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(54) Title: POLY(HISTIDINE)-BASED MICELLES FOR COMPLEXATION AND DELIVERY OF PROTEINS AND NUCLEIC ACIDS

FIGURE 1A

PLA polumerization time	NP formation method	Mean Size (nm)
4 hours	Sonication in PBS	---
4 hours	TFR in DCM	---
4 hours	TFR in Acetonitrile	717
6 hours	Sonication in PBS	247
6 hours	TFR in DCM	230
6 hours	TFR in Acetonitrile	440
12 hours	Sonication in PBS	276
12 hours	TFR in DCM	958
12 hours	TFR in Acetonitrile	335

(57) Abstract: Disclosed are compositions for delivering gene editing molecules to a cell. Exemplary compositions comprise a micelle assembled from a plurality of triblock copolymers, wherein each triblock copolymer having at least one hydrophobic block, at least one hydrophilic block, and at least one poly (L-histidine) block, wherein: the at least one poly(L-histidine) block complexes with the at least one gene editing molecule; and the at least one poly(L-histidine) block is capable of a pH dependent release of the at least one gene editing molecule.

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POLY(HISTIDINE)-BASED MICELLES FOR COMPLEXATION AND DELIVERY OF PROTEINS AND NUCLEIC ACIDS

RELATED APPLICATIONS

[01]   This application claims priority to U.S. Patent Application No. 62/329,892, April 29, 2016, U.S. Patent Application No. 62/330,775, May 2, 2016, U.S. Patent Application No. 62/330,784, May 2, 2016, the contents of which are each herein incorporated by reference in their entirety.

FIELD OF THE DISCLOSURE

[02]   The present invention is directed to compositions and methods for delivery of proteins and nucleic acids, for use in, for example, targeted gene modification.

BACKGROUND

[03]   Current mechanisms for delivering proteins and/or nucleic acids to target cells for gene modification including, for example, the use of viral-based gene delivery has limitations including toxicity, aggregation of the protein and/or nucleic acid, payload size limits, and difficulties with large-scale production, including costs and time. Despite a long-felt need in the art, there remains a need for a method of delivering proteins and/or nucleic acids for use in gene modification that overcomes the limitations of the current technology. The disclosure provides a compositions and methods that overcome the limitations of existing technologies.

SUMMARY

[04]   The disclosure provides a composition for delivering at least one gene editing molecule to a cell, the composition comprising: a micelle assembled from a plurality of triblock copolymers, wherein each triblock copolymer having at least one hydrophobic block, at least one hydrophilic block, and at least one poly(L-histidine) block, wherein: the at least one poly(L-histidine) block complexes with the at least one gene editing molecule; and the at least one poly(L-histidine) block is capable of a pH dependent release of the at least one gene editing molecule. In certain embodiments of this composition, the at least one gene editing molecule comprises one or more of a protein or a nucleic acid encoding for a protein. In certain

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embodiments of this composition, the at least one gene editing molecule comprises a protein and the protein is selected from the group comprising a transposase, a nuclease, and an integrase. In certain embodiments of this composition, the at least one gene editing molecule comprises one or more of a protein or a nucleic acid encoding for a protein, wherein the protein is selected from the group comprising a transposase, a nuclease, and an integrase. In certain embodiments of this composition, the nuclease or the protein having nuclease activity is selected from the group comprising: a CRISPR associated protein 9 (Cas9); a type IIS restriction enzyme; a transcription activator-like effector nuclease (TALEN); and a zinc finger nuclease (ZFN).

[05]   In certain embodiments of the compositions of the disclosure, the gene editing molecule comprises a DNA-binding domain and a nuclease. In certain embodiments, the DNA-binding domain comprises a guide RNA. In certain embodiments, the DNA-binding domain comprises a DNA-binding domain of a TALEN. In certain embodiments, the DNA-binding domain comprises a DNA-binding domain of a zinc-finger nuclease.

[06]   In certain embodiments of the compositions of the disclosure, the CRISPR associated protein 9 (Cas9) is an inactivated Cas9 (dCas9). In certain embodiments, the CRISPR associated protein 9 (Cas9) is truncated or short Cas9. In certain embodiments, the CRISPR associated protein 9 (Cas9) is a short and inactivated Cas9 (dSaCas9). In certain embodiments, the dSaCas9 comprises the amino acid sequence of

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1     mkrnyilglA igitsvgygi idyetrdvid agvrlfkean vennegrrsk rgarrlkrrr
61    rhriqrvkkl lfdynlltdh selsginpye arvkglsqkl seeefsaall hlakrrgvhn
121   vneveedtgn elstkeqisr nskaleekyv aelqlerlkk dgevrgsinr fktsdyvkea
181   kqllkvqkay hqldqsfidt yidlletrrt yyegpgegsp fgwkdikewy emlmghctyf
241   peelrsvkya ynadlynaln dlnnlvitrd enekleyyek fqiienvfkq kkkptlkqia
301   keilvneedi kgyrvtstgk peftnlkvyh dikditarke iienaelldq iakiltiyqs
361   sediqeeltn lnseltqeei eqisnlkgyt gthnlslkai nlildelwht ndnqiaifnr
421   lklvpkkvdl sqqkeipttl vddfilspvv krsfiqsikv inaiikkygl pndiiielar
481   eknskdaqkm inemqkrnrq tnerieeiir ttgkenakyl iekiklhdmq egkclyslea
541   ipledllnnp fnyevdhiip rsvsfdnsfn nkvlvkqeeA skkgnrtpfq ylsssdskis
601   yetfkkhiln lakgkgrisk tkkeylleer dinrfsvqkd finrnlvdtr yatrglmnll
661   rsyfrvnnld vkvksinggf tsflrrkwkf kkernkgykh haedaliian adfifkewkk
721   ldkakkvmen qmfeekqaes mpeieteqey keifitphqi khikdfkdyk yshrvdkkpn
781   relindtlys trkddkgntl ivnnlnglyd kdndklkkli nkspekllmy hhdpqtyqkl
841   klimeqygde knplykyyee tgnyltkysk kdngpvikki kyygnklnah lditddypns
901   rnkvvklslk pyrfdvyldn gvykfvtvkn ldvikkenyy evnskcyeea kklkkisnqa
961   efiasfynnd likingelyr vigvnndlln rievnmidit yreylenmnd krppriikti
1021  asktqsikky stdilgnlye vkskkhpqii kkg (SEQ ID NO: 1).
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[07]   In certain embodiments of the compositions of the disclosure, the type IIS restriction enzyme comprises one or more of AciI, MnlI, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, HgaI, HphI, HpyAV, MboII, MyII, PleI, SfaNI, AcuI, BciVI, BfuAI,

BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, BfiI, MboII, Acc36I, FokI or Clo051. In certain embodiments, the type IIS restriction enzyme comprises Clo051.

[08]   In certain embodiments of the compositions of the disclosure, the DNA binding domain or the nuclease comprises a sequence isolated or derived from a Ralstonia TALEN or from a Xanthomonas TALEN. In certain embodiments, the DNA binding domain or the nuclease comprises a recombinant TALEN sequence derived from a Ralstonia TALEN, a Xanthomonas TALEN or a combination thereof.

[09]   In certain embodiments of the compositions of the disclosure, the at least one gene editing molecule comprises one or more transposable element(s). In certain embodiments, the one or more transposable element(s) comprise a circular DNA. In certain embodiments, the one or more transposable element(s) comprise a plasmid vector or a minicircle DNA vector.

[010]   In certain embodiments of the compositions of the disclosure, the at least one gene editing molecule comprises one or more transposable element(s). In certain embodiments, the one or more transposable element(s) comprise a linear DNA. The linear recombinant and non-naturally occurring DNA sequence encoding a transposon may be produced in vitro. Linear recombinant and non-naturally occurring DNA sequences of the disclosure may be a product of a restriction digest of a circular DNA. In certain embodiments, the circular DNA is a plasmid vector or a minicircle DNA vector. Linear recombinant and non-naturally occurring DNA sequences of the disclosure may be a product of a polymerase chain reaction (PCR). Linear recombinant and non-naturally occurring DNA sequences of the disclosure may be a double-stranded doggybone™ DNA sequence. Doggybone™ DNA sequences of the disclosure may be produced by an enzymatic process that solely encodes an antigen expression cassette, comprising antigen, promoter, poly-A tail and telomeric ends.

[011]   In certain embodiments of the compositions of the disclosure, the at least one gene editing molecule comprises one or more transposable element(s). In certain embodiments, the one or more transposable element(s) comprise a piggyBac transposon, a Sleeping Beauty transposon or a LINE-1 (L1) transposon.

[012]   In certain embodiments of the compositions of the disclosure, including those embodiments wherein the at least one gene editing molecule comprises one or more transposable

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element(s), the at least one gene editing molecule comprises further comprises one or more transposase(s). In certain embodiments, including those embodiments wherein the transposon is a piggyBac transposon, the transposase is a Super piggyBac (sPBo) transposase. In certain embodiments, the Super piggyBac (PB) transposase enzyme may comprise or consist of an amino acid sequence at least 75% identical to:

MGSSLDDEHILSALLQSDDELVGEDSDSEVSDHVSEDDVQSDTEEAFIDEVHEVQPTSSG
SEILDEQNVIEQPGSSLASNRILTLPQRTIRGKNKHCWSTSKSTRRSRVSALNIVRSQRGPT
RMCRNIYDPLLCFKLFFTDEIISEIVKWTNAEISLKRRESMTSATFRDTNEDEIYAFFGILV
MTAVRKDNHMSTDDLFDRSLSMVYVSVMSRDRFDFLIRCLRMDDKSIRPTLRENDVFT
PVRKIWDLFIHQCIQNYTPGAHLTIDEQLLGFRGRCPFRVYIPNKPSKYGIKILMMCDSGT
KYMINGMPYLGRGTQTNGVPLGEYYVKELSKPVHGSCRNITCDNWFTSIPLAKNLLQEP
YKLTIVGTVRSNKREIPEVLKNSRSRPVGTSMFCFDGPLTLVSYKPKPAKMVYLLSSCDE
DASINESTGKPQMVMYYNQTKGGVDTLDQMCSVMTCSRKTNRWPMALLYGMINIACI
NSFIIYSHNVSSKGEKVQSRKKFMRNLYMSLTSSFMRKRLEAPTLKRYLRDNISNILPKE
VPGTSDDSTEEPVMKKRTYCTYCPSKIRRKANASCKKCKKVICREHNIDMCQSCF (SEQ
ID NO: 2).

[013]   In certain embodiments of the compositions of the disclosure, including those embodiments wherein the at least one gene editing molecule comprises one or more transposable element(s), the at least one gene editing molecule comprises further comprises one or more transposase(s). In certain embodiments, including those embodiments wherein the transposon is a Sleeping Beauty transposon, the transposase is a Sleeping Beauty 100X (SB100X) transposase. In certain embodiments, the Sleeping Beauty transposase enzyme comprises an amino acid sequence at least 75% identical to:

MGKSKEISQDLRKKIVDLHKSGSSLGAISKRLKVPRSSVQTIVRKYKHHGTTQPSYRSGR
RRYLSPRDERTLVRKVQINPRTTAKDLVKMLEETGTKVSISTVKRVLYRHNLKGRSARK
KPLLQNRHKKARLRFATAHGDKDRTFWRNVLWSDETKIELFGHNDHRYVWRKKGEAC
KPKNTIPTVKHGGGSIMLWGCFAAGGTGALHKIDGIMRKENYVDILKQHLKTSVRKLKL
GRKWVFQMDNDPKHTSKVVAKWLKDNKVKVLEWPSQSPDLNPIENLWAELKKRVRA
RRPTNLTQLHQLCQEEWAKIHPTYCGKLVEGYPKRLTQVKQFKGNATKY (SEQ ID NO:
3). In certain embodiments, including those wherein the Sleeping Beauty transposase is a

hyperactive Sleeping Beauty SB100X transposase, the Sleeping Beauty transposase enzyme comprises an amino acid sequence at least 75% identical to:

MGKSKEISQDLRKRIVDLHKSGSSLGAISKRLAVPRSSVQTIVRKYKHHGTTQPSYRSGR RRYLSPRDERTLVRKVQINPRTTAKDLVKMLEETGTKVSISTVKRVLYRHNLKGHSARK KPLLQNRHKKARLRFATAHGDKDRTFWRNVLWSDETKIELFGHNDHRYVWRKKGEAC KPKNTIPTVKHGGGSIMLWGCFAAGGTGALHKIDGIMDAVQYVDILKQHLKTSVRKLK LGRKWVFQHDNDPKHTSKVVAKWLKDNKVKVLEWPSQSPDLNPIENLWAELKKRVR ARRPTNLTQLHQLCQEEWAKIHPNYCGKLVEGYPKRLTQVKQFKGNATKY (SEQ ID NO: 4).

[014]   The disclosure provides a pharmaceutical composition for delivering at least one gene editing molecule to a cell, the composition comprising: a micelle assembled from a plurality of triblock copolymers, wherein each triblock copolymer having at least one hydrophobic block, at least one hydrophilic block, and at least one poly (L-histidine) block, wherein: the at least one poly (L-histidine) block complexes with the at least one gene editing molecule; and the at least one poly (L-histidine) block is capable of a pH dependent release of the at least one gene editing molecule.

[015]   The disclosure provides a kit, comprising: a pharmaceutical composition for delivering at least one gene editing molecule to a cell, the composition comprising: a micelle assembled from a plurality of triblock copolymers, wherein each triblock copolymer having at least one hydrophobic block, at least one hydrophilic block, and at least one poly (L-histidine) block, wherein: the at least one poly (L-histidine) block complexes with the at least one gene editing molecule; and the at least one poly (L-histidine) block is capable of a pH dependent release of the at least one gene editing molecule; and an implement for administering the pharmaceutical composition. In certain embodiments, the pharmaceutical composition is administered systemically or locally. In certain embodiments, the pharmaceutical composition is administered intravenously, via inhalation, topically, per rectum, per the vagina, transdermally, subcutaneously, intraperitoneally, intrathecally, intramuscularly or orally.

[016]   The disclosure provides a kit, comprising: a pharmaceutical composition for delivering at least one gene editing molecule to a cell, the composition comprising: a micelle assembled from a plurality of triblock copolymers, wherein each triblock copolymer having at least one hydrophobic block, at least one hydrophilic block, and at least one poly (L-histidine) block,

wherein: the at least one poly (L-histidine) block complexes with the at least one gene editing molecule; and the at least one poly (L-histidine) block is capable of a pH dependent release of the at least one gene editing molecule; and an implement for administering the pharmaceutical composition intravenously, via inhalation, topically, per rectum, per the vagina, transdermally, subcutaneously, intraperitoneally, intrathecally, intramuscularly or orally.

[017] In certain embodiments of the compositions of the disclosure, including pharmaceutical compositions of the disclosure, the compositions comprise a micelle structure comprising a triblock copolymer capable of complexing with at least one protein or nucleic acid, wherein the triblock copolymer comprises a hydrophilic block a hydrophobic block, and a poly(L-histidine) block. In certain embodiments of the triblock copolymer, the hydrophilic block comprises poly(ethylene oxide) (PEO). In certain embodiments of the triblock copolymer, the hydrophilic block comprises at least one aliphatic polyester. In certain embodiments of the triblock copolymer, the hydrophilic block comprises a poly(lactic acid), a poly(glycolic acid) (PGA), a poly(lactic-co-glycolic acid) (PLGA), a poly(ε-caprolactone) (PCL), a poly(3-hydroxybutyrate) (PHB) or any combination thereof. In certain embodiments of the triblock copolymer, the hydrophilic block comprises a poly(lactic acid) having an average length of 25 units.

[018] In certain embodiments of the compositions of the disclosure, including pharmaceutical compositions of the disclosure, the compositions comprise a micelle structure comprising a triblock copolymer capable of complexing with at least one protein or nucleic acid, wherein the triblock copolymer comprises a hydrophilic block a hydrophobic block, and a poly(L-histidine) block. In certain embodiments of the triblock copolymer, the hydrophobic block comprises a poly(ester), a poly(anhydride), a poly(peptide), an artificial poly(nucleic acid) or any combination thereof.

[019] In certain embodiments of the compositions of the disclosure, including pharmaceutical compositions of the disclosure, the compositions comprise a micelle structure comprising a triblock copolymer capable of complexing with at least one protein or nucleic acid, wherein the triblock copolymer comprises a hydrophilic block a hydrophobic block, and a poly(L-histidine) block. In certain embodiments of the triblock copolymer, the poly(L-histidine) block enables pH-dependent release of the at least one protein or nucleic acid. Exemplary poly(L-histidine) copolymers include, but are not limited to, non-degradable and degradable diblocks. Exemplary degradable poly(L-histidine) copolymers include, but are not limited to, PEO(5000)-b-

PCL(16300) ("P2350-EOCL"); PEO(2000)-b-PMCL(11900) ("OCL"); PEO(2000)-b-

PMCL(8300) ("OMCL"); PEO(1100)-b-PTMC(5100) ("OTMC"); and PEO(2000)-b-

PTMC/PCL(11200) ("OTCL").

[020]   In certain embodiments of the compositions of the disclosure, including pharmaceutical

compositions of the disclosure, the compositions comprise a micelle structure comprising a

copolymer comprising PEO-b-PLA-b PHIS. In certain embodiments, the PEO block may

comprise at least 1 monomer, 5 monomers, 10 monomers, 100 monomers, 500 monomers, 1000

monomers, 2500 monomers, 5000 monomers, 10000 monomers, 15000 monomers or any

number of monomers in between. In certain embodiments, the PLA block may comprise at least

1 monomer, 5 monomers, 10 monomers, 100 monomers, 500 monomers, 1000 monomers, 2500

monomers, 5000 monomers, 10000 monomers, 15000 monomers or any number of monomers in

between. In certain embodiments, the PHIS block may comprise at least 1 monomer, 5

monomers, 10 monomers, 100 monomers, 500 monomers, 1000 monomers, 2500 monomers,

5000 monomers, 10000 monomers, 15000 monomers or any number of monomers in between.

[021]   In certain embodiments of the compositions of the disclosure, including pharmaceutical

compositions of the disclosure, the compositions comprise a micelle structure comprising a

copolymer comprising PEO-b-PLA-b PHIS. In certain embodiments, the molar ratio of polymer

to cargo is 20:1, 15:1, 10:1, 5:1, or 2:1. In certain embodiments, the cargo is at least one gene

editing molecule of the disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

[022]   Figure 1A is a table depicting PLA polymerization times, micelle formation techniques,

and mean diameter sizes of nanoparticles in the diblock copolymer micelle model of Example 1.

As shown, using the particular test combination of PLA polymerization for 6 hours (25 PLA

units) and sonication of the copolymers in phosphate-buffered saline (PBS), the mean diameter

of the resulting micelles was 247 nm.

[023]   Figure 1B is a graph depicting the size distribution for the PEO-b-PLA micelles

generated using the same test combination (i.e., 6 hours PLA polymerization and sonication in

PBS) shown in Figure 1A and Example 1.

[024]   Figure 1C is a graph showing the ζ- potential distribution of the PEO-b-PLA micelles

generated using the same test combination (i.e., 6 hours PLA polymerization and sonication in

PBS) shown in Figures 1A-B and Example 1. As demonstrated, the ζ- potential of the tested
PEO-b-PLA micelle is about -12.20 mV.

[025]   Figure 2 is a graph depicting the absorbance of light at a wavelength of 560 nm by the
micelles with different concentrations of the DIL dye in solution. In particular, the graph may be
used to quantify how much DIL dye can be bound to the hydrophobic portion of the micelles.
Specifically, it was found that 1 mg of the PEO-b-PLA micelles was able to load around 4 μM of
the DIL dye.

[026]   Figure 3A is a table depicting PHIS polymerization times, micelle formation techniques,
and mean diameter sizes of the resulting nanoparticles of the diblock copolymer micelle model
of Example 1. Using the particular combination of PHIS polymerization for 48 hours and thin
film rehydration (TFR) of the block copolymers in dichloromethane (DCM) of the copolymers in
PBS, the mean diameter of the resulting micelles was 248 nm.

[027]   Figure 3B is graph showing the size distribution (around 248 nm in diameter) for the
PEO-b-PLA-b-PHA micelles generated using the same preparation parameters (i.e., 6 hours PLA
polymerization, 48 hours PHIS polymerization, and THS in DCM).

[028]   Figure 3C is a graph of the ζ-potential distribution of the PEO-b-PLA-b-PHIS micelles
generated using the same preparation parameters (i.e., 6hours PLA polymerization, 48 hours
PHIS polymerization, and THS in DCM). As demonstrated, the ζ-potential of the tested PEO-b-
PLA-b-PHIS micelle is about -18 mV.

[029]   Figure 4 is a table depicting the variation in properties of the PEO-b-PLA-b-PHIS
micelles in different pHs was tested. As shown, the micelles were the smallest at a pH of around
7, with a mean diameter size of around 316 nm. When the pH was substantially raised or
lowered, the mean diameter size increases. At the lower pH, such increase is likely due to the
micelle swelling based on poly(histidine) chains gaining positive charges and growing.

[030]   Figure 5 is a photograph of a gel electrophoresis depicting DNA + mRNA encapsulation
and release from PEO-PLA-PHIS particles. 1% agarose gel electrophoresis was used to
demonstrate the encapsulation of DNA and mRNA into PEO-PLA-PHIS particles (well 1).
Exposure of particles to acidic pH of 4.6 causes protonation of PHIS and disruption of particle
conformation to result in plasmid release as observed in the DNA band from well 2 in the gel
image. Plasmid release can be also triggered by surfactant exposure from the loading dye

containing SDS as can be seen in the well 3.The DNA band from release was compared to the band resulting from running DNA alone in the gel (well 4).

[031] Figure 6A is a graph of the average diameter of PEO-b-PLA-b-PHIS micelles complexed with BSA as a function of pH as discussed in Example 1.

[032] Figure 6B is a graph of the amount of released BSA as a function of pH as discussed in Example 1.

[033] Figure 7 is a series of photographs and FACS plots showing the transfection efficiency results from Example 1. HepG2 cells were seeded overnight in 24-well plates at 50,000 cells/well. Cell were exposed to different formulations in Opti-MEM Media (DNA alone, Lipofectamine + DNA + mRNA and PEO-PLA-PHIS + DNA + mRNA) at a final concentration of 500ng of DNA per well. At 48 hours post-incubation, cells were analyzed for GFP expression by microscopy and flow cytometry to determine the transfection efficiency for each condition.

[034] Figure 8 is a graph depicting piggyBac delivery via polymeric micelles. Evaluation of complexation of PEO-b-PLA-b-PHIS micelles with an pEF-GFP DNA vector (GFP), GFP-piggyBac transposon (GFP-Transposon), which was delivered with a second micelle that was complexed with piggyBac transposase mRNA or a DNA vector containing luciferase on a sleeping beauty transposon as well as the sleeping beauty transposase. Micelles were purified on a GPC column and a second fraction was detected as micelles containing DNA. Molar ratio of polymer to DNA cargo was 20:1.

[035] Figure 9 is a graph depicting piggyBac delivery via polymeric micelles. Evaluation of *in vitro* toxicity of PEO-b-PLA-b-PHIS micelles at different concentrations. Micelle toxicity in HepG2 cells was evaluated by an MTT assay. Empty micelles were incubated with cells over 3 days at the typical transfection concentration of DNA (1%) and at 10x the typical concentrations (i.e. 10%).

[036] Figure 10 is a graph depicting piggyBac delivery via polymeric micelles. Evaluation of transfection efficiency in HepG2 cells. HepG2 cells were incubated with plasmid or micelle formulation containing plasmid for 3 days. Flow cytometer was to detect transfected cells.

DETAILED DESCRIPTION

[037] A new era for genome editing technologies has recently emerged based on the development of sequence-specific nucleases. In particular, such nucleases may be used to

generate DNA double strand breaks (DSBs) in precise genomic locations, and cellular repair machinery then exploited to silence or replace nucleotides and/or genes. Targeted editing of nucleic acid sequences is a highly promising approach for the study of gene function and also has the potential to provide new therapies for human genetic diseases.

[038]   Current gene editing tools include, for example, various enzymes, such as endonucleases, and mobile genetic elements, such as transposons.

[039]   The these tools provide the potential, for example, to remove, replace, or add nucleotide bases to native DNA in order to correct or induce a point mutation, as well as to change a nucleotide base in order to correct or induce a frame shift mutation. Further, such tools may enable removing, inserting or modifying pieces of DNA containing a plurality of codons as part of one or more gene(s).

[040]   Currently, mechanisms for delivering proteins and/or nucleic acids to target cells include using viral vectors. However, viral-based gene delivery has limitations including toxicity, aggregation of the protein and/or nucleic acid, payload size limits, and difficulties with large-scale production, including costs and time.

[041]   Progress has been made in the delivery of functional nucleic acids, using both viral vectors (e.g., retrovirus, adenovirus, etc.) and non-viral vectors. For example, wild-type AAV has attracted considerable interest from gene therapy researchers due to a number of features, such as the virus's apparent lack of pathogenicity. It can also infect non- dividing cells and has the ability to stably integrate into the host cell genome at a specific site (designated AAVS 1) in the human chromosome 19. The feature makes it somewhat more predictable than retroviruses, which present the threat of a random insertion and of mutagenesis, which is sometimes followed by development of a cancer. AAV-based gene therapy vectors form episomal concatemers in the host cell nucleus. In non-dividing cells, these concatemers remain intact for the life of the host cell. In dividing cells, AAV DNA is lost through cell division, since the episomal DNA is not replicated along with the host cell DNA Random integration of AAV DNA into the host genome is detectable but occurs at very low frequency. AAVs also present very low immunogenicity, seemingly restricted to generation of neutralizing antibodies, while they induce no clearly defined cytotoxic response. These features, along with the ability to infect quiescent cells, demonstrate that AAVs are dominant over adenoviruses as vectors for human gene therapy. However, the use of viral vectors (including AAVs) is also associated with some disadvantages,

in particular the limited size of viral genomes. For example, the AAV genome is only 4.8 kilobase (kb), and therefore is unable to be used for single- vehicle delivery of the multitude of gene editing tools of the various embodiments.

[042]   Further drawbacks to the use of viruses to deliver gene editing tools may include targeting only dividing cells, random insertion into the host genome, risk of replication, and possible host immune reaction, as well as limitations on payload size imposed by the viral capsid.

[043]   In general, non-viral vectors are typically easy to manufacture, less likely to produce immune reactions, and do not produce replication reactions compared to viral vectors; existing methods are generally ineffective for in vivo introduction of genetic material into cells and have resulted in relatively low gene expression. Specifically, a number of existing non-viral systems have been recently explored for delivery of gene editing tools in the form of proteins and/or nucleic acids to cells. Such system may be broadly classified as: "nanocapsules" in which a slurry of free DNA/RNA/protein is wrapped with polymer peptide; "bioconjugates" (e.g., lipids, synthetic macromolecules, etc.) that target the nucleic acid, including via binding to specific proteins expressed by target cells to enable cellular internalization; and "lipid-based vehicles" (e.g., liposomes, lipid-based nanoparticles, etc.) modified with cationic amphiphilic polymers to self-assemble with the nucleic acids based on charge. Each of these non-viral systems presents its own set of issues with respect to encapsulating either single or a multitude of gene editing tools in a single delivery vehicle. For example, in a nanocapsule system, the structure is highly unstable and may leak its contents into the vasculature after intravenous administration. As such, the capability to achieve intracellular delivery and release of a sufficient quantity of material components necessary for effective gene editing is unlikely.In a bioconjugate system, the use of a vector of sufficient size will expose the protein or nucleic acid directly to nucleases in the blood stream/cytosol and can cause fragmentation and destruction of the payload. In lipid-based vehicles, the charged delivery systems have demonstrated poor loading capacity and difficult release of encapsulated payload.

[044]   Polymeric micelles have been extensively studied for their potential applications in the drug delivery field. Polymeric micelles are formed by amphiphilic block copolymers, which can self-assemble into nano-sized core/shell structures in an aqueous environment via hydrophobic or ion pair interactions between polymer segments. Such micelles generally are able to solubilize

the insoluble drugs, avoid non-selective uptake by the reticuloendothelial system (RES), and utilize the enhanced permeability and retention (EPR) effect for passive targeting. In this manner, a drug's solubility and pharmacokinetic profiles may be significantly improved through the use of micelles.

[045]   Polymeric micelles used for drug delivery have in some cases shown capabilities in attenuating nonspecific toxicities and enhancing drug delivery to desired sites resulting in improved therapeutic efficacy. Synthetic amphiphilic copolymers may be beneficial tools for drug delivery because they are highly versatile in terms of composition and architecture. Further, micelles may be customized, for example, by modifying the hydrophilic block using functional groups. Such functional group may include, for example, targeting ligands, such as monoclonal antibody, or intracellular drug delivery moieties, such as cell-penetrating peptides (CPPs), etc.

[046]   While nanoparticles have been reported to accumulate preferably in certain regions due to passive and/or active targeting, their inefficient drug release can be another barrier that may significantly lower drug's efficacy. For example, surface PEO chains may inhibit the cellular uptake of long circulating nanoparticles following intracellular events. Therefore, quicker and more controllable payload release remains a target for nanoparticle systems such as micelles.

[047]   Therefore, an effective vehicle for delivering nucleic acids, such as mRNA and/or large DNA plasmids, to target cells is needed.

[048]   The various embodiments will be described in detail with reference to the accompanying drawings. Wherever possible, the same reference numbers will be used throughout the drawings to refer to the same or like parts. References made to particular examples and implementations are for illustrative purposes, and are not intended to limit the scope of the claims.

[049]   It is to be appreciated that certain features that are, for clarity, described herein in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features that are, for brevity, described in the context of a single embodiment, may also be provided separately or in any sub-combination. Further, reference to values stated in ranges includes each and every value within that range.

[050]   As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise.

[051]   The word "plurality" is used herein to mean more than one. When a range of values is expressed, another embodiment includes from the one particular value and/or to the other

particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. All ranges are inclusive and combinable.

[052] The terms "subject" and "patient" are used interchangeably herein to refer to human patients, whereas the term "subject" may also refer to any animal. It should be understood that in various embodiments, the subject may be a mammal, a non-human animal, a canine and/or a vertebrate.

[053] The term "monomeric units" is used herein to mean a unit of polymer molecule containing the same or similar number of atoms as one of the monomers. Monomeric units, as used in this specification, may be of a single type (homogeneous) or a variety of types (heterogeneous).

[054] The term "polymer" is used according to its ordinary meaning of a macromolecule comprising connected monomeric molecules.

[055] The term "amphiphilic" is used herein to mean a substance containing both polar (water-soluble) and hydrophobic (water-insoluble) groups.

[056] The term "an effective amount" is used herein to refer to an amount of a compound, material, or composition effective to achieve a particular biological result such as, but not limited to, biological results disclosed, described, or exemplified herein. Such results may include, but are not limited to, the effective reduction of symptoms associated with any of the disease states mentioned herein, as determined by any means suitable in the art. As recognized by those of ordinary skill in the art, the effective amount of an agent, e.g., a nuclease, an integrase, a transposase, a recombinase, a hybrid protein, a fusion protein, a protein dimer, a complex of a protein (or protein dimer) and a polynucleotide, or a polynucleotide, may vary depending on various factors as, for example, on the desired biological response, the specific allele, genome, target site, cell, or tissue being targeted, and the agent being used.

[057] The term "membrane" is used herein to mean a spatially distinct collection of molecules that defines a two-dimensional surface in three-dimensional space, and thus separates one space from another in at least a local sense.

[058] The term "active agent" is used herein to refer to any a protein, peptide, sugar, saccharide, nucleoside, inorganic compound, lipid, nucleic acid, small synthetic chemical compound, or

organic compound that appreciably alters or affects the biological system to which it is introduced.

[059]   The term, "vehicle" is used herein to refer to agents with no inherent therapeutic benefit but when combined with an active agent for the purposes of delivery into a cell result in modification of the active agent's properties, including but not limited to its mechanism or mode of in vivo delivery, its concentration, bioavailability, absorption, distribution and elimination for the benefit of improving product efficacy and safety, as well as patient convenience and compliance.

[060]   The term "carrier" is used herein to describe a delivery vehicle that is used to incorporate a pharmaceutically active agent for the purposes of drug delivery.

[061]   The term "homopolymer" is used herein to refer to a polymer derived from one monomeric species of polymer.

[062]   The term "copolymer" is used herein to refer to a polymer derived from two (or more) monomeric species of polymer, as opposed to a homopolymer where only one monomer is used. Since a copolymer consists of at least two types of constituent units (also structural units), copolymers may be classified based on how these units are arranged along the chain.

[063]   The term "block copolymers" is used herein to refer to a copolymer that includes two or more homopolymer subunits linked by covalent bonds in which the union of the homopolymer subunits may require an intermediate non-repeating subunit, known as a junction block. Block copolymers with two or three distinct blocks are referred to herein as "diblock copolymers" and "triblock copolymers," respectively.

[064]   The term "loading capacity" is used herein to refer to the weight of a particular compound within a carrier divided by the total weight of carrier. The terms "complexation efficiency" and "loading efficiency" are interchangeably used herein to refer to the weight a particular compound that is complexed with and/or incorporated within a carrier suspension divided by the weight of the original compound in solution prior to forming a complex (expressed as a %).

[065]   The terms "nucleic acid" and "nucleic acid molecule" are used interchangeably herein to refer to a compound with a nucleobase and an acidic moiety, e.g., a nucleoside, a nucleotide, or a polymer of nucleotides. Typically, polymeric nucleic acids, e.g., nucleic acid molecules comprising three or more nucleotides are linear molecules, in which adjacent nucleotides are linked to each other via a phosphodiester or a phosphorothioate linkage. In some embodiments,

"nucleic acid" refers to individual nucleic acid residues (e.g. nucleotides and/or nucleosides). In some embodiments, "nucleic acid" refers to an oligonucleotide chain comprising three or more individual nucleotide residues. As used herein, the terms "oligonucleotide" and "polynucleotide" can be used interchangeably to refer to a polymer of nucleotides (e.g., a string of at least three nucleotides). In some embodiments, "nucleic acid" encompasses RNA as well as single and/or double-stranded DNA Nucleic acids may be naturally occurring, for example, in the context of a genome, a transcript, an mRNA, tRNA, rRNA, siRNA, snRNA, a plasmid, cosmid, chromosome, chromatid, or other naturally occurring nucleic acid molecule. On the other hand, a nucleic acid molecule may be a non-naturally occurring molecule, e.g., a recombinant DNA or RNA, an artificial chromosome, an engineered genome, or fragment thereof, or a synthetic DNA, RNA, DNA/RNA hybrid, or including non-naturally occurring nucleotides or nucleosides. Furthermore, the terms "nucleic acid," "DNA," "RNA," and/or similar terms include nucleic acid analogs, e.g., analogs having other than a phosphodiester backbone including a phosphorothioate linkage. Nucleic acids can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, etc. Where appropriate, e.g., in the case of chemically synthesized molecules, nucleic acids can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, and backbone modifications. A nucleic acid sequence is presented in the 5' to 3' direction unless otherwise indicated. In some embodiments, a nucleic acid is or comprises natural nucleosides (e.g. adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine); nucleoside analogs (e.g., 2- aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5- methylcytidine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propyny 1-uridine, C5-propyny 1-cytidine, C5-methy 1cytidine, 2-aminoadenosine, 7- deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 0(6)- methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (e.g., methylated bases); intercalated bases; modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (e.g., phosphorothioates and 5'-N-phosphoramidite linkages).

[066] The term "nuclease" is used interchangeably herein to refer to an enzyme that forms a complex with (e.g., binds or associates with) one or more nucleic acid to provide a target for cleavage, or indirect guide to another site for cleavage.

[067] The terms "treatment," "treat," and "treating," refer to a clinical intervention aimed to reverse, alleviate, delay the onset of, or inhibit the progress of a disease or disorder, or one or more symptoms thereof, as described herein. As used herein, the terms "treatment," "treat," and "treating" refer to a clinical intervention aimed to reverse, alleviate, delay the onset of, or inhibit the progress of a disease or disorder, or one or more symptoms thereof, as described herein. In some embodiments, treatment may be administered after one or more symptoms have developed and/or after a disease has been diagnosed. In other embodiments, treatment may be administered in the absence of symptoms, e.g., to prevent or delay onset of a symptom or inhibit onset or progression of a disease. For example, treatment may be administered to a susceptible individual prior to the onset of symptoms (e.g., in light of a history of symptoms and/or in light of genetic or other susceptibility factors). Treatment may also be continued after symptoms have resolved, for example, to prevent or delay their recurrence.

[068] In order to develop nanoparticles with controllable release, micellar systems with triggered release mechanisms may be developed that enable the delivery drugs or other treatment agents in response to specific stimuli. In particular, pH-sensitive polymeric micelles may be useful therapeutic agents since changes in pH occur in a variety of cellular processes and locations. For example, once the micelle enters cells via endocytosis where pH can drop as low as 5.5-6.0 in endosomes and 4.5-5.0 in lysosomes.

[069] Poly(histidine) (i.e., poly(L-histidine)), is a pH-sensitive polymer due to the imidazole ring providing an electron lone pair on the unsaturated nitrogen. That is, poly(histidine) has amphoteric properties through protonation-deprotonation.

[070] The various embodiments enable intracellular delivery of gene editing tools by complexing with poly(histidine)-based micelles. In particular, the various embodiments provide triblock copolymers made of a hydrophilic block, a hydrophobic block, and a charged block. In some embodiments, the hydrophilic block may be poly(ethylene oxide) (PEO), and the charged block may be poly(L-histidine). An example tri-block copolymer that may be used in various embodiments is a PEO-b-PLA-b-PHIS, with variable numbers of repeating units in each block varying by design. The gene editing tools may be various molecules that are recognized as capable of modifying, repairing, adding and/or silencing genes in various cells.

[071] The correct and efficient repair of double-strand breaks (DSBs) in DNA is critical to maintaining genome stability in cells. Structural damage to DNA may occur randomly and

unpredictably in the genome due to any of a number of intracellular factors (e.g., nucleases, reactive oxygen species, etc.) as well as external forces (e.g., ionizing radiation, ultraviolet (UV) radiation, etc.). In particular, correct and efficient repair of double-strand breaks (DSBs) in DNA is critical to maintaining genome stability. Accordingly, cells naturally possess a number of DNA repair mechanisms, which can be leveraged to alter DNA sequences through controlled DSBs at specific sites. Genetic modification tools may therefore be composed of programmable, sequence-specific DNA-binding modules associated with a nonspecific DNA nuclease, introducing DSBs into the genome. For example CRISPR, mostly found in bacteria, are loci containing short direct repeats, and are part of the acquired prokaryotic immune system, conferring resistance to exogenous sequences such as plasmids and phages. RNA-guided endonucleases are programmable genetic engineering tools that are adapted from the CRISPR/CRISPR-associated protein 9 (Cas9) system, which is a component of prokaryotic innate immunity.

[072] Diblock copolymers that may be used as intermediates for making triblock copolymers of the embodiment micelles may have hydrophilic biocompatible poly(ethylene oxide) (PEO), which is chemically synonymous with PEG, coupled to various hydrophobic aliphatic poly(anhydrides), poly(nucleic acids), poly(esters), poly(ortho esters), poly(peptides), poly(phosphazenes) and poly(saccharides), including but not limited by poly(lactide) (PLA), poly(glycolide) (PLGA), poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), and poly (trimethylene carbonate) (PTMC). Polymeric micelles comprised of 100% PEGylated surfaces possess improved in vitro chemical stability, augmented in vivo bioavailablity, and prolonged blood circulatory half-lives. For example, aliphatic polyesters, constituting the polymeric micelle's membrane portions, are degraded by hydrolysis of their ester linkages in physiological conditions such as in the human body. Because of their biodegradable nature, aliphatic polyesters have received a great deal of attention for use as implantable biomaterials in drug delivery devices, bioresorbable sutures, adhesion barriers, and as scaffolds for injury repair via tissue engineering.

[073] In various embodiments, molecules required for gene editing (i.e., gene editing tools) may be delivered to cells using one or more micelle formed from self-assembled triblock copolymers containing poly(histidine). The term "gene editing" as used herein refers to the insertion, deletion or replacement of nucleic acids in genomic DNA so as to add, disrupt or

modify the function of the product that is encoded by a gene. Various gene editing systems require, at a minimum, the introduction of a cutting enzyme (e.g., a nuclease or recombinase) that cuts genomic DNA to disrupt or activate gene function.

[074] Further, in gene editing systems that involve inserting new or existing nucleotides/nucleic acids, insertion tools (e.g. DNA template vectors, transposable elements (transposons or retrotransposons) must be delivered to the cell in addition to the cutting enzyme (e.g. a nuclease, recombinase, integrase or transposase). Examples of such insertion tools for a recombinase may include a DNA vector. Other gene editing systems require the delivery of an integrase along with an insertion vector, a transposase along with a transposon/retrotransposon, etc. In some embodiments, an example recombinase that may be used as a cutting enzyme is the CRE recombinase. In various embodiments, example integrases that may be used in insertion tools include viral based enzymes taken from any of a number of viruses including, but not limited to, AAV, gamma retrovirus, and lentivirus. Example transposons/retrotransposons that may be used in insertion tools include, but are not limited to, the piggyBac transposon, Sleeping Beauty transposon, and the L1 retrotransposon.

[075] In various embodiments, nucleases that may be used as cutting enzymes include, but are not limited to, Cas9, transcription activator-like effector nucleases (TALENs) and zinc finger nucleases.

[076] In various embodiments, the gene editing systems described herein, particularly proteins and/or nucleic acids, may be complexed with nanoparticles that are poly(histidine)-based micelles. In particular, at certain pHs, poly(histidine)-containing triblock copolymers may assemble into a micelle with positively charged poly(histidine) units on the surface, thereby enabling complexing with the negatively-charged gene editing molecule(s). Using these nanoparticles to bind and release proteins and/or nucleic acids in a pH-dependent manner may provide an efficient and selective mechanism to perform a desired gene modification.

[077] In particular, this micelle-based delivery system provides substantial flexibility with respect to the charged materials, as well as a large payload capacity, and targeted release of the nanoparticle payload. In one example, site-specific cleavage of the double stranded DNA may be enabled by delivery of a nuclease using the poly(histidine)-based micelles.

[078] The various embodiments enable intracellular delivery of gene editing tools by complexing with poly(histidine)-based micelles. In particular, the various embodiments provide

triblock copolymers made of a hydrophilic block, a hydrophobic block, and a charged block. In some embodiments, the hydrophilic block may be poly(ethylene oxide) (PEO), and the charged block may be poly(L-histidine). An example tri-block copolymer that may be used in various embodiments is a PEO-b-PLA-b-PHIS, with variable numbers of repeating units in each block varying by design. Without wishing to be bound by a particular theory, it is believed that believed that in the micelles that are formed by the various embodiment triblock copolymers, the hydrophobic blocks aggregate to form a core, leaving the hydrophilic blocks and poly(histidine) blocks on the ends to form one or more surrounding layer.

[079]   In various embodiments, poly(histidine)-based micelles may be formed at a pH higher than the pKa of poly(histidine) (e.g., pH of about 7). At a pH of around 6, the amine groups of the poly(histidine) block may be protonated, imparting a positive charge and enabling the poly(histidine) block to complex with negatively charged molecules (e.g., proteins and nucleic acids). If the pH is dropped substantially, such as a pH of around 3-4, the bound protein and/or nucleic acid may be released due to protonation of the poly(histidine). Various applications of the embodiment poly(histidine)-based micelles may exploit the controllable pH-dependent release of the payload molecules to target particular cells and/or pathways.

[080]   Additional applications of the embodiment micelles may include conjugating molecules to the hydrophilic block in order to target particular cell types. For example, Apoliprotein E or N-Acetylgalactosamine (GalNAc) may be conjugated to a PEO block for specific targeting of the micelles to hepatocytes.

[081]   The particular methods of creating the block copolymers used in the various embodiments, as well as the techniques of forming the micelles, may be varied based on the composition. In particular, these methods and techniques may be optimized to achieve the most desirable block and nanoparticle properties. For example, the polymerization times may be altered to change the molecular weight of a block, and therefore the overall nanoparticle size, as described in further detail in the examples below.

[082]   In various embodiments, the hydrophobic block of the triblock copolymers used to form the micelles may be a polyester, a polyanhydride, a polypeptide, or an artificial polynucleic acid. For example, the hydrophobic block may be an aliphatic polyester, including, but not limited to, poly(lactic acid), poly(glycolic acid) (PGA), poly(lactic-co- glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), and/or poly(3-hydroxybutyrate) (PHB).

[083] Various embodiments may be DNA-based systems that are complexed with the poly(histidine)-based micelles. In some embodiments, an expression vector that expresses a nuclease or other protein may be complexed with poly(histidine)-based micelles. The expression vector may be, for example, a plasmid constructed to contain DNA encoding nuclease as well as a promoter region. Once inside the target cell, the DNA encoding the nuclease may be transcribed and translated to create the enzyme.

[084] Various embodiment systems may also be designed to integrate DNA into the genome of a target cell using a transposon provided on a vector, such as an artificially constructed plasmid. Applications of such systems may include introducing (i.e., "knocking in") a new gene to perform a particular function through the inserted DNA, or inactivating (i.e., "knocking out") a mutated gene that is functioning improperly through interruption in the target DNA.

[085] In some embodiments, the DNA may be transposon that is directly transposed between vectors and chromosomes via a "cut and paste" mechanism. In some embodiments, the transposon may be a retrotransposon, e.g., a DNA that is first transcribed into an RNA intermediate, followed by reverse transcription into the DNA that is transposed.

[086] In various embodiments, the poly(histidine)-based micelles may complex with a vector that includes the transposon, as well as a transposase that catalyzes the integration of the transposon into specific sites in the target genome. The transposase that is used is specific to the particular transposon that is selected, each of which may have particular properties are desirable for use in various embodiments. One example transposon is the piggyBac transposon, which is transposed into a target genome by the piggyBac transposase. Specifically, the piggyBac transposase recognizes transposon-specific inverted terminal repeat sequences (ITRs) on the ends of the transposon, and moves the contents between the ITRs into TTAA chromosomal sites. The piggyBac transposon system has no payload limit for the genes of interest that can be included between the ITRs. Another example transposon system is the sleeping beauty transposon, which is transposed into the target genome by the sleeping beauty transposase that recognizes ITRs, and moves the contents between the ITRs into TA chromosomal sites. In various embodiments, SB transposon-mediated gene transfer, or gene transfer using any of a number of similar transposons, may be used for long-term expression of a therapeutic gene.

[087] Similar to the RNA-directed nucleases discussed here, poly(histidine)-based micelles may complex with the transposase in its native protein for, as mRNA that is transcribed into

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protein in the target cell, or as an expression vector containing DNA to express the transposase protein. For example, genes encoding the transposase may be provided in the same vector as the transposon itself, or on a different vector.

[088] Various embodiments may further enable complexing a nuclease and a transposon system in a poly(histidine)-based micelles for delivery to a target cell. Such micelle systems may be used for example, to replace a mutated gene that causes disease with a healthy copy of the gene that is inserted at a specific site dictated by the activity of the nuclease. Specifically, a transposon may be created that includes one or more gene to be inserted, which is surrounded by the ITRs for recognition by the transposase. The transposon and ITRs may be provided on a vector that contains homology arms on each end of the ITRs. The transposon system (i.e., the transposon vector and corresponding transposase), when delivered with the nuclease, may serve the function of the DNA repair template used in HDR. That is, following the creation of one or more DSB by the nuclease, the transposon may be inserted into the target DNA based on the homology arms. In some embodiments, the transposon insertion may occur between the two ends generated by a DSB. In other embodiments, the transposon may be inserted between one arm of a first DSB and the other arm at a second DSB in the target DNA (i.e., replacing the sequence between two DSBs).

[089] While a variety poly(histidine)-based micelle formulations that complex with proteins and/or nucleic acids may be designed for different uses, each complexing system may include common characteristics in order to be effective. For example, nucleic acids may be complexed with poly(histidine)-based micelles with at least 40% efficiency. Such minimum efficiency ensures delivery of enough active molecule to achieve efficient DNA cleavage and/or other modification, and that the product can be reproducibly generated at a low cost. In another example, the poly(histidine)-based micelles may be designed to be stable, yet to provide facile release of the complexed payload once the micelle has been taken up intracellularly, thereby avoiding endosomal retrafficking and ensuring release of the nucleic acids. Moreover, in various gene therapy systems, the vector (i.e., transposon) may be designed to provide stable expression.

[090] The gene editing tools provided in the poly(histidine)-based micelles described herein may be beneficial for a number of in vivo applications. For example, the embodiment materials may be delivered to various cell types in order to cut or to repair gene defects. Such cells include, but are not limited to, hepatocytes, hepatic endothelial cells, immune cells, neurons, etc. The

embodiment poly(histidine)-based micelles may also be delivered to various cell types in order to silence defective genes that cause diseases (for example, delivery to retinal cells to silence mutations underlying Leber's Congenital Amaurosis).

[091]   Various methods may be used to generate the poly(histidine)-based micelles and/or complexation of micelles and proteins and/or nucleic acids described herein. In some embodiments, conventional preparation techniques such as thin-film rehydration, direct-hydration, and electro-formation may be used to form polymeric micelles that complex with nucleic acids and/or proteins with gene editing functions into various degradable and non-degradable micelles.

[092]   Creation of various poly(histidine)-based micelles complexed with model proteins and model nucleic acids may be created using conventional techniques. For example, bovine serum albumin (BSA; Mw = about 66 kDa), which has a size and thermal stability (i.e., denaturation above 60 °C) comparable to other medium size proteins with therapeutic potential, was used as a model protein. Other model proteins that may be used in such compositions are myoglobin (Mb; Mw = about 17 kDa) and catalase (Mw = about 250 kDa). The complexing of model proteins having various sizes provides a range of sizes of functional proteins that may be used in various embodiments. Further, various DNA plasmids may be used as model nucleic acids for poly(histidine)-based micelles, such as plasmid DNA encoding the mammalian expression vector for expression of green fluorescent protein (GFP) using the elongation factor I alpha (EF la) promoter) (i.e., pEF-GFP DNA). The pEF-GFP DNA is about 5000 base-pairs, and has a molecular weight of about 3283 kDa.

[093]   In the micelles that are formed by the various embodiment triblock copolymers, the hydrophobic blocks may aggregate to form a core, leaving the hydrophilic blocks and poly(histidine) blocks on the ends to form one or more surrounding layer.

EXAMPLES

[094]   Different micelle formulations may be used to compare the properties between resulting particles. For example, poly(histidine)-containing triblock copolymers are used to form micelles that complex with negatively charged particles, including nucleic acids and some proteins.

[095]   Details about comparative and quantitative studies that were performed are provided below.

Experimental Procedures

[096] *Formation and Characterization of Polymeric Micelles.* PEO-b-PLA-b-PHIS micelles were prepared by a thin-film rehydration method.

[097] In overview, 20 mg of polymer was dissolved in 1 mL of DCM. The organic solvent was evaporated to form a polymer thin film by flushing Nitrogen gas. The polymer thin film was rehydrated in PBS and particles were formed by 30 minutes ice-water bath sonication at 30 kHz. Particle sizes and zeta potentials were measured using a Delsa Nano Submicron Particle Size and Zeta Potential Analyzer (Beckman Coulter).

[098] *Complexation of protein or DNA with Polymeric Micelles:* Suspensions of polymeric micelles were diluted with Opti-MEM media® (Invitrogen) to different concentrations, varying the final numbers of amino groups in solution. Equal volume solutions containing protein or DNA where then added to the micelles. For DNA, the primary parameters that were varied included the initial ratios of free amines to phosphates (NIP) in suspension, which ranged from 5:1 to 40:1. Protein- or DNA- complexed micelles were then formed by gentle pipetting and allowed to equilibrate for 30 min at RT. To determine the maximal loading of protein/DNA, the efficiency of micellar complexation, and the rates of release within different pH solutions, the micro- BSA assay (for protein concentrations), a fluorescence standard curve (for flurophore-conjugated protein), and/or ICP-MS was utilized (to determine the amount of platinum- bound DNA in solution).

Example 1: Diblock copolymer micelle model

[099] In a first copolymer micelle model, micelles were created using the diblock copolymer PEO-b-PLA. Various time durations for polymerizing the PLA block were tested in combination with different techniques for forming the micelles (i.e., "test combinations"). The of PLA polymerization times, micelle formation techniques, and mean diameter sizes of the resulting nanoparticles are shown in FIG. 1A. As shown, using the particular test combination of PLA polymerization for 6 hours (25 PLA units) and sonication of the copolymers in phosphate-buffered saline (PBS), the mean diameter of the resulting micelles was 247 nm.

[0100] As also shown, increasing the amount of time for PLA polymerization resulted in larger mean diameters of the resulting nanoparticles. FIG. 1B is graph showing the size distribution for the PEO-b-PLA micelles generated using the same test combination (i.e., 6 hours PLA

polymerization and sonication in PBS). FIG. 1C is a graph showing the ζ- potential distribution of the PEO-b-PLA micelles generated using the same test combination (i.e., 6 hours PLA polymerization and sonication in PBS). As demonstrated, the ζ- potential of the tested PEO-b-PLA micelle is about -12.20 mV.

[0101] The block copolymer micelles in the various embodiments may also encapsulate water-insoluble molecules in the hydrophobic block. This capability was shown by encapsulating a lipophilic carbocyanine fluorescent dye (DIL dye) in the hydrophobic portion (i.e., PLA) of the PEO-b-PLA micelles described with respect to FIGs 1B and 1C (i.e., prepared by 6 hours PLA polymerization and sonication in PBS). FIG. 2 is a graph showing the absorbance of light at a wavelength of 560 nm by the micelles with different concentrations of the DIL dye in solution. In particular, the graph may be used to quantify how much DIL dye can be bound to the hydrophobic portion of the micelles. Specifically, it was found that 1 mg of the PEO-b-PLA micelles was able to load around 4 μM of the DIL dye.

[0102] In various embodiments, creating a triblock copolymer for use in micelle formation involves attaching a poly(histidine) block to a diblock copolymer that has a hydrophobic and a hydrophilic block. The poly(histidine) block may be attached to the hydrophobic block, such that the resulting polymer contains the hydrophobic block in between a hydrophilic block and the poly(histidine) block.

[0103] In an embodiment, poly(histidine)-based micelles were created using the triblock copolymer PEO-b-PLA-b-PHIS. As described above with respect to FIGS. 1B and 2, creating the PEO-b-PLA portion of the copolymers involved PLA polymerization for 6 hours, and sonicating the diblock copolymers in PBS. Various time durations for creating the poly(histidine) block and adding it to the PEO-b-PLA copolymer were used in combination with different techniques for forming the triblock copolymer micelles. The PHIS polymerization times, micelle formation techniques, and mean diameter sizes of the resulting nanoparticles are shown in FIG. 3A. Using the particular combination of PHIS polymerization for 48 hours and thin film rehydration (TFR) of the block copolymers in dichloromethane (DCM) of the copolymers in PBS, the mean diameter of the resulting micelles was 248 nm. FIG. 3B is graph showing the size distribution (around 248 nm in diameter) for the PEO-b-PLA-b-PHA micelles generated using the same preparation parameters (i.e., 6 hours PLA polymerization, 48 hours PHIS polymerization, and THS in DCM). FIG. 3C is a graph of the ζ-potential distribution of

the PEO-b-PLA-b-PHIS micelles generated using the same preparation parameters (i.e., 6hours PLA polymerization, 48 hours PHIS polymerization, and THS in DCM). As demonstrated, the ζ-potential of the tested PEO-b-PLA-b-PHIS micelle is about -18 mV.

[0104] FIG. 4 is a chart showing the variation in properties of the PEO-b-PLA-b-PHIS micelles in different pHs was tested. As shown, the micelles were the smallest at a pH of around 7, with a mean diameter size of around 316 nm. When the pH was substantially raised or lowered, the mean diameter size increases. At the lower pH, such increase is likely due to the micelle swelling based on poly(histidine) chains gaining positive charges and growing.

[0105] To demonstrate the capability of poly(histidine)-based micelles to complex with negatively charged proteins, bovine serum albumin (BSA) was added to a solution with PEO-b-PLA-b-PHIS micelles at a low pH (i.e., lower than 6.6). In this manner, the BSA may complex with positively charged PHIS blocks, thereby creating neutrally charged nanoparticle-protein complexes.

[0106] When the BSA was added at a ratio of 1:3 polymer-to-protein, the complexation efficiency was around 50%. Without wishing to be bound by a particular theory, it is believe that the micelle core was formed by the hydrophobic PLA blocks. It is also believed that the BSA complexed with the poly(histidine) created a "shell" layer on the surface of the PLA core, while the PEO created a dispersed second "shell" layer around the BSA/poly(histidine) layer.

[0107] To demonstrate the capability of poly(histidine)-based micelles to complex with a nucleic acid, a model plasmid DNA encoding the mammalian DNA vector for expression of green fluorescent protein (GFP) using the elongation factor 1 alpha (EF1a) promoter) (i.e., pEF-GFP DNA) was added to a solution with PEO-b-PLA-b-PHIS micelles at a low pH (i.e., lower than 6.6). Similar to BSA, the pEF-GFP DNA may complex with positively charged PHIS blocks, thereby creating neutrally charged nanoparticle-DNA complexes.

[0108] Without wishing to be bound by a particular theory, it is believe that the micelle cores were formed by the hydrophobic PLA blocks. It is also believed that the BSA or pEF-GFP complexed with the poly(histidine) created a "shell" layer on the surface of the PLA core, while the PEO created a dispersed second "shell" layer around the BSA/poly(histidine) or pEF-GFP DNA/poly(histidine) layer.

[0109] Figure 5 demonstrates DNA + mRNA encapsulation and release from PEO-PLA-PHIS particles. 1% agarose gel electrophoresis was used to demonstrate the encapsulation of DNA and

mRNA into PEO-PLA-PHIS particles (well 1). Exposure of particles to acidic pH of 4.6 causes protonation of PHIS and disruption of particle conformation to result in plasmid release as observed in the DNA band from well 2 in the gel image. Plasmid release can be also triggered by surfactant exposure from the loading dye containing SDS as can be seen in the well 3. The DNA band from release was compared to the band resulting from running DNA alone in the gel (well 4).

[0110] The properties of the poly(histidine)-based micelles complexed with protein and/or nucleic acid also vary based on the pH. FIG. 6A is a graph of the average diameter of PEO-b-PLA-b-PHIS micelles complexed with BSA as a function of pH, and FIG. 6B is a graph of the amount of released BSA as a function of pH.

[0111] As shown, the nanoparticles are smallest at a pH of around 7 (around 400 nm). When the pH is raised above 7 (e.g., up to around 10), the overall micelle size also increases, and BSA remains complexed with the micelle. When the pH is lowered below 7 (e.g., to about 3-4), the overall micelle size also increases (e.g., swells), but at a pH of about 3-4, BSA is released from the micelles.

[0112] In another example, HepG2 cells were seeded overnight into a 24-well plate at a confluence of 50,000 cells per well. Bare DNA and the different formulations containing DNA were added to the cells to achieve a final concentration of DNA of 5μg per well. The formulations used were: Lipofectamine (a traditional method in the market used to transfect cells in vitro), PEO-PLA-PHIS particles, and PEO-PLL-PHIS particles. After 2 days of co-incubation, the cells were detached from the surface by trypsin, diluted with PBS and analyzed by flow cytometry. Analysis in flow cytometry was done to measure GFP fluorescence of transfected cells in a cell population of 10,000 cells per sample. Each condition was measured for 5 biological repetitions.

[0113] Figure 7 demonstrates transfection efficiency. HepG2 cells were seeded overnight in 24-well plates at 50,000 cells/well. Cell were exposed to different formulations in Opti-MEM Media (DNA alone, Lipofectamine + DNA + mRNA and PEO-PLA-PHIS + DNA + mRNA) at a final concentration of 500ng of DNA per well. At 48 hours post-incubation, cells were analyzed for GFP expression by microscopy and flow cytometry to determine the transfection efficiency for each condition.

[0114] Further poly(histidine)-containing triblock copolymers using the same protocols have been and continue to be developed. Such copolymers include, in addition to poly(histidine), non-degradable and degradable diblocks such as: degradable polymers include, but are not limited to: PEO(5000)-b-PCL(16300) ("P2350-EOCL"); PEO(2000)-b-PMCL(11900) ("OCL"); PEO(2000)-b-PMCL(8300) ("OMCL"); PEO(1100)-b-PTMC(5100) ("OTMC"); and PEO(2000)-b-PTMC/PCL(11200) ("OTCL").

[0115] The various embodiments include a micelle structure containing a triblock copolymer capable of complexing with at least one protein or nucleic acid, the triblock copolymer including a hydrophilic block including poly(ethylene oxide), a hydrophobic block, and a poly(L-histidine) block, wherein the poly(L-histidine) block enables pH- dependent release of the at least one protein or nucleic acid. In an embodiment, the hydrophobic block is selected from the group including poly(esters), poly(anhydrides), poly(peptides), and artificial poly(nucleic acids). In an embodiment, the hydrophobic block includes at least one aliphatic polyester selected from the group of including poly(lactic acid), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), and poly(3-hydroxybutyrate) (PHB). In an embodiment, the hydrophobic block includes poly(lactic acid) having an average length of 25 units.

[0116] Various embodiments may include a composition for delivering at least one gene editing molecule to a cell, the composition including, a micelle assembled from a plurality of triblock copolymers in which each triblock copolymer having at least one hydrophobic block, at least one hydrophilic block, and at least one poly(L-histidine) block in which the at least one poly(L-histidine) block complexes with the at least one gene editing molecule, and the at least one poly(L-histidine) block is capable of a pH dependent release of the at least one gene editing molecule.

[0117] In an embodiment, the at least one gene editing molecule may include one or more protein or nucleic acid encoding for a protein in which the protein is selected from a group that includes transposases, nucleases, and integrases. In an embodiment, the protein may be a nuclease selected from a group that includes CRISPR associated protein 9 (Cas9), transcription activator-like effector nucleases and zinc finger nucleases. In an embodiment, the at least one gene editing molecule may include one or more transposable elements. In an embodiment, the one or more transposable elements may include, but are not limited to, a piggyBac transposon, a

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Sleeping Beauty transposon, or a LINE-1 (L1) retrotransposon. In an embodiment, the at least one gene editing molecule may further include one or more transposase.

[0118] Further embodiments may include a kit including a pharmaceutical composition for delivering at least one gene editing molecule to a cell. The composition may include a micelle assembled from a plurality of triblock copolymers in which each triblock copolymer having at least one hydrophobic block, at least one hydrophilic block, and at least one poly(L-histidine) block in which the at least one poly(L-histidine) block complexes with the at least one gene editing molecule, and the at least one poly(L- histidine) block is capable of a pH dependent release of the at least one gene editing molecule, and an implement for administering the pharmaceutical composition intravenously, via inhalation, topically, per rectum, per the vagina, transdermally, subcutaneously, intraperitoneally, intrathecally, intramuscularly, or orally.

[0119] What is claimed is:

CLAIMS

1.   A composition for delivering at least one gene editing molecule to a cell, the composition comprising:

a micelle assembled from a plurality of triblock copolymers, wherein each triblock copolymer having at least one hydrophobic block, at least one hydrophilic block, and at least one poly(L-histidine) block,

wherein:

the at least one poly(L-histidine) block complexes with the at least one gene editing molecule; and

the at least one poly(L-histidine) block is capable of a pH dependent release of the at least one gene editing molecule.

2.   The composition of claim 1, wherein the at least one gene editing molecule comprises one or more of a protein or a nucleic acid encoding for a protein, wherein the protein is selected from the group comprising a transposase, a nuclease, and an integrase.

3.   The composition of claim 2, wherein the nuclease is selected from the group comprising:

a CRISPR associated protein 9 (Cas9);

a type IIS restriction enzyme;

a transcription activator-like effector nuclease (TALEN); and

a zinc finger nuclease (ZFN).

4.   The composition of claim 1, wherein the at least one gene editing molecule comprises one or more transposable element.

5.   The composition of claim 4, wherein the one or more transposable element comprises a piggyBac transposon, a Sleeping Beauty transposon or a LINE-1 (L1) transposon.

6.   The composition of claim 4, wherein the at least one gene editing molecule comprises further comprises one or more transposase(s).

7.   A kit, comprising:

a pharmaceutical composition for delivering at least one gene editing molecule to a cell, the composition comprising:

a micelle assembled from a plurality of triblock copolymers, wherein each triblock copolymer having at least one hydrophobic block, at least one hydrophilic block, and at least one poly (L-histidine) block, wherein:

the at least one poly (L-histidine) block complexes with the at least one gene editing molecule; and

the at least one poly (L-histidine) block is capable of a pH dependent release of the at least one gene editing molecule; and

an implement for administering the pharmaceutical composition intravenously, via inhalation, topically, per rectum, per the vagina, transdermally, subcutaneously, intraperitoneally, intrathecally, intramuscularly or orally.

FIGURE 1A

PLA polumerization time	NP formation method	Mean Size (nm)
4 hours	Sonication in PBS	---
4 hours	TFR in DCM	---
4 hours	TFR in Acetonitrile	717
6 hours	Sonication in PBS	247
6 hours	TFR in DCM	230
6 hours	TFR in Acetonitrile	440
12 hours	Sonication in PBS	276
12 hours	TFR in DCM	958
12 hours	TFR in Acetonitrile	335

FIGURE 1B

ζ-Potential= -12.2043 mV
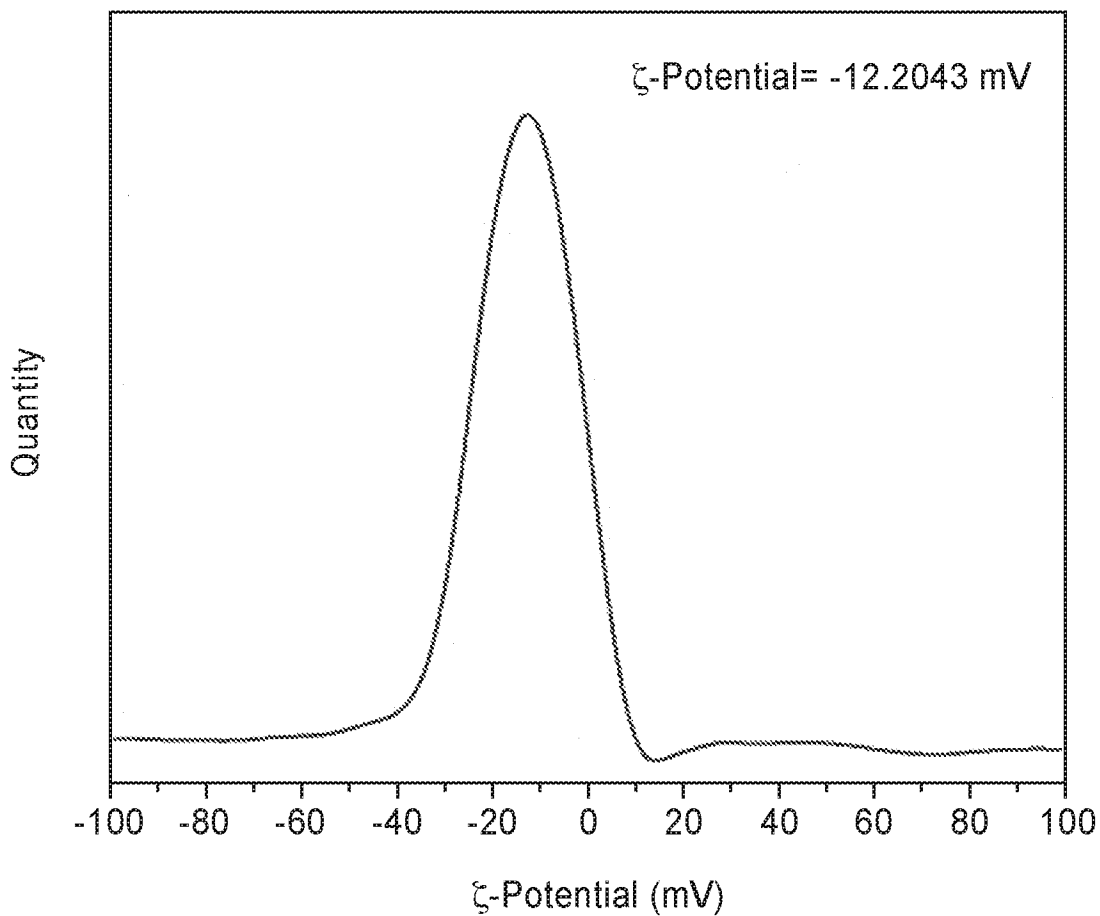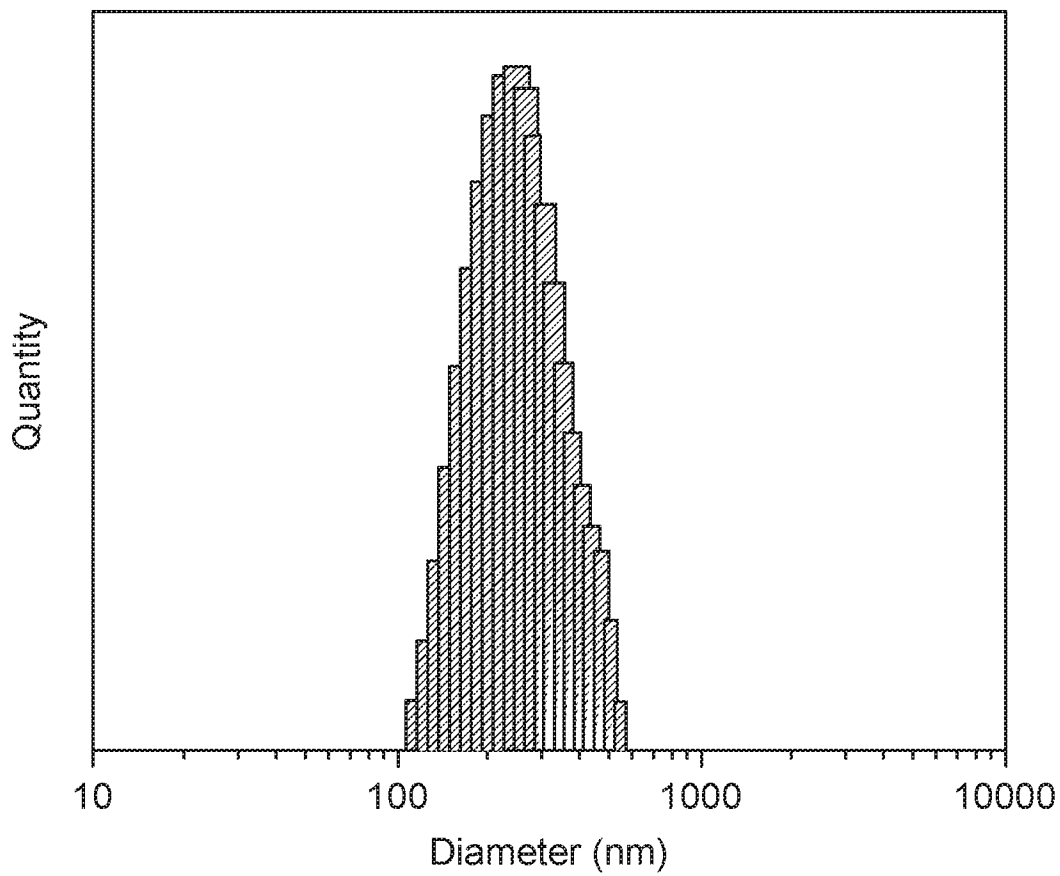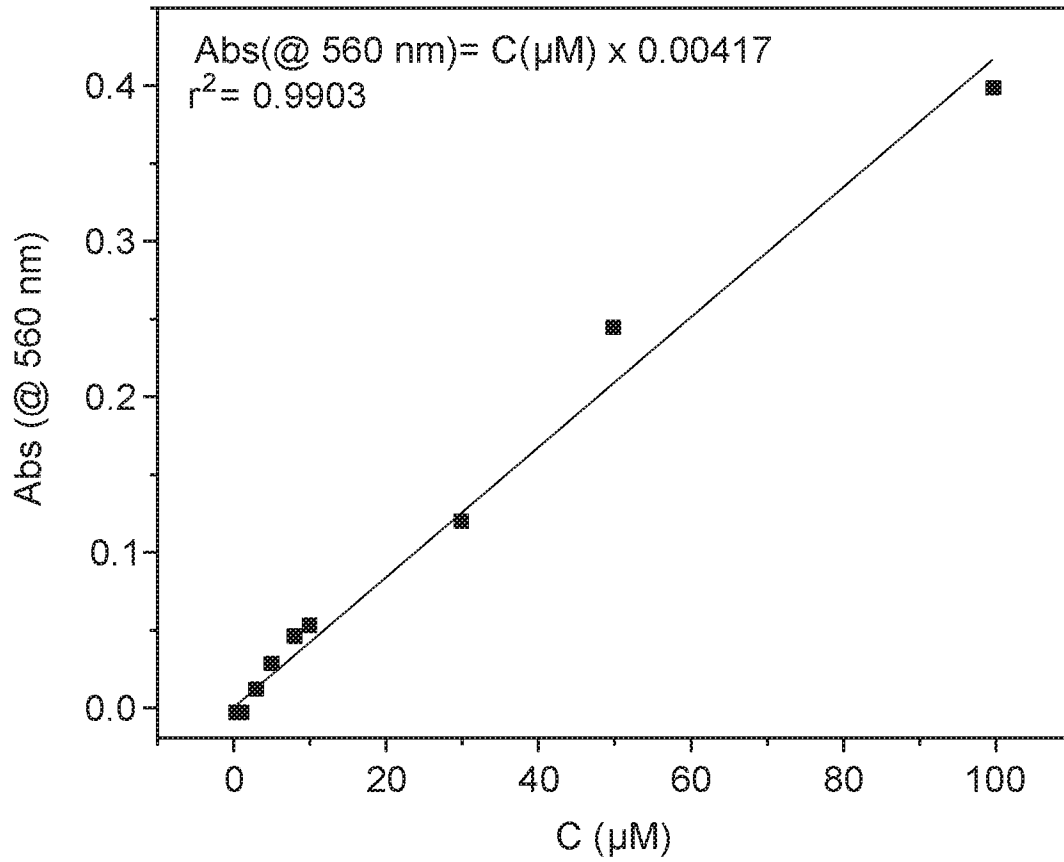
FIGURE 1C

FIGURE 2

FIGURE 3A

PHIS polymerization time	NP formation method	Mean Size (nm)
48 hours	Sonication in PBS	789
48 hours	TFR in DCM	248
72 hours	Sonication in PBS	1680
72 hours	TFR in DCM	569

FIGURE 3B
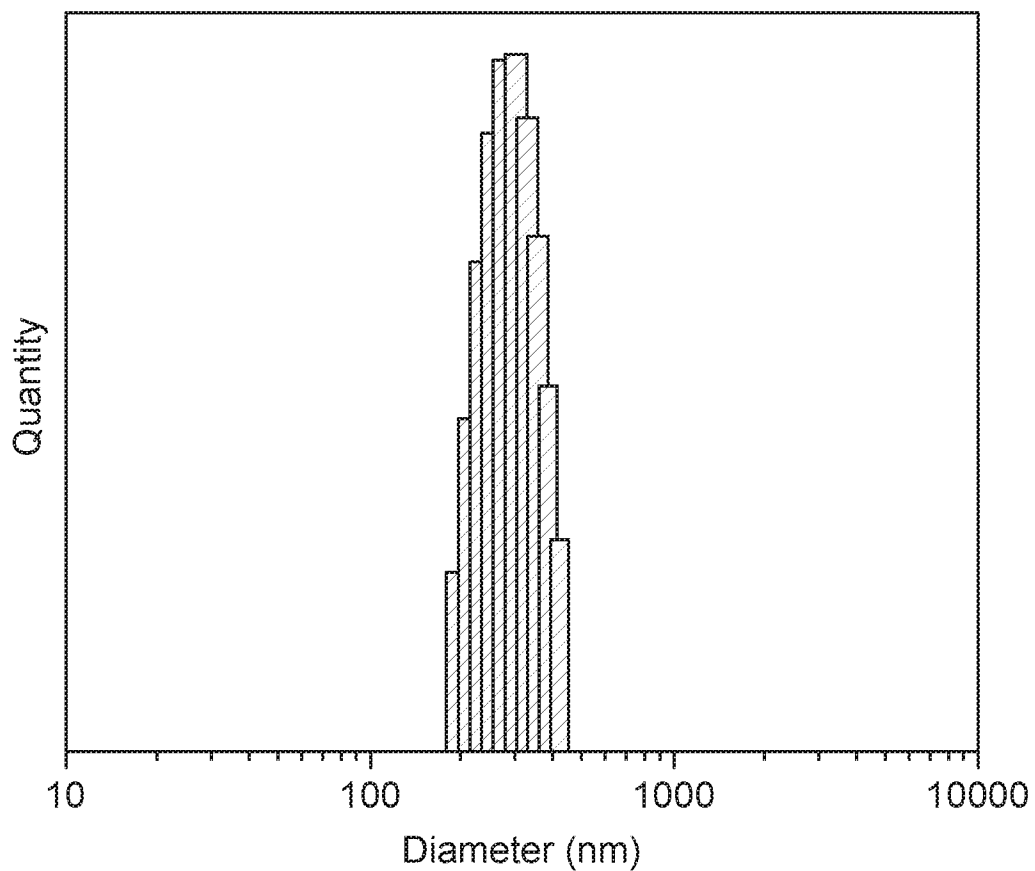
FIGURE 3C
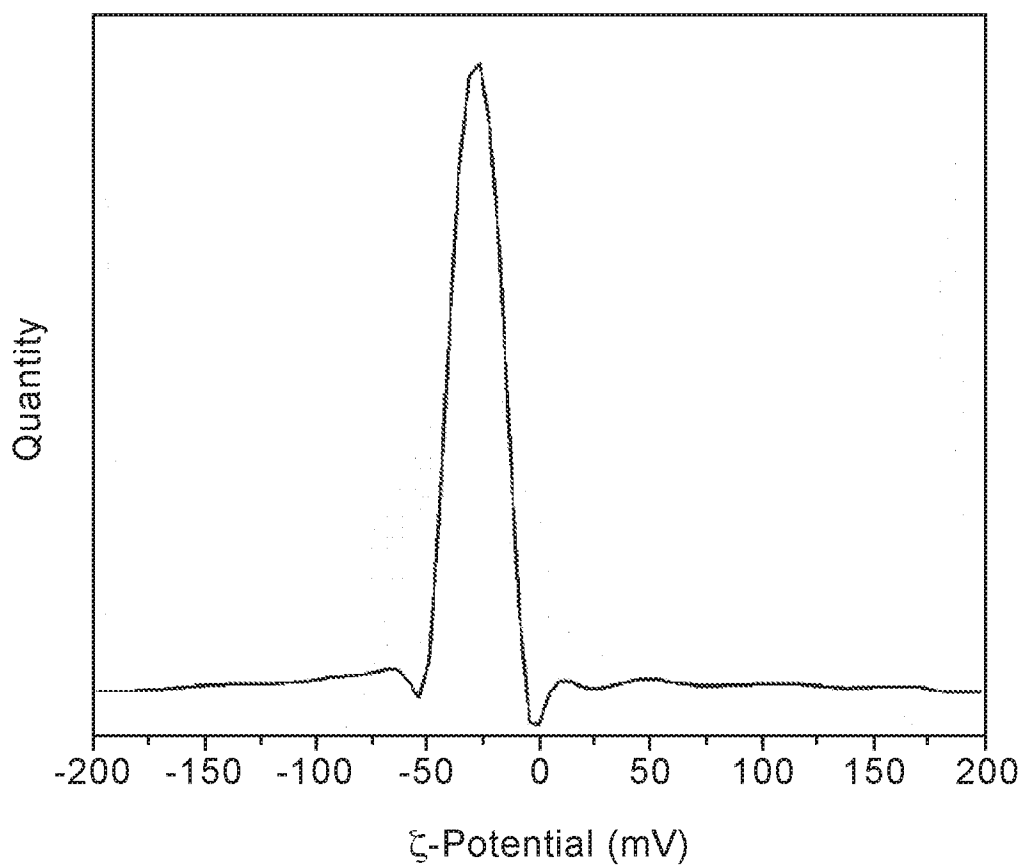
FIGURE 4

pH	Mean Size (nm)	ζ-Potential (mV)
4	952 ± 158	-----
5	785 ± 374	60.94
6	954 ± 26	-2.04
7	316 ± 37	-2.93
8	432 ± 28	-5.74
9	980 ± 26	-24.43
10	934 ± 122	-17.65
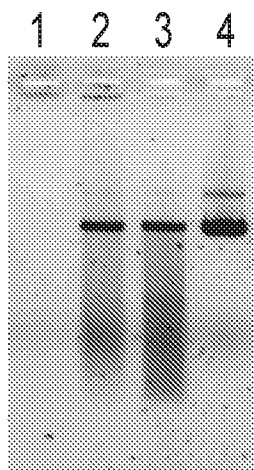
FIGURE 5

1. PEO-PLA-PHIS + DNA + mRNA

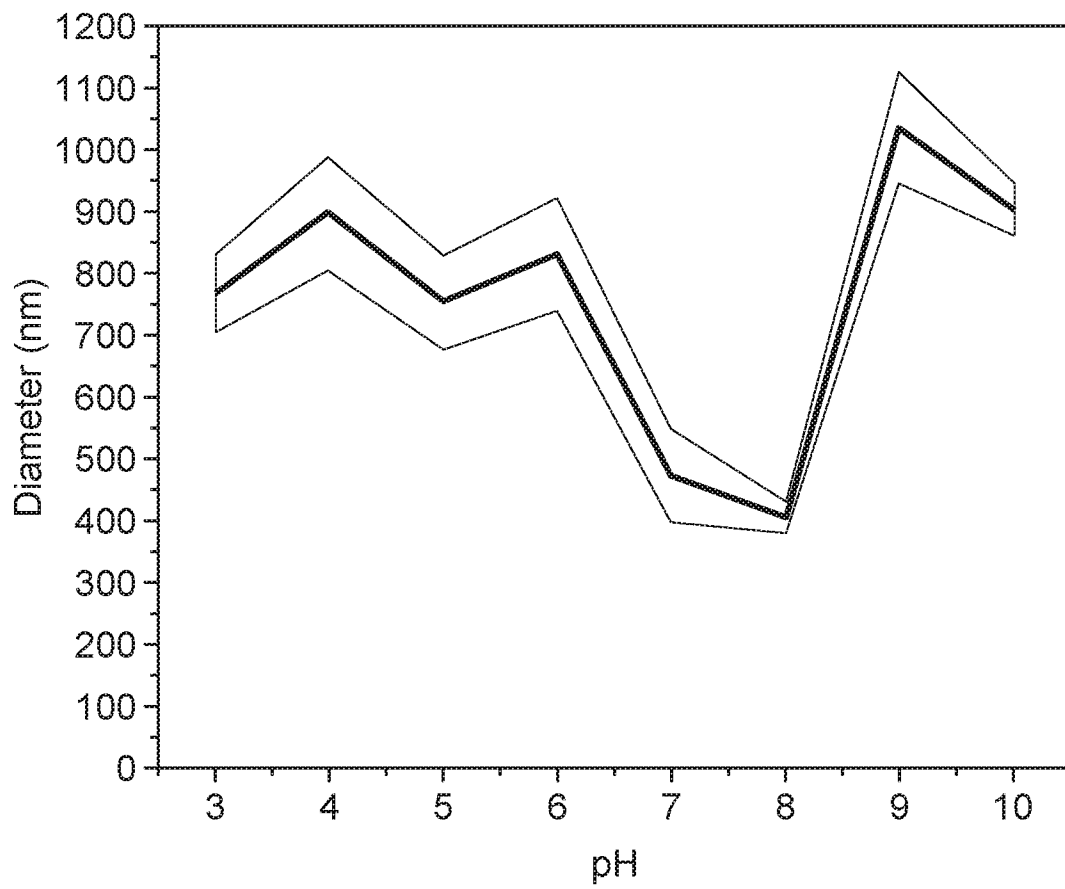2. PEO-PLA-PHIS + DNA + mRNA at PH 4.6

3. PEO-PLA-PHIS + DNA + mRNA with SDS

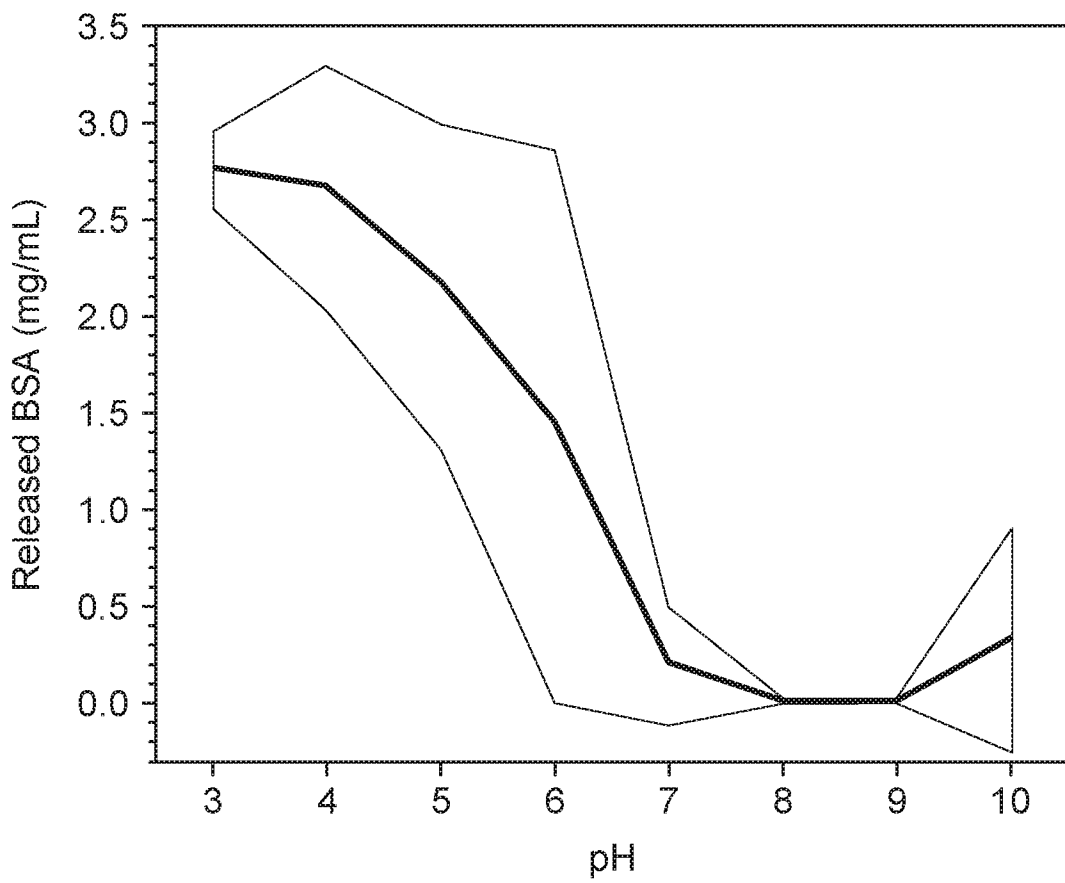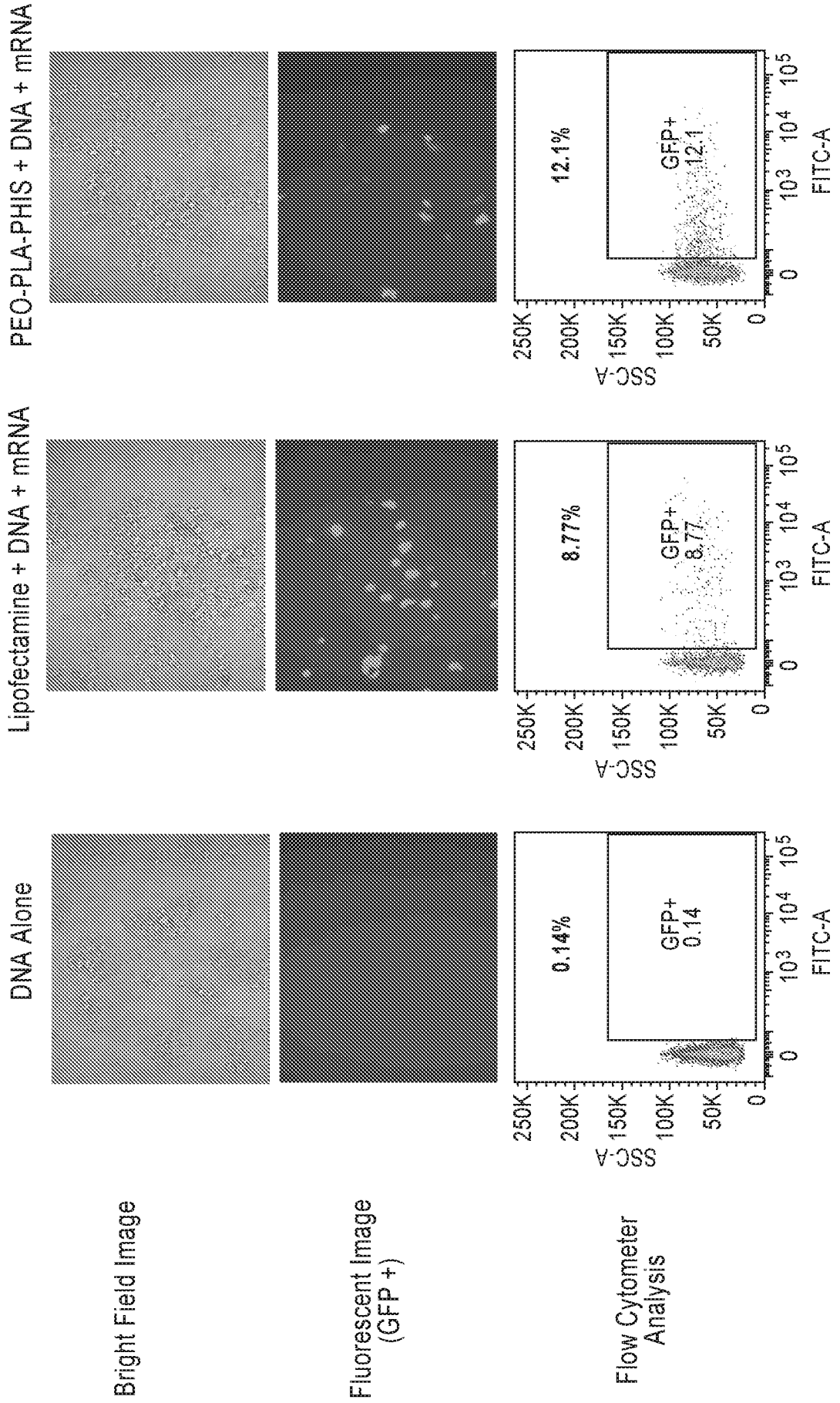4. DNA Alone

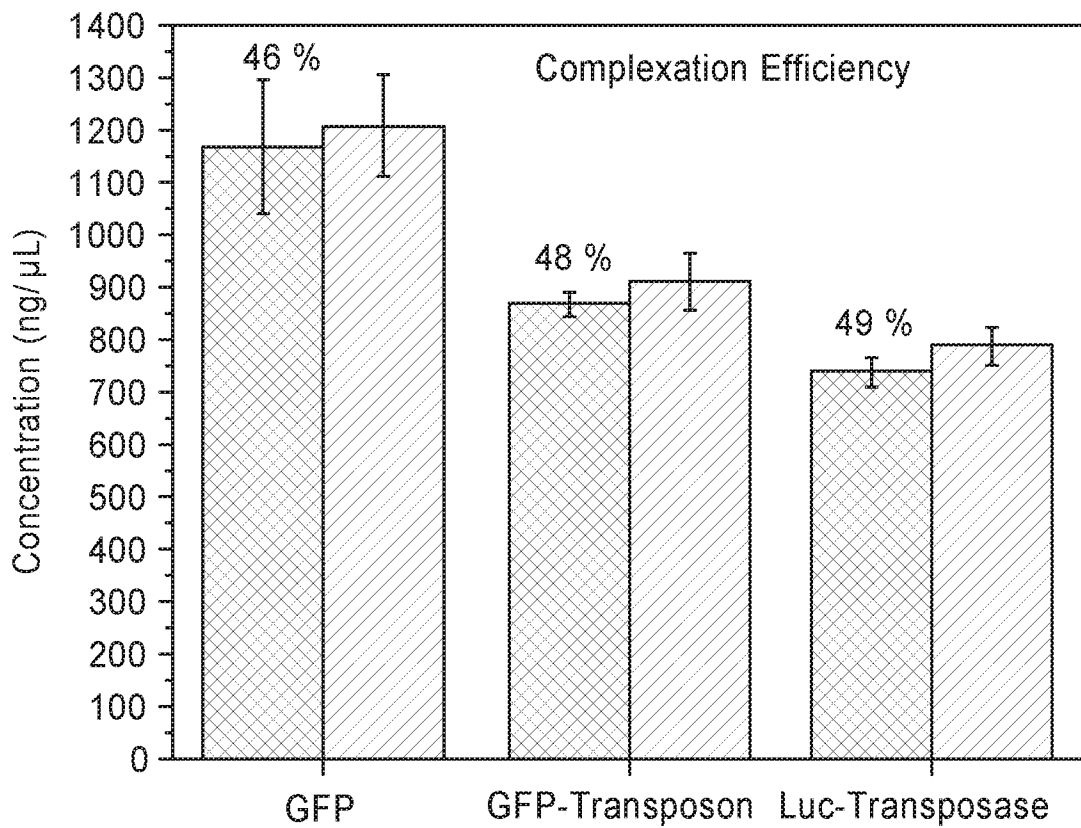## FIGURE 6A

FIGURE 6B

FIGURE 7

FIGURE 8

FIGURE 9
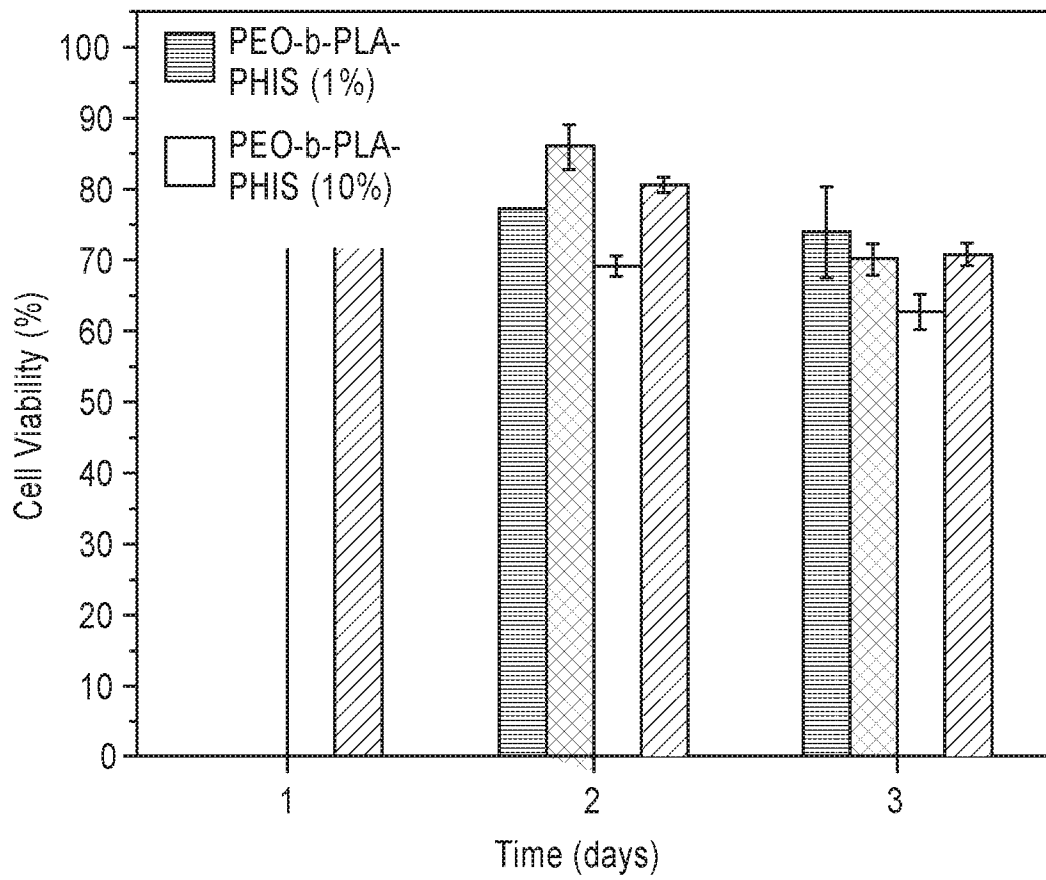
FIGURE 10

Formulation	GFP	GFP-Transposon	Luc-Transposase
None	0.2%	0.4 ± 0.22 %	0.2 %
Lipofectamine	5.4 ± 0.75 %	12.88 ± 1.38 %	15.33 ± 1.94 %
PEO-PLA-PHIS	5.43 ± 0.5 %	10.48 ± 0.67 %	14.85 ± 0.79 %

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/88   A61K9/107   A61K38/43   A61K47/56
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CAB Data, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2015/089462 A1 (BROAD INST INC [US]; MASSACHUSETTS INST TECHNOLOGY [US]) 18 June 2015 (2015-06-18) column [0174] - column [0175] column [0186] - column [0188] column [0195] - column [0199] column [0211] - column [0213] column [0005] column [0084] -----	1-7
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[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 2017	08/08/2017

Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Hornig, Horst

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C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	HU JUN ET AL: "pH-responsive and charge shielded cationic micelle of poly(l-histidine)-block-short branched PEI for acidic cancer treatment", JOURNAL OF CONTROLLED RELEASE, vol. 172, no. 1, 14 August 2013 (2013-08-14), pages 69-76, XP028772918, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2013.08.007 the whole document -----	1-7
A	STEFAN EGLI ET AL: "Functionalization of Block Copolymer Vesicle Surfaces", POLYMERS, vol. 3, no. 1, 11 January 2011 (2011-01-11), pages 252-280, XP055342438, DOI: 10.3390/polym3010252 the whole document -----	1-7
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