutravidin), hapten and an antibody, an antigen and an antibody, a primary antibody and a secondary antibody, and lectin and a glycoprotein.

[0161] In yet another instance, the reactive group is a click-chemistry group. In another non-limiting instance, the reactive pair is a click-chemistry reaction pair, which includes a first click-chemistry group and a second click-chemistry group that reacts with that first click-chemistry group. Exemplary click-chemistry groups include, e.g., a click-chemistry group, e.g., one of a click-chemistry reaction pair selected from the group consisting of a Huisgen 1,3-dipolar cycloaddition reaction between an alkynyl group and an azido group to form a triazole-containing spacer; a Diels-Alder reaction between a diene having a 4π electron system (e.g., an optionally substituted 1,3-unsaturated compound, such as optionally substituted 1,3-butadiene, 1-methoxy-3-trimethylsilyloxy-1,3-butadiene, cyclopentadiene, cyclohexadiene, or furan) and a dienophile or heterodienophile having a 2π electron system (e.g., an optionally substituted alkenyl group or an optionally substituted alkynyl group); a ring opening reaction with a nucleophile and a strained heterocyclyl electrophile; and a splint ligation reaction with a phosphorothioate group and an iodo group; and a reductive amination reaction with an aldehyde group and an amino group.

[0162] Exemplary reactive groups include an amino (e.g., —NH_2), a thio (e.g., a thioalkoxy group or a thiol group), a hydroxyl, an ester (e.g., an acrylate), an imido (e.g., a maleimido or a succinimido), an epoxide, an isocyanate, an isothiocyanate, an anhydride, an amido, a carbamido (e.g., a urea derivative), an azide, an optionally substituted alkynyl, or an optionally substituted alkenyl.

[0163] Exemplary linking groups include any moiety, including any useful subunit, which can be optionally repeated, that provides a spacer having any useful property. Exemplary linking groups include a bond (e.g., a covalent bond), optionally substituted alkylene, optionally substituted heteroalkylene (e.g., poly(ethylene glycol)), optionally substituted arylene, and optionally substituted heteroarylene. Yet other exemplary linking groups are those including an ethylene glycol group, e.g., —OCH_2CH_2—, including a poly(ethylene glycol) (PEG) group —$(OCH_2CH_2)_n$—, a four-arm PEG group (such as $C(CH_2O(CH_2CH_2O)_n—)_4$ or $C(CH_2O(CH_2CH_2O)_nCH_2—)_4$ or $C(CH_2O(CH_2CH_2O)_nCH_2CH_2—)_4$ or $C(CH_2O(CH_2CH_2O)_nCH_2CH_2NHC(O)CH_2CH_2)_4$ $C(CH_2O(CH_2CH_2O)_nCH_2C(O)O—)_4$), an eight-arm PEG group (such as —$(OCH_2CH_2)_nO$ $[CH_2CHO((CH_2CH_2O)_n—)CH_2O]_6(CH_2CH_2O)_n$ or —CH_2

$(OCH_2CH_2)_nO[CH_2CHO((CH_2CH_2O)_n CH_2)CH_2O]_6(CH_2CH_2O)_nCH_2$— or —$CH_2CH_2(OCH_2CH_2)_nO[CH_2CHO((CH_2CH_2O)_n CH_2CH_2)CH_2O]_6(CH_2CH_2O)_nCH_2CH_2$— or $R(O(CH_2CH_2O)_n—)_8$ or $R(O(CH_2CH_2O)_nCH_2—)_8$ or $R(O(CH_2CH_2O)_nCH_2CH_2—)_8$, in which R includes a tripentaerythritol core), or a derivatized PEG group (e.g., methyl ether PEG (mPEG), a propylene glycol group, etc.); including dendrimers thereof, copolymers thereof (e.g., having at least two monomers that are different), branched forms thereof, start forms thereof, comb forms thereof, etc., in which n is any useful number in any of these (e.g., any useful n to provide any useful number average molar mass M_n). Yet other linking groups can include a nucleic acid, a peptide, as well as modified forms thereof.

[0164] Exemplary linking agents can include a poly(ethylene glycol) group (e.g., a multivalent poly(ethylene glycol) precursor having a reactive functional group, such as an amino group, an ester group, an acrylate group, a hydroxyl group, a carboxylic acid group, etc.), such as eight arm-PEG amine (8-arm PEG-NH_2, e.g., catalog nos. PSB-811, PSB-812, or PSB-814 available from Creative PEGWorks, Chapel Hill, NC) or an eight-arm PEG succinimidyl ester (such as 8-arm PEG succinimidyl NHS ester or 8-arm PEG-SCM (succinimidyl carboxyl methyl ester), e.g., catalog nos. PSB-841, PSB-842, or PSB-844 available from Creative PEGWorks) or an eight-arm PEG vinylsulfone or an eight-arm PEG hydroxyl or a linear PEG thiol or a linear PEG hydroxyl or poly(ethylene glycol diacrylate) (PEG-DA) or triethylene glycol acrylate (TEGA) or 2-carboxyethyl acrylate (CEA) or 2-hydroxyethylacrylate (HEA), as well as copolymers thereof and/or combinations thereof; an amino acid (e.g., a poly(amino acid) precursor or a protein, such as a poly(lysine) precursor, a poly(arginine) precursor, lysozyme, avidin, or albumin); a glycerol group (e.g., a poly(glycerol) precursor); a vinyl group (e.g., a poly(vinyl) precursor or a poly(vinyl alcohol) precursor); a hydroxyacid group (e.g., a poly(lactic acid) precursor, a poly(glycolic acid) precursor, or a poly(lactic-co-glycolic acid) precursor); an acrylate group (e.g., a poly(acrylic acid) precursor or a poly(methacrylic acid) precursor); a silyl ether group (e.g., a poly(silyl ether) precursor); an olefin group (e.g., a poly (acetylene) precursor); and/or an aromatic group (e.g., a poly(pyrrole) precursor, a poly(aniline) precursor, or a poly (thiophene) precursor).

[0165] Other exemplary, non-limiting linking agents include 3-aminopropyltrimethoxysilane (3-APTMS); (R,S)-1-(3,4-(methylenedioxy)-6-nitrophenyl)ethyl chloroformate (MenPOC); 1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl (3-(trimethoxysilyl)propyl)carbamate; phenyltrichlorosilane (PTCS); an epoxysilane; sulfo-NHS-acetate; 1-(3-(trimethoxysilyl)propyl)-1H-pyrrole-2,5-dione; 3-glycidoxypropyltrimethoxysilane (3-GPTMS); N-(3-(trimethoxysilyl)propyl)-1H-imidazole-1-carboxamide; N-(6-aminohexyl)-1H-imidazole-1-carboxamide; anhydrides; isocyanotopropyltrimethoxysilane (IPTMS); isocyanates; isothiocyanates; and maleimides.

[0166] Yet other non-limiting linking agents include a covalent spacer or a non-covalent spacer. In some embodiments: the spacer may comprise a flexible arm, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 carbon atoms. Exemplary spacers include BS3 ([bis(sulfosuccinimidyl) suberate]; BS3 is a homobifunctional N-hydroxysuccinimide ester that targets accessible primary amines), NHS/EDC (N-hydroxysuccinimide and N-ethyl-'(dimethylaminopropyl)carbodimide; NHS/EDC allows for the conjugation of primary amine groups with carboxyl groups), sulfo-EMCS ([N-e-Maleimidocaproic acid]hydrazide; sulfo-EMCS are heterobifunctional reactive groups (maleimide and NHS-ester) that are reactive toward sulfhydryl and amino groups), hydrazide (most proteins contain exposed carbohydrates and hydrazide is a useful reagent for linking carboxyl groups to primary amines), and SATA (N-succinimidyl-S-acetylthioacetate; SATA is reactive towards amines and adds protected sulfhydryls groups). Examples of other suitable spacers are succinic acid, Lys, Glu, Asp, a dipeptide such as Gly-Lys, an α-helical spacer (e.g., $A(EAAAK)_nA$, where n is 1, 2, 3, 4, or 5), an alkyl chain (e.g., an optionally substituted C_{1-12} alkylene or alkynyl chain), or a polyethylene glycol (e.g., $(CH_2CH_2O)_m$, where m is from 1 to 50).

[0167] Protecting groups can be employed to protect a reactive group and/or to provide reduced reactivity (e.g., binding) of an agent (e.g., a capture probe). Exemplary protecting groups include any described herein, including optionally substituted aryl groups, a poly(ethylene glycol) group, UV-labile groups, etc.).

[0168] Functional groups can be present on a spacer, a core, or a cargo. In addition, a functional group can include any useful chemical group, such as a reactive group or a protecting group. In some instances, the linking agent reacts with a functional group (e.g., present on the cargo or the core), thereby forming an attached spacer that can be further reacted with another functional group.

[0169] Cargos

[0170] The construct can include CRISPR components, as well as other cargos (e.g., associated with the nanoparticle core, with a pore (e.g., by way of a spacer), and/or within the outer layer). Cargos can include a variety of molecules, including peptides, proteins (e.g., including protein complexes, such as a ribonucleoprotein (RNP) complex including a nucleic acid and a protein), nucleic acids (e.g., a plasmid, mRNA), aptamers, including antisense oligonucleotides, antibodies, small molecule drugs, such as antimicrobials and/or antivirals, alpha/flavi inhibitors, coronavirus (CoV) inhibitors, carbohydrates, dyes, markers, or any other agent described herein.

[0171] The cargo can be characterized by a dimension (e.g., a cargo dimension). Exemplary dimensions for the cargo include cargo circumference, cargo diameter, cargo length, and cargo width. Exemplary dimensions (e.g., cargo circumference, diameter, length, or width) are about 2 nm to about 5000 nm (e.g., from 2 nm to 500 nm, 2 nm to 1000 nm, 2 nm to 2500 nm, 5 nm to 500 nm, 5 nm to 1000 nm, 5 nm to 2500 nm, 5 nm to 5000 nm, 25 nm to 500 nm, 25 nm to 1000 nm, 25 nm to 2500 nm, 25 nm to 5000 nm, 50 nm to 500 nm, 50 nm to 1000 nm, 50 nm tvary byo 2500 nm, 50 nm to 5000 nm, 75 nm to 500 nm, 75 nm to 1000 nm, 75 nm to 2500 nm, 75 nm to 5000 nm, 100 nm to 500 nm, 100 nm to 1000 nm, 100 nm to 2500 nm, 100 nm to 5000 nm, 500 nm to 1000 nm, 500 nm to 2500 nm, 500 nm to 5000 nm, 750 nm to 1000 nm, 750 nm to 2500 nm, 750 nm to 5000 nm, 1000 nm to 2500 nm, 1000 nm to 5000 nm, 2500 nm to 5000 nm, or 4000 nm to 5000 nm). Cargo size can be determined by dynamic light scattering (DLS) by methods disclosed above.

[0172] Exemplary cargos include an acidic, basic, and hydrophobic drug (e.g., antiviral agents, antibiotic agents, etc.); a protein (e.g., antibodies, carbohydrates, etc.); a

nucleic acid (e.g., DNA, RNA, small interfering RNA (siRNA), minicircle DNA (mcDNA), small hairpin RNA (shRNA), complementary DNA (cDNA), naked DNA, and plasmid, as well as chimeras, single-stranded forms, duplex forms, and multiplex forms thereof and including nucleic acid sequences encoding any of these and including one or more modified nucleic acids); a CRISPR component (e.g., any described herein, including a guiding component (e.g., any described herein), a nuclease, a plasmid, a plasmid that encodes a CRISPR component, a ribonucleoprotein complex, a Cas enzyme or an ortholog or homolog thereof, a guide RNA, as well as a nucleic acid sequence encoding any of these or a complement thereof); a diagnostic/contrast agent, like quantum dots, iron oxide nanoparticles, gadolinium, and indium-111; a small molecule; a carbohydrate; a drug, a pro-drug, a vitamin, an antibody, a protein, a hormone, a growth factor, a cytokine, a steroid, an anticancer agent, a fungicide, an antimicrobial, an antibiotic, an antiviral agent, etc.; a morphogen; a toxin, e.g., a bacterial protein toxin; a peptide, e.g., an antimicrobial peptide; an antigen; an antibody; a detection agent (e.g., a particle, such as a conductive particle, a microparticle, a nanoparticle, a quantum dot, a latex bead, a colloidal particle, a magnetic particle, a fluorescent particle, etc.; or a dye, such as a fluorescent dye, a luminescent dye, a chemiluminescent dye, a colorimetric dye, a radioactive agent, an electroactive detection agent, etc.); a label (e.g., a quantum dot, a nanoparticle, a microparticle, a barcode, a fluorescent label, a colorimetric label, a radio label (e.g., an RF label or barcode), avidin, biotin, a tag, a dye, a marker, an electroactive label, an electrocatalytic label, and/or an enzyme that can optionally include one or more linking agents and/or one or more dyes); a capture agent (e.g., such as a protein that binds to or detects one or more markers (e.g., an antibody or an enzyme), a globulin protein (e.g., bovine serum albumin), a nanoparticle, a microparticle, a sandwich assay reagent, a catalyst (e.g., that reacts with one or more markers), and/or an enzyme (e.g., that reacts with one or more markers, such as any described herein)); as well as combinations thereof.

[0173] The nucleic acid can be provided in any useful form, such as RNA, DNA, DNA/RNA hybrids, phage, plasmid, linear forms thereof, concatenated forms thereof, circularized forms thereof, modified forms thereof, single stranded forms thereof, double stranded forms thereof, complements thereof, and encoded forms thereof.

[0174] In some instances, the cargo includes a plasmid. The plasmid can encode any useful CRISPR component (e.g., a guiding component or a nuclease). In addition, the plasmid can express any useful polypeptide and/or nucleic acid sequence, including a nuclear localization sequence, a cell penetrating peptide, a targeting peptide, a polypeptide toxin, a small hairpin RNA (shRNA), a small interfering RNA (siRNA), a reporter (e.g., a reporter protein), etc. Additional reporters include polypeptide reporters which may be expressed by plasmids (such as histone-packaged supercoiled DNA plasmids) and include polypeptide reporters such as fluorescent green protein and fluorescent red protein. Reporters pursuant to the disclosed technology are utilized principally in diagnostic applications including diagnosing the existence or progression of a disease state (e.g., diseased tissue) in a subject or patient and/or the progress of therapy in a patient or subject. The plasmid can be of any useful form (e.g., supercoiled and/or packaged plasmid). For instance, the plasmid can be a histone-packaged supercoiled plasmid including a mixture of histone proteins. Additional CRISPR components are described herein.

[0175] Exemplary anticancer agents include chemotherapeutic agents, such as an agent selected from the group consisting of microtubule-stabilizing agents, microtubule-disruptor agents, alkylating agents, antimetabolites, epidophyllotoxins, antineoplastic enzymes, topoisomerase inhibitors, inhibitors of cell cycle progression, and platinum coordination complexes, as well as functionalized or modified forms thereof (e.g., including polyethylene glycol (PEG)). These may be selected from the group consisting of everolimus, trabectedin, abraxane, TLK 286, AV-299, DN-101, pazopanib, GSK690693, RTA 744, ON 0910.Na, AZD 6244 (ARRY-142886), AMN-107, TKI-258, GSK461364, AZD 1152, enzastaurin, vandetanib, ARQ-197, MK-0457, MLN8054, PHA-739358, R-763, AT-9263, a FLT-3 inhibitor, a VEGFR inhibitor, an EGFR TK inhibitor, an aurora kinase inhibitor, a PIK-1 modulator, a Bcl-2 inhibitor, an HDAC inhibitor, a c-MET inhibitor, a PARP inhibitor, a Cdk inhibitor, an EGFR TK inhibitor, an IGFR-TK inhibitor, an anti-HGF antibody, a PI3 kinase inhibitors, an AKT inhibitor, a JAK/STAT inhibitor, a checkpoint-1 or 2 inhibitor, a focal adhesion kinase inhibitor, a Map kinase kinase (mek) inhibitor, a VEGF trap antibody, pemetrexed, erlotinib, dasatanib, nilotinib, decatanib, panitumumab, amrubicin, oregovomab, Lep-etu, nolatrexed, azd2171, batabulin, ofatumumab, zanolimumab, edotecarin, tetrandrine, rubitecan, tesmilifene, oblimersen, ticilimumab, ipilimumab, gossypol, Bio 111, 131-I-TM-601, ALT-110, BIO 140, CC 8490, cilengitide, gimatecan, IL13-PE38QQR, INO 1001, $IPdR_1$ KRX-0402, lucanthone, LY 317615, neuradiab, vitespan, Rta 744, Sdx 102, talampanel, atrasentan, XR 311, romidepsin, ADS-100380, sunitinib, 5-fluorouracil, vorinostat, etoposide, etoposide phosphate, gemcitabine, doxorubicin, liposomal doxorubicin, 5'-deoxy-5-fluorouridine, vincristine, temozolomide, ZK-304709, seliciclib, PD0325901, AZD-6244, capecitabine, L-glutamic acid, N-[4-[2-(2-amino-4,7-dihydro-4-oxo-1 H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl]-, disodium salt, heptahydrate, camptothecin, PEG-labeled irinotecan, tamoxifen, toremifene citrate, anastrazole, exemestane, letrozole, DES (diethylstilbestrol), estradiol, estrogen, conjugated estrogen, bevacizumab, IMC-1C11, CHIR-258, 3-[5-(methylsulfonylpiperadinemethyl)-indolyl]-quinolone, vatalanib, AG-013736, AVE-0005, the acetate salt of [D-Ser(But)6, Azgly 10] (pyro-Glu-His-Trp-Ser-Tyr-D-Ser(But)-Leu-Arg-Pro-Azgly-NH_2 acetate [$C_{59}H_{84}N_{18}O_{14}$—$(C_2H_4O_2)_X$ where x=1 to 2.4], goserelin acetate, leuprolide acetate, triptorelin pamoate, medroxyprogesterone acetate, hydroxyprogesterone caproate, megestrol acetate, raloxifene, bicalutamide, flutamide, nilutamide, megestrol acetate, CP-724714, TAK-165, HKI-272, erlotinib, lapatanib, canertinib, ABX-EGF antibody, erbitux, EKB-569, PKI-166, GW-572016, lonafarnib, BMS-214662, tipifarnib, amifostine, NVP-LAQ824, suberoyl analide hydroxamic acid, valproic acid, trichostatin A, FK-228, SU11248, sorafenib, KRN951, adriamycin, aminoglutethimide, arnsacrine, anagrelide, L-asparaginase, *Bacillus* Calmette-Guerin (BCG) vaccine, bleomycin, buserelin, busulfan, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clodronate, cyproterone, cytarabine, dacarbazine, dactinomycin, daunorubicin, diethylstilbestrol, epirubicin, fludarabine, fludrocortisone, fluoxymesterone, flutamide, gemcitabine, hydroxyurea, idarubicin, ifosf-

amide, imatinib (e.g., including imatinib mesylate), leuprolide, levamisole, lomustine, mechlorethamine, melphalan, 6-mercaptopurine, mesna, methotrexate, mitomycin, mitotane, mitoxantrone, nilutamide, octreotide, oxaliplatin, pamidronate, pentostatin, plicamycin, porfimer, procarbazine, raltitrexed, rituximab, streptozocin, teniposide, testosterone, thalidomide, thioguanine, thiotepa, tretinoin, vindesine, 13-cis-retinoic acid, phenylalanine mustard, uracil mustard, estramustine, altretamine, floxuridine, 5-deooxyuridine, cytosine arabinoside, 6-mecaptopurine, deoxycoformycin, calcitriol, valrubicin, mithramycin, vinblastine, vinorelbine, topotecan, razoxin, marimastat, COL-3, neovastat, BMS-275291, squalamine, endostatin, SU5416, SU6668, EMD121974, interleukin-12, IM862, angiostatin, vitaxin, droloxifene, idoxyfene, spironolactone, finasteride, cimitidine, trastuzumab, denileukin diftitox, gefitinib, bortezimib, paclitaxel, cremophor-free paclitaxel, docetaxel, epithilone B, BMS-247550, BMS-310705, droloxifene, 4-hydroxytamoxifen, pipendoxifene, ERA-923, arzoxifene, fulvestrant, acolbifene, lasofoxifene, idoxifene, TSE-424, HMR-3339, ZK186619, topotecan, PTK787/ZK 222584, VX-745, PD 184352, rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, temsirolimus, AP-23573, RAD001, ABT-578, BC-210, LY294002, LY292223, LY292696, LY293684, LY293646, wortmannin, ZM336372, L-779,450, PEG-filgrastim, darbepoetin, erythropoietin, granulocyte colony-stimulating factor, zolendronate, prednisone, cetuximab, granulocyte macrophage colony-stimulating factor, histrelin, pegylated interferon alfa-2a, interferon alfa-2a, pegylated interferon alfa-2b, interferon alfa-2b, azacitidine, PEG-L-asparaginase, lenalidomide, gemtuzumab, hydrocortisone, interleukin-1, dexrazoxane, alemtuzumab, all-transretinoic acid, ketoconazole, interleukin-2, megestrol, immune globulin, nitrogen mustard, methylprednisolone, ibritgumomab tiuxetan, androgens, decitabine, hexamethylmelamine, bexarotene, tositumomab, arsenic trioxide, cortisone, editronate, mitotane, cyclosporine, liposomal daunorubicin, Edwina-asparaginase, strontium 89, casopitant, netupitant, an NK-1 receptor antagonists, palonosetron, aprepitant, diphenhydramine, hydroxyzine, metoclopramide, lorazepam, alprazolam, haloperidol, droperidol, dronabinol, dexamethasone, methylprednisolone, prochlorperazine, granisetron, ondansetron, dolasetron, tropisetron, pegfilgrastim, erythropoietin, epoetin alfa, and darbepoetin alfa, among others. In some embodiments, the anticancer agent is selected from the group of doxorubicin, melphalan, bevacizumab, dactinomycin, cyclophosphamide, doxorubicin liposomal, amifostine, etoposide, gemcitabine, altretamine, topotecan, cyclophosphamide, paclitaxel, carboplatin, cisplatin, and taxol.

[0176] Exemplary antiviral agents (e.g., anti-HV agents) include, for example, nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, among others, exemplary compounds of which may include, for example, 3TC (Lamivudine), AZT (Zidovudine), (−)-FTC, ddI (Didanosine), ddC (zalcitabine), abacavir (ABC), tenofovir (PMPA), D-D4FC (Reverset), D4T (Stavudine), Racivir, L-FddC, L-FD4C, NVP (Nevirapine), DLV (Delavirdine), EFV (Efavirenz), SQVM (Saquinavir mesylate), RTV (Ritonavir), IDV (Indinavir), SQV (Saquinavir), NFV (Nelfinavir), APV (Amprenavir), LPV (Lopinavir), fusion inhibitors such as T20, among others, fuseon and mixtures thereof, including anti-HIV compounds presently in clinical trials or in development. Exemplary anti-HBV agents include, for example, hepsera (adefovir dipivoxil), lamivudine, entecavir, telbivudine, tenofovir, emtricitabine, clevudine, valtoricitabine, anidoxovir, pradefovir, racivir, BAM 205, nitazoxanide, UT 231-B, Bay 41-4109, EHT899, zadaxin (thymosin alpha-1) and mixtures thereof. Anti-HCV agents include, for example, interferon pegylated intergeron, ribavirin, NM 283, VX-950 (telaprevir), SCH 50304, TMC435, VX-500, BX-813, SCH503034, R1626, ITMN-191 (R7227), R7128, PF-868554, TT033, CGH-759, GI 5005, MK-7009, SIRNA-034, MK-0608, A-837093, GS 9190, ACH-1095, GSK625433, TG4040 (MVA-HCV), A-831, F351, NS5A, NS4B, ANA598, A-689, GNI-104, IDX102, ADX184, GL59728, GL60667, PSI-7851, TLR9 Agonist, PHX1766, SP-30 and mixtures thereof.

[0177] Other exemplary antiviral agents include broad spectrum antiviral agents, antibodies, small molecule antiviral agents, antiretroviral agents, etc. Further non-limiting antiviral agents include abacavir, ACH-3102, acyclovir (acyclovir), acyclovir, adefovir, amantadine, amprenavir, ampligen, arbidol, asunaprevir, atazanavir, atripla, balavir, BCX4430, boceprevir, brincidofovir, brivudine, cidofovir, clevudine, combivir, cytarabine, daclatasvir, dasabuvir, deleobuvir, dolutegravir, darunavir, delavirdine, didanosine, docosanol, edoxudine, efavirenz, elbasvir, emtricitabine, enfuvirtide, entecavir, ecoliever, faldaprevir, famciclovir, favipiravir, fomivirsen, fosamprenavir, foscarnet, fosfonet, ganciclovir, grazoprevir, ibacitabine, imunovir, idoxuridine, imiquimod, indinavir, interferon type III, interferon type II, interferon type I, interferon, interferon alfa 2b, lamivudine, laninamivir, ledipasvir (with or without sofosbuvir), lopinavir, loviride, maraviroc, moroxydine, methisazone, MK-3682, MK-8408, nelfinavir, nevirapine, nexavir, novir, ombitasvir (with or without paritaprevir and/or ritonavir), oseltamivir (Tamiflu), paritaprevir, peginterferon alfa-2a, penciclovir, peramivir, pleconaril, podophyllotoxin, raltegravir, resiquimod, ribavirin, rifampicin, rimantadine, ritonavir, pyramidine, samatasvir, saquinavir, simeprevir, sofosbuvir, stavudine, taribavirin, tecovirimat (ST-246), telaprevir, telbivudine, tenofovir, tenofovir disoproxil, tipiracil, tipranavir, trifluridine (with or without tipiracil), trizivir, tromantadine, truvada, umifenovir, valaciclovir (Valtrex), valganciclovir, vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir (Relenza), zidovudine, including prodrugs, salts, and/or combinations thereof.

[0178] Exemplary antibiotics or antibacterial agents include gentamicin, kanamycin, neomycin, netilmicin, tobramycin, paromomycin, spectinomycin, geldanamycin, herbimycin, rifaximin, streptomycin, ertapenem, doripenem, imipenem/cilastatin, meropenem, cefadroxil, cefazolin, cephalothin, cephalexin, cefaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, cefixime, cefdinir, cefditoren, cefoperazone cefotaxime, cefpodoxime, ceftazadime, ceftibuten, ceftizoxime ceftriaxone, cefepime, ceftaroline fosamil, ceftobiprole, teicoplanin, vancomycin, telavancin, daptomycin, oritavancin, WAP-8294A, azithromycin, clarithromycin, dirithromycin, erythromycin, roxithromycin, telithromycin, spiramycin, clindamycin, lincomycin, aztreonam, furazolidone, nitrofurantoin, oxazolidonones, linezolid, posizolid, radezolid, torezolid, amoxicillin, ampicillin, azlocillin, carbenicillin, cloxacillin dicloxacillin, flucloxacillin, mezlocillin, methicillin, nafcillin, oxacillin, penicillin G, penicillin V, piperacillin, temocillin, ticarcillin, amoxicillin/clavulanate, ampicillin/sulbactam, piperacillin/tazobactam,

ticarcillin/clavulanate, bacitracin, colistin, polymyxin B, ciprofloxacin, enoxacin, gatifloxacin, gemifloxacin, levofloxacin, lomefloxacin, moxifloxacin, nalidixic acid, norfloxacin, ofloxacin, trovafloxacin, grepafloxacin, sparfloxacin, mafenide, sulfacetamide, sulfadiazine, sulfadimethoxine, sulfamethizole, sulfamethoxazole, sulfasalazine, sulfisoxazole, trimethoprim-sulfamethoxazole, sulfonamidochrysoidine, demeclocycline, doxycycline, vibramycin minocycline, tigecycline, oxytetracycline, tetracycline, clofazimine, capreomycin, cycloserine, ethambutol, rifampicin, rifabutin, rifapentine, arsphenamine, chloramphenicol, fosfomycin, fusidic acid, metronidazole, mupirocin, platensimycin, quinupristin/dalfopristin, thiamphenicol, tigecycline, and tinidazole and combinations thereof.

[0179] CRISPR Components

[0180] In an embodiment, the cargo includes a CRISPR component. CRISPR component includes any employing a nucleic acid sequence capable of recruiting a CRISPR-associated (Cas) protein to achieve genetic modification. An exemplary CRISPR component includes those having a trans-acting CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA) fused into a single, synthetic 'guide RNA' that directs a Cas nuclease (e.g., Cas9) to virtually any desired DNA sequence (see, e.g., FIG. **6**). The synthetic guide RNA (gRNA) can include at least three different portions: a first portion including the tracrRNA sequence, a second portion including the crRNA sequence, and a third portion including a targeting portion or a genomic specific sequence (gsRNA) that binds to a desired genomic target sequence (e.g., genomic target DNA sequence, including a portion or a strand thereof). The chimeric tracrRNA-crRNA sequence facilitates binding and recruitment of the endonuclease (e.g., Cas9), and the gsRNA sequence provides site-specificity to the target nucleic acid, thereby allowing Cas9 to selectively introduce site-specific breaks in the target.

[0181] In any embodiment herein, the cargo can include a CRISPR component. Exemplary CRISPR components can include a guide RNA, a Cas enzyme, and a nucleic acid sequence (e.g., a plasmid) encoding any of these. Yet other exemplary CRISPR components are shown in FIGS. **12**, **13**A-**13**C, **14**A-**14**H, **15**, **16**A-**16**C, **17**, **18**, and **19**, including, as applicable, a nucleic acid sequence encoding any of these (e.g., a nucleic acid sequence encoding any polypeptide sequence therein, such as SEQ ID Nos: 110-117 or a fragment thereof), a polypeptide generated by any nucleic acid sequence therein, as well as a complement of any nucleic acid sequence therein (e.g., a nucleic acid sequence that is a complement of any one of SEQ ID Nos: 20-32, 40-54, 60-65, 80-93, 100-103, or a fragment thereof).

[0182] In particular embodiments, the particle can include one or more CRISPR components (e.g., associated with or within a pore of the core (e.g., by way of a spacer), associated with a surface of the core, and/or within the outer layer).

[0183] FIG. **6** and FIG. **7**A-**7**C show exemplary CRISPR components. FIG. **6** shows an exemplary CRISPR component that includes a guiding component **90** to bind to the target sequence **97**, as well as a nuclease **98** (e.g., a Cas nuclease or an endonuclease, such as a Cas endonuclease) that interacts with the guiding component and the target sequence.

[0184] FIG. **7**(A) shows a non-limiting guiding component **300** having a targeting portion **304**, a first portion **301**, a second portion **302**, and a linker **303** disposed between the first and second portions. FIG. **7**(B) shows another non-limiting guiding component **350** having a targeting portion **354**, a first portion **351**, a second portion **352** having a hairpin, and a linker **353** disposed between the first and second portions. Fig. (C) shows non-limiting interactions between the guiding component **400**, the genomic sequence **412**, and the first and second portion **401**,**402**. As can be seen, the target sequence **411** of the genomic sequence **412** is targeted by way of non-covalent binding **421** to the targeting portion **404**, and secondary structure can be optionally implemented by way of non-covalent binding **422** between the first portion **401** and the second portion **402**. The targeting portion **404**, first portion **401**, linker **403**, and second portion **402** can be attached in any useful manner (e.g., to provide a 5' end **405** and a 3' end **406**).

[0185] This CRISPR/Cas system can be adapted to control genetic expression in targeted manner, such as, e.g., by employing synthetic, non-naturally occurring constructs that use crRNA nucleic acid sequences, tracrRNA nucleic acid sequences, and/or Cas polypeptide sequences, as well as modified forms thereof.

[0186] In an embodiment, a CRISPR component includes a guiding component. In general, the guiding component includes a nucleic acid sequence (e.g., a single polynucleotide) that includes at least two portions: a targeting portion, which is a nucleic acid sequence that imparts specific targeting to the target genomic locus (e.g., a guide RNA or gRNA); and an interacting portion, which is another nucleic acid sequence that binds to a nuclease (e.g., a Cas endonuclease). In some instances, the interacting portion includes two particular sequences that bind the nuclease, e.g., a short crRNA sequence attached to the guide nucleic acid sequence; and a tracrRNA sequence attached to the crRNA sequence. Exemplary targeting CRISPR components include a minicircle DNA vector optimized for in vivo expression.

[0187] Another CRISPR component includes a nuclease (e.g., that binds the targeting nucleic acid sequence). The nuclease CRISPR component can either be an enzyme, or a nucleic acid sequence that encodes for that enzyme. Exogenous endonuclease (e.g., Cas9) can be encoded by a cargo stored within the construct. In an embodiment, a nuclease such as Cas9 (e.g., SEQ ID NO:110) is employed, as well as nickase forms and deactivated forms (e.g., SEQ ID NO:111) thereof (e.g., including one or more mutations, such as D10A, H840A, N854A, and N863A in SEQ ID NO:110 or in an amino acid sequence sufficiently aligned with SEQ ID NO:110), including nucleic acid sequences that encode for such nuclease. Pathogen-directed and host-directed CRISPR components (e.g., guiding components and/or nuclease), as well as minicircle DNA vectors that encode Cas and guiding components can be developed. The nuclease can be configured to bind the target sequence and/or cleave the target sequence.

[0188] Non-limiting examples of nucleases are described in FIG. **8**A-**8**H. In some embodiments, a vector comprises a regulatory element operably linked to an enzyme-coding sequence encoding a nuclease (e.g., a CRISPR enzyme, such as a Cas protein). Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16,

CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologs thereof, or modified versions thereof. These enzymes are known; for example, the amino acid sequence of *S. pyogenes* Cas9 protein may be found in the SwissProt database under accession number Q99ZW2. In some embodiments, the unmodified CRISPR enzyme has DNA cleavage activity, such as Cas9. In some embodiments the CRISPR enzyme is Cas9, and may be Cas9 from *S. pyogenes* or *S. pneumoniae*. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence.

[0189] The nuclease may be a Cas9 homolog or ortholog. In some embodiments, the nuclease is codon-optimized for expression in a eukaryotic cell. In some embodiments, the nuclease directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the nuclease lacks DNA strand cleavage activity. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter.

[0190] Any useful Cas protein or complex can be employed. Exemplary Cas proteins or complexes include those involved in Type I, Type II, or Type III CRISPR/Cas systems, including but not limited to the CRISPR-associated complex for antiviral defense (Cascade, including a RAMP protein), Cas3 and/or Cas 7 (e.g., for Type I systems, such as Type I-E systems), Cas9 (formerly known as Csn1 or Csx12, e.g., such as in Type II systems), Csm (e.g., in Type III-A systems), Cmr (e.g., in Type III-B systems), Cas10 (e.g., in Type III systems), as well as subassemblies or sub-components thereof and assemblies including such Cas proteins or complexes. Additional Cas proteins and complexes are described in Makarova K S et al., "Evolution and classification of the CRISPR-Cas systems," *Nat. Rev. Microbiol.* 2011; 9:467-77, which is incorporated herein by reference in its entirety.

[0191] In some embodiments, a vector encodes a CRISPR enzyme that is mutated with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9 from *S. pyogenes* converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). Other examples of mutations that render Cas9 a nickase include, without limitation, H840A, N854A, and N863A. In aspects of the disclosed technology, nickases may be used for genome editing via homologous recombination. In some instances, the Cas protein includes a modification of one of more of D10A, H840A, N854A, and N863A in SEQ ID NO:110 or in an amino acid sequence sufficiently aligned with SEQ ID NO:110.

[0192] As a further example, two or more catalytic domains of Cas9 (RuvC I, RuvC II, and RuvC III) may be mutated to produce a mutated Cas9 substantially lacking all DNA cleavage activity. In some embodiments, a D10A mutation is combined with one or more of H840A, N854A, or N863A mutations to produce a Cas9 enzyme substantially lacking all DNA cleavage activity. In some embodiments, a CRISPR enzyme is considered to substantially lack all DNA cleavage activity when the DNA cleavage activity of the mutated enzyme is less than about 25%, 10%, 5%, 1%, 0.1%, 0.01%, or lower with respect to its non-mutated form. Other mutations may be useful; where the Cas9 or other CRISPR enzyme is from a species other than *S. pyogenes*, mutations in corresponding amino acids may be made to achieve similar effects.

[0193] In some embodiments, the guiding component comprises a modification or sequence that provides for an additional desirable feature (e.g., modified or regulated stability; subcellular targeting; tracking, e.g., a fluorescent label; a binding site for a protein or protein complex; etc.). Non-limiting examples include: a short motif (referred to as the protospacer adjacent motif (PAM)); a 5' cap (e.g., a 7-methylguanylate cap (m7G)); a 3' polyadenylated tail (i.e., a 3' poly(A) tail); a riboswitch sequence (e.g., to allow for regulated stability and/or regulated accessibility by proteins and/or protein complexes); a stability control sequence; a sequence that forms a dsRNA duplex (i.e., a hairpin)); a modification or sequence that targets the RNA to a subcellular location (e.g., nucleus, mitochondria, and chloroplasts); a modification or sequence that provides for tracking (e.g., direct conjugation to a fluorescent molecule, conjugation to a moiety that facilitates fluorescent detection, a sequence that allows for fluorescent detection, etc.); a modification or sequence that provides a binding site for proteins (e.g., proteins that act on DNA, including transcriptional activators, transcriptional repressors, DNA methyltransferases, DNA demethylases, histone acetyltransferases, and histone deacetylases); and combinations thereof.

[0194] A guiding component and a nuclease can form a complex (i.e., bind via non-covalent interactions). The guiding component provides target specificity to the complex by comprising a nucleotide sequence that is complementary to a sequence of a target sequence. The nuclease of the complex provides the site-specific activity. In other words, the nuclease is guided to a target sequence (e.g., a target sequence in a chromosomal nucleic acid; a target sequence in an extrachromosomal nucleic acid, e.g., an episomal nucleic acid, a minicircle, etc.; a target sequence in a mitochondrial nucleic acid; a target sequence in a chloroplast nucleic acid; a target sequence in a plasmid; etc.) by virtue of its association with the protein-binding segment (e.g., the interacting portion) of the guiding component.

[0195] In some embodiments, the guiding component comprises two separate nucleic acid molecules (e.g., a separate targeting portion and a separate interacting portion; a separate first portion and a separate second portion; or a separate targeting portion-first portion that is covalently bound and a separate second portion). In other embodiments, the guiding component is a single nucleic acid molecule including a covalent bond or a linker between each separate portion (e.g., a targeting portion covalently linked to an interacting portion).

[0196] FIG. **6** shows an exemplary CRISPR component that includes a guiding component **90** to bind to the target sequence **97**, as well as a nuclease **98** (e.g., a Cas nuclease or an endonuclease, such as a Cas endonuclease) that interacts with the guiding component and the target sequence. As can be seen, the guiding component **90** includes a targeting portion **94** configured to bind to the target sequence **97** of a genomic sequence **96** (e.g., a target sequence having substantially complementarity with the

genomic sequence or a portion thereof). In this manner, the targeting portion confers specificity to the guiding component, thereby allowing certain target sequences to be activated, inactivated, and/or modified.

[0197] The guiding component **90** also includes an interacting portion **95**, which in turn is composed of a first portion **91**, a second portion **92**, and a linker **93** that covalently links the first and second portions. The interacting portion **95** is configured to recruit the nuclease (e.g., a Cas nuclease) in proximity to the site of the target sequence. Thus, the interacting portion includes nucleic acid sequences that provide preferential binding (e.g., specific binding) of the nuclease. Once in proximity, the nuclease **98** can bind and/or cleave the target sequence or a sequence in proximity to the target sequence in a site-specific manner.

[0198] The first portion, second portion, and linker can be derived in any useful manner. In one instance, the first portion can include a crRNA sequence, a consensus sequence derived from known crRNA sequences, a modified crRNA sequence, or an entirely synthetic sequence known to bind a Cas nuclease or determined to competitively bind a Cas nuclease when compared to a known crRNA sequence. Exemplary sequences for a first portion are described in FIG. **9** (SEQ ID NOs:20-32). Another exemplary sequence for a first portion is 5'-GUUUUAGAGCUA-3' (SEQ ID NO:70). In some embodiments, the first portion is a nucleic acid sequence having at least 80% sequence identity (e.g., at least 85%, 90%, 95%, or 99% sequence identity) to any one of SEQ ID NOs:20-32 and 70 or a complement of any of these, or a fragment thereof (e.g., having a length of about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, or more nucleotides).

[0199] In some embodiments, the first portion is a crRNA sequence that exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity to any one of SEQ ID NOs:20-32 and 70. In other embodiments, the first portion is a fragment (e.g., having a length of about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, or more nucleotides) of a crRNA sequence that exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity to any one of SEQ ID NOs:20-32 and 70.

[0200] In another instance, the second portion can include a tracrRNA sequence, a consensus sequence derived from known tracrRNA sequences, a modified tracrRNA sequence, or an entirely synthetic sequence known to bind a Cas nuclease or determined to competitively bind a Cas nuclease when compared to a known tracrRNA sequence. Exemplary sequences for a second portion are described in FIG. **10**A-**10**C (SEQ ID NOs:40-54) and in FIG. **11** (SEQ ID NOs: 60-65). Another exemplary sequence for a second portion is 5'-UAGCAAGUUAAAA UAAGGCUAGUCCG-3' (SEQ ID NO:71).

[0201] In some embodiments, the second portion is a nucleic acid sequence having at least 80% sequence identity (e.g., at least 85%, 90%, 95%, or 99% sequence identity) to any one of SEQ ID NOs:40-54, 60-65, and 71 or a complement of any of these, or a fragment thereof (e.g., having a length of about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, or more nucleotides).

[0202] In some embodiments, the second portion is a tracrRNA sequence that exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity to any one of SEQ ID NOs:40-54, 60-65, and 71. In other embodiments, the second portion is a fragment (e.g., having a length of about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, or more nucleotides) of a tracrRNA sequence that exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity to any one of SEQ ID NOs:40-54, 60-65, and 71.

[0203] The linker can be, for example, one or more transcribable elements, such as a nucleotide or a nucleic acid, or including one or more chemical linkers. Further, the linker can be derived from a fragment of any useful tracrRNA sequence (e.g., any described herein). The first and second portions can interact in any useful manner. For example, the first portion can have a sequence portion that is sufficiently complementary to a sequence portion of the second portion, thereby facilitating duplex formation or non-covalent bonding between the first and second portion. In another example, the second portion can include a first sequence portion that is sufficiently complementary to a second sequence portion, thereby facilitating hairpin formation within the second portion. Further CRISPR components are described in FIG. **7**A-**7**C.

[0204] In another embodiment, the guiding component has a structure of A-L-B, in which A includes a first portion (e.g., any one of SEQ ID NOs:20-32 and 70, or a fragment thereof), L is a linker (e.g., a covalent bond, a nucleic acid sequence, a fragment of any one of SEQ ID NOs:40-54, 60-65, and 71, or any other useful linker or spacer described herein), and B is a second portion (e.g., any one of SEQ ID NOs:40-54, 60-65, and 71, or a fragment thereof) (FIG. **12**). In another embodiment, the guiding component is a sequence having at least 80% sequence identity (e.g., at least 85%, 90%, 95%, or 99% sequence identity) to any one SEQ ID NOs:80-93, or a fragment thereof.

[0205] In yet another embodiment, the guiding component is a sequence that exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity to any one SEQ ID NOs:100-103, or a fragment thereof (FIG. **13**). In another embodiment, the guiding component is a sequence having at least 80% sequence identity (e.g., at least 85%, 90%, 95%, or 99% sequence identity) to any one SEQ ID NOs:100-103, or a fragment thereof.

[0206] In some embodiments, the CRISPR component includes ds plasmid DNA, which is modified to express RNA and/or a protein. In other embodiments, the CRISPR component is supercoiled and/or packaged (e.g., within a complex, such as those containing histones, lipids (e.g., lipoplexes), proteins (e.g., cationic proteins), cationic carrier, nanoparticles (e.g., gold or metal nanoparticles), etc.), which may be optionally modified with a nuclear localization sequence (e.g., a peptide sequence incorporated or otherwise crosslinked into histone proteins, which comprise the histone-packaged supercoiled plasmid DNA). Other exemplary histone proteins include H1, H2A, H2B, H3 and H4, e.g., in a ratio of 1:2:2:2:2 with optional nuclear localization sequences (e.g., any described herein, such as SEQ ID NOs:9-12).

[0207] The CRISPR component can include any useful promoter sequence(s), expression control sequence(s) that controls and regulates the transcription and translation of another DNA sequence, and signal sequence(s) that encodes a signal peptide. The promoter sequence can include a DNA regulatory region capable of binding RNA polymerase in a

cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of the present disclosed technology, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgamo sequences in addition to the −10 and −35 consensus sequences.

[0208] In addition, the CRISPR components can be formed from any useful combination of one or more nucleic acids (or a polymer of nucleic acids, such as a polynucleotide). Exemplary nucleic acids or polynucleotides of the disclosed technology include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β-D-ribo configuration, α-LNA having an α-L-ribo configuration (a diastereomer of LNA), 2'-amino-LNA having a 2'-amino functionalization, and 2'-amino-α-LNA having a 2'-amino functionalization) or hybrids, chimeras, or modified forms thereof. Exemplary modifications include any useful modification, such as to the sugar, the nucleobase, or the internucleoside linkage (e.g., to a linking phosphate/to a phosphodiester linkage/to the phosphodiester backbone). One or more atoms of a pyrimidine nucleobase may be replaced or substituted with optionally substituted amino, optionally substituted thiol, optionally substituted alkyl (e.g., methyl or ethyl), or halo (e.g., chloro or fluoro). In certain embodiments, modifications (e.g., one or more modifications) are present in each of the sugar and the internucleoside linkage. Modifications according to the present disclosure may be modifications of ribonucleic acids (RNAs) to deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs) or hybrids thereof). Additional modifications are described herein.

[0209] Toxicity of CRISPR components, to the host, can be minimized. For instance, toxicity can result from protocells or carriers due to expression of Cas9 products or immune responses. Specifically, the lifetime of CRISPR components in the cell can be controlled by adding features that are stabilized or destabilized with cellular proteases, by inducing expression only under a microbial or viral promoter, and by using guiding components with modified backbones (e.g., 2-OMe) to minimize immune recognition.

[0210] Resistance to CRISPR components can be minimized. Any single antibiotic or antiviral countermeasure is prone to the development of resistance, so pathogens will likely mutate around individual guiding component targets. However, the development of resistance can be prevented by targeting orthogonal mechanisms via multiplexed guiding components in combination with current antivirals/antimicrobials.

[0211] Off-target mutations or genetic modification can be minimized. For instance, bioinformatic guiding component design programs can be used to determine minimal effective CRISPR component doses. If needed, the nickase version of Cas9 can be employed.

[0212] The CRISPR component can be employed to target a nucleic acid sequence (e.g., present in the host's genomic sequence and/or the pathogen's genomic sequence). In one instance, the target sequence can include a sequence present in the host's genomic sequence in order, e.g., activate, inactive, or modify expression of factor or proteins within the host's cellular machinery. For instance, the target sequence can bind to one or more genomic sequences for an immunostimulatory protein that, upon expression, would enhance the immune response by the host to an infection. Pathogens are known to down-regulate proteins that would otherwise assist in recognizing non-self protein motifs. Thus, in another instance, the target sequence can bind to one or more regulator proteins and enhance their transcription and expression. In yet another instance, one or more polypeptides may be up-regulated, as compared to the normal basal rate, and such up-regulation may be modified by the presence of the pathogen. Accordingly, the target sequence can be employed to bind to one or more up-regulated polypeptides in order to inactivate or repress transcription/expression of those polypeptides.

[0213] An exemplary target sequence (e.g., in a host or subject) includes, without limitation, a nucleic acid sequence encoding an immunostimulatory protein, a cluster of differentiation protein, a cell surface protein, a pathogen receptor protein (e.g., a pathogen recognition receptor, such as TLR9), a glycoprotein (e.g., granulocyte-colony stimulating factor), a cytokine (e.g., interferon or transforming growth factor beta (TGF-beta)), a pattern recognition receptor protein, a hormone (e.g., a prostaglandin), or a helicase enzyme.

[0214] In yet another instance, the target sequence can be employed to activate, inhibit, and/or modify a target sequence (e.g., associated with the presence of a pathogen, a tumor, etc.). For instance, the target sequence can be configured to activate one or more target sequences encoding proteins that promote programmed cell death or apoptosis (e.g., of the pathogen or of particular tissue types, such as metastatic growths, tumors, lesions, etc.). For instance, the target sequence can be configured to inactivate or modify one or more target sequences encoding proteins that are suppressed by the pathogen. Exemplary target sequence (e.g., in a pathogen) includes, without limitation, a nucleic acid sequence encoding a virulence factor (e.g., a lipase, a protease, a nuclease (e.g., a DNAse or an RNase), a hemolysin, a hyaluronidase, an immunoglobulin protease, an endotoxin, or an exotoxin), a cell surface protein (e.g., an adhesion), an envelope protein (e.g., a phospholipid, a lipopolysaccharide, a lipoprotein, or a polysaccharide), a glycoprotein, a polysaccharide protein, a transmembrane protein (e.g., an invasin), or a regulatory protein.

[0215] The CRISPR component can be employed to activate the target sequence (e.g., the Cas polypeptide can include one or more transcriptional activation domains, which upon binding of the Cas polypeptide to the target sequence, results in enhanced transcription and/or expression of the target sequence), inactivate the target sequence (e.g., the Cas polypeptide can bind to the target sequence, thereby inhibiting expression of one or more proteins encoded by the target sequence; the Cas polypeptide can introduce double-stranded or single-stranded breaks in the target sequence, thereby inactivating the gene; or the Cas polypeptide can include one or more transcriptional repressor domains, which upon binding of the Cas polypeptide to the target sequence, results in reduced transcription and/or

expression of the target sequence), and/or modify the target sequence (e.g., the Cas polypeptide can cleave the target sequence of the pathogen and optionally inserts a further nucleic acid sequence).

[0216] Any useful transcriptional activation domains can be employed (e.g., VP64, VP16, HIV TAT, or a p65 subunit of nuclear factor KB). In particular, such activation domains are useful when employed with a deactivated or modified form of the Cas polypeptide with minimized cleavage activity. In this way, specific recruitment of the Cas polypeptide to the target sequence is enabled by the interacting portion of the target component, and transcriptional activity is controlled by the activation domains.

[0217] Further, transcriptional repressor domains can be employed (e.g., a Krüppel-associated box domain, a SID domain, an Engrailed repression domain (EnR), or a SID4X domain). In particular, such repressor domains can be employed with a deactivated or modified form of the Cas polypeptide with minimized cleavage activity or with an active Cas polypeptide with retained endonuclease activity.

[0218] A guiding component may be selected to target any target sequence. In some embodiments, the target sequence is a sequence within a genome of a host (e.g., a host cell) or a pathogen (e.g., a pathogen cell). In some embodiments, the guiding component is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guiding component is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. The ability of a guiding component to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guiding component to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay. Similarly, cleavage of a target sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guiding component to be tested and a control guiding component different from the test guiding component, and comparing binding or rate of cleavage at the target sequence between the test and control guiding component reactions.

[0219] Outer Layer

[0220] The constructs disclosed herein include an outer layer (also referred to as a coating herein) disposed around the core. In particular embodiments, the outer layer includes a combination of lipids supported by or bonded to the surface of the core. The outer layer or coating may also include one or more moieties (e.g., one or more targeting ligands, such as a pegylated lipid). In other embodiments, the outer layer can include a polymer layer (e.g., supported by the surface of the core) that can optionally include one or more moieties (e.g., one or more targeting ligands).

[0221] The outer layer or coating, (lipid bilayer) can be characterized by its thickness (e.g., about 5 nm to about 3 to about 40 nm, such as about 4 to about 25 nm, or about 5 to about 15 nm), the number of layers within the outer layers (e.g., two, three, four, five, six, seven, or more lipid and/or polymer layers within the outer layer), and/or the net charge of the outer layer (e.g., a net non-negative charge, such as a net positive charge; or as determined by the composition of the lipid layer, such as one formed by use of a liposome formulation having more than about 20 mol. % of a cationic lipid, such as any herein (e.g., DOTAP)).

[0222] In an embodiment, the outer layer includes a cationic lipid, a pegylated lipid, a zwitterionic lipid, and a sterol, as well as salts of any of these (e.g., pharmaceutically acceptable salts).

[0223] The lipid layer can include one or more lipids selected from the group of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-[phosphor-L-serine](DOPS), 1,2-dioleoyl-3-trimethylammonium-propane (18:1 DOTAP), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (18:1 PEG-2000 PE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (16:0 PEG-2000 PE), 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl]-sn-glycero-3-phosphocholine (18:1-12:0 NBD PC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] ($DSPE\text{-}PEG_{2000}$), 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl}-sn-glycero-3-phosphocholine (16:0-12:0 NBD PC), a sterol (e.g., cholesterol, desmosterol, diplopterol, cholestanol, cholic acid, 12-deoxycholic acid, 7-deoxycholic acid, or a derivative thereof, such as cholesterol sulfate), and mixtures thereof and conjugated forms thereof (e.g., conjugated to PEG moieties, peptides, polypeptides, including immunogenic peptides, proteins and antibodies, and nucleic acids (e.g., RNA and DNA) by way of a covalent bond or by way of a linker or spacer (e.g., any described herein).

[0224] In an embodiment, the outer layer also includes a polymer, including, for example, polyethylene glycol (PEG) or polyethylene oxide (PEO) (e.g., a PEG-polyester), or a copolymer (e.g., a diblock copolymer, such as an amphiphilic diblock copolymer). Non-limiting polymers include a PEG-lactic acid polymer (PEG-LA, e.g., poly(ethyleneglycol)-b-poly(lactic acid) copolymer or PEG-b-poly(D,L-lactic acid)); a polycarbonate-polyglutamic acid polymer (PC-PGA, e.g., poly(trimethylene carbonate)-b-poly(glutamic acid); a poly(lactic acid) (PLA, e.g., methoxy poly(ethylene glycol)-Gly-Phe-Leu-Gly-Phe-poly(D,L-lactide), PEG-PLA, or maleimide-PEG-PLA); a poly(butadiene) (PBD, e.g., PEO-b-PBD or PEG-PBD); a poly(caprolactone) (PCL, e.g., PEG-PCL, PEO-PCL, PEG-b-poly(ε-caprolactone), mPEG-poly(ε-caprolactone), α-carboxyl PEG-poly(3-caprolactone)/PEG-PLA, or PEO-b-poly(γ-methyl-3-caprolactone)); and a PEG- or PEO-polypeptide (e.g., PEG-b-poly(2-hydroxyethyl aspartamide) substituted with octadecyl chains, poly(carboxyl ethylene glycol-γ-glutamate)-co-poly(distearin-γ-glutamate), or poly(ethylene glycol)-γ-glutamate)-co-poly (distearin-γ-glutamate)).

[0225] The outer layer can be a hybrid layer (e.g., including one or more lipids and one or more polymers). Exem-

plary hybrid layers can include a lipid (e.g., any described herein), an optional sterol, and a polymer (e.g., any described herein, such as a polymer including PEG or PEO).

[0226] Cores, lipids, polymers, and cargos can be PEGylated with a variety of polyethylene glycol-containing compositions as described herein. PEG molecules can have a variety of lengths and molecular weights and include, but are not limited to, PEG 200, PEG 1000, PEG 1500, PEG 2000, PEG 4600, PEG 5000, PEG 10,000, PEG-peptide conjugates or combinations thereof.

[0227] In one instance, the outer layer includes a cationic lipid (e.g., DOTAP), a zwitterionic lipid (e.g., DOPE), a sterol (e.g., cholesterol), and a PEGylated lipid (e.g., 1,2-distearoyl-sn-157 glycero-3-phosphoethanolamine-N-[carboxy-(polyethylene glycol)-2000 (DSPE-PEG2000)). These four components may be in a molar ratio of about 1 cationic lipid (e.g., DOTAP) to 1 zwitterionic lipid (e.g., DOPE) to 0.9 sterol (e.g., cholesterol) to 0.15 PEGylated lipid (e.g., DSPE-PEG2000). In an instance, each of these ratios may optionally vary by plus or minus 10%, or plus or minus 5%, or plus or minus 3%. Accordingly, each number recited in the ratio above, may range from a multiple of about 0.9 to 1.1, 0.95 to 1.05, or 0.97 to 1.03.

[0228] In an instance, the outer layer includes about 10 to about 50 mol. % cationic lipid (e.g., DOTAP), about 10 to 50 mol. % zwitterionic lipid (e.g., DOPE), about 5 to about 45 mol. % sterol (e.g., cholesterol), and about 2 to 8 mol. % of a PEGylated lipid (e.g., DSPE-PEG2000). In another instance, the outer layer includes about 20 to about 40 mol. % cationic lipid (e.g., DOTAP), about 20 to 40 mol. % zwitterionic lipid (e.g., DOPE), about 10 to about 35 mol. % sterol (e.g., cholesterol), and about 2.5 to 6 mol. % of a PEGylated lipid (e.g., DSPE-PEG2000). In another instance, the outer layer includes about 30 to about 35 mol. % cationic lipid (e.g., DOTAP), about 30 to 35 mol. % zwitterionic lipid (e.g., DOPE), about 27 to about 33 mol. % sterol (e.g., cholesterol), and about 3 to 5 mol. % of a PEGylated lipid (e.g., DSPE-PEG2000).

[0229] In particular embodiments, the outer layer includes about 33 mol % of the cationic lipid, about 33 mol % of the zwitterionic lipid, about 30 mol % of the sterol, and about 4% of the PEGylated lipid.

[0230] Exemplary cationic lipids include 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-stearoyl-3-trimethylammonium-propane (18:0 TAP), 1,2-dipalmitoyl-3-trimethylammonium-propane (16:0 TAP), 1,2-dimyristoyl-3-trimethylammonium-propane (14:0 TAP), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), N1-[2-((1S)-1-[(3-aminopropyl)amino]-4-[di(3-amino-propyl)amino]butylcarboxamido)ethyl]-3,4-di[oleyloxy]-benzamide (MVL5), ethylphosphocholine (ethyl PC) (e.g., 1,2-dimyristoleoyl-sn-glycero-3-ethylphosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine, 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine, 1,2-distearoyl-sn-glycero-3-ethylphosphocholine, 1,2-dipalmitoyl-sn-glycero-3-ethylphosphocholine, 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine, or 1,2-dilauroyl-sn-glycero-3-ethylphosphocholine), dimethyldioctadecylammonium (DDAB), 1,2-dipalmitoyl-sn-glycero-O-ethyl-3-phosphocholine (EDPPC), or any described herein.

[0231] Exemplary zwitterionic lipids include DOPC, DPPC, DOPE, DPPE, POPC, DLPC, DSPC, DMPC, SOPC, or any described herein.

[0232] Exemplary, non-limiting sterols include cholesterol (e.g., from ovine wool or from plant sources), campestanol, campesterol, cholestanol, cholestenone, desmosterol, 7-dehydrodesmosterol, dehydroepiandrosterone (DHEA), desmosterol, diosgenin, FF-MAS (14-demethyl-14-dehydrolanosterol), lanosterol, lathosterol, pregnenolone, sitostanol, sitosterol, stigmasterol, zymosterol, zymostenol, zymosterone, as well as derivatives thereof, such as sulfates thereof, esters thereof, stereoisomers thereof, deuterated forms thereof, sulfonated forms thereof, phosphorylated forms thereof, unsaturated forms thereof, keto forms thereof, oxidized forms thereof, an oxysterol thereof, PEGylated forms thereof (e.g., cholesterol-(polyethylene glycol-600)), or substituted forms thereof (e.g., having one or more hydroxyl, epoxy, alkyl, phospho, and/or halo, such as fluoro).

[0233] Exemplary PEGylated lipids (e.g., a lipid having a poly(ethylene glycol moiety)) include PEGylated DSPE (e.g., 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-X] (DSPE X) or N-[carbonyl-2',3'-bis(methoxypolyethyleneglycol X)]-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-2arm PEGX)), PEGylated phosphoethanolamine (PE) (e.g., 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-X] (18:1 PEGX PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-X] (18:0 PEGX PE), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-X] (14:0 PEGX PE), or 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (16:0 PEGX PE)), PEGylated DPPE (e.g., N-(carbonyl-methoxypolyethyleneglycol X)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), PEGylated DMPE (e.g., N-(carbonyl-methoxypolyethyleneglycol X)-1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), PEGylated DPG (e.g., 1,2-dipalmitoyl-sn-glycerol, methoxypolyethylene glycol), PEGylated DSG (e.g., 1,2-distearoyl-sn-glycerol, methoxypolyethylene glycol), PEGylated DOG (e.g., 1,2-dioleoyl-sn-glycerol, methoxypolyethylene glycol), or PEGylated DMG (e.g., 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol), where X indicates an approximate number average molecular weight (Mn) as measured by Gel Permeation Chromotography (GPC) with appropriate standards, and where X is 500, 3000, 2000, 1000, 750, 550, or 350.

[0234] The outer layer of the particle can be composed of lipids, polymers, and/or components in an amount similar to that provided by the lipid formulation. For instance, an exemplary lipid formulation comprising about 47 mol. % of a cationic lipid can provide a lipid layer (for a construct) that comprises 47 mol. % of that cationic lipid. Thus, any composition provided for a lipid formulation herein also provides a composition for the outer layer.

[0235] Targeting Ligands

[0236] Optionally the construct can include one or more cell targeting species, cell receptor ligands, cell penetrating peptides, fusogenic peptides, and/or targeting peptides. Such species can be included within the cargo, configured to be expressed by a plasmid of the cargo, located within the outer layer, and/or provided by an external surface of the outer layer (e.g., provided by the outer lipid layer). In an embodiment the targeting ligand can be added to the construct via the pegylated lipid, PEG2000 and derivatives thereof. The composition of the outer layer can include one or more

components that facilitate ligand orientation, maximize cellular interaction, provide lipid stability, and/or confer enhanced cellular entry.

[0237] In some instances, the targeting ligand can be a cell penetration peptide, a fusogenic peptide, or an endosomolytic peptide, which are peptides that aid a particle in translocating across a lipid bilayer, such as a cellular membrane or endosome lipid bilayer of the host cell. In one embodiment, the targeting ligand is optionally crosslinked onto a lipid layer surface of the outer layer.

[0238] Endosomolytic peptides are a sub-species of fusogenic peptides as described herein. Representative and preferred electrostatic cell penetration (fusogenic) peptides include an 8 mer polyarginine (NH_2—RRRRRRRR—COOH, SEQ ID NO:1), among others known in the art, which are included in or on particles in order to enhance the penetration of into cells. Representative endosomolytic fusogenic peptides ("endosomolytic peptides") include H5WYG peptide (NH_2-GLFHAIAHFIHGGWHG-LIHGWYGGC-COOH, SEQ ID NO:2), RALA peptide (NH_2-WEARLARALARALARHLARALARALRAGEA-COOH, SEQ ID NO:3), KALA peptide (NH_2-WEAK-LAKALAKALAKHLAKALAKALKAGEA-COOH), SEQ ID NO:4), GALA (NH_2-WEAALAEALAEALAEHLAEA-LAEALEALAA-COOH, SEQ ID NO:5) and INF7 (NH_2-GLFEAIEGFIENGWEGMIDGWYG-COOH, SEQ ID NO:6), or fragments thereof, among others. In one instance, the targeting ligand includes an amino acid sequence having at least 80% sequence identity (e.g., at least 85%, 90%, 95%, or 99% sequence identity) to any one of SEQ ID NOs:1-6, or a fragment thereof.

[0239] Proteins gain entry into the nucleus through the nuclear envelope. Yet other ligands can include a nuclear localization sequence (NLS), e.g., N112-GNQSSNFGPMKGGNFGGRSSGPY GGGGQYFAK-PRNQGGYGGC-COOH (SEQ ID NO:9), RRMKWKK (SEQ ID NO:10), PKKKRKV (SEQ ID NO:11), and KR[PAATKKAGQA]KKKK (SEQ ID NO:12), the NLS of nucleoplasmin, a prototypical bipartite signal comprising two clusters of basic amino acids, separated by a spacer of about 10 amino acids. Numerous other nuclear localization sequences are well known in the art. See, for example, LaCasse E C et al., "Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins," Nucl. Acids Res. 1995; 23:1647-56; Weis, K, "Importins and exportins: how to get in and out of the nucleus," [*published erratum appears in Trends Biochem. Sci.* 1998 July; 23(7):235] Trends Biochem. Sci. 1998; 23:185-9; and Cokol M et al., *EMBO Rep.* 2000 Nov. 15; 1(5): 411-5, each of which is incorporated herein by reference in its entirety.

[0240] Preferred ligands which may be used to target cells include peptides, affibodies, and antibodies (including monoclonal and/or polyclonal antibodies). In certain embodiments, targeting ligands selected from the group consisting of Fcγ from human IgG (which binds to Fcγ receptors on macrophages and dendritic cells), human complement C3 (which binds to CR1 on macrophages and dendritic cells), ephrin B2 (which binds to EphB4 receptors on alveolar type II epithelial cells), SP94 peptide (which binds to unknown receptor(s) on hepatocyte-derived cells), and MET receptor binding peptide. Exemplary, non-limiting SP94 peptides include SP94 free peptide (H2N-SFSIILT-PILPL-COOH, SEQ ID NO:126), a SP94 peptide modified with C-terminal Cys for conjugation (H2N-SFSIILTPIL-PLGGC-COOH, SEQ ID NO:127), and a further modified SP94 peptide (H2N-SFSIILTPILPLEEEGGC-COOH, SEQ ID NO:128). Exemplary MET binding peptides include ASVHFPP (SEQ ID NO:121), TATFWFQ (SEQ ID NO:122), TSPVALL (SEQ ID NO:123), IPLKVHP (SEQ ID NO:124), and WPRLTNM (SEQ ID NO:125).

[0241] Other exemplary targeting ligands include poly-L-arginine, including $(R)_n$, where $6<n<12$, such as an R12 peptide (e.g., RRRRRRRRRRRR (SEQ ID NO:210)) or an R9 peptide (e.g., RRRRRRRRR (SEQ ID NO:211)); a poly-histidine-lysine, such as a $(KH)_9$ (e.g., KHKHKHKHKHKHKHKHKH (SEQ ID NO:212)); a Tat protein or derivatives and fragments thereof, such as RKKRRQRRR (SEQ ID NO:213), GRKKRRQRRRPQ (SEQ ID NO:214), GRKKRRQRRR (SEQ ID NO:215), GRKKRRQRRRPPQ (SEQ ID NO:216), YGRKKRRQRRR (SEQ ID NO:217), and RKKRRQRRRRKKRRQRRR (SEQ ID NO:218); a Cady protein or derivatives and fragments thereof, such as Ac-GLWRALWRLLRSLWRLLWRA-cysteamide (SEQ ID NO:219); a penetratin protein or derivatives and fragments thereof, such as RQIKIWFQNRRMKWKKGG (SEQ ID NO:220), RQIRIWFQNRRMRWRR (SEQ ID NO:221), and RQIKIWFQNRRMKWKK (SEQ ID NO:222); an anti-trypsin protein or derivatives and fragments thereof, such as CSIPPEVKFNKPFVYLI (SEQ ID NO:223); a temporin protein or derivatives and fragments thereof, such as FVQWFSKFLGRIL-NH_2 (SEQ ID NO:224); a MAP protein or derivatives and fragments thereof, such as KLA-LKLALKALKAALKLA (SEQ ID NO:225); a RW protein or derivatives and fragments thereof, such as RRWWRRWRR (SEQ ID NO:226); a pVEC protein or derivatives and fragments thereof, such as LLIILRR-RIRKQAHAHSK (SEQ ID NO:227); a transportan protein or derivatives and fragments thereof, such as GWTLN-SAGYLLGKIN LKALAALAKKIL (SEQ IDNO:228); a MPG protein or derivatives and fragments thereof, such as GALFLGFLGAAGSTMGAWSQPKKKRKV (SEQ ID NO:229); a Pep protein or derivatives and fragments thereof, such as KETWWETWWTEWSQPKKKRKV (SEQ ID NO:230), Ac-KETWWETWWTEWSQPKKKRKV-cysteamine (SEQ ID NO:231), and WKLFKKILKVL-amide (SEQ ID NO:232); a Bp100 protein or derivatives and fragments thereof, such as KKLFKKILKYL (SEQ ID NO:233) and KKLFKKILKYL-amide (SEQ ID NO:234); a maurocalcine protein or derivatives and fragments thereof, such as GDC(acm)LPHLKLC (SEQ ID NO:235); a calcitonin protein or derivatives and fragments thereof, such as LGTYTQDFNKFHTFPQTAIGVGAP (SEQ ID NO:236); a neurturin protein or derivatives and fragments thereof, such as GAAEAAARVYDLGLRRLRQRRRLRRERVRA (SEQ ID NO:237); and a human P1 protein or derivatives and fragments thereof, such as MGLGLHLLV-LAAALQGAWSQPKKKRKV (SEQ ID NO:238).

[0242] In one instance, the targeting ligand includes an amino acid sequence having at least 80% sequence identity (e.g., at least 85%, 90%, 95%, or 99% sequence identity) to any one of SEQ ID NOs:10-12 and 210-238 or a fragment thereof (e.g., having a length of about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, or more amino acids).

[0243] Exemplary ligands also include a peptide that binds to ephrin B2, to target Vero cells; Fcγ to target THP-1 cells

and primary alveolar macrophages; the 'GE11' peptide (see, e.g., Li Z et al., *FASEB J* 2006; 19: 1978-85) to target A549 cells and primary alveolar epithelial cells; the 'SP94' peptide (see, e.g., Lo A et al., *Molec. Cancer Therap.* 2008; 7:579-89) to target HepG2 cells and primary hepatocytes; human complement C3, which binds to receptors on macrophages and dendritic cells; or the 'H5WYG' peptide, which ruptures the membranes of acidic intracellular vesicles via the 'proton sponge' mechanism (see, e.g., Moore N M et al., *J. Gene. Med.* 2008 10: 1134-49).

[0244] Other ligands include a peptide (e.g., a peptide zip code or a cell penetrating peptide), an endosomolytic peptide, an antibody (including fragments thereof), affibodies, a carbohydrate, an aptamer, a cluster of differentiation (CD) protein, or a self-associated molecular pattern (SAMP) (e.g., as described in Lambris J D et al., *Nat. Rev. Microbiol.* 2008; 6(2):132; and Poon I K H, *Cell Death Differ.* 2010; 17:381-97, each of which is incorporated herein by reference in its entirety). Exemplary CD proteins include CD47 (OMIM Entry No. 601028, a marker of self that allows RBC to avoid phagocytosis), CD59 (OMIM Entry No. 107271, a marker that prevents lysis by complement), C1 inhibitor (C1INH, OMIM Entry No. 606860, a marker that suppresses activation of the host's complement system), CD200 (OMIM Entry No. 155970, an immunosuppressive factor), CD55 (OMIM Entry No. 125240, a marker that inhibits the complement cascade), CD46 (OMIM Entry No. 120920, a marker that inhibits the complement cascade), and CD31 (OMIM Entry No. 173445, an adhesion regulator and a negative regulator of platelet-collagen interactions). Each recited OMIM Entry is incorporated herein by reference in its entirety.

[0245] Other useful ligands can be employed, such as those identified by the 'BRASIL' (Biopanning and Rapid Analysis of Selective Interactive Ligands) method (see, e.g., Giordano R J et al., *Nat. Med.* 2001; 7:1249-53; Giordano R J et al., *Proc. Natl Acad. Sci. USA* 2010; 107(11):5112-7; and Kolonin M G et al., *Cancer Res.* 2006; 66:34-40) to identify novel targeting peptides and single-chain variable fragments (scFvs) via phage display (see, e.g., Giordano R J et al., *Chem. Biol.* 2005; 12:1075-83; Giordano R J et al., *Proc. Natl Acad. Sci. USA* 2010; 107(11):5112-7; Kolonin M G et al., *Cancer Res.* 2006; 66:34-40; Tonelli R R et al., *PLoS Negl. Dis.* 2010; 4:e864; Lionakis M S et al., *Infect. Immun.* 2005; 73:7747-58; and Barbu E M et al., *PLoS Pathog.* 2010; 6:e1000726).

[0246] Particle Characteristics and Surface Properties

[0247] The construct (or particle) can be characterized by, e.g., overall charge, dimension, or dispersity. In some embodiments, one or more optional targeting ligands can be present in or on an outer layer. The particle can have a diameter, circumference, length, width, height, etc. Exemplary values for dimensions include, without limitation, greater than about 10 nm (e.g., greater than about 20 nm, 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 125 nm, 150 nm, 200 nm, 300 nm, 500 nm, 750 nm, 1 m, 2 m, 5 m, 10 m, 20 m) or of about 2 nm to 500 nm (e.g., from 2 nm to 50 nm, 2 nm to 100 nm, 2 nm to 150 nm, 2 nm to 200 nm, 2 nm to 300 nm, 2 nm to 400 nm, 10 nm to 50 nm, 10 nm to 100 nm, 10 nm to 150 nm, 10 nm to 200 nm, 10 nm to 300 nm, 10 nm to 400 nm, 10 nm to 500 nm, 20 nm to 50 nm, 20 nm to 100 nm, 20 nm to 150 nm, 20 nm to 200 nm, 20 nm to 300 nm, 20 nm to 400 nm, 20 nm to 500 nm, 50 nm to 100 nm, 50 nm to 150 nm, 50 nm to 200 nm, 50 nm to 300 nm, 50 nm to 400 nm, 50 nm to 500 nm, 100 nm to 150 nm, 100 nm to 200 nm, 100 nm to 300 nm, 100 nm to 400 nm, 100 nm to 500 nm, 150 nm to 200 nm, 150 nm to 300 nm, 150 nm to 400 nm, 150 nm to 500 nm, 200 nm to 300 nm, 200 nm to 400 nm, or 200 nm to 500 nm). In each case, the dimension of particle or construct is larger than the core dimension of the same type.

[0248] In particular embodiments, a plurality of particles is monodisperse in diameter, such as by having a polydispersity index (PdI) that is less than about 0.2 or by having a PdI that is of about 0.05 to about 0.2 (e.g., from 0.05 to 0.1, 0.05 to 0.15, 0.1 to 0.15, 0.1 to 0.2, or 0.15 to 0.2). The calculations used for the determination of size and PDI parameters are defined in the ISO standard documents 13321:1996 E and ISO 22412:2008. In some embodiments, the monodisperse particles range in a size of from about 50 nm to about 475 nm (e.g., from 150 nm (+/−10 nm) to 350 nm (+/−15 nm)).

[0249] In embodiments, the particle (or a plurality of particles) has a charge (or a net charge) that is near neutral (e.g., a zeta potential of about +5 mV to −5 mV, or about +10 to about −10 mV). As mentioned above, the construct can include appropriate targeting ligands to promote their cell-specific binding and internalization, and can include a useful ligand to promote endosomal escape or nuclear localization within host cells.

[0250] Compositions and Formulations

[0251] The present constructs can be formulated, for example, for subcutaneous (SC), intranasal (IN), aerosol, intravenous (IV), intramuscular (IM), intraperitoneal (IP), oral, topical, transdermal, or retro-orbital delivery. Exemplary dosages include, e.g., about 0.01 g (construct)/kg (body wt.) to about 0.2 g/kg, such as, 0.05 g/kg to about 0.15 g/kf, or about 0.07 to 0.1 g/kg. A dose of 0.1 g/kg was well tolerated in mice and could translate to humans. A dose of 0.1 g LC-MSNs/kg/day was tested up to four days in mice. Daily doses can be given from 2 to 10 days, such as 2 to 8, or 3 to 5 days. Certain cargos may vary the dosage, for example, RNP delivery may be applied only up to four days.

[0252] The formulation or composition can include a plurality of particles (e.g., an effective amount thereof) and an optional pharmaceutically acceptable excipient (e.g., any described herein). In some instances, the pharmaceutical composition includes a population of particles (e.g., any described herein) in an amount effective for modulating or modifying a target gene within a subject in combination with a pharmaceutically acceptable carrier, additive, or excipient. In other instances, the composition further includes a drug, a therapeutic agent, etc., which is not disposed as cargo within the particle.

[0253] The composition can be formulated in any useful manner with a plurality of particles. Such formulations can be included with a medium, excipient (e.g., lactose, saccharide, carbohydrate, mannitol, leucine, PEG, or trehalose), additive, propellant, solution (e.g., aqueous solution, such as a buffer), additive, preservative, carrier (e.g., aqueous saline, aqueous dextrose, glycerol, or ethanol), binder (e.g., saccharide, cellulose preparation, starch paste, or methyl cellulose), filler, or disintegrator.

[0254] Pharmaceutical compositions according to the present disclosure include an effective population of constructs herein formulated to effect an intended result (e.g., immunogenic result, therapeutic result and/or diagnostic analysis, including the monitoring of therapy) formulated in

combination with a pharmaceutically acceptable carrier, additive, or excipient. The particles within the population of the composition may be the same or different depending upon the desired result to be obtained. Pharmaceutical compositions according to the present disclosure may also comprise an addition bioactive agent or drug, such as an antibiotic or antiviral agent.

[0255] Formulations and compositions containing the particles according to the present disclosure may take the form of liquid, solid, semi-solid or lyophilized powder forms, such as, for example, solutions, suspensions, emulsions, sustained-release formulations, tablets, capsules, powders, suppositories, creams, ointments, lotions, aerosols, or patches, in unit dosage forms suitable for simple administration of precise dosages.

[0256] Methods for preparing such dosage forms are known or apparent to those skilled in the art; for example, see Remington's Pharmaceutical Sciences (17th Ed., Mack Pub. Co., 1985). The composition to be administered will contain a quantity of the selected compound in a pharmaceutically effective amount for therapeutic use in a biological system, including a patient or subject according to the disclosed technology.

[0257] Methods

[0258] The constructs herein can be adapted to recognize the target and, if needed, deliver the one or more cargos to treat that target. Exemplary targets include a cell, a pathogen, an organ (e.g., dermis, vasculature, lymphoid tissue, liver, lung, spleen, kidneys, heart, brain, bone, muscle, etc.), a cellular target (e.g., targets of the subject, such as a human subject, including host tissue, host cytoplasm, host nucleus, etc., in any useful cell, such as e.g., hepatocytes, alveolar epithelial cells, and innate immune cells, etc.); as well as targets for exogenous cells and organisms, such as extracellular and/or intracellular components of a pathogen, e.g., bacteria), a molecular target (e.g., within the subject or the exogenous cell/organism, such as pathogen DNA, host DNA, pathogen RNA, pathogen proteins, surface proteins or carbohydrates of any subject or exogenous cell).

[0259] In one instance, the particle is employed to target a host (e.g., a subject), a pathogen, or both (e.g., thereby treating the subject and/or the target). Exemplary pathogens include a bacterium, such as *Bacillus* (e.g., *B. anthracis*), Enterobacteriaceae (e.g., *Salmonella, Escherichia coli, Yersinia pestis, Klebsiella*, and *Shigella*), *Yersinia* (e.g., *Y. pestis* or *Y. enterocolitica*), *Staphylococcus* (e.g., *S. aureus*), *Streptococcus, Gonorrheae, Enterococcus* (e.g., *E. faecalis*), *Listeria* (e.g., *L. monocytogenes*), *Brucella* (e.g., *B. abortus, B. melitensis*, or *B. suis*), *Vibrio* (e.g., *V. cholerae*), *Corynebacterium diphtheria, Pseudomonas* (e.g., *P. pseudomallei* or *P. aeruginosa*), *Burkholderia* (e.g., *B. mallei* or *B. pseudomallei*), *Shigella* (e.g., *S. dysenteriae*), *Rickettsia* (e.g., *R. rickettsii, R. prowazekii*, or *R. typhi*), *Francisella tularensis, Chlamydia psittaci, Coxiella burnetii, Mycoplasma* (e.g., *M. mycoides*), etc.; mycotoxins, mold spores, or bacterial spores such as *Clostridium botulinum* and *C. perfringens*; a virus, including DNA or RNA viruses, such as Adenoviridae (e.g., adenovirus), Arenaviridae (e.g., Machupo virus), Bunyaviridae (e.g., Hantavirus or Rift Valley fever virus), Coronaviridae (e.g., SARS-Cov-2), Orthomyxoviridae (e.g., influenza viruses), Filoviridae (e.g., Ebola virus and Marburg virus), Flaviviridae (e.g., Japanese encephalitis virus, hepatitis C virus, and Yellow fever virus), Hepadnaviridae (e.g., hepatitis B virus), Herpesviridae (e.g., herpes simplex viruses, herpesvirus, cytomegalovirus, Epstein-Barr virus, or varicella zoster viruses), Papillomaviridae (e.g., papilloma viruses), Papovaviridae (e.g., papilloma viruses), Paramyxoviridae (e.g., respiratory syncytial virus, measles virus, mumps virus, or parainfluenza virus), Parvoviridae, Picornaviridae (e.g., polioviruses and hepatitis A virus), Polyomaviridae, Poxviridae (e.g., variola viruses or vaccinia virus), Reoviridae (e.g., rotaviruses), Retroviridae (e.g., human T cell lymphotropic viruses (HTLV) and human immunodeficiency viruses (HIV)), Rhabdoviridae (e.g., rabies virus), and Togaviridae (e.g., encephalitis viruses, yellow fever virus, and rubella virus)); a protozoon, such as *Cryptosporidium parvum, Encephalitozoa, Plasmodium, Toxoplasma gondii, Acanthamoeba, Entamoeba histolytica, Giardia lamblia, Trichomonas vaginalis, Leishmania*, or *Trypanosoma* (e.g., *T. brucei* and T. *Cruzi*); a helminth, such as cestodes (tapeworms), trematodes (flukes), or nematodes (roundworms, e.g., *Ascaris lumbricoides, Trichuris trichiura, Necator americanus*, or *Ancylostoma duodenale*); a parasite (e.g., any protozoa or helminths described herein); or a fungus, such as *Aspergilli, Candidae, Coccidioides immitis*, and *Cryptococci*. Other pathogens include a multi-drug resistant (MDR) pathogen, such as MDR forms of any pathogen described herein. Additional pathogens are described in Cello J et al., *Science* 2002; 297:1016-8; Gibson D G et al., *Science* 2010; 329: 52-6; Jackson R J et al., *J. Virol.* 2001; 75:1205-10; Russell C A et al., *Science* 2012; 336:1541-7; Tumpey T M et al., *Science* 2005; 310:77-80; and Weber N D et al., *Virology* 2014; 454-455c:353-61, each of which is incorporated herein by reference in its entirety.

[0260] The constructs of the disclosed technology can be employed to treat any useful disease that would benefit from genetic knock-out of a known protein. For instance, the particles can be employed to treat a subject from a disease correlated with the presence of that known protein (e.g., a known protein expressed within the subject or within a pathogen infecting that subject). Other diseases include a genetic disorder (e.g., Huntington's disease, hemophilia, sickle cell anemia, metabolic disorders, etc.), in which expression of a known protein is correlated with the disease or its symptoms.

[0261] The constructs can be employed to transform a subject (e.g., by genetically modifying a target gene within the subject by employing a CRISPR component configured to bind to that target gene). Thus, in one instance, the particle can be configured to bind to a target sequence in a genomic sequence of the subject in order to modulate that target sequence. Modulation can include activating, inactivating, deactivating, and/or modifying expression or activity of the target sequence. For example, the cargo can bind to the target sequence, e.g., thereby inhibiting expression of one or more proteins encoded by the target sequence. In another example, the cargo cleaves the target sequence and optionally inserts a further nucleic acid sequence into the genomic sequence of the subject. In yet another example, the cargo activates the target sequence. Any useful target sequence can be modulated.

[0262] Methods of treating patients or subjects in need for a particular disease state or infection can include administration an effective amount of a pharmaceutical composition having a plurality of constructs (e.g., any described herein). Additional methods include diagnostic methods, which can include administering an effective amount of a population of

diagnostic particles to a subject in need thereof. In some embodiments, the population of particles, or a portion thereof, includes a ligand (e.g., to bind to target cells) and a reporter (e.g., to indicate binding to the target cell), whereupon the binding of one or more particles to cells as evidenced by the reporter component (moiety) will enable a diagnosis of the existence of a disease state in the subject.

[0263] In accordance with the present disclosure, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, 2001, "Molecular Cloning: A Laboratory Manual"; Ausubel, ed., 1994, "Current Protocols in Molecular Biology" Volumes I-III; Celis, ed., 1994, "Cell Biology: A Laboratory Handbook" Volumes I-III; Coligan, ed., 1994, "Current Protocols in Immunology" Volumes I-III; Gait ed., 1984, "Oligonucleotide Synthesis"; Hames & Higgins eds., 1985, "Nucleic Acid Hybridization"; Hames & Higgins, eds., 1984, "Transcription And Translation"; Freshney, ed., 1986, "Animal Cell Culture"; IRL.

[0264] The present disclosure also relates to methods of fabricating a construct (e.g., or a population of particles). The method can include, e.g., providing a core (including a plurality of cores) having any useful characteristic (e.g., any described herein, such as having a dimension greater than about 50 nm, having a negative charge, having one or more pores, and/or including a silica); incubating the core with one or more cargo (e.g., any herein, including a plasmid, a CRISPR component, etc.), thereby providing a loaded core; and exposing the loaded core to a lipid formulation (e.g., any described herein).

[0265] In other embodiments, the method can include providing a core and then expanding the pores present on the core. In some instance, a method can include: providing a core including an external surface and a plurality of pores in fluidic communication with the external surface (e.g., where an average dimension of the plurality of pores is characterized by a first dimension); expanding the pores (e.g., thereby providing a core comprising a plurality of expanded pores, wherein an average dimension of the plurality of expanded pores is characterized by a second dimension that is greater than the first dimension); incubating the core with one or more cargo, thereby providing a loaded core; and exposing the loaded core to a polymer formulation or a lipid formulation to form an outer layer supported upon the external surface of the core (e.g., thereby providing the construct).

[0266] FIG. **3**A-**3**C shows exemplary methods for providing a construct and its use for in vitro gene editing. Provided are schematics of (A) an exemplary method for loading RNP within a construct and (B) an exemplary method for in vitro gene editing by use of an RNP-loaded construct. Also provided are (C) fluorescence photomicrographs showing delivery of an RNP-loaded construct to a reporter cell line with an AAVS1 target site, in which effective gene-editing by the RNP results in a frameshift mutation and GFP expression.

EXAMPLES

[0267] Numerous experiments were run with various LCMSN (lipid-coated mesoporous nanoparticle) constructs comprising lipid compositions and mesoporous nanoparticles (MSNs) until a surprisingly effective combination was found. In particular, a stellate MSN with a 33 mol %, DOTAP, 33 mol % DOPE, 30 mol % cholesterol and 4 mol % DSPE-PEG2000 synthesized in Example 3 below was chosen after trial and error experimentation to achieve surprisingly effective delivery of CRISPR-Cas9 RNP packaged in LC-MSN. To assess the LC-MSN with the selected coating, negative staining and cryo-EM was performed on CRISPR-Cas9 RNP-loaded LC-MSN. A complete lipid coat was visible on microscopic analysis. Further details are provided below.

Example 1: MSN Fabrication and Characterization

[0268] In Example 1A Stellate MSN synthesis was carried out in a 50 mL round bottom flask, by combining triethanolamine (70 mg), CTATos (0.300 g, cas #138-32-9) with 20 mL water (Sigma). The solution was stirred at 75-80° C. for 30 min to ensure complete dissolution. Condensation was achieved by adding TEOS (2.9 mL) dropwise to the solution over 5 minutes stirring at 850 rpm. The reaction was then carried out for 2 hours at 75-80° C. with a condenser. MSN solution was removed from heat and cooled for 15 minutes then spun down for 15 minutes at 50,000×g. The collected MSN were washed twice by pure then 190 proof ethanol. Surfactant removal was carried out by suspending the MSN in 5% v (volume) HCl (12 N, 37%) in ethanol in a 100 mL round bottom flask and refluxing for 2 hours (using a cooling column, refrigerant). This was repeated with HCl 1% instead of 5% and then 190 proof ethanol then pure ethanol. The resulting MSN were resuspended in pure ethanol and passed through a 1 μm filter to remove any aggregates.

[0269] Size and zeta potential were assessed using a Zetasizer instrument (Malvern Instruments, Ltd). Morphology was assessed by TEM (JEOL 2010).

[0270] In addition, hexagonal prism small pore particles were prepared as previously described (LaBauve, et al, *Lipid-Coated Mesoporous Silica Nanoparticles for the Delivery of the ML*336 *Antiviral to Inhibit Encephalitic Alphavirus Infection*, Sci Rep. 2018; 8: 13990, 2018 Sep. 18. doi: 10.1038/s41598-018-32033-w) incorporated herein by reference. These were stored in pure ethanol and quantified. The average size (diameter) by DLS was 160 nm and the zeta potential was −37 mV.

Example 2: Preparation of Liposomes

[0271] A 7.27 mg/mL liposome solution in 50:50 PBS: water (4 mM $MgCl_2$) composed of 33 mol % 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) 33 mol % 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) 30 mol % Cholesterol and 4 mol % 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy-(polyethyleneglycol)-2000] (DPSE-PEG2000) (Avanti Polar Lipids) was used. Stock lipids were diluted in chloroform (Sigma) and 7.27 mg total lipids were combined in a glass scintillation vial then dried down using a rotary evaporator (Buchi Corp.). The resulting lipid films were placed under vacuum overnight to ensure complete solvent removal then resuspended in 1 mL 0.5×PBS 4 mM $MgCl_2$ solution. The suspension was placed in a sonication bath (Branson) and sonicated for 30 minutes at 30° C., then immediately extruded with 21 passes through a 100 nm filter (Whatman). Liposome size was assessed by DLS. See FIG. **18**B, column labeled "lipid".

Example 3: Cargo Loading and LC-MSN Fabrication

[0272] 100 μg of MSN stored in ethanol was spun down in a 1.5 mL Eppendorf tube at 21,000×g for 10 minutes and

washed with 1 mL of water under sonication. Washed MSN were collected by centrifugation and resuspended in 10 μL of water. CRISPR-Cas-9/gRNA complex from ribonucleoproteins (RNPs) was complexed in 53.3 μL 100 mM NaCl, 50 mM Tris, 10% glycerol, at pH 8.0 by adding 20 pg of SpyCas9 and 6.67 μg sgRNA (a 1:3 ratio gRNA:Cas9) for a final Cas-9 concentration of 0.375 μg/uL and incubated at 30° C. for 15 minutes to achieve a ratio of 1:5 Cas9:MSN. The RNP solution was mixed with the MSN with pipetting and sonication then incubated at room temperature for 30 minutes. Liposomes were fused to RNP loaded MSN by adding 100 μL of extruded liposomes in a mix of 50:50 PBS:water supplemented with 4 mM $MgCl_2$ and pipetting with occasional sonication. The resulting LC-MSN were collected by centrifugation for 15 minutes at 15,000×g and washed with 1 mL PBS then resuspended in 100 μL PBS with pipetting and sonication.

[0273] FIG. **18**D shows comparison of the RNP-loaded MSN with and without lipids. FIG. **18**C shows size measurements during LC-MSN assembly.

Example 4: LC-MSN Loading

[0274] Immediately after LC-MSN formation, 25 μg of LC-MSN in PBS was collected in 1.5 mL Eppendorf tubes. Either a 1.8% solution of TX-100 in PBS, or PBS (12.5 μL) was added and the solution was incubated at 37° C. shaking at 400 rpm for 4 hours. The LC-MSN was then spun down at 21,000×g for 10 minutes to separate the supernatant from remaining MSN. The pellets were resuspended in 37.5 μL of PBS and 4× Laemmli buffer was added and all samples were boiled for 10 minutes. Samples were run on a 10% polyacrylamide gel (Biorad) with standard known amounts on Cas-9 protein.

[0275] Loading and release was assessed by densitometry. See FIG. **16**. Western blot gel images were taken and analyzed by ImageJ software where the density of signal across protein bands were quantified and normalized to Cas9 protein standards.

Example 5: Reporter Cell Line

[0276] The reporter cell line was generating in A549 cells (ATCC) using a fluorescent reporter system from PNA Bio. The gene was cloned into a lentivirus vector, and particles derived from a single clone were used to transduce human A549 lung epithelial cells. The transduced cells constitutively express red fluorescent protein (RFP). The RFP signal is linked to a gene encoding green fluorescent protein (EGFP) gene. Expression of EGFP is dependent on double strand breaks that lead to a frame-shift mutation. See FIG. **19** illustrating this Example. Reporter and parental cell lines were maintained in F-K12 media (Gibco) supplemented with 10% FBS (Gibco) and penicillin/streptomycin.

Example 6: In Vitro Editing Experiments

[0277] Cells were seeded at 50,000 cells per well in 12-well plates (Costar) in 1 mL of F-K12 media supplemented with 5% FBS (Gibco) and pen/strep (Gibco) and were incubated overnight. The RNP loaded LC-MSNs were resuspended in PBS at 1 mg/mL. LC-MSNs were added directly to the 1 mL of media in each well at increasing concentrations of 20 μg/mL, 40 μg/mL, 60 μg/mL and 80 μg/mL. Media was replaced after 16 hours and editing efficiency was assessed at 72 hours by microscopy and flow cytometry.

Example 7: Flow Cytometry

[0278] Editing was assessed by flow cytometry. Cells in 12-well plates were lifted with 250 μL trypsin (Gibco) collected by centrifugation at 4000×g in 1.5 mL tubes, washed with 1 mL PBS (Gibco) and resuspended in 1 mL of PBS supplementing with 4% paraformaldehyde. Fixed cells were assessed with an Accuri C3 flow cytometer (BD). Untreated and RNP-CRISPR Max treated A549R cells were used as negative and positive controls respectively for gating.

Example 8: Cryo-EM Analysis

[0279] For cryo-EM analysis, freshly prepared RNP loaded and unloaded LC-MSNs were vitrified using an automatic plunge freezer EM (Leica). 4 μL of LC-MSN solution was added to a C-flat grid (Protochips, Inc.) with 2 μm holes and blotted with filter paper. The grid was plunged into liquid ethane for flash freezing. Frozen grids were stored in liquid nitrogen and transferred to a JEM 2200FS electron microscope (JEOL Ltd.). Grids were imaged at 200 keV using DE-20 (Direct Detector Inc.) direct electron detector camera. The energy selecting slit was set to 20 eV and the microscope had a field emission electron source and omega-type electron energy filter to remove inelastically scattered electrons from the image formation.

[0280] A DE-20 camera was used to collect images in movie mode with a frame rate of 25 frames/sec. After image collection, frame alignment was performed using the E_process_frames.py script provided by Direct Electron Inc. Images were collected at 40,000× magnification and the pixel size on the specimen scale corresponded to 1.5 A/pixel. See FIG. **14**C.

Example 9: Phosphotungstic Acid-Based Negative Staining of LC-MSN for TEM

[0281] RNP-loaded LC-MSNs were prepared as described above, diluted in PBS to 0.1 mg/mL then added (5 μL) to a TEM copper grid (Sigma Aldrich). After a 5 minute drying period, a phosphotungstinic acid solution (2% in water) was added (5 μL) to the grid and removed by dabbing with the corner of a Kimwipe (Kimtech) after 10-15 seconds. The grid was washed with 15 μL water and allowed to dry at room temperature prior to TEM imaging as described above. See FIG. **14**B.

Example 10: In Vivo Delivery of CRISPR-Cas9 RNP LC-MSN

[0282] To assess the efficiency of the LC-MSN delivery vehicle in vivo a murine liver NPC was utilized.

Example 11: Comparison of Different Core Particles

[0283] Delivery of the CRISPR-Cas-9 cargo was tested in vitro with varying types of particles (cores) that were able to form an LC-MSN of under 400 nm in average diameter (DLS by methods described herein) after loading of CRISPR-Cas9/gRNA cargo. See FIG. **18**A. All particle types that were tested had an average diameter of 2 nm pores

or greater. Hexagonal prisms had pore size of 2.5 nm, Stober particles are non-porous, Stellate particules were 12 nm and dendritic particles were either 8 nm or 18 nm in average pore size. These Examples were assayed by measuring % Green Fluorescent Protein (GFP) positive cells after 72-hour exposure of A549 reporter cells to 40, 60, and 80 μg/mL of the CRISPR-Cas9 loaded LC-MSN (FIG. **17**A).

[0284] While several of the other particle types, including the dendritic 8 nm pore and 18 nm pore, resulted in a good sized stable LC-MSN, editing efficiency was surprisingly high (FIG. **17**C) in the stellate core types. A stellate particle core with pore size range of 6-10.5 nm was selected to move forward with studies on CRISPR-RNP delivery.

Examples 12: Comparison of CRISPR-Cas9 Loaded LC-MSN (Example 3) to CRISPR-Cas-9 and Free Lipid Composition from Example 2 with No Core Particles

[0285] While the liposome composition in Example 2 mixed with CRISPR-Cas9 RNP resulted in a low amount of editing in the absence of the MSN, editing increased substantially with the use of the stellate LC-MSNs of Example 3. See FIG. **17**B. The figure also includes a comparison with NiNTA functionalized particles. The NiNTA particles were MSN cores functionalized to capture Cas9 that is 6×HIS tagged. This is another method used to load the particles with CRISPR RNPs.

[0286] All publications, patents, and patent applications mentioned in this specification are incorporated herein by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

[0287] What has been described above includes examples of one or more embodiments. It is, of course, not possible to describe every conceivable modification and alteration of the above devices or methodologies for purposes of describing the aforementioned aspects, but one of ordinary skill in the art can recognize that many further modifications and permutations of various aspects are possible. Accordingly, the described aspects are intended to embrace all such alterations, modifications, and variations that fall within the spirit and scope of the appended claims. Furthermore, to the extent that the term "includes" is used in either the detailed description or the claims, such term is intended to be inclusive in a manner similar to the term "comprising" as "comprising" is interpreted when employed as a transitional word in a claim. The term "consisting essentially" as used herein means the specified materials or steps and those that do not materially affect the basic and novel characteristics of the material or method. Unless the context indicates otherwise, all percentages and averages are by weight. If not specified above, the properties mentioned herein may be determined by applicable NIST standards, or if an NIST standard does not exist for the property, then NCL, and then ASTM standards may be used, if none of the above standards are available, the most commonly used standard known by those of skill in the art may be used. The articles "a," "an," and "the," should be interpreted to mean "one or more" unless the context indicates the contrary.

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variation 8
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variation 9
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variation 11
note = may be T
variation 12
note = n = A,T,C,G, or U (e.g., A,C, or G)
variation 13
note = may be T
variation 14
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variation 24
note = n = A,T,C,G, or U (e.g., A or G)
variation 25
note = may be T
variation 26
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variation 8
note = may be T
variation 9
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variation 10
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variation 11
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variation 21
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variation 22
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SEQUENCE: 33
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SEQ ID NO: 38 moltype = length =
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SEQ ID NO: 39 moltype = length =
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variation 4
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variation 7
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variation 15
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note = n = A,T,C,G, or U (e.g., T,C, or U)

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variation	33 note = n = A,T,C,G, or U (e.g., A or absent)
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                       note = n = A,T,C,G, or U (e.g., T,U, or absent)
variation              5
                       note = n = A,T,C,G, or U (e.g., A or absent)
variation              6
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variation              8
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variation              22
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                       note = n = A,T,C,G, or U (e.g., T,C, or U)
```

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variation	34
	note = n = A,T,C,G, or U (e.g., A,T, or U)
variation	35
	note = n = A,T,C,G, or U (e.g., A or G)
variation	37
	note = n = A,T,C,G, or U (e.g., A,T, or U)
variation	38
	note = n = A,T,C,G, or U (e.g., T,C, or U)
variation	39..41
	note = n = A,T,C,G, or U (e.g., A or absent)
variation	42
	note = n = A,T,C,G, or U (e.g., T,U, or absent)
variation	43
	note = n = A,T,C,G, or U (e.g., C or absent)
variation	44
	note = n = A,T,C,G, or U (e.g., T,U, or absent)
variation	45
	note = n = A,T,C,G, or U (e.g., A,T, or U)
variation	48
	note = n = A,T,C,G, or U (e.g., A or absent)
variation	49
	note = n = A,T,C,G, or U (e.g., G or absent)
variation	50
	note = n = A,T,C,G, or U (e.g., A or G)
variation	53
	note = n = A,T,C,G, or U (e.g., T,C, or U)
variation	54
	note = may be T
variation	56
	note = n = A,T,C,G, or U (e.g., C or absent)
variation	57..58
	note = n = A,T,C,G, or U (e.g., A or absent)
variation	60
	note = n = A,T,C,G, or U (e.g., A or G)
variation	62
	note = may be T
variation	68
	note = may be T
variation	69
	note = n = A,T,C,G, or U (e.g., T,U, or absent)
variation	70
	note = n = A,T,C,G, or U (e.g., A,T,C, or U)
variation	71
	note = n = A,T,C,G, or U (e.g., A or G)
variation	72
	note = may be T
variation	73
	note = n = A,T,C,G, or U (e.g., G or absent)
variation	77..78
	note = n = A,T,C,G, or U (e.g., A,T, or U)
variation	79
	note = n = A,T,C,G, or U (e.g., A or C)
variation	80
	note = may be T
variation	85
	note = n = A,T,C,G, or U (e.g., A or absent)
variation	86..87
	note = n = A,T,C,G, or U (e.g., C or absent)
variation	88
	note = n = A,T,C,G, or U (e.g., T,C, or U)
variation	89
	note = may be T
variation	90
	note = n = A,T,C,G, or U (e.g., T,G, or U)
variation	91
	note = n = A,T,C,G, or U (e.g., A,T, or U)
variation	92
	note = n = A,T,C,G, or U (e.g., A or C)
variation	94..95
	note = n = A,T,C,G, or U (e.g., T,U, or absent)
variation	96
	note = n = A,T,C,G, or U (e.g., A,T, or U)
variation	97
	note = n = A,T,C,G, or U (e.g., A,T,G, or U)
variation	98
	note = n = A,T,C,G, or U (e.g., A or G)

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variation	99 note = may be T
variation	100 note = n = A,T,C,G, or U (e.g., A or G)
variation	103 note = n = A,T,C,G, or U (e.g., A or G)
variation	104 note = n = A,T,C,G, or U (e.g., C or G)
variation	105 note = n = A,T,C,G, or U (e.g., T,C,G, or U)
variation	107 note = n = A,T,C,G, or U (e.g., A,T, or U)
variation	108 note = n = A,T,C,G, or U (e.g., T,G, or U)
variation	109 note = may be T
variation	110 note = n = A,T,C,G, or U (e.g., T,C, or U)
variation	111..112 note = n = A,T,C,G, or U (e.g., T,G, or U)
variation	113 note = n = A,T,C,G, or U (e.g., T,C, or U)
variation	115 note = n = A,T,C,G, or U (e.g., T,C,U, or absent)
variation	116..117 note = may be T
variation	118 note = n = A,T,C,G, or U (e.g., A,T, or U)
variation	119..121 note = may be T
source	1..121 mol_type = other RNA organism = synthetic construct

SEQUENCE: 64

nnnnnntnnn ntgnnannnn nnnnntncnn annnncnnnn nnnnngcnnn agntannnan 60
ataaggctnn ntnccgnnnt caacnnnntn nnannnnntn gcnnngnntn nnngnttntt 120
t 121

SEQ ID NO: 65	moltype = RNA length = 39
FEATURE	Location/Qualifiers
misc_feature	1..39 note = Synthetic construct
variation	1 note = n = A,T,C,G, or U (e.g., T,C, or U)
variation	2..4 note = n = A,T,C,G, or U (e.g., A or absent)
variation	5 note = n = A,T,C,G, or U (e.g., T,U, or absent)
variation	6 note = n = A,T,C,G, or U (e.g., C or absent)
variation	7 note = n = A,T,C,G, or U (e.g., T,U, or absent)
variation	8 note = n = A,T,C,G, or U (e.g., A,T, or U)
variation	11 note = n = A,T,C,G, or U (e.g., A or absent)
variation	12 note = n = A,T,C,G, or U (e.g., G or absent)
variation	13 note = n = A,T,C,G, or U (e.g., A or G)
variation	16 note = n = A,T,C,G, or U (e.g., T,C, or U)
variation	17 note = may be T
variation	19 note = n = A,T,C,G, or U (e.g., C or absent)
variation	20..21 note = n = A,T,C,G, or U (e.g., A or absent)
variation	23 note = n = A,T,C,G, or U (e.g., A or G)
variation	25 note = may be T
variation	31 note = may be T
variation	32 note = n = A,T,C,G, or U (e.g., T,U, or absent)

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variation	33 note = n = A,T,C,G, or U (e.g., A,T,C, or U)
variation	34 note = n = A,T,C,G, or U (e.g., A or G)
variation	35 note = may be T
variation	36 note = n = A,T,C,G, or U (e.g., G or absent)
source	1..39 mol_type = other RNA organism = synthetic construct

SEQUENCE: 65
nnnnnnnngc nnnagntann nanataaggc tnnntnccg 39

SEQ ID NO: 66 moltype = length =
SEQUENCE: 66
000

SEQ ID NO: 67 moltype = length =
SEQUENCE: 67
000

SEQ ID NO: 68 moltype = length =
SEQUENCE: 68
000

SEQ ID NO: 69 moltype = length =
SEQUENCE: 69
000

SEQ ID NO: 70 moltype = RNA length = 12

FEATURE	Location/Qualifiers
misc_feature	1..12 note = Synthetic construct
source	1..12 mol_type = other RNA organism = synthetic construct

SEQUENCE: 70
gttttagagc ta 12

SEQ ID NO: 71 moltype = RNA length = 26

FEATURE	Location/Qualifiers
misc_feature	1..26 note = Synthetic construct
source	1..26 mol_type = other RNA organism = synthetic construct

SEQUENCE: 71
tagcaagtta aaataaggct agtccg 26

SEQ ID NO: 72 moltype = length =
SEQUENCE: 72
000

SEQ ID NO: 73 moltype = length =
SEQUENCE: 73
000

SEQ ID NO: 74 moltype = length =
SEQUENCE: 74
000

SEQ ID NO: 75 moltype = length =
SEQUENCE: 75
000

SEQ ID NO: 76 moltype = length =
SEQUENCE: 76
000

SEQ ID NO: 77 moltype = length =
SEQUENCE: 77
000

SEQ ID NO: 78 moltype = length =
SEQUENCE: 78

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000

SEQ ID NO: 79 moltype = length =
SEQUENCE: 79
000

SEQ ID NO: 80 moltype = RNA length = 38
FEATURE Location/Qualifiers
misc_feature 1..38
note = Synthetic construct
misc_feature 12..13
note = Location of linker, L
source 1..38
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 80
gttttagagc tatagcaagt taaaataagg ctagtccg 38

SEQ ID NO: 81 moltype = length =
SEQUENCE: 81
000

SEQ ID NO: 82 moltype = length =
SEQUENCE: 82
000

SEQ ID NO: 83 moltype = length =
SEQUENCE: 83
000

SEQ ID NO: 84 moltype = RNA length = 51
FEATURE Location/Qualifiers
misc_feature 1..51
note = Synthetic construct
misc_feature 12..13
note = Location of linker, L
source 1..51
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 84
nnnnnnnnnn tnnnnnnnnn gcnnnagnta nnnanataag gctnnntncc g 51

SEQ ID NO: 85 moltype = RNA length = 55
FEATURE Location/Qualifiers
misc_feature 1..55
note = Synthetic construct
misc_feature 16..17
note = Location of linker, L
source 1..55
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 85
nnnnnnnnnn tntnnnnnnn nnnngcnnna gntannnana taaggctnnn tnccg 55

SEQ ID NO: 86 moltype = RNA length = 73
FEATURE Location/Qualifiers
misc_feature 1..73
note = Synthetic construct
misc_feature 36..37
note = Location of linker, L
source 1..73
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 86
gttttngnnc tntnnnnntt nnantnnnnn nanaacnnnn nnnnnnnant nnaannnnnn 60
nagnnnntnn aaa 73

SEQ ID NO: 87 moltype = RNA length = 49
FEATURE Location/Qualifiers
misc_feature 1..49
note = Synthetic construct
misc_feature 12..13
note = Location of linker, L
source 1..49
mol_type = other RNA
organism = synthetic construct

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SEQUENCE: 87
gttttngnnc tnnnnnnnnn nnnantnnaa nnnnnnnagn nnntnnaaa 49

SEQ ID NO: 88 moltype = RNA length = 25
FEATURE Location/Qualifiers
misc_feature 1..25
note = Synthetic construct
misc_feature 12..13
note = Location of linker, L
source 1..25
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 88
gttttngnnc tnnagnnnnt nnaaa 25

SEQ ID NO: 89 moltype = RNA length = 51
FEATURE Location/Qualifiers
misc_feature 1..51
note = Synthetic construct
misc_feature 12..13
note = Location of linker, L
source 1..51
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 89
gttttngnnc tnnnnnnnnn gcnnnagnta nnnanataag gctnnntncc g 51

SEQ ID NO: 90 moltype = RNA length = 75
FEATURE Location/Qualifiers
misc_feature 1..75
note = Synthetic construct
misc_feature 38..39
note = Location of linker, L
source 1..75
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 90
nnaacantnn tntancaaat nnnntnancn nntgaaacnn nnnnnnnnna ntnnaannnn 60
nnnagnnnnt nnaaa 75

SEQ ID NO: 91 moltype = RNA length = 55
FEATURE Location/Qualifiers
misc_feature 1..55
note = Synthetic construct
misc_feature 16..17
note = Location of linker, L
source 1..55
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 91
nnaacantnn tntancnntn nnnnnnntnn nannnnnttn ntannnnnnt nnnnn 55

SEQ ID NO: 92 moltype = RNA length = 32
FEATURE Location/Qualifiers
misc_feature 1..32
note = Synthetic construct
misc_feature 16..17
note = Location of linker, L
source 1..32
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 92
nnaacantnn tntancnnta nnnnnntnnn nn 32

SEQ ID NO: 93 moltype = RNA length = 55
FEATURE Location/Qualifiers
misc_feature 1..55
note = Synthetic construct
misc_feature 16..17
note = Location of linker, L
source 1..55
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 93
nnaacantnn tntancnnnn nnnngcnnna gntannnana taaggctnnn tnccg 55

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SEQ ID NO: 94 moltype = length =
SEQUENCE: 94
000

SEQ ID NO: 95 moltype = length =
SEQUENCE: 95
000

SEQ ID NO: 96 moltype = length =
SEQUENCE: 96
000

SEQ ID NO: 97 moltype = length =
SEQUENCE: 97
000

SEQ ID NO: 98 moltype = length =
SEQUENCE: 98
000

SEQ ID NO: 99 moltype = length =
SEQUENCE: 99
000

SEQ ID NO: 100 moltype = RNA length = 218
FEATURE Location/Qualifiers
misc_feature 1..218
note = Synthetic construct
variation 1..80
note = where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and n is a, c, t, g, u, or modified forms thereof
variation 93..192
note = where n at each of positions 93-192 can be present or absent such that this region can contain anywhere from 3 to 100 nucleotides and n is a, c, t, g, u, or modified forms thereof
source 1..218
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 100
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 60
nnnnnnnnnn nnnnnnnnnn gttttagagc tannnnnnnn nnnnnnnnnn nnnnnnnnnn 120
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 180
nnnnnnnnnn nntagcaagt taaaataagg ctagtccg 218

SEQ ID NO: 101 moltype = RNA length = 219
FEATURE Location/Qualifiers
misc_feature 1..219
note = Synthetic construct
variation 1..80
note = where n at each of positions 1-80 can be present or absent such that this region can contain anywhere from 12 to 80 nucleotides and n is a, c, t, g, u, or modified forms thereof
variation 93..192
note = where n at each of positions 93-192 can be present or absent such that this region can contain anywhere from 3 to 100 nucleotides and n is a, c, t, g, u, or modified forms thereof
source 1..219
mol_type = other RNA
organism = synthetic construct
SEQUENCE: 101
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 60
nnnnnnnnnn nnnnnnnnnn gttttagagc tannnnnnnn nnnnnnnnnn nnnnnnnnnn 120
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 180
nnnnnnnnnn nntagcaagt taaaataagg ctttgtccg 219

SEQ ID NO: 102 moltype = RNA length = 163
FEATURE Location/Qualifiers
misc_feature 1..163
note = Synthetic construct
variation 1..80
note = where n at each of positions 1-80 can be present or

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                      absent such that this region can contain anywhere from 12
                      to 80 nucleotides and n is a, c, t, g, u, or modified
                      forms thereof
source                1..163
                      mol_type = other RNA
                      organism = synthetic construct
SEQUENCE: 102
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  60
nnnnnnnnnn nnnnnnnnnn gttttagagc tagaaatagc aagttaaaat aaggctagtc  120
cgttatcaac ttgaaaaagt ggcaccgagt cggtgctttt ttt                   163

SEQ ID NO: 103        moltype = RNA  length = 163
FEATURE               Location/Qualifiers
misc_feature          1..163
                      note = Synthetic construct
variation             1..80
                      note = where n at each of positions 1-80 can be present or
                      absent such that this region can contain anywhere from 12
                      to 80 nucleotides and n is a, c, t, g, u, or modified
                      forms thereof
source                1..163
                      mol_type = other RNA
                      organism = synthetic construct
SEQUENCE: 103
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn  60
nnnnnnnnnn nnnnnnnnnn gttttagagc tagaaatagc aagttaaaat aaggctagtc  120
cgttatcaac ttgaaaaagt ggcaccgagt cggtgctttt ttt                   163

SEQ ID NO: 104        moltype =   length =
SEQUENCE: 104
000

SEQ ID NO: 105        moltype =   length =
SEQUENCE: 105
000

SEQ ID NO: 106        moltype =   length =
SEQUENCE: 106
000

SEQ ID NO: 107        moltype =   length =
SEQUENCE: 107
000

SEQ ID NO: 108        moltype =   length =
SEQUENCE: 108
000

SEQ ID NO: 109        moltype =   length =
SEQUENCE: 109
000

SEQ ID NO: 110        moltype = AA  length = 1368
FEATURE               Location/Qualifiers
source                1..1368
                      mol_type = protein
                      organism = Streptococcus pyogenes
SEQUENCE: 110
MDKKYSIGLD IGTNSVGWAV ITDEYKVPSK KFKVLGNTDR HSIKKNLIGA LLFDSGETAE  60
ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG  120
NIVDEVAYHE KYPTIYHLRK KLVDSTDKAD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD  180
VDKLFIQLVQ TYNQLFEENP INASGVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN  240
LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAI  300
LLSDILRVNT EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA  360
GYIDGGASQE EFYKFIKPIL EKMDGTEELL VKLNREDLLR KQRTFDNGSI PHQIHLGELH  420
AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE  480
VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL  540
SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI  600
IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG  660
RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL  720
HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER  780
MKRIEEGIKE LGSQILKEHP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDH  840
IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL  900
TKAERGGLSE LDKAGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVKVITLKS  960
KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK  1020
MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF  1080
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ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA  1140
YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKNPID FLEAKGYKEV KKDLIIKLPK  1200
YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE  1260
QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA  1320
PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ SITGLYETRI DLSQLGGD               1368

SEQ ID NO: 111          moltype = AA  length = 1368
FEATURE                 Location/Qualifiers
REGION                  1..1368
                        note = Synthetic construct
source                  1..1368
                        mol_type = protein
                        organism = synthetic construct
SEQUENCE: 111
MDKKYSIGLA IGTNSVGWAV ITDEYKVPSK KFKVLGNTDR HSIKKNLIGA LLFDSGETAE  60
ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG  120
NIVDEVAYHE KYPTIYHLRK KLVDSTDKAD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD  180
VDKLFIQLVQ TYNQLFEENP INASGVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN  240
LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAI  300
LLSDILRVNT EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA  360
GYIDGGASQE EFYKFIKPIL EKMDGTEELL VKLNREDLLR KQRTFDNGSI PHQIHLGELH  420
AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE  480
VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL  540
SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI  600
IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG  660
RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL  720
HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER  780
MKRIEEGIKE LGSQILKEHP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDA  840
IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL  900
TKAERGGLSE LDKAGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVKVITLKS  960
KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK  1020
MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF  1080
ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA  1140
YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKNPID FLEAKGYKEV KKDLIIKLPK  1200
YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE  1260
QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA  1320
PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ SITGLYETRI DLSQLGGD               1368

SEQ ID NO: 112          moltype = AA  length = 1368
FEATURE                 Location/Qualifiers
source                  1..1368
                        mol_type = protein
                        organism = Streptococcus pyogenes
SEQUENCE: 112
MDKKYSIGLD IGTNSVGWAV ITDDYKVPSK KLKGLGNTDR HGIKKNLIGA LLFDSGETAE  60
ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG  120
NIVDEVAYHE KYPTIYHLRK KLADSTDKVD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD  180
VDKLFIQLVQ TYNQLFEENP INASRVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN  240
LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAT  300
LLSDILRVNS EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA  360
GYIDGGASQE EFYKFIKPIL EKMDGTEELL AKLNREDLLR KQRTFDNGSI PYQIHLGELH  420
AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE  480
VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL  540
SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI  600
IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG  660
RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL  720
HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER  780
MKRIEEGIKE LGSDILKEYP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDH  840
IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL  900
TKAERGGLSE LDKVGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVRVITLKS  960
KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK  1020
MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF  1080
ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA  1140
YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKDPID FLEAKGYKEV RKDLIIKLPK  1200
YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE  1260
QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA  1320
PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ SITGLYETRI DLSQLGGD               1368

SEQ ID NO: 113          moltype = AA  length = 1629
FEATURE                 Location/Qualifiers
source                  1..1629
                        mol_type = protein
                        organism = Francisella tularensis
SEQUENCE: 113
MNFKILPIAI DLGVKNTGVF SAFYQKGTSL ERLDNKNGKV YELSKDSYTL LMNNRTARRH  60
QRRGIDRKQL VKRLFKLIWT EQLNLEWDKD TQQAISFLFN RRGFSFITDG YSPEYLNIVP  120
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EQVKAILMDI FDDYNGEDDL DSYLKLATEQ ESKISEIYNK LMQKILEFKL MKLCTDIKDD  180
KVSTKTLKEI TSYEFELLAD YLANYSESLK TQKFSYTDKQ GNLKELSYYH HDKYNIQEFL  240
KRHATINDRI LDTLLTDDLD IWNFNFEKFD FDKNEEKLQN QEDKDHIQAH LHHFVFAVNK  300
IKSEMASGGR HRSQYFQEIT NVLDENNHQE GYLKNFCENL HNKKYSNLSV KNLVNLIGNL  360
SNLELKPLRK YFNDKIHAKA DHWDEQKFTE TYCHWILGEW RVGVKDQDKK DGAKYSYKDL  420
CNELKQKVTK AGLVDFLLEL DPCRTIPPYL DNNNRKPPKC QSLILNPKFL DNQYPNWQQY  480
LQELKKLQSI QNYLDSFETD LKVLKSSKDQ PYFVEYKSSN QQIASGQRDY KDLDARILQF  540
IFDRVKASDE LLLNEIYFQA KKLKQKASSE LEKLESSKKL DEVIANSQLS QILKSQHTNG  600
IFEQGTFLHL VCKYYKQRQR ARDSRLYIMP EYRYDKKLHK YNNTGRFDDD NQLLTYCNHK  660
PRQKRYQLLN DLAGVLQVSP NFLKDKIGSD DDLFISKWLV EHIRGFKKAC EDSLKIQKDN  720
RGLLNHKINI ARNTKGKCEK EIFNLICKIE GSEDKKGNYK HGLAYELGVL LFGEPNEASK  780
PEFDRKIKKF NSIYSFAQIQ QIAFAERKGN ANTCAVCSAD NAHRMQQIKI TEPVEDNKDK  840
IILSAKAQRL PAIPTRIVDG AVKKMATILA KNIVDDNWQN IKQVLSAKHQ LHIPIITESN  900
AFEFEPALAD VKGKSLKDRR KKALERISPE NIFKDKNNRI KEFAKGISAY SGANLTDGDF  960
DGAKEELDHI IPRSHKKYGT LNDEANLICV TRGDNKNKGN RIFCLRDLAD NYKLKQFETT  1020
DDLEIEKKIA DTIWDANKKD FKFGNYRSFI NLTPQEQKAF RHALFLADEN PIKQAVIRAI  1080
NNRNRTFVNG TQRYFAEVLA NNIYLRAKKE NLNTDKISFD YFGIPTIGNG RGIAEIRQLY  1140
EKVDSDIQAY AKGDKPQASY SHLIDAMLAF CIAADEHRND GSIGLEIDKN YSLYPLDKNT  1200
GEVFTKDIFS QIKITDNEFS DKKLVRKKAI EGFNTHRQMT RDGIYAENYL PILIHKELNE  1260
VRKGYTWKNS EEIKIFKGKK YDIQQLNNLV YCLKFVDKPI SIDIQISTLE ELRNILTTNN  1320
IAATAEYYYI NLKTQKLHEY YIENYNTALG YKKYSKEMEF LRSLAYRSER VKIKSIDDVK  1380
QVLDKDSNFI IGKITLPFKK EWQRLYREWQ NTTIKDDYEF LKSFFNVKSI TKLHKKVRKD  1440
FSLPISTNEG KFLVKRKTWD NNFIYQILND SDSRADGTKP FIPAFDISKN EIVEAIIDSF  1500
TSKNIFWLPK NIELQKVDNK NIFAIDTSKW FEVETPSDLR DIGIATIQYK IDNNSRPKVR  1560
VKLDYVIDDD SKINYFMNHS LLKSRYPDKV LEILKQSTII EFESSGFNKT IKEMLGMKLA  1620
GIYNETSNN                                                          1629

SEQ ID NO: 114          moltype = AA  length = 1409
FEATURE                 Location/Qualifiers
source                  1..1409
                        mol_type = protein
                        organism = Streptococcus thermophilus
SEQUENCE: 114
MLFNKCIIIS INLDFSNKEK CMTKPYSIGL DIGTNSVGWA VITDNYKVPS KKMKVLGNTS  60
KKYIKKNLLG VLLFDSGITA EGRRLKRTAR RRYTRRRNRI LYLQEIFSTE MATLDDAFFQ  120
RLDDSFLVPD DKRDSKYPIF GNLVEEKVYH DEFPTIYHLR KYLADSTKKA DLRLVYLALA  180
HMIKYRGHFL IEGEFNSKNN DIQKNFQDFL DTYNAIFESD LSLENSKQLE EIVKDKISKL  240
EKKDRILKLF PGEKNSGIFS EFLKLIVGNQ ADFRKCFNLD EKASLHFSKE SYDEDLETLL  300
GYIGDDYSDV FLKAKKLYDA ILLSGFLTVT DNETEAPLSS AMIKRYNEHK EDLALLKEYI  360
RNISLKTYNE VFKDDTKNGY AGYIDGKTNQ EDFYVYLKNL LAEFEGADYF LEKIDREDFL  420
RKQRTFDNGS IPYQIHLQEM RAILDKQAKF YPFLAKNKER IEKILTFRIP YYVGPLARGN  480
SDFAWSIRKR NEKITPWNFE DVIDKESSAE AFINRMTSFD LYLPEEKVLP KHSLLYETFN  540
VYNELTKVRF IAESMRDYQF LDSKQKKDIV RLYFKDKRKV TDKDIIEYLH AIYGYDGIEL  600
KGIEKQFNSS LSTYHDLLNI INDKEFLDDS SNEAIIEEII HTLTIFEDRE MIKQRLSKFE  660
NIFDKSVLKK LSRRHYTGWG KLSAKLINGI RDEKSGNTIL DYLIDDGISN RNFMQLIHDD  720
ALSFKKKIQK AQIIGDEDKG NIKEVVKSLP GSPAIKKGIL QSIKIVDELV KVMGGRKPES  780
IVVEMARENQ YTNQGKSNSQ QRLKRLEKSL KELGSKILKE NIPAKLSKID NNALQNDRLY  840
LYYLQNGKDM YTGDDLDIDR LSNYDIDHII PQAFLKDNSI DNKVLVSSAS NRGKSDDFPS  900
LEVVKKRKTF WYQLLKSKLI SQRKFDNLTK AERGGLLPED KAGFIQRQLV ETRQITKHVA  960
RLLDEKFNNK KDENNRAVRT VKIITLKSTL VSQFRKDFEL YKVREINDFH HAHDAYLNAV  1020
IASALLKKYP KLEPEFVYGD YPKYNSFRER KSATEKVYFY SNIMNIFKKS ISLADGRVIE  1080
RPLIEVNEET GESVWNKESD LATVRRVLSY PQVNVVKKVE EQNHGLDRGK PKGLFNANLS  1140
SKPKPNSNEN LVGAKEYLDP KKYGGYAGIS NSFAVLVKGT IEKGAKKKIT NVLEFQGISI  1200
LDRINYRKDK LNFLLEKGYK DIELIIELPK YSLFELSDGS RRMLASILST NNKRGEIHKG  1260
NQIFLSQKFV KLLYHAKRIS NTINENHRKY VENHKKEFEE LFYYILEFNE NYVGAKKNGK  1320
LLNSAFQSWQ NHSIDELCSS FIGPTGSERK GLFELTSRGS AADFEFLGVK IPRYRDYTPS  1380
SLLKDATLIH QSVTGLYETR IDLAKLGEG                                    1409

SEQ ID NO: 115          moltype = AA  length = 1388
FEATURE                 Location/Qualifiers
source                  1..1388
                        mol_type = protein
                        organism = Streptococcus thermophilus
SEQUENCE: 115
MTKPYSIGLD IGTNSVGWAV TTDNYKVPSK KMKVLGNTSK KYIKKNLLGV LLFDSGITAE  60
GRRLKRTARR RYTRRRNRIL YLQEIFSTEM ATLDDAFFQR LDDSFLVPDD KRDSKYPIFG  120
NLVEEKAYHD EFPTIYHLRK YLADSTKKAD LRLVYLALAH MIKYRGHFLI EGEFNSKNND  180
IQKNFQDFLD TYNAIFESDL SLENSKQLEE IVKDKISKLE KKDRILKLFP GEKNSGIFSE  240
FLKLIVGNQA DFRKCFNLDE KASLHFSKES YDEDLETLLG YIGDDYSDVF LKAKKLYDAI  300
LLSGFLTVTD NETEAPLSSA MIKRYNEHKE DLALLKEYIR NISLKTYNEV FKDDTKNGYA  360
GYIDGKTNQE DFYVYLKKLL AEFEGADYFL EKIDREDFLR KQRTFDNGSI PYQIHLQEMR  420
AILDKQAKFY PFLAKNKERI EKILTFRIPY YVGPLARGNS DFAWSIRKRN EKITPWNFED  480
VIDKESSAEA FINRMTSFDL YLPEEKVLPK HSLLYETFNV YNELTKVRFI AESMRDYQFL  540
DSKQKKDIVR LYFKDKRKVT DKDIIEYLHA IYGYDGIELK GIEKQFNSSL STYHDLLNII  600
NDKEFLDDSS NEAIIEEIIH TLTIFEDREM IKQRLSKFEN IFDKSVLKKL SRRHYTGWGK  660
LSAKLINGIR DEKSGNTILD YLIDDGISNR NFMQLIHDDA LSFKKKIQKA QIIGDEDKGN  720
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IKEVVKSLPG SPAIKKGILQ SIKIVDELVK VMGGRKPESI VVEMARENQY TNQGKSNSQQ 780
RLKRLEKSLK ELGSKILKEN IPAKLSKIDN NALQNDRLYL YYLQNGKDMY TGDDLDIDRL 840
SNYDIDHIIP QAFLKDNSID NKVLVSSASN RGKSDDVPSL EVVKKRKTFW YQLLKSKLIS 900
QRKFDNLTKA ERGGLSPEDK AGFIQRQLVE TRQITKHVAR LLDEKFNNKK DENNRAVRTV 960
KIITLKSTLV SQFRKDFELY KVREINDFHH AHDAYLNAVV ASALLKKYPK LEPEFVYGDY 1020
PKYNSFRERK SATEKVYFYS NIMNIFKKSI SLADGRVIER PLIEVNEETG ESVWNKESDL 1080
ATVRRVLSYP QVNVVKKVEE QNHGLDRGKP KGLFNANLSS KPKPNSNENL VGAKEYLDPK 1140
KYGGYAGISN SFTVLVKGTI EKGAKKKITN VLEFQGISIL DRINYRKDKL NFLLEKGYKD 1200
IELIIELPKY SLFELSDGSR RMLASILSTN NKRGEIHKGN QIFLSQKFVK LLYHAKRISN 1260
TINENHRKYV ENHKKEFEEL FYYILEFNEN YVGAKKNGKL LNSAFQSWQN HSIDELCSSF 1320
IGPTGSERKG LFELTSRGSA ADFEFLGVKI PRYRDYTPSS LLKDATLIHQ SVTGLYETRI 1380
DLAKLGEG 1388

SEQ ID NO: 116 moltype = AA length = 1334
FEATURE Location/Qualifiers
source 1..1334
mol_type = protein
organism = Listeria innocua
SEQUENCE: 116
MKKPYTIGLD IGTNSVGWAV LTDQYDLVKR KMKIAGDSEK KQIKKNFWGV RLFDEGQTAA 60
DRRMARTARR RIERRRNRIS YLQGIFAEEM SKTDANFFCR LSDSFYVDNE KRNSRHPFFA 120
TIEEEVEYHK NYPTIYHLRE ELVNSSEKAD LRLVYLALAH IIKYRGNFLI EGALDTQNTS 180
VDGIYKQFIQ TYNQVFASGI EDGSLKKLED NKDVAKILVE KVTRKEKLER ILKLYPGEKS 240
AGMFAQFISL IVGSKGNFQK PFDLIEKSDI ECAKDSYEED LESLLALIGD EYAELFVAAK 300
NAYSAVVLSS IITVAETETN AKLSASMIER FDTHEEDLGE LKAFIKLHLP KHYEEIFSNT 360
EKHGYAGYID GKTKQADFYK YMKMTLENIE GADYFIAKIE KENFLRKQRT FDNGAIPHQL 420
HLEELEAILH QQAKYYPFLK ENYDKIKSLV TFRIPYFVGP LANGQSEFAW LTRKADGEIR 480
PWNIEEKVDF GKSAVDFIEK MTNKDTYLPK ENVLPKHSLC YQKYLVYNEL TKVRYINDQG 540
KTSYFSGQEK EQIFNDLFKQ KRKVKKKDLE LFLRNMSHVE SPTIEGLEDS FNSSYSTYHD 600
LLKVGIKQEI LDNPVNTEML ENIVKILTVF EDKRMIKEQL QQFSDVLDGV VLKKLERRHY 660
TGWGRLSAKL LMGIRDKQSH LTILDYLMND DGLNRNLMQL INDSNLSFKS IIEKEQVTTA 720
DKDIQSIVAD LAGSPAIKKG ILQSLKIVDE LVSVMGYPPQ TIVVEMAREN QTTGKGKNNS 780
RPRYKSLEKA IKEFGSQILK EHPTDNQELR NNRLYLYYLQ NGKDMYTGQD LDIHNLSNYD 840
IDHIVPQSFI TDNSIDNLVL TSSAGNREKG DDVPPLEIVR KRKVFWEKLY QGNLMSKRKF 900
DYLTKAERGG LTEADKARFI HRQLVETRQI TKNVANILHQ RFNYEKDDHG NTMKQVRIVT 960
LKSALVSQFR KQFQLYKVRD VNDYHHAHDA YLNGVVANTL LKVYPQLEPE FVYGDYHQFD 1020
WFKANKATAK KQFYTNIMLF FAQKDRIIDE NGEILWDKKY LDTVKKVMSY RQMNIVKKTE 1080
IQKGEFSKAT IKPKGNSSKL IPRKTNWDPM KYGGLDSPNM AYAVVIEYAK GKNKLVFEKK 1140
IIRVTIMERK AFEKDEKAFL EEQGYRQPKV LAKLPKYTLY ECEEGRRRML ASANEAQKGN 1200
QQVLPNHLVT LLHHAANCEV SDGKSLDYIE SNREMFAELL AHVSEFAKRY TLAEANLNKI 1260
NQLFEQNKEG DIKAIAQSFV DLMAFNAMGA PASFKFFETT IERKRYNNLK ELLNSTIIYQ 1320
SITGLYESRK RLDD 1334

SEQ ID NO: 117 moltype = AA length = 1059
FEATURE Location/Qualifiers
source 1..1059
mol_type = protein
organism = Wolinella succinogenes
SEQUENCE: 117
MIERILGVDL GISSLGWAIV EYDKDDEAAN RIIDCGVRLF TAAETPKKKE SPNKARREAR 60
GIRRVLNRRR VRMNMIKKLF LRAGLIQDVD LDGEGGMFYS KANRADVWEL RHDGLYRLLK 120
GDELARVLIH IAKHRGYKFI GDDEADEESG KVKKAGVVLR QNFEAAGCRT VGEWLWRERG 180
ANGKKRNKHG DYEISIHRDL LVEEVEAIFV AQQEMRSTIA TDALKAAYRE IAFFVRPMQR 240
IEKMVGHCTY FPEERRAPKS APTAEKFIAI SKFFSTVIID NEGWEQKIIE RKTLEELLDF 300
AVSREKVEFR HLRKFLDLSD NEIFKGLHYK GKPKTAKKRE ATLFDPNEPT ELEFDKVEAE 360
KKAWISLRGA AKLREALGNE FYGRFVALGK HADEATKILT YYKDEGQKRR ELTKLPLEAE 420
MVERLVKIGF SDFLKLSLKA IRDILPAMES GARYDEAVLM LGVPHKEKSA ILPPLNKTDI 480
DILNPTVIRA FAQFRKVANA LVRKYGAFDR VHFELAREIN TKGEIEDIKE SQRKNEKERK 540
EAADWIAETS FQVPLTRKNI LKKRLYIQQD GRCAYTGDVI ELERLFDEGY CEIDHILPRS 600
RSADDSFANK VLCLARANQQ KTDRTPYEWF GHDAARWNAF ETRTSAPSNR VRTGKGKIDR 660
LLKKNFDENS EMAFKDRNLN DTRYMARAIK TYCEQYWVFK NSHTKAPVQV RSGKLTSVLR 720
YQWGLESKDR ESHTHHAVDA IIIAFSTQGM VQKLSEYYRF KETHREKERP KLAVPLANFR 780
DAVEEATRIE NTETVKEGVE VKRLLISRPP RARVTGQAHE QTAKPYPRIK QVKNKKKWRL 840
APIDEEKFES FKADRVASAN QKNFYETSTI PRVDVYHKKG KFHLVPIYLH EMVLNELPNL 900
SLGTNPEAMD ENFFKFSIFK DDLISIQTQG TPKKPAKIIM GYFKNMHGAN MVLSSINNSP 960
CEGFTCTPVS MDKKHKDKCK LCPEENRIAG RCLQGFLDYW SQEGLRPPRK EFECDQGVKF 1020
ALDVKKYQID PLGYYYEVKQ EKRLGTIPQM RSAKKLVKK 1059

SEQ ID NO: 118 moltype = length =
SEQUENCE: 118
000

SEQ ID NO: 119 moltype = length =
SEQUENCE: 119
000

-continued

SEQ ID NO: 120 moltype = length =
SEQUENCE: 120
000

SEQ ID NO: 121 moltype = AA length = 7
FEATURE Location/Qualifiers
REGION 1..7
note = Synthetic construct
source 1..7
mol_type = protein
organism = synthetic construct
SEQUENCE: 121
ASVHFPP 7

SEQ ID NO: 122 moltype = AA length = 7
FEATURE Location/Qualifiers
REGION 1..7
note = Synthetic construct
source 1..7
mol_type = protein
organism = synthetic construct
SEQUENCE: 122
TATFWFQ 7

SEQ ID NO: 123 moltype = AA length = 7
FEATURE Location/Qualifiers
REGION 1..7
note = Synthetic construct
source 1..7
mol_type = protein
organism = synthetic construct
SEQUENCE: 123
TSPVALL 7

SEQ ID NO: 124 moltype = AA length = 7
FEATURE Location/Qualifiers
REGION 1..7
note = Synthetic construct
source 1..7
mol_type = protein
organism = synthetic construct
SEQUENCE: 124
IPLKVHP 7

SEQ ID NO: 125 moltype = AA length = 7
FEATURE Location/Qualifiers
REGION 1..7
note = Synthetic construct
source 1..7
mol_type = protein
organism = synthetic construct
SEQUENCE: 125
WPRLTNM 7

SEQ ID NO: 126 moltype = AA length = 12
FEATURE Location/Qualifiers
REGION 1..12
note = Synthetic construct
source 1..12
mol_type = protein
organism = synthetic construct
SEQUENCE: 126
SFSIILTPIL PL 12

SEQ ID NO: 127 moltype = AA length = 15
FEATURE Location/Qualifiers
REGION 1..15
note = Synthetic construct
source 1..15
mol_type = protein
organism = synthetic construct
SEQUENCE: 127
SFSIILTPIL PLGGC 15

SEQ ID NO: 128 moltype = AA length = 18
FEATURE Location/Qualifiers

-continued

REGION	1..18 note = Synthetic construct
source	1..18 mol_type = protein organism = synthetic construct

SEQUENCE: 128
SFSIILTPIL PLEEEGGC 18

SEQ ID NO: 129 moltype = length =
SEQUENCE: 129
000

SEQ ID NO: 130 moltype = length =
SEQUENCE: 130
000

SEQ ID NO: 131 moltype = length =
SEQUENCE: 131
000

SEQ ID NO: 132 moltype = length =
SEQUENCE: 132
000

SEQ ID NO: 133 moltype = length =
SEQUENCE: 133
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SEQ ID NO: 134 moltype = length =
SEQUENCE: 134
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SEQ ID NO: 135 moltype = length =
SEQUENCE: 135
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SEQ ID NO: 136 moltype = length =
SEQUENCE: 136
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SEQ ID NO: 137 moltype = length =
SEQUENCE: 137
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SEQ ID NO: 138 moltype = length =
SEQUENCE: 138
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SEQ ID NO: 139 moltype = length =
SEQUENCE: 139
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SEQ ID NO: 140 moltype = length =
SEQUENCE: 140
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SEQ ID NO: 141 moltype = length =
SEQUENCE: 141
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SEQ ID NO: 142 moltype = length =
SEQUENCE: 142
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SEQ ID NO: 143 moltype = length =
SEQUENCE: 143
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SEQ ID NO: 144 moltype = length =
SEQUENCE: 144
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SEQ ID NO: 145 moltype = length =
SEQUENCE: 145
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-continued

SEQ ID NO: 146 moltype = length =
SEQUENCE: 146
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SEQ ID NO: 147 moltype = length =
SEQUENCE: 147
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SEQ ID NO: 148 moltype = length =
SEQUENCE: 148
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SEQ ID NO: 149 moltype = length =
SEQUENCE: 149
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SEQ ID NO: 150 moltype = length =
SEQUENCE: 150
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SEQ ID NO: 151 moltype = length =
SEQUENCE: 151
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SEQ ID NO: 152 moltype = length =
SEQUENCE: 152
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SEQ ID NO: 153 moltype = length =
SEQUENCE: 153
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SEQ ID NO: 154 moltype = length =
SEQUENCE: 154
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SEQ ID NO: 155 moltype = length =
SEQUENCE: 155
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SEQ ID NO: 156 moltype = length =
SEQUENCE: 156
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SEQ ID NO: 157 moltype = length =
SEQUENCE: 157
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SEQ ID NO: 158 moltype = length =
SEQUENCE: 158
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SEQ ID NO: 159 moltype = length =
SEQUENCE: 159
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SEQ ID NO: 160 moltype = length =
SEQUENCE: 160
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SEQ ID NO: 161 moltype = length =
SEQUENCE: 161
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SEQ ID NO: 162 moltype = length =
SEQUENCE: 162
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SEQ ID NO: 163 moltype = length =
SEQUENCE: 163
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SEQ ID NO: 164 moltype = length =
SEQUENCE: 164
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-continued

SEQ ID NO: 165 moltype = length =
SEQUENCE: 165
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SEQ ID NO: 166 moltype = length =
SEQUENCE: 166
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SEQ ID NO: 167 moltype = length =
SEQUENCE: 167
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SEQ ID NO: 168 moltype = length =
SEQUENCE: 168
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SEQ ID NO: 169 moltype = length =
SEQUENCE: 169
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SEQ ID NO: 170 moltype = length =
SEQUENCE: 170
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SEQ ID NO: 171 moltype = length =
SEQUENCE: 171
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SEQ ID NO: 172 moltype = length =
SEQUENCE: 172
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SEQ ID NO: 173 moltype = length =
SEQUENCE: 173
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SEQ ID NO: 174 moltype = length =
SEQUENCE: 174
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SEQ ID NO: 175 moltype = length =
SEQUENCE: 175
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SEQ ID NO: 176 moltype = length =
SEQUENCE: 176
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SEQ ID NO: 177 moltype = length =
SEQUENCE: 177
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SEQ ID NO: 178 moltype = length =
SEQUENCE: 178
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SEQ ID NO: 179 moltype = length =
SEQUENCE: 179
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SEQ ID NO: 180 moltype = length =
SEQUENCE: 180
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SEQ ID NO: 181 moltype = length =
SEQUENCE: 181
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SEQ ID NO: 182 moltype = length =
SEQUENCE: 182
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SEQ ID NO: 183 moltype = length =
SEQUENCE: 183
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SEQ ID NO: 184 moltype = length =
SEQUENCE: 184
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SEQ ID NO: 185 moltype = length =
SEQUENCE: 185
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SEQ ID NO: 186 moltype = length =
SEQUENCE: 186
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SEQ ID NO: 187 moltype = length =
SEQUENCE: 187
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SEQ ID NO: 188 moltype = length =
SEQUENCE: 188
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SEQ ID NO: 189 moltype = length =
SEQUENCE: 189
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SEQ ID NO: 190 moltype = length =
SEQUENCE: 190
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SEQ ID NO: 191 moltype = length =
SEQUENCE: 191
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SEQ ID NO: 192 moltype = length =
SEQUENCE: 192
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SEQ ID NO: 193 moltype = length =
SEQUENCE: 193
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SEQ ID NO: 194 moltype = length =
SEQUENCE: 194
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SEQ ID NO: 195 moltype = length =
SEQUENCE: 195
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SEQ ID NO: 196 moltype = length =
SEQUENCE: 196
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SEQ ID NO: 197 moltype = length =
SEQUENCE: 197
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SEQ ID NO: 198 moltype = length =
SEQUENCE: 198
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SEQ ID NO: 199 moltype = length =
SEQUENCE: 199
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SEQ ID NO: 200 moltype = length =
SEQUENCE: 200
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SEQ ID NO: 201 moltype = length =
SEQUENCE: 201
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SEQ ID NO: 202 moltype = length =
SEQUENCE: 202
000

-continued

SEQ ID NO: 203 moltype = length =
SEQUENCE: 203
000

SEQ ID NO: 204 moltype = length =
SEQUENCE: 204
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SEQ ID NO: 205 moltype = length =
SEQUENCE: 205
000

SEQ ID NO: 206 moltype = length =
SEQUENCE: 206
000

SEQ ID NO: 207 moltype = length =
SEQUENCE: 207
000

SEQ ID NO: 208 moltype = length =
SEQUENCE: 208
000

SEQ ID NO: 209 moltype = length =
SEQUENCE: 209
000

SEQ ID NO: 210 moltype = AA length = 12
FEATURE Location/Qualifiers
REGION 1..12
note = Synthetic construct
source 1..12
mol_type = protein
organism = synthetic construct
SEQUENCE: 210
RRRRRRRRR RR 12

SEQ ID NO: 211 moltype = AA length = 9
FEATURE Location/Qualifiers
REGION 1..9
note = Synthetic construct
source 1..9
mol_type = protein
organism = synthetic construct
SEQUENCE: 211
RRRRRRRRR 9

SEQ ID NO: 212 moltype = AA length = 18
FEATURE Location/Qualifiers
REGION 1..18
note = Synthetic construct
source 1..18
mol_type = protein
organism = synthetic construct
SEQUENCE: 212
KHKHKHKHKH KHKHKHKH 18

SEQ ID NO: 213 moltype = AA length = 9
FEATURE Location/Qualifiers
REGION 1..9
note = Synthetic construct
source 1..9
mol_type = protein
organism = synthetic construct
SEQUENCE: 213
RKKRRQRRR 9

SEQ ID NO: 214 moltype = AA length = 12
FEATURE Location/Qualifiers
REGION 1..12
note = Synthetic construct
source 1..12
mol_type = protein
organism = synthetic construct
SEQUENCE: 214

-continued

GRKKRRQRRR PQ		12
SEQ ID NO: 215	moltype = AA length = 10	
FEATURE	Location/Qualifiers	
REGION	1..10	
	note = Synthetic construct	
source	1..10	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 215		
GRKKRRQRRR		10
SEQ ID NO: 216	moltype = AA length = 13	
FEATURE	Location/Qualifiers	
REGION	1..13	
	note = Synthetic construct	
source	1..13	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 216		
GRKKRRQRRR PPQ		13
SEQ ID NO: 217	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
REGION	1..11	
	note = Synthetic construct	
source	1..11	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 217		
YGRKKRRQRR R		11
SEQ ID NO: 218	moltype = AA length = 18	
FEATURE	Location/Qualifiers	
REGION	1..18	
	note = Synthetic construct	
source	1..18	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 218		
RKKRRQRRRR KKRRQRRR		18
SEQ ID NO: 219	moltype = AA length = 20	
FEATURE	Location/Qualifiers	
REGION	1..20	
	note = Synthetic construct	
MOD_RES	1	
	note = Acetyl	
MOD_RES	20	
	note = Cysteamide	
source	1..20	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 219		
GLWRALWRLL RSLWRLLWRA		20
SEQ ID NO: 220	moltype = AA length = 18	
FEATURE	Location/Qualifiers	
REGION	1..18	
	note = Synthetic construct	
source	1..18	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 220		
RQIKIWFQNR RMKWKKGG		18
SEQ ID NO: 221	moltype = AA length = 15	
FEATURE	Location/Qualifiers	
REGION	1..15	
	note = Synthetic construct	
source	1..15	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 221		
RQIRIWFQNR RMRWR		15

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SEQ ID NO: 222	moltype = AA length = 16	
FEATURE	Location/Qualifiers	
REGION	1..16	
	note = Synthetic construct	
source	1..16	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 222		
RQIKIWFQNR RMKWKK		16
SEQ ID NO: 223	moltype = AA length = 17	
FEATURE	Location/Qualifiers	
REGION	1..17	
	note = Synthetic construct	
source	1..17	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 223		
CSIPPEVKFN KPFVYLI		17
SEQ ID NO: 224	moltype = AA length = 13	
FEATURE	Location/Qualifiers	
REGION	1..13	
	note = Synthetic construct	
source	1..13	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 224		
FVQWFSKFLG RIL		13
SEQ ID NO: 225	moltype = AA length = 18	
FEATURE	Location/Qualifiers	
REGION	1..18	
	note = Synthetic construct	
source	1..18	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 225		
KLALKLALKA LKAALKLA		18
SEQ ID NO: 226	moltype = AA length = 9	
FEATURE	Location/Qualifiers	
REGION	1..9	
	note = Synthetic construct	
source	1..9	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 226		
RRWWRRWRR		9
SEQ ID NO: 227	moltype = AA length = 18	
FEATURE	Location/Qualifiers	
REGION	1..18	
	note = Synthetic construct	
source	1..18	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 227		
LLIILRRRIR KQAHAHSK		18
SEQ ID NO: 228	moltype = AA length = 27	
FEATURE	Location/Qualifiers	
REGION	1..27	
	note = Synthetic construct	
source	1..27	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 228		
GWTLNSAGYL LGKINLKALA ALAKKIL		27
SEQ ID NO: 229	moltype = AA length = 27	
FEATURE	Location/Qualifiers	
REGION	1..27	
	note = Synthetic construct	
source	1..27	
	mol_type = protein	

-continued

	organism = synthetic construct	
SEQUENCE: 229		
GALFLGFLGA AGSTMGAWSQ PKKKRKV		27
SEQ ID NO: 230	moltype = AA length = 21	
FEATURE	Location/Qualifiers	
REGION	1..21	
	note = Synthetic construct	
source	1..21	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 230		
KETWWETWWT EWSQPKKKRK V		21
SEQ ID NO: 231	moltype = AA length = 21	
FEATURE	Location/Qualifiers	
REGION	1..21	
	note = Synthetic construct	
MOD_RES	1	
	note = Acetyl	
MOD_RES	21	
	note = Cysteamide	
source	1..21	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 231		
KETWWETWWT EWSQPKKKRK V		21
SEQ ID NO: 232	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
REGION	1..11	
	note = Synthetic construct	
MOD_RES	11	
	note = Amide	
source	1..11	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 232		
WKLFKKILKV L		11
SEQ ID NO: 233	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
REGION	1..11	
	note = Synthetic construct	
source	1..11	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 233		
KKLFKKILKY L		11
SEQ ID NO: 234	moltype = AA length = 11	
FEATURE	Location/Qualifiers	
REGION	1..11	
	note = Synthetic construct	
MOD_RES	11	
	note = Amide	
source	1..11	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 234		
KKLFKKILKY L		11
SEQ ID NO: 235	moltype = AA length = 10	
FEATURE	Location/Qualifiers	
REGION	1..10	
	note = Synthetic construct	
MOD_RES	3	
	note = Acm modification	
source	1..10	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 235		
GDCLPHLKLC		10
SEQ ID NO: 236	moltype = AA length = 24	
FEATURE	Location/Qualifiers	

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REGION	1..24	
	note = Synthetic construct	
source	1..24	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 236		
LGTYTQDFNK FHTFPQTAIG VGAP		24
SEQ ID NO: 237	moltype = AA length = 30	
FEATURE	Location/Qualifiers	
REGION	1..30	
	note = Synthetic construct	
source	1..30	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 237		
GAAEAAARVY DLGLRRLRQR RRLRRERVRA		30
SEQ ID NO: 238	moltype = AA length = 27	
FEATURE	Location/Qualifiers	
REGION	1..27	
	note = Synthetic construct	
source	1..27	
	mol_type = protein	
	organism = synthetic construct	
SEQUENCE: 238		
MGLGLHLLVL AAALQGAWSQ PKKKRKV		27

1. A construct comprising:

a core comprising an external surface and a plurality of pores, wherein an average dimension of the plurality of pores is greater than about 2 nm;

a cargo disposed in a pore of the plurality of the pores, the cargo comprising one or more selected from the group consisting of: peptides, proteins, nucleic acids, mRNA, aptamers, antibodies, pharmaceuticals, antisense oligonucleotides, alpha/flavi virus inhibitors, coronavirus inhibitors, carbohydrates, dyes, and markers; and

a coating coupled to the core, wherein the coating comprises a cationic lipid, a pegylated lipid, a zwitterionic lipid, and a sterol;

wherein the coating comprises a molar ratio of about 1 cationic lipid to 1 zwitterionic lipid to 0.9 sterol to 0.15 PEGylated lipid, wherein each molar ratio optionally varies by about plus or minus 10%; or

wherein the cationic lipid is 1,2-dioleoyl-3-trimethylammonium-propane, the zwitterionic lipid is 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, the sterol is cholesterol, and the PEGylated lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000].

2. The construct of claim **1**, wherein the coating comprises a molar ratio of about 1 cationic lipid to 1 zwitterionic lipid to 0.9 sterol to 0.15 PEGylated lipid, wherein each molar ratio optionally varies by about plus or minus 10%.

3. (canceled)

4. The construct of claim **1**, further comprising a pharmaceutically acceptable excipient.

5. The construct of claim **1**, wherein the core is a stellate mesoporous silica nanoparticle.

6. The construct of claim **1**, wherein the average diameter of the plurality of pores is of from about 3 nm to about 20 nm as determined by porosimetry with nitrogen adsorption-desorption analysis.

7. The construct of claim **6**, wherein the core has an average diameter of about 75 to about 400 nm.

8. A method of treating a subject, the method comprising:

administering to a subject in need thereof, an effective amount of a construct, the construct including:

a core comprising an external surface and a plurality of pores, wherein an average dimension of the plurality of pores is greater than about 2 nm;

a cargo disposed in a pore of the plurality of pores, the cargo comprising a CRISPR Cas9 component, or a nucleic acid sequence encoding a CRISPR Cas9 component; and

a coating coupled to the core, wherein the coating comprises a cationic lipid, a PEGylated lipid, a zwitterionic lipid, and a sterol.

9. The method of claim **8**, wherein the core is a stellate mesoporous silica nanoparticle.

10. The method of claim **8**, wherein the average diameter of the plurality of pores is of from about 3 nm to about 20 nm as determined by porosimetry with nitrogen adsorption-desorption analysis.

11. The method of claim **8**, wherein the core has an average diameter of about 75 to about 400 nm.

12. The method of claim **9**, wherein the mesoporous silica nanoparticle is monodisperse in particle diameter, wherein about 90% of the distribution lies within about 5% of the median diameter, as measured by dynamic light scattering.

13. The method of claim **8**, wherein the coating comprises about 10 to about 50 mol % of the cationic lipid and zwitterionic lipid, about 5 to about 45 mol. % of the sterol, and about 2 to 8 mol. % of the PEGylated lipid.

14. The method of claim **8**, wherein the coating comprises about 20 to about 40 mol % of the cationic lipid and zwitterionic lipid, about 10 to about 35 mol. % of the sterol, and about 2.5 to 6 mol. % of the PEGylated lipid.

15. The method of claim **8**, wherein the coating comprises a molar ratio of about 1 cationic lipid to 1 zwitterionic lipid to 0.9 sterol to 0.15 PEGylated lipid, wherein each molar ratio optionally varies by about plus or minus 10%.

16. The method of claim **8**, wherein the cationic lipid is 1,2-dioleoyl-3-trimethylammonium-propane, the zwitterionic lipid is 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, the sterol is cholesterol, and the PEGylated lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000].

17. The method of claim **8**, wherein the sterol is selected from the group consisting of: cholesterol, desmosterol, diplopterol, cholestanol, cholic acid, 12-deoxycholic acid, 7-deoxycholic acid, or a derivative thereof, and mixtures thereof and conjugated forms thereof.

18. The method of claim **8**, wherein the coating comprises a molar ratio of 1 cationic lipid to 1 zwitterionic lipid to 0.9 sterol to 0.15 PEGylated lipid, wherein each molar ratio optionally varies by about plus or minus 10%; and

wherein the cationic lipid is 1,2-dioleoyl-3-trimethylammonium-propane, the zwitterionic lipid is 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, the sterol is cholesterol, and the PEGylated lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000].

19. The method of claim **8**, wherein the cargo of the construct is configured to bind to a target sequence of the subject.

20. The method of claim **8**, wherein the cargo is a coronavirus inhibitor.

21. The construct of claim **1**, wherein the cargo is a coronavirus inhibitor.

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