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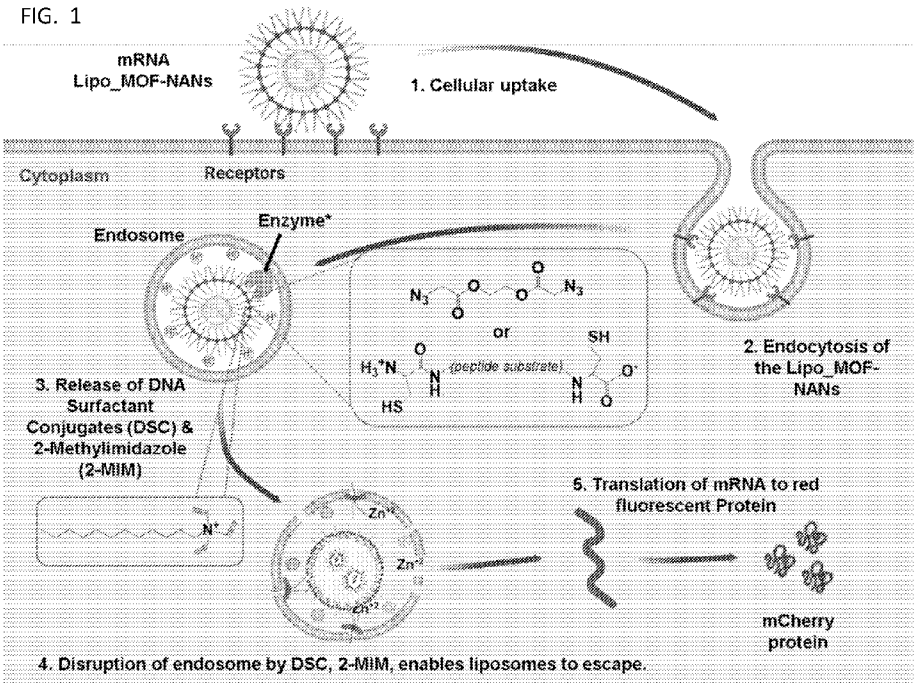
(54) **Title:** NUCLEIC ACID NANOCAPSULES FOR DRUG DELIVERY

FIG. 1



(57) **Abstract:** The present disclosure describes a multi-layered multifunctional liposomal metal organic framework (MOF) nucleic acid nanocapsule capable of stabilizing therapeutic nucleic acid cargo, particularly mRNA. More particularly, the present disclosure relates to a multifunctional nanoparticle that functions as a nucleic acid delivery material which can both deliver and release mRNA to cells in an enzyme specific fashion; and methods of using such nanoparticles for treatment and/or diagnosis of diseases and conditions.

[Continued on next page]

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NUCLEIC ACID NANOCAPSULES FOR DRUG DELIVERY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 63/521,369, filed June 16, 2023, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under GM138226 awarded by the National Institutes of Health, and 1847869 awarded by the National Science Foundation. The government has certain rights in the invention.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0003] A computer readable form of the Sequence Listing is filed with this application by electronic submission and is incorporated into this application by reference in its entirety. The Sequence Listing is contained in the file created on June 16, 2024, having the file name "24-0740-WO_Sequence_Listing_ST26.xml," and being 24,218 bytes in size.

FIELD OF THE DISCLOSURE

[0004] The present disclosure provides multilayered, multifunctional nanoparticles that can deliver biomacromolecules, such as mRNA, into cells to alter gene expression. More specifically, the present disclosure relates to multilayered, multifunctional nanoparticles having one or more nucleic acid ligands and/or internalized nucleic acid cargo, as well as methods making such nanoparticle carriers, and methods of using such nanoparticles for treatment and/or diagnosis of diseases and conditions.

BACKGROUND

[0005] The potential for the use of nucleic acids, and mRNA specifically, as both a vaccine and a general therapeutic has garnered significant attention and research focus. Lipid

nanoparticle formulations used to deliver mRNA vaccines to date required significant supporting infrastructure be provided as the formulations required advanced cryopreservation conditions (-20 °C to -80 °C) for handling and storage. Additionally, beyond viral vaccine development, mRNA (and certain nucleic acid moieties, generally) hold great promise as a therapeutic for a variety of diseases including cancer and inflammatory diseases. However, in order to be more broadly applied, both in terms of disease type and practical accessibility, new formulations that are more stable and specific to cell types and tissues are needed.

[0006] Well known delivery systems, such as liposomes and lipid nanoparticles, although clinically approved, suffer from many drawbacks. The lipid formulations developed to date suffer from off target tissue distribution, non-specific cellular uptake, an inability to efficiently escape endosomes/bypass lysosomes and, in some cases, are hepatotoxic. Additionally, many lipid formulations have a low loading capacity and have lipids that tend to oxidize or hydrolyze in biological fluids, limiting the overall stability of the formulation and the mRNA cargo. Therefore more effective delivery vehicles that can both stabilize as well as target the delivery of mRNA and other therapeutic nucleic acid cargos are needed.

SUMMARY

[0007] In an aspect, the disclosure provides a multifunctional nanoparticle that includes a nucleic acid nanocapsule (NAN), a metal organic framework (MOF), a liposome, and one or more therapeutic agents, or a pharmaceutically acceptable salt, hydrate or solvate thereof. In such multifunctional nanoparticles, the therapeutic agent is encased in the liposome, the liposome is encased in the MOF to make a lipid-MOF complex; and the lipid-MOF is encased in the NAN. In embodiments, the NAN includes one or more of nucleic acid ligands covalently attached to a particle comprising non-polymeric amphiphiles, in which the hydrophobic groups of the amphiphiles are arranged toward the particle interior, and the hydrophilic groups of the amphiphiles are at the particle surface and are crosslinked with one or more linkers cleavable by one or more intracellular or extracellular release agents. In some embodiments, the hydrophilic groups of the amphiphiles are crosslinked through a triazole, thioether, or alkenyl sulfide group with one or more cleavable linkers.

[0008] In another aspect, the disclosure provides a composition for treating or preventing a disease, a disorder, or symptom in a subject, including at least one of the multifunctional

nanoparticles of the disclosure, or a pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable vehicle and/or excipient.

[0009] In another aspect, the disclosure provides a method of treating or preventing a disease, a disorder, or symptom associated with the disease or disorder in a subject in a need thereof, the method including administering a therapeutically effective amount of a multifunctional nanoparticle disclosed herein, a pharmaceutically acceptable salt thereof, or the composition disclosed herein to the subject. In embodiments, the therapeutic agent (for example, an oligonucleotide such as mRNA) is released by one or more intracellular or extracellular release agent present in the subject, thus releasing the therapeutic agent.

[0010] These and other aspects of the present invention are described in more detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] The accompanying drawings are included to provide a further understanding of the methods and compositions of the disclosure and are incorporated in and constitute a part of this specification. The drawings illustrate one or more embodiment(s) of the disclosure, and together with the description serve to explain the principles and operation of the disclosure.

[0012] **FIG. 1** shows a schematic representation of proposed mechanism of mRNA delivery using multilayered Lipo_MOF-NANs to the cytosol of the cell. mRNA loaded Lipo_MOF-NAN enters cell through receptor mediated endocytosis followed by enzyme responsive endosomal escape to successfully deliver mCherry encoding mRNA to the cytosol. First the particles can bind to the cells outer receptors resulting in the endocytosis of the Lipo_MOF-NANs. Next, depending on the nature of the crosslinker used in the synthesis of the formulation, endosomal esterases or proteases can degrade the outer NAN and MOF layers of the particle and release a mixture of DNA surfactant conjugates (DSCs), 2-Methylimidazole (2-MIM), and cationic lipids. The result of these endosomal disrupting agents is the cytosolic delivery of mRNA which results in mRNA translation and the production of mCherry, a red fluorescent protein. The type of enzyme that will degrade the particle is programmed by the chemical nature of the micelle surface crosslinker. An ester crosslinker or a peptide crosslinker (structures shown in box in middle of **FIG. 1**) can be utilized for targeted cleavage by either endosomal esterases or

proteases (e.g. MMP9). Peptide crosslinkers generally demonstrate greater stability than ester linkers *in vivo* likely due to the greater serum stability of the amide bonds.

[0013] **FIG. 2A** to **FIG. 2C** shows a schematic detailing the assembly of a mRNA Lipo_MOF-NAN. (**FIG. 2A**) Stepwise assembly of a Lipo_MOF-NAN, a multi-layered nucleic acid delivery vehicle. First, cationic liposomes are formed within which are encapsulated anionic mRNA. Next, DNA surfactant conjugates (C_{12} surfactant tail length) are added, wherein the hydrophobic tails intercalate into the liposomal outer layer, presenting negatively charged nucleic acids. Next, 2-methyl imidazole and zinc salt are added to form a MOF cage around the liposome. This layer stabilizes the liposome to prevent premature rupture during administration. Lastly the mRNA Lipo_MOF is then formulated within a nucleic acid nanocapsule (NAN), first by templating of a cationic surfactant around the MOF cage, followed by crosslinking the surfactants head groups into a surface crosslinked micelle (SCM). These mRNA Lipo_MOF-SCMs are then subsequently functionalized at their surface with a polyT_{20} DNA for enhanced cellular uptake by scavenger receptors. The chemical nature of the crosslinker (see **FIG. 1** for example chemical structures) can be either an ester containing linker or a peptide linker to regulate the release of the mRNA by either an esterase or a protease, respectively. The mRNA molecule used herein encodes mCherry protein to facilitate detection of translation and expression via red fluorescence both in cell culture and in vivo. (**FIG. 2B**) TEM images showing the nanoscale characterization of each layer of the particle as it is assembled into a mRNA Lipo_MOF-NAN. All scale bars are 500 nm. (**FIG. 2C**) Temperature ramping experienced by the mRNA throughout the synthesis of the mRNA Lipo_MOF NANs, highlighting the amount of time the mRNA and the formulation spend above -20 °C while maintaining stability.

[0014] **FIG. 3A** to **FIG. 3F** show a characterization of mRNA loaded Lipo_MOF-NANs. (**FIG. 3A**) DLS based hydrodynamic size of 1 - liposomes (A), 2 - mRNA loaded liposomes (B), 3 - mRNA loaded liposomes functionalized with DNA ligands to make its surface anionic (C), 4 - mRNA loaded liposomal MOFs, mRNA Lipo_MOFs (D), 5 - mRNA loaded liposomal MOF surface crosslinked micelles, mRNA Lipo_MOF-SCMs (E), and 6 - mRNA loaded liposomal MOF nucleic acid nanocapsules mRNA Lipo_MOF-NANs (F), showing that changes in hydrodynamic size change occur at every step of addition of a new layer. (**FIG. 3B**) Changes in surface potential occur depending on the surface ligand functionality indicating the successful synthesis of the materials layers at each step. (**FIG. 3C**) Powder XRD based diffraction indicates

the crystallinity of the mRNA loaded Lipo_MOF (**FIG. 3D**) High resolution TEM EDS elemental mapping of mRNA loaded Lipo_MOF-NANs with signals for zinc, phosphorus and nitrogen present representing the lipids, MOF and mRNA components. The bottom left shows a schematic of the particles being analyzed in (**FIG. 3D**). (**FIG. 3E**) TEM and (**FIG. 3F**) SEM micrographs of mRNA loaded Lipo_MOF-NANs. Scale bars in both (**FIG. 3E**) and (**FIG. 3F**) are 500nm.

[0015] **FIG. 4** shows characterization of an anionic surfactant of the disclosure. In order to modify the surface of the cationic liposomes to be more anionic in character, a thiol modified T_{20} DNA (synthesized using a 5' thiol modifier C_6 phosphoramidite) was reacted with an in-house synthesized C_{12} surfactant used to assemble certain NAN formulations of the disclosure. The surfactant conjugation step was added in an attempt to anchor the DNA into the liposome in addition to the electrostatic interaction between the negatively charged DNA and the cationic liposome surface. After reacting the thiolated DNA with the surfactant at a ratio of 3:1 (DNA to surfactant), it was added to a solution of the cationic liposomes, incubated for an hour, and purified using Sephadex-25 NAP-10 column. Mass spectrometry indicated that on average there was one DNA added to each surfactant, likely due to the charge repulsion from neighboring DNA strands. DNA that reacted to form a conjugate with the surfactant is referred to as a DNA surfactant conjugate (DSC). A change in zeta potential from +55.7 to -34.6 mV was observed for the liposome post treatment with the DSC mixture.

[0016] **FIG. 5** shows TEM images of cationic liposomes with increasing DNA surfactant conjugate concentrations from 5 μM to 75 μM. Results indicated that that 50 μM DNA surfactant conjugate solution added to an 8 mg/mL solution of liposomes, the cationic liposomes were a suitable size and stable enough for further encapsulation.

[0017] **FIG. 6A** to **FIG. 6D** show a TEM analysis of liposomes of the disclosure in stages of construction versus control liposomes. (**FIG. 6A**) mRNA loaded control liposomes. (**FIG. 6B**) mRNA loaded DNA modified liposomes. (**FIG. 6C**) Liposomal MOF encapsulating surface crosslinked micelles (mRNA Lipo_MOF-SCMs). The synthesized liposomes have a large range of sizes post extrusion through a 0.2-micron filter; whereas, post purification, the mRNA loaded Lipo-MOF-SCMs are relatively uniform in size. All images are stained with uranyl acetate to be able to observe the organic materials under TEM. A TEM image of mRNA Lipo_MOF however

can be seen without uranyl acetate staining (**FIG. 6D**) due to the inorganic surface of the MOF layer.

[0018] **FIG. 7A** to **FIG. 7D** show the dual responsive nature of Lipo_MOF-NANs. (**FIG. 7A**) mCherry mRNA-loaded Lipo_MOF-NANs were treated with esterase followed by acetate buffer (pH=5.5) to lower the pH to a condition at which the MOF layer should be protonated and subsequently degraded. Total mRNA release was visualized by a reverse transcription (RT) PCR assay run on an 8% PAGE gel. (**FIG. 7B**) 8% denaturing PAGE gel indicating the release of mRNA is greatest when Lipo_MOF-SCMs are exposed to both esterase and low pH conditions. (**FIG. 7C**) Chloroquine inhibitor experiments to investigate the role of the MOF layer in gating protein expression from mRNA Lipo_MOF-NANs. HeLa and A549 cells were pretreated with chloroquine to prevent the maturation and thus acidification of endosomes. Confocal imaging in both HeLa and A549 cell lines in both serum-added or serum-free media shows that the MOF layer is only degraded inside endosomes allowed to acidify (non-chloroquine treated) as indicated by mCherry expression levels. Scale bars are 50 μm. mCherry expression appears as brighter white regions among darker gray regions in the black and white image. (**FIG. 7D**) MFI calculations shown below the confocal images indicate the large differences in fluorescence intensities between chloroquine and non-chloroquine treated samples. The significance was called for $p < 0.05$ (ns $p > 0.05$, * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

[0019] **FIG. 8** shows fluorescein loaded Lipo_MOF NANs after 4 hours (hr) of incubation with A549 cells in both 10% serum-added (F12-K) and serum-free (opti-MEM media). Green emission (appears as brighter white, regions with a more particulate character in black and white) represents Fluorescein dye embedded/ released within/ from the Lipo_MOF-NANs, and blue emission (appears in black and white as light gray regions, with a more diffuse character as compared to the green emission) is from Hoechst stained nuclei. Scale bars are 50 μm.

[0020] **FIG. 9A** to **FIG. 9C** shows confocal microscopy imaging of mCherry expression when mRNA formulations samples were kept at 4 °C and incubated with HeLa cell for 8 hr in serum added DMEM media. Samples were treated 1, 7, 14, 21 days after synthesis. Treated cells were imaged keeping all conditions same. mCherry expression appears as brighter white regions among darker gray regions in the black and white image. (**FIG. 9A**) Confocal microscopy imaging in HeLa cells and Mean Fluorescent Intensity (MFI) calculated from different fields in

HeLa (**FIG. 9B**) and A549 (**FIG. 9C**) cell lines. (Corresponding confocal images of A549 shown in **FIG. 12**). M-Cherry mRNA delivered by Lipo_MOF-NANs resulted in higher expression of mCherry than when the mRNA is delivered through cationic liposomes or in water solution -- even after 14 days. This result is supported by at least **FIGS. 12-14**. Similar observations were found when samples were treated in HeLa and A549 cells in serum free opti-MEM media. All experiments were performed in triplicate in different biological sets and sample sets. Here the bar diagram of MFI is representative of one trial and considering 8-10 cells from different field considering. The significance was called for $p < 0.05$ (ns $p > 0.05$, * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

[0021] **FIG. 10A** to **FIG. 10C** shows confocal microscopy imaging of mCherry mRNA expression over time. Imaging of mCherry expression when mRNA formulations were stored at room temperature for the indicated times and incubated with HeLa cells for 8 hr in serum added DMEM media. (**FIG. 10A**) Confocal microscopy imaging in HeLa cells. (**FIG. 10B**, **FIG. 10C**) Mean Fluorescent Intensity (MFI) calculated from different fields in HeLa (**FIG. 10B**) and A549 (**FIG. 10C**) cell lines. (Additional confocal images of A549 are shown in **FIG. 15**). mCherry mRNA delivered by Lipo_MOF-NANs resulted in higher expression of mCherry than when the mRNA was delivered via cationic liposomes or in water solution even after 7-14 days. This result is supported by at least **FIGS. 15-17**, where similar observations were found when samples were treated in HeLa and A549 cells in serum free opti-MEM media. All experiments were performed in triplicate in different biological sets and sample sets. Here the bar diagram of MFI is representative of one trial and considering 8-10 cells from different field considering. The significance was called for $p < 0.05$ (ns $p > 0.05$, * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

[0022] **FIG. 11A** to **FIG. 11D** show Confocal microscopy time course assay imaging of mCherry expression that resulted when samples were stored at 4 °C overnight and treated in HeLa or A549 cells in corresponding serum added media and serum free media conditions. mCherry expression in HeLa (**FIG. 11A** and **FIG. 11B**) or A549 (**FIG. 11C** and **FIG. 11D**) cells after incubation of cells with mRNA Lipo_MOF-NANs with serum (**FIG. 11A**, **FIG. 11C**) and without serum (**FIG. 11B**, **FIG. 11D**). Samples were stored overnight at 4 °C prior to addition to cells. Confocal images were taken just after 4 hr (left-most panels), 8 hr (middle panels) incubation and after another 16 hr passed after 8 hr incubation (right-most panels) (for **FIG. 11A**, **FIG. 11B**). Similarly, confocal images were taken just after 4 hr (left-most panels), 6 hr

(middle panels) incubation and after another 16 hr transpired post 6 hr incubation (right-most panels) (for **FIG. 11C**, **FIG. 11D**). Results show that mCherry expression (red fluorescence; expressed as brighter white regions among darker gray regions in the black and white images) is significant within 8 hr for HeLa and 4 hr for A549. Images also indicate that emission remains significant even at longer time points (24 hr) in both cells.

[0023] **FIG. 12** shows confocal imaging of mCherry protein expression when mRNA loaded samples were kept at 4 °C for indicated times and incubated with A549 cells for 4 hr in serum added F-12k media. Expression of mRNA delivered by Lipo_MOF-NANs was higher than when delivered through cationic liposomes or in PBS solution and expression persisted for ~2 weeks. There was no significant expression of mCherry after 21 days. This result is consistent with the results shown in **FIG. 9A – FIG. 9C**. Experiments were performed three times with different sets of samples and biological cells. Scale bars are 50 μm.

[0024] **FIG. 13** shows confocal imaging of mCherry protein expression when mRNA loaded samples were kept at 4 °C for indicated times and incubated with A549 cell for 4 hr in serum free opti-MEM media. Expression of mCherry delivered by liposomal-MOF-NANs (Lipo_MOF-NANs) was higher than when delivered through cationic liposomes or in PBS solution and expression persisted for ~2 weeks. There was no significant expression of mCherry after 21 days. This result is consistent with **FIG. 9A – FIG. 9C**. Experiments were performed three times with different sets of samples and biological cells.

[0025] **FIG. 14** shows imaging of mCherry expression when mRNA loaded samples were kept at 4 °C at the indicated times and incubated with HeLa cells for 4 hr in serum free DMEM media. Whether kept at 4 °C or room temperature, expression of mRNA delivered by Lipo_MOF-NANs was higher than when delivered through cationic liposomes or in PBS solution. This result supports the results shown in **FIG. 9A – FIG. 9C**. Experiments were performed three times with different sets of samples and biological cells.

[0026] **FIG. 15** shows imaging of mCherry protein expression when mRNA loaded samples were kept at room temperature for the indicated times and incubated with A549 cell for 4 hr in serum added F-12K media. Expression of mCherry mRNA delivered by Lipo_MOF-NANs expression was higher than when delivered through cationic liposomes or in PBS solution even after 7 days. There is no significant emission of mCherry expression after 21 days. This result

supports **FIG. 10A – FIG. 10C**. Experiments were performed three times with different sets of sample and biological cells. Scale bars are 50 μm.

[0027] **FIG. 16** shows imaging of mCherry expression when mRNA loaded samples were kept at room temperature at the indicated times and incubated with A549 cells for 4 hr in serum free opti-MEM media. Expression of mCherry mRNA delivered by Lipo_MOF-NANs was higher than when delivered through cationic liposome or in PBS solution even after 13 days. There was no significant emission of mCherry expression after 21 days. This result supports the results shown in **FIG. 10A – FIG. 10C**. Experiments were performed three times with different sets of sample and biological cells.

[0028] **FIG. 17** shows imaging of mCherry protein expression when mRNA loaded samples were kept at room temperature for the indicated times and incubated with HeLa cell for 8 hr in serum free DMEM media. Whether samples were kept at 4 °C or room temperature, expression of mCherry mRNA delivered by liposomal-MOF-NANs was higher than when delivered through cationic liposome or in PBS solution. This result supports the results of **FIG. 9A – FIG. 9C**. Experiments were performed three times with different sets of sample and biological cells.

[0029] **FIG. 18** shows results of a MTS cell viability assay used to assess the toxicity profile of the Lipo_MOF NANs relative to liposomes and mRNA transfected into cells using lipofectamine 2000. A549 cells were treated with equal concentrations of mCherry loaded Lipo_MOF NANs, cationic liposomes and mCherry mRNA in lipofectamine. Samples were incubated with cells for 24 hr followed by MTS treatment. No toxicity of the Lipo_MOF-NANs was seen even at 10 μM. In contrast, liposomes and transfected mRNA showed toxicity at lower concentrations. A lower concentration of Lipo_MOF NANs was screened for a peptide crosslinked formulation as such lower concentrations were used for the *in vivo* studies described herein. Here, untreated cells were set as 100% cell viability and as control for this experiment. Error bars are the average of three different experiments.

[0030] **FIG. 19A** to **FIG. 19D** show Annexin 2 aptamer functionalization of Lipo_MOF-NANs for inducing cell specific targeting. mCherry loaded Lipo_MOF-NANs were functionalized with an annexin 2 specific aptamer referred to as ACE-4 and the particles were incubated with MCF-7 cells in serum free opti-MEM media for 30 mins. (**FIG. 19A**) and (**FIG. 19B**) Sequence and schematic representation of the design and assembly of mRNA loaded

aptamer functionalized Lipo_MOF-NANs. (**FIG. 19C**) Comparison of mCherry expression after incubation of MCF-7 cells with bare mRNA, mRNA loaded liposomes, mRNA loaded Lipo_MOF-NANs and mRNA loaded Lipo_MOF-NANs functionalized with either aptamer or a scramble sequence. Results show that aptamer functionalization increases the mCherry expression, outcompeting the other conditions after short incubation times as evidenced by the higher red emission (expressed as brighter white regions among darker gray regions in the black and white images) for the aptamer functionalized Lipo_MOF-NANs. (**FIG. 19D**) mCherry expression as seen by confocal microscopy imaging of 7 μm thick tumor tissue-sections of MCF-7 tumor bearing mice 24 hr post injection of mRNA loaded aptamer functionalized Lipo_MOF-NANs synthesized with an ester crosslinker. Imaging of control samples is summarized in **FIG. 20** and **FIG. 21**. All experiments were performed three times in different biological sets and sample sets.

[0031] **FIG. 20A** to **FIG. 20C** show peptide crosslinked Lipo_MOF-NANs for enzyme triggered release of mRNA to tumor bearing mice. (**FIG. 20A**) Schematic representation of protease responsive delivery of mRNA by MMP-9 peptide crosslinked Lipo_MOF-NANs. (**FIG. 20B**) mCherry mRNA expression in A549 and HeLa cells in serum added DMEM media was observed when cells were treated with mRNA loaded peptide crosslinked Lipo_MOF-NANs overnight (16 hr), indicating greater expression was achieved using a protease responsive release as a trigger for mRNA delivery. Scale bars are 50 μm. (**FIG. 20C**) MCF-7 tumor bearing mice injected with mRNA loaded peptide crosslinked Lipo_MOF-NAN functionalized with an annexin 2 specific aptamer (**FIG. 20C, panel row I**) and mRNA loaded peptide crosslinked Lipo_MOF-NAN with a scrambled sequence (**FIG. 20C, panel row II**). A 5 nmol/kg injection of annexin 2 specific aptamer or scrambled sequence was injected into mice bearing subcutaneous MCF-7 tumors via tail vein injection. The mice were sacrificed 24 hr post injection and 7 μm cross sections of tumor were imaged under confocal microscopy. Results showed a reasonably homogeneous mRNA expression (red emission; expressed as brighter white regions among darker gray regions in the black and white images) throughout the tumor observed when mRNA is delivered using the peptide crosslinked Lipo_MOF-NAN aptamer formulation (**FIG. 20C, panel row I**). In contrast, the scrambled aptamer formulation (**FIG. 20C, panel row II**) showed fluorescence largely localized to the tumor periphery and discrete intra-tumoral regions. Control tumor tissues collected from mice treated with 1X PBS, empty aptamer modified

Lipo_MOF-NANs or empty scrambled aptamer modified Lipo_MOF-NANs (**FIG. 20C, panel row III**) showed limited to no cellular emission in the red channel. Further detailed analysis of mRNA expression within the tumor tissue can be found, e.g., in **FIG. 22 – FIG. 24**.

[0032] **FIG. 21** shows representative confocal fluorescent image of tumor taken from a 7 μm tumor section prepared from a subcutaneous MCF-7 tumor 24 hr post IV injection of 1X PBS buffer. Results show that there is no significant emission from the tissue sections, which can be considered as a control experiment of **FIGS. 19-20**.

[0033] **FIG. 22** shows representative confocal fluorescent images of tumor edge and tumor core taken from a 7 μm tumor section prepared from a subcutaneous MCF-7 tumor 24 hr post IV injection. Results show that there is significant mRNA expression in the tumor tissue when mice were treated with mRNA Lipo_MOF-NANs with both aptamer and scrambled aptamer functionalization. However, mCherry expression (expressed as brighter white or gray regions in the black and white images) was more homogenously distributed throughout the tumor when the mRNA was delivered by the aptamer functionalized particles. This result is consistent with the homogeneous expression of mRNA observed in **FIG. 20**.

[0034] **FIG. 23** shows ACE4 aptamer induced homogeneous delivery of mRNA throughout a subcutaneous MCF-7 seeded tumor growing on the thigh of mice. Two different tissue sections of the tumors are represented here and mean fluorescent intensity (MFI) of different areas (marked as white boxes) were calculated by ImageJ software. MFI values are indicated above marked areas. Results show that mRNA expression is reasonably homogeneously distributed when delivered using ACE4 aptamer functionalized Lipo_MOF-NANs. In contrast, scrambled sequence functionalized particles showed large differences in expression levels throughout the tumor.

[0035] **FIG. 24** shows H&E-stained tumor sections showing homogeneous distribution of MCF-7 cells in the tumor. A region of interest was marked within the H&E-stained tissue section and the section was imaged under confocal microscopy. Analysis confirmed the fluorescent images were within regions of tumor cells. The H&E staining suggests homogeneous distribution of tumor cells and that the heterogeneity in fluorescent emission observed throughout the tumor may be attributed to the difference in mRNA delivery formulations (having an aptamer-modified (a) vs scramble sequence-modified (b) particle surface) and not due to a difference in cell type

distribution. These data support the analysis in **FIG. 20** and **FIGS. 22-23**. Selected area of interest is not to the exact scale to the fluorescent images, but an approximate area is marked.

DETAILED DESCRIPTION

[0036] Herein, multi-layered nanoparticles ("nanocapsules") are provided that offer targeting capability, enhanced stability and highly controlled release of nucleic acid cargo (e.g., mRNA), and a prolonged shelf life. Nanocapsules of the disclosure comprise a liposomal layer to encapsulate nucleic acid cargo, a metal organic framework (MOF) layer to stabilize the liposome, and a nucleic acid nanocapsule (NAN) outer layer to, e.g., influence the targeting of the nanocapsule and delivery of nucleic acid cargo. Thus, the nanocapsules as described herein, as well as related compositions and methods of use, offer significant advantages over other delivery systems.

[0037] A number of terms are introduced below, which are used to describe the invention of the present disclosure. In instances where a technical or scientific term is not specifically defined herein, they will have the same meaning as commonly understood by one of ordinary skill in the art. For example, any nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics, and protein and nucleic acid chemistry and hybridization described herein are well known and commonly used in the art. In case of conflict, the present disclosure, including definitions, will control. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing of the embodiments and aspects described herein.

[0038] As used herein, the terms "nanocapsule", "nanoparticle", "nanocarrier", "nanoparticle carrier", "Liposomal Metal Organic Framework-Nucleic Acid Nanocapsule", and "Lipo_MOF-NAN", and the like, refer to multilayer nanoscale complexes capable of encapsulating within or otherwise encompassing or carrying one or more cargo molecules for delivery, e.g., to one or more locations in a body (e.g., cell, tissue, compartment). Nanocapsules of the disclosure can comprise layers including, e.g., a cationic liposome layer comprising a nucleic acid cargo for delivery into a cell, a metal organic framework (MOF) layer that helps stabilize the liposome, a surface crosslinked micelle (SCM) layer formed by the templating of a cationic surfactant around the MOF cage, followed by crosslinking the surfactant head groups; and a layer formed by

modifying the surface of the SCM layer with a functional group, e.g., a polyT_{20} DNA for enhanced cellular uptake by scavenger receptors, to form a nucleic acid nanocapsule (NAN) layer.

[0039] The term "nanoparticle", as used herein, refers to any particle having a diameter of less than about 1000 nm. Nanoparticles can comprise, e.g., one or more natural and/or synthetic lipids, polymers, or mixtures thereof. Disclosed nanoparticles may include nanoparticles having a diameter of about 1nm to about 1000 nm, with further specific detail provided elsewhere herein.

[0040] The term "liposome" encompasses any compartment enclosed by at least one lipid bilayer. The term liposome includes unilamellar structures, which comprise a single lipid bilayer, and multilamellar structures, which comprise more than one lipid bilayer. As used herein "liposomes" can refer to unilamellar vesicles (UV) (and, more specifically, small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV)), double bilayer vesicles (DBV), oligolamellar vesicles (OLV) and multilamellar vesicles (MLV). As used herein, a liposome is a type of nanoparticle.

[0041] The term "lipid" refers to lipid molecules that can include fats, waxes, steroids, cholesterol, fat-soluble vitamins, monoglycerides, diglycerides, phospholipids, sphingolipids, glycolipids, cationic or anionic lipids, derivatized lipids, and the like, as described in detail below. Lipids can form micelles, monolayers, and bilayer membranes. The lipids can self-assemble into liposomes.

[0042] As used herein, the term “administering” refers to the actual physical introduction of a composition into or onto (as appropriate) a subject, a host, or cell. Any and all methods of introducing the composition into the subject, host or cell are contemplated according to the invention; the method is not dependent on any particular means of introduction and is not to be so construed. Means of introduction are well-known to those skilled in the art, and also are exemplified herein. “Providing” means giving, administering, selling, distributing, transferring (for profit or not), manufacturing, compounding, or dispensing.

[0043] As used herein, the term “pharmaceutically acceptable” refers to compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction when administered to a subject, preferably a human or a non-human subject. Preferably,

as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of a federal or state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

[0044] As used herein, the terms "treat," "treating," and "treatment" include inhibiting the pathological condition, disorder, or disease, *e.g.*, arresting or reducing the development of the pathological condition, disorder, or disease or its clinical symptoms; or relieving the pathological condition, disorder, or disease, *e.g.*, causing regression of the pathological condition, disorder, or disease or its clinical symptoms. Treatment means any way the symptoms of a pathological condition, disorder, or disease are ameliorated or otherwise beneficially altered. Preferably, a subject in need of such treatment is a mammal, and particularly a human, but is not so limited as defined below. Treatment also means providing an active compound to a patient in an amount sufficient to measurably reduce any disease symptom, slow disease progression or cause disease regression. These terms also encompass therapy and cure.

[0045] As used herein, the term "effective amount" or "therapeutically effective amount" refers to the amount of a therapy, which is sufficient to reduce or ameliorate the severity and/or duration of a disorder or one or more symptoms thereof, inhibit or prevent the advancement of a disorder, cause regression of a disorder, inhibit or prevent the recurrence, development, onset or progression of one or more symptoms associated with a disorder, detect a disorder, or enhance or improve the prophylactic or therapeutic effect(s) of another therapy (*e.g.*, prophylactic or therapeutic agent). An effective amount can require more than one dose. As used herein, the term "prophylaxis" refers to preventing or reducing the progression of a disorder, either to a statistically significant degree or to a degree detectable by a person of ordinary skill in the art.

[0046] Effective amounts may vary depending upon the biological effect desired in the individual, condition to be treated, and/or the specific characteristics of the composition according to the present invention and the individual. In this respect, any suitable dose of the composition can be administered to the patient (*e.g.*, human), according to the type of disease to be treated. Various general considerations taken into account in determining the "effective amount" are known to those of skill in the art and are described, *e.g.*, in Gilman et al., eds., Goodman And Gilman's: *The Pharmacological Bases of Therapeutics*, 8th ed., Pergamon Press,

1990; and Remington's Pharmaceutical Sciences, 17th Ed., Mack Publishing Co., Easton, Pa., 1990, each of which is herein incorporated by reference.

[0047] As used herein, the term "dose" denotes any form of an active ingredient formulation or composition, including cells, that contains an amount sufficient to initiate or produce a therapeutic effect with at least one or more administrations. "Formulation" and "composition" are used interchangeably herein. As used herein, the terms "control," or "reference" are used herein interchangeably. A "reference" or "control" level may be a predetermined value or range, which is employed as a baseline or benchmark against which to assess a measured result. "Control" also refers to control experiments or control cells.

[0048] The terms "therapeutic" or "therapeutic agent" refer to a compound or molecule that, when present in an effective amount, produces a desired therapeutic effect on a subject in need thereof. The present invention contemplates a broad range of therapeutic agents and their use in conjunction with the liposome compositions, as further described herein.

[0049] The term "pharmaceutical compositions" means compositions comprising at least one active agent, such as a compound or salt of Formula (I), and at least one other substance, such as a carrier. Pharmaceutical compositions meet the U.S. FDA's GMP (good manufacturing practice) standards for human or non-human drugs.

[0050] The term "carrier" refers to a diluent, excipient, or vehicle with which an active compound is administered. A "pharmaceutically acceptable carrier" means a substance, e.g., excipient, diluent, or vehicle, that is useful in preparing a pharmaceutical composition that is generally safe, non-toxic and neither biologically nor otherwise undesirable, and includes a carrier that is acceptable for veterinary use as well as human pharmaceutical use. A "pharmaceutically acceptable carrier" includes both one and more than one such carrier.

[0051] The term "subject" or "patient" is used herein to refer to an animal, such as a mammal, including a primate (such as a human, a non-human primate, *e.g.*, a monkey, and a chimpanzee), a non-primate (such as a cow, a pig, a camel, a llama, a horse, a goat, a rabbit, a sheep, a hamster, a guinea pig, a cat, a dog, a rat, a mouse, and a whale), a bird (*e.g.*, a duck or a goose), and a shark. In an embodiment, the subject or patient is a human subject or a human patient, such as a human being treated or assessed for a disease, disorder or condition, a human at risk for a disease, disorder

or condition, a human having a disease, disorder or condition, and/or human being treated for a disease, disorder or condition as described herein.

[0052] As used herein, a subject is "in need of treatment" if such subject would benefit biologically, medically, or in quality of life from such treatment. A subject in need of treatment does not necessarily present symptoms, particular in the case of preventative or prophylaxis treatments.

[0053] All percentages and ratios are calculated by weight unless otherwise indicated. All percentages are calculated based on the total composition unless otherwise indicated. Generally, unless otherwise expressly stated herein, "weight" or "amount" as used herein with respect to the percent amount of an ingredient refers to the amount of the raw material comprising the ingredient, wherein the raw material may be described herein to comprise less than and up to 100% activity of the ingredient. Therefore, weight percent of an active in a composition is represented as the amount of raw material containing the active that is used and may or may not reflect the final percentage of the active, wherein the final percentage of the active is dependent on the weight percent of active in the raw material.

[0054] All methods described herein can be performed in a suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, "such as"), is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention as used herein. Unless defined otherwise, technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art of this disclosure.

[0055] Furthermore, the disclosure encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, and descriptive terms from one or more of the listed claims are introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Where elements are presented as lists, *e.g.*, in Markush group format, each subgroup of the elements is also disclosed, and any element(s) can be removed from the group.

[0056] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited. For example, if a size range is stated as 1 nm to 100 nm (or concentrations, degrees, mass amounts, and the like), it is intended that values such as 2 nm to 90 nm, 10 nm to 70 nm, 30 nm to 95 nm, 75 nm to 100 nm, or 2 nm to 27 nm, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended, and all possible combinations of numerical values between and including the lowest value and the highest value enumerated are to be considered to be expressly stated in this disclosure.

[0057] Furthermore, when "about", "approximately" and/or "substantially" is/are utilized to describe a value, this is meant to encompass minor variations (up to +/- 10%) from the stated value. Where no stated value is provided, an element described as "substantially" means at least about 60%, 70%, 80%, 90%, 95%, 99%, or more of the element, as is logically coherent within in the context. Unless specifically stated to the contrary, for ranges specified using "about" language, the about applies to both ends of the recited range whether specified or not. For example, "between about 10 mM and 10 μM" is equivalent to "between about 10 mM and about 10 μM".

[0058] As used herein, the terms “comprising”, “having”, “including”, and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to”) unless otherwise noted.

[0059] When introducing elements of the present disclosure or the aspects and embodiment thereof, the articles "a," "an," and "the" are intended to mean that there are one or more of the elements. Similarly, the adjective "another," when used to introduce an element, is intended to mean one or more elements.

[0060] The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically

identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

[0061] As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e., "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of."

[0062] As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from anyone or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified. Thus, as a nonlimiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, w, ith no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

[0063] The phrase "one or more," as used herein, means at least one, and thus includes individual components as well as mixtures/combinations of the listed components in any combination.

[0064] As used herein, "optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

[0065] As used herein, "alkyl" refers to a straight or branched chain hydrocarbon containing from 1 to 10 carbon atoms unless otherwise specified. Representative examples of alkyl include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl, iso-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, n-hexyl, 3-methylhexyl, 2,2-dimethylpentyl, 2,3-dimethylpentyl, n-heptyl, n-octyl, n-nonyl, and n-decyl. When an "alkyl" group is a linking group between two other moieties, then it may also be a straight or branched chain; examples include, but are not limited to $-CH_2-$, $-CH_2CH_2-$, $-CH_2CH_2CHC(CH_3)-$, and $-CH_2CH(CH_2CH_3)CH_2-$.

[0066] As used herein, "alkenyl" refers to means a straight or branched chain hydrocarbon containing from 2 to 10 carbons, unless otherwise specified, and containing at least one carbon-carbon double bond. Representative examples of alkenyl include, but are not limited to, ethenyl, 2-propenyl, 2-methyl-2-propenyl, 3-butenyl, 4-pentenyl, 5-hexenyl, 2-heptenyl, 2-methyl-1-heptenyl, 3-decenyl, and 3,7-dimethylocta-2,6-dienyl.

[0067] As used herein, "alkynyl" refers to a straight or branched chain hydrocarbon group containing from 2 to 10 carbon atoms and containing at least one carbon-carbon triple bond. Representative examples of alkynyl include, but are not limited, to acetylenyl, 1-propynyl, 2-propynyl, 3-butynyl, 2-pentynyl, and 1-butynyl.

[0068] As used herein, "alkoxy" refers to an alkyl group, as defined herein, appended to the parent molecular moiety through an oxygen atom. Representative examples of alkoxy include, but are not limited to, methoxy, ethoxy, propoxy, 2-propoxy, butoxy, tert-butoxy, pentyloxy, and hexyloxy.

[0069] As used herein, "halo" or "halogen" refers to -Cl, -Br, -I or -F.

[0070] Substituents are intended to be read "left to right" (i.e., the attachment is via the last portion of the name) unless a dash indicates otherwise. For example, C_1-C_6 alkoxycarbonyl

and -C(O)C_1-C_6alkyl indicate the same functionality; similarly arylalkyl and –alkylaryl indicate the same functionality.

[0071] It should also be understood that, in certain methods described herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited unless the context indicates otherwise. Furthermore, the terms first, second, etc., as used herein are not meant to denote any particular ordering, but simply for convenience to denote a plurality of, for example, layers.

[0072] Moving to the specifics of the subject matter, disclosed herein are multilayered, multifunctional nanoparticles (or nanocapsules, henceforth described as multifunctional nanoparticles) that are capable of encapsulating one or more therapeutic agents/cargos such as small molecule drugs, biologics, or dyes or rapidly functionalized with therapeutic nucleic acid ligands (such as a DNAzyme or siRNA sequence), or a pharmaceutically acceptable salt, hydrate or solvate thereof. In addition, the multilayered, multifunctional nanoparticles of the disclosure are capable of nucleic acid delivery and targeted gene knockdown. These multifunctional nanoparticles can be targeted to specific cell receptors by incorporating DNA or RNA aptamers as the nucleic acid ligands. For example, mucin-1, annexin-2, transferrin and nucleolin receptors can be targeted using their published aptamer sequences. In addition, the targeted breakdown of the NAN and release of the internalized drug cargo can be programmed for degradation by specific proteases (i.e., cathepsins, matrix metalloproteases, for example MMP9) depending on the need for intracellular or extracellular release, by utilizing the unique crosslinker design of the NAN.

[0073] The benefits of the multilayered, multifunctional nanoparticles of the disclosure address a number of current and important drug delivery hurdles present in the art, such as the ability to easily functionalize the surface of a drug delivery vehicle for therapeutic or targeting applications, biodegradability, and the capacity for combination therapy, as the interior can be loaded with one or more drugs, and the surface modified with a separate therapeutic biomolecule.

[0074] In one aspect, a multifunctional nanoparticle is provided, including a nucleic acid nanocapsule (NAN), a metal organic framework (MOF), a liposome, and one or more therapeutic agents, or a pharmaceutically acceptable salt, hydrate or solvate thereof, in which the therapeutic

agent is encased in the liposome; the liposome is encased in the MOF to make a lipid-MOF complex; and the lipid-MOF is encased in the NAN.

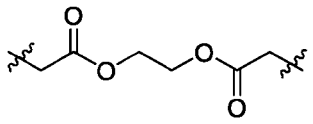
[0075] In some embodiments, the NAN includes non-polymeric amphiphiles, in which the hydrophobic groups of the amphiphiles are arranged toward the particle interior (i.e., toward the MOF), and the hydrophilic groups of the amphiphiles are arranged toward the particle surface and are crosslinked with one or more linkers cleavable by one or more intracellular or extracellular release agents (such as peptidase, protease, esterase, or elastase), to form a surface crosslinked micelle (SCM), to which one or more nucleic acid ligands are covalently attached to form a NAN. In some embodiments, the hydrophilic groups of the amphiphiles are crosslinked through a triazole, thioether, or alkenyl sulfide group with one or more cleavable linkers.

[0076] In some embodiments, the multifunctional nanoparticle further includes a targeting nucleic acid ligand (e.g., aptamers, therapeutic oligonucleotides, antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), microRNAs (miRNAs), ribozymes, DNAzymes, peptide nucleic acids (PNAs), locked nucleic acids (LNAs), and the like, or combinations thereof) attached to the exterior of the NAN.

[0077] In some embodiments, the triazole, thioether, or alkenyl sulfide group crosslinking the hydrophilic groups of the amphiphile and the linker results from a reaction of alkyne or alkene moiety on the hydrophilic group of the amphiphile (e.g., on the ammonium group) and an azide or thiol moiety on the linker. In some embodiments, the alkyne or alkene moiety on the hydrophilic group of the amphiphile is one or two of prop-2-ynyl or prop-2-enyl; one or two of prop-2-ynyl, or one or two of prop-2-enyl, and in some embodiments, the hydrophilic group of the amphiphile is tri(prop-2-yn-1-yl)amino or triallylamino.

[0078] In some embodiments, the linker includes a peptide (for example, cleavable with a peptidase or protease), in which the peptide is at least two amino acids long, or at least three amino acids long, or at least four amino acids long; or the peptide is between two and twenty-five amino acids long. In some embodiments, the peptide linker includes CGPLGLAGGERDGC (SEQ ID NO:1), GPLGLAGGERDG (SEQ ID NO:14), GFLG (SEQ ID NO:15), GPMGIAGQ (SEQ ID NO:16), CGFLGC (SEQ ID NO:17), CGPMGIAGQC (SEQ ID NO:18), Phe-Leu, Val-Ala, Val-Cit, Val-Lys, Val-Arg, or Phe-Lys.

[0079] In other embodiments, the linker includes one or more of ester groups (for example, cleavable with an esterase), which, in some embodiments, includes the following group



[0080] In some embodiments, the linker includes an acid-labile group hydrolysable in the lysosome, including one or more of hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, or thioether groups, or a combination thereof, or other acid-labile groups.

[0081] In some embodiments, the linker includes a disulfide group, while in some embodiments, the linker excludes disulfide group or other group cleavable under reducing conditions.

[0082] In some embodiments, the linker is cleavable by one intracellular or extracellular release agent, and in other embodiments, the linker is cleavable by two or more intracellular or extracellular release agents (e.g., wherein the linker comprises two or more different chemical groups each cleavable by a different release agent). In some embodiments, the linker includes at least two groups selected from an ester, hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, thioether, disulfide, and a peptide, wherein the peptide is at least two amino acids long, or at least three amino acids long, or at least four amino acids long; or the peptide is between about two to thirty amino acids long, for example, about two and twenty-five amino acids long, about two to twenty amino acids long, about two to fifteen amino acids long, about two to ten amino acids long, or about two to five amino acids long.

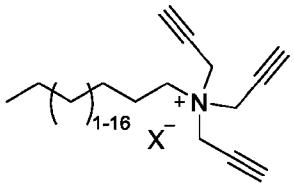
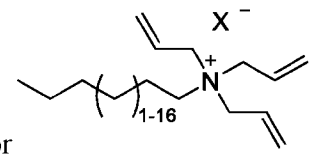
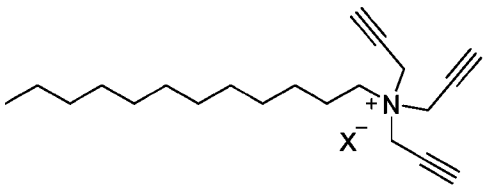
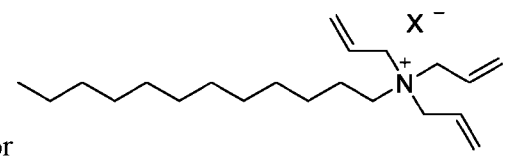
[0083] In some embodiments, the nucleic acid ligands are covalently attached to the hydrophilic groups of the amphiphiles through a thioether or alkenyl sulfide group. In some embodiments, the thioether or alkenyl sulfide group attaching the nucleic acid ligands to the hydrophilic group of the amphiphile results from a reaction of alkyne or alkene moiety on the hydrophilic group of the amphiphile (e.g., on the ammonium group) and a thiol moiety (e.g., Cys) on the nucleic acid ligand.

[0084] In some embodiments, the alkyne or alkene moiety on the hydrophilic groups of the amphiphiles is prop-2-ynyl or prop-2-enyl, or prop-2-ynyl, or prop-2-enyl, and in some

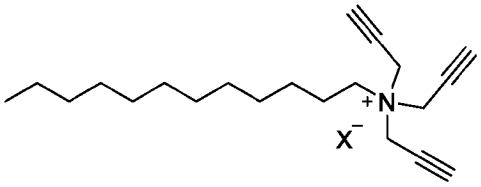
embodiments, the alkyne or alkene moiety on the hydrophilic group of the amphiphile is prop-2-yn-1-ylamino or allylamino.

[0085] In some embodiments, nucleic acid ligands are capable of selectively binding to a cell surface antigen, a protein, a carbohydrate, or to a nucleic acid. In some embodiments in which the nucleic acid ligand selectively binds to a protein, the protein is selected from the group consisting of tumor-markers, integrins, cell surface receptors, transmembrane proteins, ion channels, membrane transport protein, enzymes, antibodies, and chimeric proteins. In some embodiments in which the nucleic acid ligand selectively binds to a carbohydrate, the carbohydrate is selected from the group consisting of glycoproteins, sugar residues, and glycocalyx. In some embodiments in which the nucleic acid ligand selectively binds to a nucleic acid, the nucleic acid includes binding DNA, RNA, modified DNA, modified RNA, DNAzymes, ribozymes, mRNA, siRNA, microRNA, shRNA, and combinations thereof.

[0086] In some embodiments, the nucleic acid ligand is capable of gene regulation, and wherein the nucleic acid is siRNA, DNAzyme, ribozyme, microRNA, or other therapeutic oligonucleotide. In some embodiments, the nucleic acid ligand is capable of selectively binding to a cell receptor, or to a cell during a specific developmental stage (e.g., stage having developmentally specific cell surface antigens) or to a cell in a specific disease state (e.g., a tumor cell that has tumor-associated antigens or tumor-specific antigens). Thus, in some embodiments, the nanoparticle is targeted to a specific cell receptor, non limiting examples of such receptors including nucleolin, transferrin, annexin 2 and mucin-1 receptors.

[0087] Regarding non-polymeric amphiphiles, in some embodiments, the amphiphiles are derived from  or  , where X is halogen, and in some embodiments, the non-polymeric amphiphiles are derived from  or  , where X is

also a halogen. In some embodiments, the non-polymeric amphiphile is



, where X is again a halogen.

[0088] Regarding the metal organic framework, in some embodiments, the MOF includes a plurality of metal ions and a plurality of organic ligands, in which the metal ions and organic ligands are coordinated to form a porous, three-dimensional framework around the liposome.

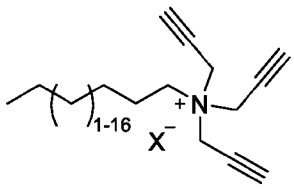
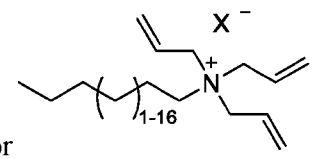
[0089] In some embodiments, the metal ion includes one or more of Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, Fe^{2+}, Ti^{4+}, Zr^{4+}, Hf^{4+}, Ni^{2+}, Ni^{+}, V^{4+}, V^{3+}, V^{2-}, and Al^{3+}, while in some embodiments, the metal ion is Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, or Fe^{2+}, and in some embodiments, the metal ion is Zn^{2+}.

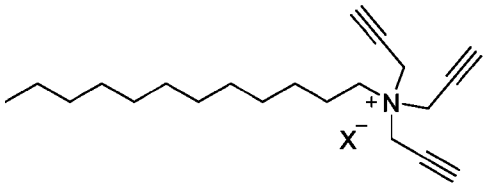
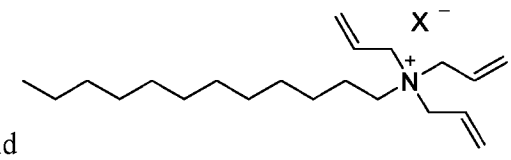
[0090] In some embodiments, the organic ligand includes one or more of 1-methylimidazole, 2-methylimidazole, 4-methyl-1H-imidazole, imidazole, benzimidazole, 1-methyl-1,2,4-triazole, 1-methyl-1H-tetrazole, or 2-imidazolcarbonitrile. In some embodiments, the organic ligand includes 1-methylimidazole, 2-methylimidazole, 4-methyl-1H-imidazole, imidazole, or benzimidazole. In some embodiments, the organic ligand comprises 1-methylimidazole, or 2-methylimidazole, and in some embodiments, the organic ligand is 1-methylimidazole. In certain embodiments, the metal ion is Zn^{2+}, and the organic ligand is 1-methylimidazole, and in some embodiments, the MOF formed by Zn^{2+} and 1-methylimidazole is a Zeolitic Imidazolate Framework-8 (ZIF-8) MOF.

[0091] Regarding the liposome, in some embodiments, the liposome comprises one or more cationic lipids and a one or more non-cationic (helper) lipids. In some embodiments, the one or more cationic lipids is a cholesterol-based lipid, which in some embodiments is 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol).

[0092] In some embodiments, the one or more non-cationic (helper) lipids includes a lipid that is neutral or zwitterionic at physiological pH, and in some embodiments, the one or more non-cationic (helper) lipids comprises 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE).

[0093] In some embodiments, the nanoparticle of the disclosure further includes a DNA Surfactant Conjugate (DSC) incorporated into the liposome layer, e.g., liposome bilayer. In some

embodiments, the nanoparticle of the disclosure further includes DNA-surfactant conjugate, a DNA-cholesterol conjugate, DNA-tocopherol conjugate, or any combination thereof incorporated into the liposome layer. In some embodiments, the nanoparticle includes a bilayer that is anionic. In some embodiments, the DSC includes a non-polymeric amphiphile according to  or  , where X is halogen; and a thiolated oligonucleotide.

[0094] In some embodiments, the DSC includes a non-polymeric amphiphile selected from  and  , where X is halogen; and a thiolated oligonucleotide.

[0095] In some embodiments, the thiolated oligonucleotide is between about 6 to 30 nucleotides in length, about 8 and 30 nucleotides in length, about 10 and 25 nucleotides in length, about 10 and 20 nucleotides in length, about 12 and 25 nucleotides in length, about 14 and 25 nucleotides in length, about 16 and 30 nucleotides in length, about 16 and 25 nucleotides in length, about 18 and 25 nucleotides in length, or about 18 and 20 nucleotides in length, and in some embodiments, the thiolated oligonucleotide is polyT_{20} DNA.

[0096] In some embodiments, the thiolated oligonucleotide is reacted with the non-polymeric amphiphile via a thiolyne click chemistry reaction.

[0097] Regarding the therapeutic agent(s), in some embodiments the therapeutic agent includes one or more of a therapeutic nucleic acid, a hydrophobic small molecule drug including an anti-cancer agent, an antibiotic, an antiviral, an antiparasitic agent, an anticoagulant, an analgesic agent, an anesthetic agent, an ion channel potentiator, an ion channel inhibitor, an anti-inflammatory, a metallodrug, and any combination thereof.

[0098] In some embodiments, the therapeutic agent includes camptothecin, doxorubicin, daunorubicin, vincristine, paclitaxel, neocarzinostatin, calicheamicin, cisplatin, carboplatin,

oxaliplatin, satraplatin, picoplatin, lurtotecan, annamycin, docetaxel, tamoxifen, epirubicin, methotrexate, vinblastin, vincristin, topotecan, prednisone, prednisolone, abt-737, or a pharmaceutically acceptable salt, hydrate or solvate thereof, or any combination thereof.

[0099] In some embodiments, the therapeutic agent is a nucleic acid comprising one or more of Small Interfering RNA (siRNA), MicroRNA (miRNA), Antisense Oligonucleotides (ASOs), ribozymes, aptamers, mRNA, Splice-Switching Oligonucleotides (SSOs), or any combination thereof.

[0100] In some embodiments, the nanoparticle comprises a diagnostic agent, which includes a fluorophore, a radiolabeled nucleotide, a radioisotope, biotin, tocopherol, cholesterol, a steroid, or an electron dense tag and a metal chelator, or any combination thereof.

[0101] In an aspect, a composition for treating or preventing a disease, a disorder, or symptom in a subject is provided, the composition including at least one of the nanoparticles of the disclosure or a pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable vehicle and/or excipient.

[0102] In an aspect, a method of treating or diagnosing a disease or disorder is provided, including administering to a subject in need thereof an effective amount of the nanoparticle of the disclosure, in which the linker is cleavable by one or more intracellular or extracellular release agent present in the subject, thus releasing the therapeutic agent or diagnostic agent.

[0103] In some embodiments, a method of treating or preventing a disease, a disorder, or symptom associated with the conditions which require mRNA and thus require an increase in gene expression. In certain embodiments, the condition includes communicable diseases such as influenza or SARS CoV2; cancer; or a combination thereof. In certain embodiments, the method is delivered through a process of vaccination, for example, SARS CoV2 vaccine.

[0104] In certain embodiments, the method modulates the immune response of a subject by modulating the immune response of the subject by altering gene expression.

[0105] In certain embodiments, the multifunctional nanoparticle of the disclosure as described herein, are particularly applicable to the delivery of hydrophobic small molecule drugs in conjunction with therapeutic oligonucleotides (some nonlimiting examples are mRNA, siRNA, antisense oligonucleotides, microRNA, aptamers, DNAzymes, Ribozymes, etc.) that are

useful for intracellular gene knockdown and altering protein expression levels. For example, the multifunctional nanoparticles of the disclosure as described herein deliver camptothecin, a topoisomerase inhibitor and cancer drug, along with a DNAzyme specifically designed to target the cleavage of GATA-3 mRNA. GATA-3 is a transcription factor that plays an important role in inflammation pathways by initiating downstream TH1 and TH2 cell differentiation. Cleavage of mRNA can result in blocked protein translation.

[0106] In some embodiments, no more than about 20%, no more than about 15%, no more than about 10%, no more than about 5%, no more than about 3%, or no more than about 1% of the linker is cleaved in an extracellular environment, and in some embodiments, no less than about 40%, no less than about 50%, no less than about 60%, no less than about 70%, no less than about 80%, or no less than about 90% of the linker is cleaved in an intracellular environment.

[0107] In some embodiments, the release mechanisms is an enzyme expressed by tumor cells, and in some embodiments, the release agent is a lysosome agent, endosome agent, and/or caveolae agent.

[0108] Nanoparticles of the disclosure demonstrate enhanced stability of deliverable oligonucleotide. In some embodiments, the stability of the nanoparticles disclosed herein can be from at least 7 days at 4 °C to at least 60 days at 4 °C. In some embodiments, the stability of the nanoparticles disclosed herein can be from at least 7 days at room temperature to at least 30 days at room temperature; for example the stability can be at least 7 days, 15 days, 21 days, or at least 30 days. In an embodiment, the stability of the nanoparticles can be at least 7 days, 10 days, 15 days, 21 days, 30 days, 40 days, 45 days, 50 days, 55 days, or at least 60 days at 4 °C. In an embodiment, the stability of the nanoparticles can be at least 7 days, 10 days, 15 days, 21 days, or at least 30 days at room temperature.

[0109] Liposomes

[0110] Liposomes, in general, are spherical vesicles composed of one or more layers (e.g., bilayers) which can encapsulate aqueous solutions. Liposomes are typically formed by amphiphilic molecules, such as lipids of synthetic or natural origin that comprise spatially separated hydrophilic and hydrophobic domains. Cationic liposomes are positively charged lipid vesicles designed to encapsulate and deliver therapeutic agents, particularly negatively charged molecules like nucleic acids, e.g., DNA and RNA.

[0111] In some embodiments, liposomes comprise one or more cationic lipids. As used herein, the phrase "cationic lipid" refers to any of a number of lipid species that have a net positive charge at a selected pH, such as physiological pH. In some embodiments, the one or more cationic lipids may be cholesterol-based. A suitable cholesterol-based cationic lipids is 3ß-[N-(N',N'- dimethylaminoethane)-carbamoyl]cholesterol hydrochloride or "DC-Chol".

[0112] In some embodiment, the one or more cationic lipids may be N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride or "DOTMA", 5-carboxyspermylglycinedioctadecylamide or "DOGS", 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium or "DOSPA", 1,2-Dioleoyl-3-Dimethylammonium-Propane or "DODAP", 1,2-Dioleoyl-3-Trimethylammonium-Propane or "DOTAP".

[0113] Additional exemplary cationic lipids also include 1,2-distearyloxy-N,N-dimethyl-3-aminopropane or "DSDMA", 1,2-dioleyloxy-N,N-dimethyl-3-aminopropane or "DODMA", 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane or "DLinDMA", 1,2-dilinolenyloxy-N,N-dimethyl-3-aminopropane or "DLenDMA", N-dioleyl-N,N-dimethylammonium chloride or "DODAC", N,N-distearyl-N,N-dimethylammonium bromide or "DDAB", N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide or "DMRIE", 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane or "CLinDMA", 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy 1-1-(cis,cis-9',1-2'-octadecadienoxy)propane or "CpLinDMA", N,N-dimethyl-3,4-dioleyloxybenzylamine or "DMOBA", 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane or "DOcarbDAP", 2,3-Dilinoleoyloxy-N,N-dimethylpropylamine or "DLinDAP", 1,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane or "DLincarbDAP", 1,2-Dilinoleoylcarbamyl-3-dimethylaminopropane or "DLinCDAP", 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane or "DLin-1-DMA", 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane or "DLin-K-XTC2-DMA", and 2-(2,2-di((9Z,12Z)-octadeca-9,12-dien-1-yl)-1,3-dioxolan-4-yl)-N,N-dimethylethanamine (DLin-KC2-DMA), (2,2-Dilinoley 1-4-dimethylaminoethyl-[1,3]-dioxolane) or "XCT", (((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate), or "MC3", ((3aR,5s,6aS)—N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine)), or "ALNY-100", (4,7,13-tris(3-oxo-3-(undecylamino)propyl)-N1,N16-diundecyl-

4,7,10,13-tetraazahexadecane-1,16-diamide), or "NC98-5", (1,2-dioleyl-3-dimethylammonium propane), or "DODAP", or mixtures thereof.

[0114] In certain embodiments, the liposomes comprise an ionizable cationic lipid, such as, e.g, (15Z,18Z)-N,N-dimethyl-6-(9Z,12Z)-octadeca-9,12-dien-1-yl)tetracosa-15,18-dien-1-amine (HGT5000), (15Z,18Z)-N,N-dimethyl-6-((9Z,12Z)-octadeca-9,12-dien-1-yl)tetracosa-4,15,18-trien-1-amine (HGT5001), and (15Z,18Z)-N,N-dimethyl-6-((9Z,12Z)-octadeca-9,12-dien-1-yl)tetracosa-5,15,18-trien-1-amine (HGT5002), or mixtures thereof.

[0115] In some embodiments, the cationic lipids comprise DC-Chol.

[0116] In some embodiments, the percentage of cationic lipid in a liposome may be greater than 20%, greater than 30%, greater than 40%, greater than 50%, greater than 60%, greater than 70%, greater than 80%, or greater than 90% by weight. In some embodiments, the cationic lipid(s) constitute between about 20-65% (e.g., about 25-60%, about 20-40%, about 40-60%, about 30-45%, about 35-45%, and the like) of the liposome by mole fraction. In some embodiments, the cationic lipid (e.g., DC-Chol) constitutes about 30%, about 35%, about 40%, about 45%, or about 50% of the liposome by mole ratio.

[0117] In some embodiments, liposomes comprise one or more non-cationic ("helper") lipids. As used herein, the phrase "non-cationic lipid" refers to any neutral or zwitterionic lipid. Non-cationic lipids include, but are not limited to, distearoylphosphatidylcholine (DSPC; zwitterionic), dioleoylphosphatidylcholine (DOPC; neutral), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), and the like, or a mixture thereof.

[0118] In some embodiments, such helper lipids may be used alone, but are preferably used in combination with other excipients, for example, cationic lipids. In some embodiments, the percentage of helper lipid in a liposome may be greater than 20%, greater than 30%, greater than 40%, greater than 50%, greater than 60%, greater than 70%, greater than 80%, or greater than 90% by weight. In some embodiments, the helper lipid(s) constitute between about 25-85% (e.g., about 25-75%, about 30-70%, about 50-70%, about 25-55%, about 40-60%, and the like) of the liposome by mole fraction. In some embodiments, the helper lipid (e.g., DOPE) constitutes about

40%, about 45%, about 50%, about 55%, about 60%, or about 65% of the liposome by mole ratio.

[0119] Metal organic frameworks (MOFs).

[0120] Metal organic frameworks (MOFs) are a class of crystalline materials composed of metal ions or clusters coordinated to organic ligands, forming a porous, three-dimensional framework. MOFs are known for their high surface area, tunable pore sizes, and exceptional chemical and thermal stability.

[0121] Sources for metal ions may be a metal salt and/or a metal oxide. Metal ions include at least one of the groups consisting of Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, Fe^{2+}, Ti^{4+}, Zr^{4+}, Hf^{4+}, Ni^{2+}, Ni^{+}, V^{4+}, V^{3+}, V^{2-}, Al^{3+}, Sr^{2+}, Ba^{2+}, Sc^{3+}, Y^{3+}, V^{4+}, V^{3+}, V^{2-}, Nb^{3+}, Ta^{3+}, Cr^{3+}, Mo^{3+}, W^{3+}, Mn^{3+}, Mn^{2+}, Re^{3-}, Re^{2+}, Ru^{3+}, Ru^{2+}, Os^{3+}, Os^{2-}, Co^{3+}, Co^{2+}, Rh^{2+}, Rh^{+}, Ir^{2+}, Ir^{-}, Pd^{2+}, Pd^{+}, Pt^{2+}, Cu^{2+}, Cu^{+}, Ag^{+}, Au^{+}, Cd^{2+}, Hg^{2+}, Ga^{3+}, In^{3+}, Tl^{3+}, Si^{4+}, Si^{2-}, Ge^{4+}, Ge^{2+}, Sn^{4+}, Sn^{2+}, Pb^{4+}, Pb^{2+}, As^{5+}, As^{3-}, As^{+}, Bi^{5+}, Bi^{3+} and Bi^{+}. In some embodiments the metal ions include at least one of the groups consisting of Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, Fe^{2+}, Ti^{4+}, Zr^{4+}, Hf^{4+}, Ni^{2+}, Ni^{+}, V^{4+}, V^{3+}, V^{2-}, and Al^{3+}, while, in some embodiments, the metal ions include at least one of the groups consisting of Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, and Fe^{2+}. In some embodiments, the metal ions are Zn^{2+}.

[0122] In various aspects and embodiments described herein, the organic ligands refer to organic moieties that contain one or more coordination functional groups in its molecular structure. In one of embodiments, the coordination functional groups in the organic ligands may be various functional groups capable of forming coordinate bonds with metal ions, including at least one of the groups consisting of —CO_2H, —NO_2, —OH, —NH2, —CN, —SO_3H, —SH, 1-methylimidazole, 2-methylimidazole, 4-methyl-1H-imidazole, imidazole, benzimidazole, 1-methyl-1,2,4-triazole, 1-methyl-1H-tetrazole, 2-imidazolcarbonitrile, —PO_4H_2, —AsO_3H, —AsO_4H, —$CH(RSH)_2$, —$C(RSH)_3$, —$CH(RNH_2)_2$, —$C(RNH_2)_3$, —$CH(ROH)_2$, —$C(ROH)_3$, —$CH(RCN)_2$, —$C(RCN)_3$, —$CH(NH_2)_2$, —$C(NH_2)_3$, —$CH(CN)_2$ and —$C(CN)_3$, wherein each R in the functional groups independently represents a hydrocarbon group containing 1 to 5 benzene rings.

[0123] In some embodiments, the coordination functional groups in the organic ligands include at least one of groups consisting of —CO_2H, benzimidazole, imidazole, 1-methylimidazole, and/or 2-methylimidazole.

[0124] The organic ligands containing the above-mentioned coordination functional groups are exemplified below. For example, the organic ligands include terephthalic acid, trimesic acid, 2-nitroterephthalic acid, 2-hydroxyterephthalic acid, 2,5-dihydroxyterephthalic acid, 2-aminoterephthalic acid, monosodium 2-sulfoterephthalate, 5-aminoisophthalic acid, 5-nitroisophthalic acid, 4-hydroxyisophthalic acid, monosodium 5-sulfoisophthalate, benzoic acid, 4,4′-biphenyldicarboxylic acid, 2,2′-dinitro-4,4′-biphenyldicarboxylic acid, 2,2′-diamino-4,4′-biphenyldicarboxylic acid, 2,2′-dihydroxy-4,4′-biphenyldicarboxylic acid, 3,3′,5,5′-biphenyltetracarboxylic acid, dimercaptosuccinic acid, 1,4,5,8-naphthalenetetracarboxylic acid, 2,6-naphthalenedicarboxylic acid, naphthalene-1,4-dicarboxylic acid, O-phospho-DL-threonine, O-phospho-L-tyrosine, barium 3-phospho-D-glycerate, 3-mercaptopropionic acid, 3-amino-5-mercapto-1,2,4-triazole, 2,3-dimercaptosuccinic acid, 5-methoxy-2-mercaptobenzimidazole, 1-methyl-5-sulfoyltetrazole, imidazole, benzimidazole, 2-mercaptobenzimidazole, N,N-carbonyldiimidazole, 1-methylimidazole, 2-ethyl-4-methylimidazole, 2,4-dimethylimidazole, 2-methylimidazole, 4-methylimidazole, 2-nitroimidazole, 2-cyanoimidazole, 1,2-dimethylimidazole, imidazole-4,5-dicarboxylic acid, 4-amino-5-imidazolecarboxamide, 2-hydroxymethyl-1H-benzimidazole, 2-methylbenzimidazole, 5,6-dimethylbenzimidazole, 4,5-dicyanoimidazole, benzimidazole-5-carboxylic acid, 1H-imidazole-4-formic acid, 2-isopropylimidazole, 1-benzyl-2-methylimidazole, 4-nitroimidazole, 5-aminotetrazole monohydrate, tetrazole acetic acid, 1,2,4-triazole, triazole-3-carboxylic acid, 4-amino-4H-1,2,4-triazole, 3-nitro-1,2,4-triazole.

[0125] In some of embodiments, the organic ligands is selected from 1-methylimidazole, 2-methylimidazole, benzimidazole, imidazole and 2-aminoterephthalic acid, and in some embodiments the organic ligand is selected from 1-methylimidazole and 2-methylimidazole. In some embodiments, the organic ligand is 1-methylimidazole.

[0126] In some embodiments, the metal ion is Zn^{2+}, and the organic ligand is 1-methylimidazole. In certain embodiments, the MOF formed by Zn^{2+} and 1-methylimidazole forms a Zeolitic Imidazolate Framework-8 (ZIF-8) MOF around the liposome. ZIF-8 is a desirable type of MOF comprised of zinc ions (Zn^{2+}) coordinated to imidazolate anions, forming a three-dimensional framework with a topology resembling zeolites, with a sodalite (SOD) topology, i.e., a porous structure with large cavities connected by narrow windows. ZIF-8 MOFs

have unique properties, including high surface area and porosity, thermal stability and chemical stability.

[0127] In the present disclosure, the amount of or ratio of the metal sources to organic acid used comprises any ratio that results in the desired MOF product and may be about 0.01 mol to 5 mol, about 0.05 mol to 4 mol, about 0.05 mol to about 3 mol, about 0.05 mol to about 2 mol, about 0.1 mol to about 1 mol, about 0.1 mol to about 0.75 mol, or about 0.2 mol to about 0.5 mol, with respect to 1 mol of the organic ligands.

[0128] Nucleic acid nanocapsules (NANs)

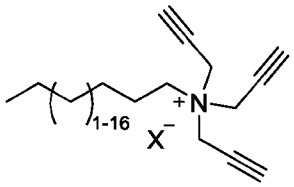
[0129] NANs of the disclosure include non-polymeric amphiphiles, in which the hydrophobic groups of the amphiphiles are arranged toward the particle interior (i.e., toward, to or interacting with the MOF), and the hydrophilic groups of the amphiphiles are oriented toward the particle surface, crosslinked with one or more linkers cleavable by one or more intracellular or extracellular release agents, to form a surface crosslinked micelle (SCM), to which one or more nucleic acid ligands (e.g., thiolated DNA ligands) are covalently attached to form a NAN. In some embodiments, the hydrophobic groups of the amphiphiles are crosslinked through a triazole, thioether, or alkenyl sulfide group with one or more cleavable linkers. The outer nucleic acid (e.g., DNA) ligand helps stabilize the particle in water and buffer and can themselves facilitate selective binding to a cell surface antigen, protein or carbohydrate, or be further functionalized (e.g., via enzymatic ligation) with additional nucleic acid ligands (e.g., targeting nucleic acids) for cell specific uptake.

[0130] Non-polymeric amphiphiles. As used herein, the term "non-polymeric" means a material that is not a polymer (i.e., a molecule composed of repeat units). The amphiphiles of the disclosure have hydrophobic groups arranged toward the particle interior, and hydrophilic groups are at the particle surface. In certain embodiments, the hydrophobic groups of the amphiphile as otherwise described herein include C_6-C_{22} alkyl, C_6-C_{22} alkenyl, or C_6-C_{22} alkynyl group, each optionally substituted with halo, C_1-C_4 alkyl, C_2-C_4 alkenyl, C_1-C_6 alkoxy, or –CO(C_1-C_{22} alkyl). In other embodiments, the hydrophobic groups of the amphiphile include unsubstituted C_6-C_{22} alkyl, C_6-C_{22} alkenyl, or C_6-C_{22} alkynyl group. In other embodiments, the hydrophobic groups of the amphiphile include unsubstituted C_6-C_{22} alkyl; or unsubstituted C_6-C_{20} alkyl; or unsubstituted C_6-C_{18} alkyl; or unsubstituted C_6-C_{15} alkyl; or unsubstituted C_6-C_{12}

alkyl; or unsubstituted C_6-C_{10} alkyl; or unsubstituted C_{10}-C_{22} alkyl; or unsubstituted C_{10}-C_{20} alkyl; or unsubstituted C_{10}-C_{18} alkyl; or unsubstituted C_{10}-C_{15} alkyl; or unsubstituted C_{12}-C_{22} alkyl; or unsubstituted C_{12}-C_{20} alkyl; or unsubstituted C_{12}-C_{18} alkyl; or unsubstituted C_{12}-C_{15} alkyl. In other embodiments, the hydrophobic groups of the amphiphile include optionally substituted C_{10} alkyl. In other embodiments, the hydrophobic groups of the amphiphile include unsubstituted C_{10} alkyl. In certain embodiments, the hydrophilic groups of the amphiphile as otherwise described herein include an ammonium group.

[0131] The amphiphiles of the disclosure as otherwise described herein are crosslinked through a triazole, thioether, or alkenyl sulfide group with one or more linkers. In certain embodiments, amphiphiles of the disclosure as otherwise described herein are crosslinked through a triazole group. In certain embodiments, amphiphiles of the disclosure as otherwise described herein are crosslinked through a thioether group. In certain embodiments, amphiphiles of the disclosure as otherwise described herein are crosslinked through an alkenyl sulfide group. In certain embodiments, the triazole, thioether, or alkenyl sulfide crosslinking group results from a reaction of alkyne or alkene moiety on the hydrophilic group of the amphiphile (e.g., on the ammonium group) and an azide or thiol moiety on the linker. In one example, the triazole crosslinker results from a reaction of alkyne moiety on the hydrophilic group of the amphiphile (e.g., on the ammonium group) with an azide moiety on the linker. In one example, the alkenyl sulfide crosslinker results from a reaction of alkyne moiety on the hydrophilic group of the amphiphile (e.g., on the ammonium group) with a thiol moiety on the linker. In one example, the thioether crosslinker results from a reaction of alkene moiety on the hydrophilic group of the amphiphile (e.g., on the ammonium group) with a thiol moiety on the linker. In certain embodiments, the alkyne or alkene moiety on the hydrophilic group of the amphiphile of the disclosure as otherwise described herein is one or two of prop-2-ynyl or prop-2-enyl; one or two of prop-2-ynyl, or one or two of prop-2-enyl. In certain embodiments, the hydrophilic group is tri(prop-2-yn-1-yl)amino or triallylamino. In certain embodiments, the crosslinking group results from a reaction of prop-2-ynyl. In certain embodiments, the crosslinking group results from a reaction of diallylamino.

[0132] In certain embodiments, the non-polymeric amphiphiles of the disclosure as otherwise described herein are derived from



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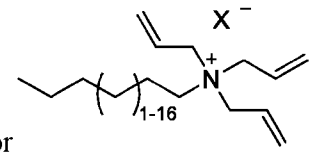
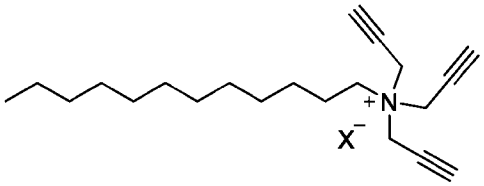


, wherein X is halogen (e.g., Br). For example, in certain embodiments, the non-polymeric amphiphiles are derived from



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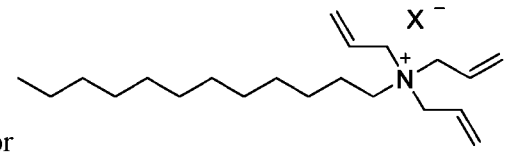


, wherein X is halogen (e.g., Br).

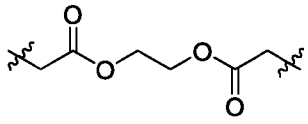
[0133] Linkers

[0134] The hydrophilic groups of the amphiphiles of the disclosure as otherwise described herein are crosslinked with one or more linkers that are cleavable by one or more intracellular or extracellular release agents. For example, in one embodiment, the linker is cleavable by one or more enzymes, such as, but not limited to, peptidases, proteases, esterases, or elastases. In some embodiments, the linker is cleavable by an esterase. In certain embodiments, the linkers of the disclosure as otherwise described herein are cleavable by one intracellular or extracellular release agent. In certain embodiments, the linkers of the disclosure as otherwise described herein are cleavable by two or more intracellular or extracellular release agents (e.g., wherein the linker comprises two or more different chemical groups each cleavable by a different release agent).

[0135] In certain embodiments, the linkers of the disclosure as otherwise described herein include a peptide (for example, cleavable with a peptidase or protease), wherein the peptide is at least two amino acids long. In certain embodiments, the peptide is at least two amino acids long. In certain embodiments, at least three amino acids long. In certain embodiments, at least four amino acids long. In certain embodiments, the peptide is between two and twenty-five amino acids long; or between three and twenty-five amino acids long; or between four and twenty-five amino acids long. In some embodiments, the peptide linker comprises GPLGLAGGERDG (SEQ ID NO:14), GFLG (SEQ ID NO:15), GPMGIAGQ (SEQ ID NO:16), Phe-Leu, Val-Ala, Val-Cit, Val-Lys, Val-Arg, or Phe-Lys. In some embodiments, the peptide linker comprises GPLGLAGGERDG (SEQ ID NO:14), GFLG (SEQ ID NO:15), or GPMGIAGQ (SEQ ID

NO:16). In some embodiments, the peptide linker is GPLGLAGGERDG (SEQ ID NO:14), GFLG (SEQ ID NO:15), GPMGIAGQ (SEQ ID NO:16), Phe-Leu, Val-Ala, Val-Cit, Val-Lys, Val-Arg, or Phe-Lys. In some embodiments, the peptide linker is GPLGLAGGERDG (SEQ ID NO:14), GFLG (SEQ ID NO:15), or GPMGIAGQ (SEQ ID NO:16). In some embodiments, the peptide linker as otherwise described herein comprises (or further comprises) two Cys groups (for example, at each end of the peptide linker, such that the sulfur on the peptide linker makes up thioether or alkenyl sulfide group crosslinking the hydrophilic group of the amphiphile and the linker). In some embodiments, the peptide linker comprises CGPLGLAGGERDGC (SEQ ID NO:1), CGFLGC (SEQ ID NO:17), CGPMGIAGQC (SEQ ID NO:18), CFLC (SEQ ID NO:19), CVAC (SEQ ID NO:20), Cys-Val-Cit-Cys (SEQ ID NO:21), CVKC (SEQ ID NO:22), CVRC (SEQ ID NO:23), or CFKC (SEQ ID NO:24). In some embodiments, the linker comprises CGPLGLAGGERDGC (SEQ ID NO:1), CGFLGC (SEQ ID NO:17), or CGPMGIAGQC (SEQ ID NO:18). In some embodiments, the linker is CGPLGLAGGERDGC (SEQ ID NO:1), CGFLGC (SEQ ID NO:17), CGPMGIAGQC (SEQ ID NO:18), CFLC (SEQ ID NO:19), CVAC (SEQ ID NO:20), Cys-Val-Cit-Cys (SEQ ID NO:21), CVKC (SEQ ID NO:22), CVRC (SEQ ID NO:23), or CFKC (SEQ ID NO:24). In some embodiments, the linker is CGPLGLAGGERDGC (SEQ ID NO:1), CGFLGC (SEQ ID NO:17), or CGPMGIAGQC (SEQ ID NO:18).

[0136] In certain embodiments, the linkers of the disclosure as otherwise described herein include one or more of ester groups (for example, cleavable with an esterase). In one embodiment, the linkers of the disclosure as otherwise described herein include



group.

[0137] In certain embodiments, the linkers of the disclosure as otherwise described herein include one or more of hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, or thioether groups, or a combination thereof, or other acid-labile groups that are hydrolyzable in the lysosome.

[0138] In certain embodiments, the linkers of the disclosure as otherwise described herein include at least two groups selected from an ester, hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, thioether, disulfide, and a

peptide, wherein the peptide is at least two amino acids long, or at least three amino acids long, or at least four amino acids long; or the peptide is between two and twenty-five amino acids long. In certain embodiments, the linker is cleavable by two or more intracellular or extracellular release agents and include at least two groups selected from an ester, hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, thioether, disulfide, and a peptide as described herein.

[0139] In certain embodiments, the linkers of the disclosure as otherwise described herein include a disulfide group.

[0140] In certain embodiments, the linkers of the disclosure as otherwise described herein exclude disulfide group or another group cleavable under reducing conditions.

[0141] The linkers of the disclosure may be selectively cleaved. For example, in one embodiment, no more than about 20%, no more than about 15%, no more than about 10%, no more than about 5%, no more than about 3%, or no more than about 1% of the linker is cleaved in an extracellular environment. In another embodiment, no less than about 20%, no less than about 15%, no less than about 10%, no less than about 5%, no less than about 3%, or no less than about 1% of the linker is cleaved in an extracellular environment.

[0142] In certain embodiments of the methods of the disclosure, the release mechanism is an enzyme expressed by tumor cells.

[0143] In certain embodiments of the methods of the disclosure, the release agent is a lysosome agent, endosome agent, and/or caveolae agent.

[0144] Nucleic acid ligands and targeting nucleic acid ligands

[0145] As provided above, the multifunctional nanoparticles of the disclosure include nucleic acid ligands covalently attached to the particle as otherwise described herein, and in some embodiments further include targeting nucleic acid ligands that are linked to one or more nucleic acid ligands. For example, in some embodiments, the nucleic acid ligands and targeting nucleic acid ligands of the disclosure are capable of selectively binding to a cell surface antigen (aptamer).

[0146] In some embodiments, the nucleic acid ligands and targeting nucleic acid ligands of the disclosure are capable of selectively binding to a protein or a carbohydrate. In some

embodiments, the nucleic acid ligands and targeting nucleic acid ligands of the disclosure are capable of selectively binding to a protein, wherein the protein is selected from the group consisting of tumor-markers, integrins, cell surface receptors, transmembrane proteins, ion channels, membrane transport protein, enzymes, antibodies, and chimeric proteins. In some embodiments, the nucleic acid ligands and targeting nucleic acid ligands of the disclosure are capable of selectively binding to a carbohydrate, wherein the carbohydrate is selected from the group consisting of glycoproteins, sugar residues, and glycocalyx.

[0147] In certain embodiments, the nucleic acid ligands and targeting nucleic acid ligands of the disclosure as otherwise described herein are capable of selectively binding DNA, RNA, modified DNA, modified RNA, DNAzymes, ribozymes, mRNA, siRNA, microRNA, shRNA, and combinations thereof.

[0148] In certain embodiments, the nucleic acid ligands and targeting nucleic acid ligands of the disclosure as otherwise described herein are capable of selectively binding to a cell during a specific developmental stage (e.g., stage having developmentally specific cell surface antigens) or to a cell in a specific disease state (e.g., a tumor cell that has tumor-associated antigens or tumor-specific antigens.)

[0149] In certain embodiments, the nucleic acid ligands and targeting nucleic acid ligands of the disclosure as otherwise described herein are capable of gene regulation. For example, in some embodiments, the nucleic acid ligands and targeting nucleic acid ligands capable of gene regulation can be siRNA, DNAzyme, ribozyme, microRNA, or any other therapeutic oligonucleotides (including antisense oligonucleotides).

[0150] In certain embodiments, the nucleic acid ligands and targeting nucleic acid ligands of the disclosure as otherwise described herein can be native or modified, including phosphorthioated backbones, and 2' prime protected ribonucleic acids, or can be an aptamer, either RNA or DNA, modified or unmodified.

[0151] The inventors have recognized that, in certain embodiments, the multifunctional nanoparticle of the disclosure can transport the nucleic acid ligand to the cytosol. Without being bound by a particular theory, it is believed that the nucleic acid ligand may be assisted in its ability to reach the cytosol due to its covalent attachment to the amphiphiles (i.e., its relationship to the particle's hydrophobic group of the amphiphiles).

[0152] As provided above, the nucleic acid ligands of the disclosure are covalently attached to the hydrophilic groups of the amphiphiles. In certain embodiments, up to two nucleic acid molecules are attached to the hydrophilic groups of the amphiphiles (e.g., up to two per alkyne). In certain embodiments, one nucleic acid molecule is attached to the hydrophilic groups of the amphiphiles. In certain embodiments, two nucleic acid molecules are attached to the hydrophilic groups of the amphiphiles.

[0153] The nucleic acid ligands, for example in certain embodiments, are covalently attached to the hydrophilic groups of the amphiphiles through a thioether or alkenyl sulfide group. Such thioether or alkenyl sulfide groups may result from a reaction of alkyne or alkene moiety on the hydrophilic group of the amphiphile (e.g., on the ammonium group) and a thiol moiety (e.g., Cys) on the nucleic acid ligand. In certain embodiments, the alkyne or alkene moiety on the hydrophilic groups of the amphiphiles is prop-2-ynyl or prop-2-enyl, or prop-2-ynyl, or prop-2-enyl; or the alkyne or alkene moiety on the hydrophilic group of the amphiphile is prop-2-yn-1-ylamino or allylamino.

[0154] The multifunctional nanoparticles as described herein can be provided in a variety of different particle sizes, depending, e.g., on the amphiphiles and crosslinkers used for making them. For example, in certain embodiments, the multifunctional nanoparticle as described herein has a particle size within the range of about 1 nm to about 1 μm in diameter, e.g., 1 nm to 750 nm, or 1 nm to 500 nm, or 5 nm to 500 nm, or 10 nm to 750 nm, or 10 nm to 500 nm, or 15 nm to 5000 nm, or 20 nm to 1 μm, or 20 nm to 500 nm, or 20 nm to 400 nm, or 20 nm to 300 nm, or 30 nm to 750 nm, or 30 nm to 500 nm, or 30 nm to 400 nm, or 30 nm to 300 nm, or 40 nm to 500 nm, or 50 nm to 400 nm, or 50 nm to 300 nm, or 100 nm to 500 nm, or 100 nm to 400 nm, or 100 nm to 350 nm, or 100 nm to 300 nm, or 100 nm to 250 nm in diameter. In certain embodiments, the multifunctional nanoparticle as described herein has a particle size within the range of about 100 nm to about 300 nm in diameter, and in some embodiments, the multifunctional nanoparticle has a particle size within the range of about 150 nm to about 250 nm in diameter, all as measured by DLS. The person of ordinary skill in the art can, in view of the materials and methods described herein, provide a desired particle size to a multifunctional nanoparticle.

[0155] In certain embodiments, the multifunctional nanoparticles as described herein have a discrete particle size and are monodisperse (i.e., uniform).

[0156] In some embodiments, the zeta for surface charge is about -50 millivolt (mV) to about 100 mV. For example, the zeta for surface charge is about -40 mV, -30 mV, -20 mV, -10 mV, 10 mV, 20 mV, 30 mV, 40 mV, 50 mV, 60 mV, 70 mV, 80 mV, or about 90 mV.

[0157] Therapeutic agents

[0158] The disclosure provides that one or more therapeutic agents is encased by, or enclosed within, the liposome compartment of the multifunctional nanoparticle.

[0159] In certain embodiments, the therapeutic agent comprises a hydrophobic small molecule drug, such as, but not limited to, an anti-cancer agent, an antibiotic, an antiviral, an antiparasitic agent, an anticoagulant, an analgesic agent, an anesthetic agent, an ion channel potentiator, an ion channel inhibitor, an anti-inflammatory, a metallodrug, and any combination thereof. For example, in certain embodiments, the therapeutic agent is selected from camptothecin, doxorubicin, daunorubicin, vincristine, paclitaxel, neocarzinostatin, calicheamicin, cisplatin, carboplatin, oxaliplatin, satraplatin, picoplatin, lurtotecan, annamycin, docetaxel, tamoxifen, epirubicin, methotrexate, vinblastin, vincristin, topotecan, prednisone, prednisolone, abt-737, or any combination thereof.

[0160] In some embodiments, the therapeutic agent is a nucleic acid comprising one or more of Small Interfering RNA (siRNA), MicroRNA (miRNA), Antisense Oligonucleotides (ASOs), ribozymes, aptamers, mRNA, circularized mRNA, Short Hairpin RNA (shRNA), plasmids, single guide RNA (sgRNA) Splice-Switching Oligonucleotides (SSOs), or any combination thereof.

[0161] In certain embodiments, the multifunctional nanoparticle includes a diagnostic agent. The diagnostic agent may be, for example, a fluorophore, a radiolabeled nucleotide, a radioisotope, biotin, tocopherol, cholesterol, a steroid, an electron dense tag and a metal chelator, or any combination thereof.

[0162] Pharmaceutical formulations

[0163] Multifunctional nanoparticles disclosed herein may be combined with pharmaceutical acceptable carriers to form a pharmaceutical composition, according to another aspect of the

invention. Typically, the physiologically acceptable carriers are present in liquid form. Examples of liquid carriers include physiological saline, phosphate buffer, normal buffered saline (135-150 mM NaCl), water, buffered water, 0.4% saline, 0.3% glycine, glycoproteins to provide enhanced stability (e.g., albumin, lipoprotein, globulin, etc.), and the like. Since physiologically acceptable carriers may be chosen, e.g., based on the route of administration as described below, the location of the target issue, the drug being delivered, the time course of delivery of the drug, and the like, there are a wide variety of suitable formulations of pharmaceutical compositions for embodiments of the present disclosure (See, e.g., Remington's Pharmaceutical Sciences, 17th ed., 1989).

[0164] The pharmaceutical compositions of this invention can be administered to a patient by any means known in the art, including oral and parenteral routes. In certain embodiments parenteral routes are desirable since they avoid contact with the digestive enzymes that are found in the alimentary canal. According to such embodiments, inventive compositions may be administered by injection (e.g., intramuscular, intravenous, subcutaneous, intraperitoneal injection), rectally, vaginally, topically (as by powders, creams, ointments, or drops), or by inhalation (as by sprays).

[0165] Injectable preparations, for example, sterile injectable aqueous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed include water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. The injectable formulations can be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[0166] It will be appreciated that the exact dosage of nanocapsules of the disclosure is chosen by the individual physician in view of the patient to be treated. Typically, dosage and administration are adjusted to provide an effective amount of the nanocapsules to the patient being treated. As will be appreciated by those of ordinary skill in this art, the effective amount of nanocapsules may vary depending on such factors as the desired biological endpoint, the

therapeutic(s) to be delivered, the target tissue, the route of administration, and the like. For example, the effective amount of nanocapsules containing a particle modifier that is also an anti-cancer therapeutic, as well as a second (or more) therapeutic in the core region, might be the amount that results in a reduction in tumor size by a desired amount over a desired period of time. Additional factors which may be considered include the severity of the disease state; age, weight and gender of the patient being treated; diet, time and frequency of administration; drug combinations; reaction sensitivities; and tolerance/response to therapy.

[0167] The nanocapsules of the disclosure may be formulated in unit dosage form for ease of administration and uniformity of dosage. The expression "unit dosage form" as used herein refers to a physically discrete unit of nanocapsule appropriate for the patient to be treated. It will be understood, however, that the total daily usage of the compositions of the present disclosure will be decided by the attending physician within the scope of sound medical judgment. For any nanocapsule or nanocapsule composition, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. Therapeutic efficacy and toxicity of nanocapsules can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED_{50} (the dose is therapeutically effective in 50% of the population) and LD_{50} (the dose is lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD_{50}/ED_{50}. Pharmaceutical compositions which exhibit large therapeutic indices may be useful in some embodiments. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for human use.

[0168] In some embodiments, a composition is provided including a plurality of nanocapsules of the various embodiments described herein, and one or more of a pharmaceutically acceptable carrier, a pharmaceutically acceptable excipient, or pharmaceutically acceptable diluent. Such compositions can further comprise any other components as appropriate for an intended use of the nanocapsule composition.

[0169] In some embodiments, a composition suitable for freezing is provided, including nanocapsules as disclosed herein and a solution suitable for freezing, e.g. a sucrose solution is

added to the nanocapsule suspension. The sucrose may act, e.g., as a cryoprotectant to prevent the particles from aggregating upon freezing. For example, provided herein is a nanocapsule formulation comprising a plurality of disclosed nanocapsules, sucrose and water; wherein the nanocapsules/sucrose/water is about 3-30%/10-30%/50-90% (w/w/w) or about 5-10%/10-15%/80-90% (w/w/w).

[0170] Method of treatment

[0171] In an aspect of the disclosure, methods of treating a disease or disorder are provided, including administering to a subject in need thereof an effective amount of the multifunctional nanoparticle of the disclosure, wherein one or more linkers is cleavable by one or more intracellular or extracellular release agent present in the subject, thus releasing the therapeutic agent or diagnostic agent.

[0172] For example, in some embodiments, the disease or disorder is cancer, infection (e.g., bacterial, viral, or parasitic), pain, asthma, inflammation, neurological disease or disorder (e.g., Alzheimer's disease, Parkinson's disease, etc.). In certain embodiments, the disease or disorder is asthma, inflammation (e.g., asthma-induced inflammation or chronic obstructive pulmonary disease (COPD)-induced inflammation), or infection (e.g., lower respiratory infections).

[0173] In some embodiments, a method for the treatment of cancer (e.g., prostate, breast cancer, and the like) is provided. In some embodiments, the treatment of cancer includes administering a therapeutically effective amount of the nanoparticles described herein, or composition thereof, to a subject having a cancer. Nanoparticles, or compositions thereof, can be administered in such amounts and for such time as is necessary to achieve the desired result as including treating, alleviating, ameliorating, relieving, delaying onset of, inhibiting progression or growth of, inhibiting the migration of, reducing severity of, and/or reducing incidence of one or more symptoms or features of cancer.

[0174] In some embodiments, a method for preventing cancer is provided. Such methods include administering a therapeutically effective amount of a nanoparticle or composition thereof to a healthy individual (i.e., a subject who does not display any symptoms of cancer and/or who has not been diagnosed with cancer). For example, healthy individuals may be "immunized" with an inventive targeted particle prior to development of cancer and/or onset of symptoms of cancer; at risk individuals (e.g., patients who have a family history of cancer; patients carrying

one or more genetic mutations associated with development of cancer; patients having a genetic polymorphism associated with development of cancer; patients infected by a virus associated with development of cancer; patients with habits and/or lifestyles associated with development of cancer; etc.) can be treated prior to or substantially contemporaneously with the onset of symptoms of cancer.

[0175] All statements herein reciting principles, aspects, and embodiments of the disclosure, as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents as well as equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure.

[0176] Various other components may be included and called upon for providing for aspects of the teachings herein. For example, additional materials, combinations of materials and/or omission of materials may be used to provide for added embodiments that are within the scope of the teachings herein. Adequacy of any particular element for practice of the teachings herein is to be judged from the perspective of a designer, manufacturer, seller, user, system operator or other similarly interested party, and such limitations are to be perceived according to the standards of the interested party.

[0177] The following examples further illustrate the present invention but should not be construed as in any way limiting its scope.

[0178] Examples

[0179] In the following examples, a multilayer, multifunctional nanocapsule is described that achieves long term stabilization and enzyme specific release of cargo mRNA. To the inventors' knowledge there are currently no other mRNA delivery formulations that have been designed to deliver mRNA in an enzyme specific manner. In addition, we show in cell culture studies that the multilayered Lipo_MOF-NAN nanocapsule of the disclosure can effectively target the mRNAs expression to a cell via aptamer functionalization when that cell has greater expression of a specific cell receptor, in this case annexin 2. The Lipo_MOF-NAN was effectively shown to require and benefit from every component built into its design. The cationic liposomal core encapsulates the mRNA cargo, and the MOF layer provides added stability, a pH responsive release profile, and additional endosomal escape capability due to the N-methyl imidazole

ligands it releases when it degrades. The final outer NAN layer provides maximal stability as well as an enzyme-gated degradation mechanism that enables enzyme specific release of the mRNA and its subsequent expression in cells. Lastly the programmable nature of the NAN enables its outer oligonucleotide ligands to be ligated with aptamers, and it is shown herein that it can be used to target cancer cells overexpressing unique cell receptors in vitro and that, when tested in vivo, a more homogeneous expression of mRNA is observed, deeper into the tumor. The highly controlled release of mRNA, targeting capabilities, and prolonged shelf life of the multilayered Lipo_MOF-NAN nanocapsule of the disclosure, can provide important translational opportunities for the delivery of therapeutically relevant nucleic acid cargo.

[0180] EXAMPLE 1: Synthesis and Characterization of mRNA Lipo_MOF-NANs

[0181] Many mRNA formulations to date are not stable at temperatures warmer than -80 °C, due to the instability of the formulation itself and mRNA susceptibility to nuclease. Liposomes, in particular, have proved to be unstable within cellular environments and, under certain conditions, are toxic to cells. Therefore, their high cationic surface charge is often masked by modification with PEG and other ligands and significant efforts are underway to target liposomes through the incorporation of different lipid headgroups that can bias localization to specific tissues and organs. However, even with surface modifications for stabilization and improved lipid choices for biasing localization to specific sites, the majority of lipid formulations still suffer from limited endosomal escape.

[0182] A design approach is described herein that can address these limitations. FIG. 2A shows how the mRNA, designed to express mCherry, a red fluorescent protein, is loaded within a cationic liposome formulation, constructed from 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Cholesterol) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) via simple electrostatic and hydrophobic interactions.

[0183] To directly address the liposome stability issue, whether a cationic lipid formulation could be encased within a metal organic framework (MOF), rather than being PEGylated, was investigated. A MOF was investigated to determine whether it could stabilize the liposome (and thus potentially prolong the stability of its mRNA cargo); secondly, whether its N-methyl imidazole could have beneficial effects on endosomal escape; and thirdly, whether it could

impart controllable degradation and targeting properties when encapsulated within a nucleic acid nanocapsule (NAN).

[0184] To begin construction of Lipo_MOF-NANs, liposomes were first prepared as follows: 25 mg/mL liposomes were synthesized by placing DOPE and DC-Cholesterol in a 2:1 molar ratio in chloroform followed by drying under an inert atmosphere. The mixture was then kept overnight under vacuum, followed by redispersion in milli-Q water and purified through a mini extruder of an appropriate size filter membrane. Successful synthesis of liposomes was determined by TEM imaging. Representative nanoscale characterization are shown in **FIG. 4** – **FIG. 6**. All oligonucleotide sequences used to assemble the particles are provided in Table 2 (Materials and Methods section, below). TEM was used to determine the average size of all particles wherein the starting hydrodynamic size of the liposomes is 73.7 ± 23.7 nm, as corroborated by dynamic light scattering (DLS). The cationic liposomes showed a zeta potential of +55.7 mV. The size and surface charge after successful synthesis of each additional layer of the formulation is summarized in Table 1.

Table 1: Summary of TEM derived size calculations and zeta potential measurements after addition of each layer of the Lipo_MOF-NANs

Sample	Average Size (nm)	PDI	Zeta Potential (mV)
Liposomes	73.7 ± 29.7	0.63	+55.7
mRNA Lipo-DSC	110.5 ± 33.6	0.092	-34.6
mRNA Lipo_MOF	94.7 ± 36.4	0.15	-13.2
mRNA Lipo_MOF-SCM	133.3 ± 21.4	0.026	+35.9
mRNA Lipo_MOF-NAN	140.3 ± 43.2	0.095	-44.0

[0185] For encapsulation of the cationic liposome within a MOF, a way to modify the liposomes surface charge was needed, as the cationic components of the ZIF-8 synthesis protocol would be incompatible with the cationic charge of the liposomes as synthesized in the previous step. In order to modify the charge of the liposome exterior, DNA surfactant conjugates were used by reacting a thiolated DNA with an alkyne presenting surfactant (FIG. 4). DNA surfactant conjugate modification of the liposome was successfully achieved and resulted in changing the net surface charge from cationic (+31.9 mV) to anionic (-34.6 mV) (FIG. 3B). It was observed

that the size and stability of liposomes is highly dependent on the concentration ratio of the DNA surfactant conjugates to liposomes. In light of this, DNA surfactant conjugate concentrations were varied from 10 to 100 μM relative to a fixed concentration of liposome (6 mg/ml). TEM analysis was performed to determine the best DNA surfactant conjugate to liposome concentration ratio and found that at 75 μM DNA surfactant conjugate (DSC) and approximately 6 mg/ml of liposomal solution, the shape and size of the liposome was most uniform and small enough to be utilized as a core for the described multi-layered approach (FIG. 5).

[0186] After successful synthesis of DNA surfactant conjugate (DSC) modified liposomes, size exclusion chromatography (SEC) purification was performed to remove all excess DSCs in solution. The size of the liposomes after DSC modification increased from 73.7± 29.7 to 110.5 ± 33.6 nm, and the surface zeta potential became more negative (-34.6 mV). Next, the MOF encapsulation step was performed using a modified ZIF-8 synthesis protocol to control the growth of the Zn-imidazole layer formed around the DNA surfactant-modified liposomes. The amount of zinc and imidazole added, and the time allowed for mineralization was varied to maintain particle size in the nanometer size range. These mRNA Lipo_MOFs were purified by multiple washes in deionized water through centrifugation and pelleting of the particles. Using TEM analysis, the particle was shown to have a size of 94.7 ± 36.4 nm that was verified by DLS-based hydrodynamic size determination. It was observed that the mRNA Lipo_MOFs surface potential became less negative (-13.2 mV) due to the MOF layer and the masking of the DNA surfactant conjugate ligands. Interestingly, unlike the organic liposomal core, the mRNA Lipo_MOF particles can be visualized in TEM (FIG. 6) without uranyl acetate staining due to the metal framework.

[0187] Lastly, the final layer, the Lipo_MOF, was encapsulated within a nucleic acid nanocapsule (NAN). This step begins with the templating of C_{12} surfactant molecules on the mRNA Lipo_MOF. To achieve this, 15mM of C_{12} surfactant is added to 2 mg/mL of mRNA Lipo_MOF in order to fully encapsulate the mRNA Lipo_MOF core particle. Once the micelle layer is formed, it is crosslinked in place using a diazido ester crosslinker which is covalently incorporated into the particle surface using copper catalyzed azide alkyne cycloaddition (CuAAC) chemistry. The result of this step is a Lipo_MOF surface crosslinked micelle particle (mRNA Lipo_MOF-SCM). A surface crosslinked micelle (SCM) is the structural core of the present inventors' previously developed nucleic acid nanocapsule (NAN) design. A unique

aspect of the SCM design is the crosslinking step which serves to stabilize the micelle preventing premature degradation and release of nucleic acid cargo (surface ligands) during cellular delivery. The crosslinker is a versatile component of the NAN design as it is highly modular; the particle's degradation by different enzymatic targets can also be programmed by modifying the chemical nature of the crosslinker. For most of the work described herein, an esterified (ester containing) crosslinker as well as a matrix metalloproteinase 9 (MMP9) responsive peptide crosslinker was used. Both were used in order to test the enzyme specificity of the design. Protease specific peptide crosslinkers were incorporated to have the NAN particles degrade in the presence of enzymes that are elevated during specific disease states due to overexpression of a specific enzyme (e.g. increased MMP9 expression during inflammation and cancer). The ester crosslinker was specifically designed to enhance endosomal degradation, as there are high levels of esterase present within the endosomes of most cells. Here, the ester crosslinker was incorporated at the surface of the micelle encapsulated Lipo_MOFs using CuAAC chemistry. Next the mRNA Lipo_MOF-SCMs were purified via size exclusion with a NAP-5 G-25 sephadex column. The particles had a size of 133.3 ± 21.4 nm and a positive surface potential of +35.9 mV, indicating the successful formation of the cationic surfactant layer assembled at the particle surface. The single peak seen in both DLS, and zeta potential readings suggest a mono disperse population of the particles at every step (FIG. 3A and FIG. 3B).

[0188] Freshly prepared mRNA Lipo_MOF-SCMs were further functionalized by a thiolated DNA ligand layer (see Table 2 for sequence) to convert them into the final mRNA Lipo_MOF-NANs. The outer DNA ligand helps stabilize the particle in water and buffer and can be further functionalized with targeting oligonucleotides for cell specific uptake via enzymatic ligation. The thiolated DNA is incorporated via a thiolyne reaction using UV light and a photoinitiator. Particles formed (mRNA Lipo_MOF-NANs) are then purified from excess free DNA using size exclusion chromatography by using NAP-5 G-25 sephadex; fractions 3 and 4 were collected. After DNA functionalization, the zeta potential of the particles changed to negative (-44 mV) confirming the addition of the DNA layer at the surface of the particle. TEM analysis indicated a final size of 140.3 ± 43.2 nm. These mRNA Lipo_MOF-NAN fractions were then used for all further experiments (see FIG. 3, FIG. 6, and Table 1). HR-TEM coupled with EDS elemental analysis of these particles indicate a significant amount of C, P, N, Zn and O present in the multi-

layered particles. The Zn and N are attributed to the Zn salt and N-methyl imidazole of the MOF; the C and P and O as well as N, to the phospholipids and mRNA and DNA outer layer.

[0189] In order to investigate the resulting crystallinity of the MOF layer within the particle, powder X-ray diffraction (XRD) analysis was performed, and it was found that the peaks observed indicated a crystalline pattern that is similar to ZIF-8, though slightly different (FIG. 3C). Whether the beneficial properties of a MOF-like layer were evident in the particle design (i.e. pH gated degradation capabilities and stabilization of the mRNA and liposome) was then investigated.

[0190] **EXAMPLE 2: Enzyme and pH Responsive Release of mRNA**

[0191] To assess the stabilizing properties of the Lipo_MOF-NANs, an *in vitro* reverse transcriptase (RT) PCR assay was developed to assess the amount of mRNA released from the formulation in response to specific enzymatic and pH dependent stimuli. The assay (FIG. 7A) included treating the mRNA loaded Lipo_MOF-SCMs with either acidic conditions (acetate buffer, pH 5.5), esterase enzyme, both esterase and acidic conditions, or PBS as a control. Total released mRNA was evaluated by reverse transcription followed by PCR amplification and visualized via 8% denaturing polyacrylamide gel electrophoresis (8% PAGE). Results are summarized in FIG. 7B, where the gel results show that the highest amount of mRNA is released from the Lipo_MOF-SCMs when the particles are treated with esterase followed by acetate buffer at pH 5.5. A clear band at 160 bases long (see Table 2 for primer and RT-PCR design) confirms mRNA release is highest when both conditions, acidic pH and enzymatic degradation are present, rather than when treated with only esterase or only acetate buffer separately. Results from this mRNA release assay indicated that the multi-layered particle would be stable when introduced in a biological system until it is endocytosed (where acidification occurs) and exposed to endosomal esterases. To test this hypothesis, A549 and HeLa cells were treated with 10 μM Lipo_MOF-NANs containing mCherry expressing mRNA in 0% and 10% FBS in F-12K/ opti-MEM media with and without chloroquine pre-treatment. Results from this assay (see FIG. 7C and FIG. 9A) indicated that insignificant amounts of mRNA were released from the Lipo_MOF-NANs into the media and higher amount of mRNA were delivered to cytosol by Lipo_MOF-NANs compared to liposomes. Additionally, FIG. 7C indicates that nonspecific

release of mRNA is minimal, suggesting a decreased chance of premature mRNA release in complex biological environments.

[0192] EXAMPLE 3: Role of the MOF Layer in Gating Protein Expression from mRNA Lipo_MOF-NANs

[0193] In order to assess the added stability and gating provided by the MOF layer, a cell study was conducted in which chloroquine was administered to cells prior to nanoparticle treatment. Chloroquine is known to effectively prevent the maturation of endosomes by preventing acidification of the endosome. FIG. 7C indicates that cells treated with chloroquine followed by the mRNA Lipo_MOF-NANs show significantly lower mCherry expression than when treated with our particles without chloroquine. This result supports the conclusion that endosomal acidification is responsible for degradation of the MOF layer – a step that is necessary for the release of the mRNA that enables mCherry protein expression. This experiment also confirms the added stability of the MOF to the particles design and ruled out nonspecific release of mRNA.

[0194] EXAMPLE 4: Lipo_MOF-NANs Achieve Enhanced mRNA Expression and Stability

[0195] To evaluate the efficiency of the multilayered nucleic acid delivery nanocapsules of the disclosure, a series of Lipo_MOF- NAN cell studies were carried out assessing the amount of red fluorescence observed as a result of the expression of the mRNA protein encoded by mCherry mRNA within cells. As an initial test, Lipo_MOF- NANs were first loaded with fluorescein as a probe to assess the successful uptake of the particle without mRNA. The fluorescein loaded Lipo_MOF-NANs were incubated with A549 cells for 4 hr in both serum free-opti MEM and 10% FBS added F-12K media. The results of this experiment are summarized in FIG. 8. The appearance of high levels of green emission of fluorescein throughout the cytoplasm of the cells is evident even in the presence of media, suggesting the delivery vehicle is capable of remaining stable even in the presence of degrading enzymes in serum.

[0196] The efficiency of mRNA delivery via the Lipo_MOF-NANs was then assessed. For these studies the extent of mRNA encoding mCherry (referred to here as mCherry mRNA) cell delivery was assessed as a function of time of incubation with A549 and Hela cells. The cells

were imaged using confocal microscopy with excitation at 558 nm and emission at 610 ± 20 nm. In brief, mRNA loaded Lipo_MOF-NANs, along with other control samples (mRNA in bare liposomes or mRNA), were stored at 4 °C overnight and then added to HeLa or A549 cells in both serum and serum free media for 4 and 8 hr followed by washing of the cells and imaging. A freshly prepared DI water solution of mCherry mRNA of the same concentration that was loaded into the particles was used as a negative control; mCherry cationic liposomes (mRNA Lipo) loaded at the same mRNA concentration were used as a positive control. Results of these treatments are summarized in FIG. 9, FIG. 10, and FIGS. 11-17.

[0197] First quantified were the relative levels of mCherry expression (indicated by the red emission channel) in both HeLa and A549 cells. (FIG. 11) Treatments were conducted in both serum added and serum free media. Samples were incubated for indicated times and imaged by confocal microscopy. Significant expression of mCherry was evident after 8 hr of incubation for HeLa cells and after 4 hr of incubation for A549 cells in serum. mCherry expression in the absence of serum was at 4 hr for both cell lines. No significant enhancement in red emission after longer time points indicated that 4 hr and 8 hr for each respective cell line is a sufficient amount of time for evaluating mRNA expression in subsequent experiments. Another important observation from this experiment was that mRNA expression was still intense even after 24 hr, which suggests that there is a persistent and continual release of mRNA and/or stabilization of the mRNA over several hours within the cell.

[0198] The relative efficiency of mRNA delivery by the Lipo_MOF-NANs compared to mRNA delivery by cationic liposomes and bare mRNA was assessed. The results (FIG. 9) clearly showed that mCherry expression in both cell lines was significantly higher when the mRNA was delivered via the Lipo_MOF-NANs of the disclosure, rather than when delivered by the liposomes or as bare mRNA, irrespective of the media conditions (serum added or not). It was noted that the expression of mCherry was comparable to, and in many instances more than, that of the mCherry mRNA loaded liposome as seen by the mean fluorescence intensity analysis. This may be due to the storage conditions and stability of each formulation – samples were kept overnight in a freezer, which, for the liposome may have made the particles less uniform and may have then affected stability in serum. In the case of the mRNA, susceptibility to serum nucleases alongside poor transfection without the aid of a transfection agent are likely the reason for its limited expression.

[0199] To further evaluate the relative stability of the mRNA within the Lipo_MOF-NANs, a time course study was carried out assessing relative mCherry expression levels after the formulation was kept either in the refrigerator (4 °C) or at room temperature for long durations of time and compared the results to the mCherry expression levels achieved by either bare liposomes, or bare mRNA as a negative control. Two different cell lines (A549 and HeLa) and two different media conditions (with or without serum) were used to assess any cell line dependency and to assess broader applicability of the results; the same media conditions as previously described were utilized. mCherry expression levels were assessed by confocal microscopy post treatment with particles after 7, 14, and 21 days for HeLa (and 8, 15, 20 for A549) storage and imaged using confocal microscopy. Results shown in FIG. 9 and FIGS. 12-14 indicate that mRNA expression is higher in both cell lines when delivered using the Lipo_MOF-NANs of the disclosure as compared to the mCherry expression observed for liposomes, or for mRNA, where little to no expression of mCherry was observed at later time points. Results were also compared to mRNA delivered using lipofectamine as a positive control, in which significant mCherry expression was apparent and expected; however, we also found significant toxicity as well as non-specificity for cell type, as is well documented. Three identical biological replicates were carried out; results showed that the Lipo_MOF-NAN of the disclosure can stabilize mRNA for up to at least 14 days (as seen by mCherry expression), when stored at 4 °C or room temperature conditions.

[0200] EXAMPLE 5: Lipo_MOF-NANs Achieve Room Temperature Preservation of mRNA

[0201] A major drawback of current mRNA formulations is the need for frozen storage, often at -80 °C, for preservation of the structure of the formulation and the mRNA. Here, mRNA expression studies were conducted after storage of all the samples (mRNA loaded Lipo_MOF-NANs, mRNA loaded liposomes and mRNA) at room temperature, followed by incubating them in A549 and HeLa cells. The total mRNA expression was assessed by confocal microscopy 1, 7, 14 and 21 days after the preparations were made (see FIG. 10, FIG. 15 - FIG. 17). mCherry expression was higher for the Lipo_MOF-NAN formulation than for that of the liposome or free mRNA. Surprisingly, the mRNA loaded Lipo_MOF-NAN continued to support the expression of mCherry protein for up to 2 weeks, which was an unexpected result considering the samples are stored at room temperature, and mRNA is notoriously unstable at such temperatures for such

time scales. This result indicates the multilayered Lipo_MOF-NANs of the disclosure offered superior stabilization of mRNA where the layers both preserve the stability of the mRNA and enable its effective delivery, which resulted in persistent ~14-day stability and expression of protein.

[0202] EXAMPLE 6: Cell Viability

[0203] Evaluation of the potential cellular toxicity of the Lipo_MOF-NAN nanocarrier was tested prior to moving into *in vivo* studies. mRNA loaded Lipo_MOF- NANs, along with mRNA-Liposomes and mRNA-lipofectamine, with varying dose were incubated with A549 and HeLa cell lines in serum added media. Results are summarized in FIG. 18, where results indicate that in both cell lines the mRNA Lipo_MOF-NANs induce no significant toxicity as indicated by an MTS assay. Importantly, Lipo-MOF-NAN was less toxic than the mRNA-Liposome and mRNA-lipofectamine preparations when tested at equivalent concentrations.

[0204] EXAMPLE 7: Cell Receptor Specific Targeting of Lipo_MOF-NANs via Aptamer Functionalization

[0205] To date there are limited examples of targeted mRNA delivery. This is the case as it is often difficult to functionalize liposomes with larger biomacromolecules such as antibodies for targeting nanocarriers to specific cell types. For particles of the disclosure, the outer layer is a nucleic acid displaying NAN, which can be functionalized with one or more targeting moieties (e.g., oligonucleotides). To assess cell specific uptake, the outer most layer of the Lipo_MOF-NAN was modified with an aptamer (FIG. 19A). Specifically, the nanocarriers were functionalized with an aptamer that can target and bind exclusively to annexin 2, a cell surface receptor that is often over-expressed in cancer cells. The aptamer was functionalized to the surface of the mRNA Lipo_MOF-NANs via enzymatic ligation.

[0206] As annexin 2 is overexpressed in MCF-7 cells, this cell line was chosen as a model cell line for assessing the ability to target the expression of the mRNA delivery platform of the disclosure. To test this, mRNA Lipo_MOF-NANs functionalized with or without aptamer were incubated with MCF-7 cells in serum free media for just 30 minutes to minimize any non-specific binding and internalization that may occur via scavenger receptors. The cells were then washed and allowed to sit for another 3.5 hr to allow any mRNA internalized into the cells to be translated into protein. mRNA-Lipo_MOF-NANs functionalized with a scrambled sequence the

same size and chemistry as the active aptamer, as well as liposomes loaded with mRNA (mRNA Lipo) and bare mRNA were also incubated with cells for this short time (30 mins) followed by 3.5 hr incubation to serve as direct comparisons. After 4 hr the cells were washed and then treated with Hoechst stain and imaged under confocal microscopy (FIG. 19C). Results clearly indicate that the aptamer functionalized nanocarrier delivered mCherry within 30 min, likely due to its rapid receptor-specific (annexin 2) binding and internalization ability. This result is in stark contrast to all the other sample conditions tested, that lacked an active targeting moiety where the absence of red emission (expressed as ___ in black and white images) indicated that mRNA was not efficiently translated in these cells.

[0207] Lipo-MOF-NANs functionalized with aptamer or the scrambled sequence were injected IV into mice that had subcutaneous MCF-7 tumors growing on their thighs. Post-injection (24 hr), the mice were euthanized, and the tumors fixed in formalin, cryopreserved in sucrose, frozen and sectioned and assayed for mCherry expression (see Methods). Red fluorescence (expressed as ___ in black and white images) was seen in both aptamer and scramble preparations indicating that mRNA was delivered to tumors in both cases and expressed.

[0208] EXAMPLE 8: Protease Responsive Delivery of mRNA to Assess Therapeutic Applicability

[0209] To combine receptor targeting with enzyme specific delivery, mCherry mRNA was incorporated into Lipo_MOF-NANs synthesized with a protease specific crosslinker at its surface, rather than the ester crosslinker described above. The peptide sequence chosen to evaluate within the Lipo_MOF-NAN design (CGPLGLAGGERDGC; SEQ ID NO:1) is targeted by the extracellularly secreted enzyme MMP9. Here, an MMP9 specific Lipo_MOF-NAN was synthesized to evaluate mRNA delivery and its expression *in vivo*. These particles were then compared with a scrambled sequence control in MCF-7 cells. Results, summarized in FIG. 20B, show bright red emission (expressed as ___ in black and white images) when both of these formulations were incubated with cells overnight (16 hr); little to no fluorescence was detected in liposome and mRNA only treated control cells.

[0210] EXAMPLE 9: In Vivo Imaging and Targeting

[0211] Lipo_MOF-NANs functionalized with both MMP9 specific peptide crosslinkages within the particle and the annexin 2 specific aptamer on its surface were evaluated for mRNA delivery to tumors after IV injection. Mice were implanted subcutaneously with $5x10^6$ MCF-7 cells. Once the tumors were sufficiently large, Lipo_MOF-NANs loaded with mCherry mRNA and functionalized with ACE-4 aptamer or scrambled sequence, or saline or Lipo-MOF-NANs lacking mCherry mRNA (empty NP) also with either aptamer or scrambled sequence (controls), were injected into the mice (12.5 nmol/kg by aptamer or 7.5ng/kg by mRNA loading) via tail vein injection. The mice were then euthanized 24 hr post injection and the tumors were processed for confocal fluorescence microscopy (see Methods). FIG. 20C shows representative images of tumor edge and tumor core for the two test groups and the control group.

[0212] Detailed confocal microscopy imaging revealed mCherry expression to be reasonably homogeneous throughout the tumor edge and core when mRNA is delivered using the aptamer functionalized Lipo_MOF-NANs (FIG. 20C, row I). However, in contrast, mRNA delivered using the scrambled sequence Lipo_MOF-NANs (FIG. 20C, row II) showed more discrete regions of mCherry expression often localized at the tumor edge as well as some limited areas within the tumor—potentially in the vicinity of intra-tumoral spaces that might represent vasculogenic mimicry (see FIG. 20C, FIG. 22, and FIG. 23). These differences in expression patterns within the tumor appear to be tied to the specificity of internalization mechanisms (active annexin 2 aptamer versus scrambled sequence) and indicate that the aptamer may have the ability to change the distribution of mRNA expression within the tumor from a more heterogenous expression to a more homogeneous expression pattern.

[0213] The *in vitro* data herein indicate that the aptamer is likely mediating the internalization of the Lipo_MOF-NAN via annexin-2 binding. It appears that after *in vivo* administration the presence of aptamer allows greater binding of annexin 2 receptors that are distributed throughout the tumor. As a significant problem in cancer therapeutics is the delivery of drug throughout the tumor, the aptamer modified formulations of the disclosure may overcome the problem of heterogenous delivery of oligonucleotide therapeutics to tumors.

[0214] It is also notable that the amount of mRNA that delivered in the particle formulations described herein was on the order of 150 picograms per mouse; whereas, it is common in the literature to use hundreds of nanograms of mRNA. The unexpectedly substantial enhancement of

mRNA stability appears to allow for the delivery of orders of magnitude less mRNA that conventional methods.

[0215] Materials and Methods

[0216] Cationic Nanoliposomes (Lipo(+)) Synthesis.

[0217] Cationic liposomes were prepared starting from commercially available 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Cholesterol) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in a 1:2 volume ratio (80 µL of DC-cholesterol from 25 mg/mL stock of chloroform and 160 µL of DOPE from 25 mg/mL stock in chloroform). These solutions were added under argon purge to an ice bath to slow down the evaporation of chloroform with constant stirring. After complete evaporation of the chloroform leaving a thin film at the bottom of the flask, it was kept in a vacuum desiccator overnight to remove the remaining chloroform. The thin film was resuspended in 500 µL of Milli Q water and stirred for 30 mins followed by sonication for an hour. Finally, it was filtered through a mini extruder by passing the suspension through a 1 µm, 400 nm and then finally 200 nm pore-sized polycarbonate membrane filter respectively, 10-15 times each. The as prepared liposomal solution was kept at 4 °C for further use.

[0218] mRNA Loaded DNA-Surfactant Conjugate (DSC) Modified Nano-liposome (mRNA Lipo (-)) Synthesis.

[0219] To prepare DSCs, a thiolated polyT_{20} oligonucleotide was reacted with N,N,N-Tripropargyl dodecylammonium bromide (C_{12}) surfactant (**FIG. 1**) via thiolyne click reaction. In brief 150 µM C_{12} surfactant, 450 µM thiolated polyT_{20} DNA, and 100 µM 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (DHEMPP-photoinitiator) were mixed and exposed to UV-light in a Rayonet reactor while stirring for 30 mins. Meanwhile, 100 µL liposomes were loaded with commercially available mCherry mRNA (10 µL of 1mg/mL stock, Trilink Biotechnologies) by incubating at room temperature for 20 min. 100 µL of synthesized DSCs was added to the mRNA loaded nanoliposomes (making a final volume 210 µL) and placed on a thermoshaker at 25 °C and 800 rpm. The resulting mRNA-Lipo (-) particles were purified using a Sephadex G-25 NAP-10 column, and 500 µL fractions were collected.

[0220] Synthesis of a Zinc Imidazole-based Metal Organic Framework (MOF) Around an mRNA Loaded Liposome (mRNA Lipo_MOF)

[0221] To build a protective layer of metal organic framework (MOF) around the liposome encasing an mRNA cargo, 350μL of an as prepared mRNA loaded liposome solution was diluted with 400μL of 20mM HEPES buffer and 150 μL of 2 M methyl imidazole solution (prepared in 20 mM HEPES buffer) was added. While stirring this solution, 100 μL of 1 M zinc nitrate hexahydrate solution (prepared in 20mM HEPES buffer) was added dropwise which resulted in an instant and observable turbidity. After 4 hr of stirring, mRNA loaded Lipo_MOF were centrifuged at 10000 rpm for 5 min and the supernatant was discarded. Following the same method, they were washed twice with DI water. Finally, the mRNA loaded Lipo_MOFs were dried under vacuum and kept at -20 °C for long term storage.

[0222] Synthesis of mRNA Loaded Liposomal-MOF-Nucleic Acid Nanocapsules (mRNA Lipo_MOF-NANs).

[0223] A 2 mg/mL solution of mRNA Lipo_MOFs was prepared by vortexing followed by sonication for 5 min. 300 μL of an as prepared solution was added to an Eppendorf tube which contained 1.8 mg (0.005 mmol) of C_{12} surfactant. The solution was sonicated for 45 min. To crosslink the micelles loaded with liposomal-MOFs, a diazido ester crosslinker (0.04 mmol) along with 10 μL of 126 mM sodium ascorbate, and 10 μl of 25 mM Cu-THPTA were added in the given order. The solution was kept in a sonication bath for 2 h while making sure that temperature of the bath remains cold. The ester crosslinked mRNA Lipo_MOF-SCMs were then purified using a Sephadex G-25 NAP-5 column. To prepare the MMP-9 crosslinked Lipo_MOF-SCMs, 3.2mg (0.00245 mmol) of MMP-9 peptide (CGPLGLAGGERDGC; SEQ ID NO:1) was dissolved in 10 μl of DMSO. This peptide solution was added to Lipo_MOFs and C_{12} surfactant solution along with 3 μl of 10 mM DHEMPP and kept under UV light irradiation for 30 min. As the peptide is modified with Cys residues to present thiol terminal ends, the peptide is able to be incorporated into the micelle surface via a thiolyne click reaction between the alkyne head groups of the C_{12} surfactant and the thiol groups of the Cys residues. The MMP-9 mRNA Lipo_MOF- SCMs used for NAN synthesis were used without any further purification.

[0224] Finally, the particles were functionalized with an oligonucleotide sequence at the surface of as prepared mRNA Lipo_MOF-SCMs by an additional thiolyne click reaction

between a thiolated DNA molecule and the alkyne head groups of the surfactant molecules. For this step, a 100 μM solution of as prepared SCMs were reacted with 150 μM of thiolated polyT_{20} oligonucleotide in the presence of 100 μM DHEMPP under UV light irradiation for 30 min. Lastly, the mRNA-Lipo_MOF-NANs were purified using Sephadex G-25 NAP-5 column.

[0225] Reverse Transcription PCR mRNA Release Assay.

[0226] Enzyme and pH responsive release of mRNA was analyzed. Two 10 μL samples of mRNA Lipo_MOF-NANs/mRNA Lipo-MOF_SCMs were treated with 5μL of 100U/mL porcine liver esterase (MilliporeSigma); and one each was treated with 5 μL Milli-Q water; 10 μL of 10% FBS added F-12K media; or 10μl of PBS buffer. In a parallel sample, 10 μL of 1 ng/ml of mCherry mRNA was incubated with 5 μL of 100U/mL esterase. All solutions were maintained for 45 mins on a thermoshaker at 37 °C and at 600 rpm. After 45 mins, 5 μL of 200 mM sodium acetate buffer was added to (1) one sample treated with porcine liver esterase, (2) the sample treated with 10% FBS added F-12K media, and (3) the parallel mCherry mRNA sample to maintain an endosomal pH of 5.5, and returned to a thermoshaker for an additional 10 min at the same conditions. The other tubes continued to shake without any further disturbance for 10 min. Subsequently, to quantify the amount of mRNA released, reverse transcription was conducted using a standard RT protocol with SuperScript III (Invitrogen) on all the samples along with an untreated mRNA, followed by RT-qPCR on a BioRad CFX Connect Real-Time PCR Detection System. Primer sequences used for amplification are provided in Table 2.

Table 2: Oligonucleotide Sequences and Linker sequences

SEQ ID NO:	Name	Sequence
2	DNA anchor for ACE4 aptamer	PO_4-GTG CAG GTC GTC TTT TTT TTT T-SH
3	ACE4 Aptamer	GGG AGA UGA UCC GUU GAU GCG AGG GAA CGC AAG AAC UGA GGG CCA UGA GGG CGC CUU CCC UUG CUC AGG ACG CAA GUC GUC GUU CGU AGG CAG AAU CGC AUU GCC CCA GCG UGA CUG CCU A
4	Scrambled ACE4 Aptamer	GGG AGA UGA UCC GUU GAU GCG AGC ACU ACA ACU GCU GGU CAG CAC UAC UGG GAC GCC AGC UGA CGG CGG AGA AGU CGU CGU UCG UAG GCA GAA UCG CAU UGC CCC AGC GUG ACU GCC UA

5	DNA Bridge for ACE4 aptamer	GAC GAC CTG CAC TAG GCA GTC ACG
6	AH DNA anchor	SH-TTT TTT TTT TCA CGT CCA GCA G
7	mCherry Forward Primer	ATG GTG AGC AAG GGC GAG GAG
8	mCherry Reverse Primer	GCC ACC CTT GGT CAC CTT CAG C
9	mCherry mRNA*	**AUGGUGAGCAAGGGCGAG**GAGGACAACAUGGCCAUCAUCAA GGAGUUCAUGCGGUUCAAGGUGCACAUGGAGGGCAGCGUGA ACGGCCACGAGUUCGAGAUCGAGGGCGAGGGCGAGGGCCGG CCCUACGAGGGCACCCAGACCGCCAA**GCUGAAGGUGACCAA GGGCGGC**CCCCUGCCCUUCGCCUGGGACAUCCUGAGCCCCC AGUUCAUGUACGGCAGCAAGGCCUACGUGAAGCACCCCGCC GACAUCCCCGACUACCUGAAGCUGAGCUUCCCCGAGGGCUU CAAGUGGGAGCGGGUGAUGAACUUCGAGGACGGCGGCGUGG UGACCGUGACCCAGGACAGCAGCCUGCAGGACGGCGAGUUC AUCUACAAGGUGAAGCUGCGGGGCACCAACUUCCCCAGCGA CGGCCCCGUGAUGCAGAAGAAGACCAUGGGCUGGGAGGCCA GCAGCGAGCGGAUGUACCCCGAGGACGGCGCCCUGAAGGGC GAGAUCAAGCAGCGGCUGAAGCUGAAGGACGGCGGCCACUA CGACGCCGAGGUGAAGACCACCUACAAGGCCAAGAAGCCCG UGCAGCUGCCCGGCGCCUACAACGUGAACAUCAAGCUGGAC AUCACCAGCCACAACGAGGACUACACCAUCGUGGAGCAGUA CGAGCGGGCCGAGGGCCGGCACAGCACCGGCGGCAUGGACG AGCUGUACAAGAGCGGCAACUGA
10	ACE4 aptamer ssDNA	GGGAGATGATCCGTTGATGCGAGGGAACGCAAAAACTGAG GCCGTGAGGCGCCT TCCCTTGCTCAGGACGCAAGTCGTC GTTCGTAGGCAGAATC
11	Scrambled ACE4 ssDNA	GGGAGATGATCCGTTGATGCGAGCACTACAACTGCTGGTC AGCACTACTGGGACGCCAGCTGACGGCGGAGAAGTCGTCG TTCGTAGGCAGAATC
12	X primer	TAGGCAGTCACGCTGGGGCAATGCGATTCTGCCTACGAAC GACGACTT
13	P70 primer	CTCGAGTAATACGACTCACTATAGGGAGATGATCCGTTGA TGCGAG
14	Linker 1	GPLGLAGGERDG
15	Linker 2	GFLG
16	Linker 3	GPMGIAGQ
17	Linker 4	CGFLGC
18	Linker 5	CGPMGIAGQC

19	Linker 6	CFLC
20	Linker 7	CVAC
21	Linker 8	CV-Cit-C (where Cit is citrulline)
22	Linker 9	CVKC
23	Linker 10	CVRC
24	Linker 11	CFKC
25	Linker 12	CGPLGLAGGERDGC
26	Linker 13	CGFLGGFLGGFLGC
*Note bold regions indicate where forward and reverse primers will bind. The resulting PCR product would be 160 bases and will serve as the read out for the presence of the mRNA in solution. All sequences written 5' to 3'		

[0227] In order to visualize and assess the relative release of mRNA from the particles under the various treatments the samples were run in an 8% polyacrylamide gel electrophoresis (PAGE) assay under denaturing conditions (8M urea) and run for 40 min at 350 V. The gel was stained with SYBR gold, excited at 472 nm and emission was visualized at 520 nm.

[0228] **Relative Cell Expression of mCherry mRNA.**

[0229] To examine mRNA delivery efficiency of Lipo_MOF-NANs, mCherry mRNA loaded cationic liposomes (mRNA Lipo) were used, as well as free mCherry (mRNA) as negative controls, maintaining the same mRNA concentrations in all cases. A549, HeLa, and MCF-7 cell were used as model cell lines. Samples were treated in the presence or absence of serum and were incubated for 4 hr (for A549 and MCF-7 cells) or 8 hr (for HeLa). These times were chosen due to the observed expression profiles shown in FIG. 11. Approximately 4×10^4 cells/mL were plated and cultured overnight in 10% serum containing media at 37 °C and 5% CO_2. Media was changed to serum added or serum free media 1 hr prior to sample treatment. After 4 hr (or 8 hr) of incubation with the samples, washed cells were imaged using a Nikon Eclipse Ti Microscope (Nikon A1R confocal). After staining the nucleus with Hoechst, mCherry expression was imaged (558 nm laser excitation and emission at 610±20 nm). Images were processed by ImageJ software using the same corrections for all images. In all *in vitro* experiments, the mRNA was delivered at a concentration of 6 ng/ml.

[0230] Determination of Stability of mRNA.

[0231] To evaluate the enhanced stability of the mRNA cargo afforded by nanocapsules described herein, synthesized mRNA Lipo_MOF-NAN, mRNA Lipo and purified mRNA were stored in deionized water at 4 °C or room temperature. Cellular expression studies were performed as described above. All the samples were incubated with A549 and HeLa cells, both in serum added and serum free media, and incubated for the corresponding times followed by nuclear staining and imaging using confocal microscopy. Cell treatments were performed 1, 7, 14 and 21 days after sample preparation.

[0232] Synthesis of ACE4 Annexin 2 Specific Aptamer.

[0233] The anti-annexin 2 aptamer (ACE4) and a scramble sequence (Scr) of the same size were synthesized. Chemically synthesized ssDNA (5'-GGGAGATGATCCGTTGATGCGAGGGAACGCAAAAACTGAG GCCGTGAGGCGCCT TCCCTTGCTCAGGACGCAAGTCGTC GTTCGTAGGCAGAATC-3' (SEQ ID NO:10) for ACE4 aptamer and 5'-GGGAGATGATCCGTTGATGCGAGCACTACAACTGCTGGTC AGCACTACTGGGACGCCAGCTGACGGCGGAGAAGTCGTCG TTCGTAGGCAGAATC-3' (SEQ ID NO:11) for the scramble sequence) were amplified by 25 cycles of PCR using Primers X (SEQ ID NO:12) and P70 (SEQ ID NO:13). The double stranded PCR products were then in vitro transcribed with 2′F-Py RNA and purified by denaturing polyacrylamide gel electrophoresis (PAGE). The ACE4 aptamer used herein contains two mutations (G33A/A44G) that have been shown to improve its affinity to its receptor target. In addition, using the GP-60 primer, a 23-nucleotide spacer is added to the 3' end of the aptamer to provide distance between the aptamer and the nanoparticle.

[0234] Aptamer Ligation to mRNA-Lipo_MOF-NANs.

[0235] The nanocarrier surface was functionalized with ACE4 aptamer as follows: 10 μM mRNA-Lipo_MOF-NANs, 10 μM aptamer/ scrambled aptamer and 40 μM bridge ligand were mixed in a microcentrifuge tube and heated at 70 °C for 10 mins. Another solution was prepared containing 10 μL ligase buffer, 5 μL of 5 mM ATP, 2 μL of T4 DNA ligase and 33 μL of milli-Q water. After heating the first solution, it was cooled to room temperature and mixed with the second solution to obtain a final volume of 100 μL. This solution was then incubated at 37 °C for 14 hr. It was diluted to 500 μL and purified through NAP-10 column. Fractions 3-6 were

collected and concentrated to exact concentration by a low temperature speed vacuum method. Prepared samples along with other control samples were used for cell imaging studies.

[0236] Confocal Imaging of Aptamer Modified mRNA Lipo_MOF-NANs.

[0237] MCF-7 cells (4×10^4 cells/mL were plated in an 8 well confocal slide overnight. Aptamer modified mRNA Lipo_MOF- NANs were added to serum free opti-MEM media at a final concentration of 2 μM and incubated for 30 mins. Then cells were washed thoroughly to remove any unbound particles and kept another 3.5 h in 37 °C and 5% CO_2 atmosphere to allow for mRNA expression. Cells were then imaged by confocal microscopy using a Nikon Eclipse Ti Microscope (Nikon A1R confocal).

[0238] Cell Viability Assay.

[0239] Cytotoxicity was tested using a conventional MTS assay (Promega) per manufacturer's instructions. 2×10^4 cells/mL were plated in 96 well plate overnight. The various formulations and control samples were incubated with A549 cells for 24 hr followed by incubation for another 3 hr in presence of MTS reagent. Absorbance at 550 nm was measured in a 96 well plate reader (Biotek Synergy H1). Untreated cells were used as controls and a baseline was obtained using a solution of MTS reagent. Three distinct sample replications were used for all data points.

[0240] Aptamer mRNA Lipo_MOF NAN Treatment in Tumor Expressing Mice.

[0241] Animal experiments were conducted according to NIH guidelines and approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee (IACUC) before the start of the study. Five million MCF-7 cells that had been purchased from the ATCC were mixed with maitrigel (Corning CLS356234, 120 μL maitrigel, 40 μL cells) and injected subcutaneously into the thighs of athymic nude mice purchased from Charles River. When tumors were 500-1000 mm^3 (about three weeks), mice were injected IV with NP formulations or saline. Mice were euthanized 24 hr later. Legs with tumors were fixed with 4% buffered formalin for ~18 hr at 4 °C, then washed 3X with PBS (15 min/wash) and placed into 30% sucrose in PBS and stored at 4 °C. After ~3 days, legs with tumors were removed, washed 3X with PBS (10 min washes). Tumors were removed from the leg, cut in half and placed into cassettes in cryomatrix and rapidly frozen in an isopentane/dry ice bath. The frozen blocks were

then cryosectioned, coverslipped (50% glycerol in PBS with DAPI and the thin sections of tumor (7 microns) subjected to confocal fluorescent microscopy, both at UCHC (Zeiss LSM 880) and at Storrs (Nikon Eclipse Ti).

[0242] Nanoscale Characterization.

[0243] Hydrodynamic size and surface potential of synthesized materials were characterized by dynamic light scattering (DLS) and zeta potential using Malvern Zetasizer Nano ZS-90. Exact sizes were measured on a ThermoFisher Tecnai G2 Spirit BioTWIN operated at 80 keV followed by calculation of average size and PDI using ImageJ software. Liposomal- MOFs were subsequently analyzed by scanning electron microscopy (SEM) and X-Ray diffraction (XRD). SEM analysis was performed on a ThermoFisher Nova NanoSEM 450 operating at 2 keV. A Rigaku Ultima IV X-ray diffractometer (Cu Ka, l = 1.5406 Å), with a working voltage of 40 keV, a current of 44 mA and a scan speed of 1° min-1 was used to obtain XRD patterns. ESI-MS- based mass spectrometric (negative mode) detection of the DNA surfactant conjugate (DSC) was conducted on an Orbitrap Exploris 480 and a Vanquish LC. DSC samples were dissolved in 150 mM ammonium acetate to reduce the impacts of sodiation.

CLAIMS

What is claimed is:

1. A multifunctional nanoparticle comprising: a nucleic acid nanocapsule (NAN), a metal organic framework (MOF), a liposome, and one or more therapeutic agents, or a pharmaceutically acceptable salt, hydrate or solvate thereof,

wherein the therapeutic agent is encased in the liposome;

wherein the liposome is encased in the MOF to make a lipid-MOF complex; and

wherein the lipid-MOF is encased in the NAN.

2. The multifunctional nanoparticle of claim 1, wherein the NAN comprises one or more nucleic acid ligands covalently attached to a particle comprising non-polymeric amphiphiles,

wherein hydrophobic groups of the amphiphiles are arranged toward the particle interior, and

wherein hydrophilic groups of the amphiphiles are at the particle surface and are crosslinked through a linker with one or more linkers cleavable by one or more intracellular or extracellular release agents.

3. The multifunctional nanoparticle of claim 1 or 2, further comprising a targeting nucleic acid ligand (aptamer) attached to the exterior of the NAN.

4. The multifunctional nanoparticle of either of claims 2 or 3, wherein the hydrophilic groups of the amphiphiles are crosslinked through a triazole, thioether, or alkenyl sulfide group with one or more linkers cleavable by one or more intracellular or extracellular release agents results from a reaction of alkyne or alkene moiety on the hydrophilic group of the amphiphile (e.g., on the ammonium group) and an azide or thiol moiety on the linker.

5. The multifunctional nanoparticle of claim 4, wherein the alkyne or alkene moiety on the hydrophilic group of the amphiphile is one or two of prop-2-ynyl or prop-2-enyl; one or two of prop-2-ynyl, or one or two of prop-2-enyl.

6. The multifunctional nanoparticle of claim 4, wherein the hydrophilic group of the amphiphile is tri(prop-2-yn-1-yl)amino or triallylamino.

7. The multifunctional nanoparticle of any one of claims 3-6, wherein the release agent comprises an enzyme (such as peptidase, protease, esterase, or elastase).

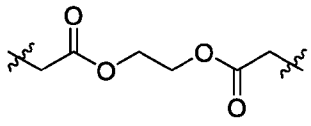
8. The multifunctional nanoparticle of any one of claims 3-7, wherein the linker comprises a peptide (for example, cleavable with a peptidase or protease), wherein the peptide is at least two amino acids long, or at least three amino acids long, or at least four amino acids long; or the peptide is between two and twenty-five amino acids long, and optionally, the linker comprises Cys residues or azide moieties on each end of the peptide .

9. The multifunctional nanoparticle of claim 8, wherein the peptide linker comprises CGPLGLAGGERDGC (SEQ ID NO:1), GPLGLAGGERDG (SEQ ID NO:14), GFLG (SEQ ID NO:15), GPMGIAGQ (SEQ ID NO:16), CGFLGC (SEQ ID NO:17), CGPMGIAGQC (SEQ ID NO:18), Phe-Leu, Val-Ala, Val-Cit, Val-Lys, Val-Arg, or Phe-Lys.

10. The multifunctional nanoparticle of claim 8, wherein the peptide linker comprises CGPLGLAGGERDGC (SEQ ID NO:1), CGFLGC (SEQ ID NO:17), or CGPMGIAGQC (SEQ ID NO:18).

11. The multifunctional nanoparticle of any one of claims 3-10, wherein the linker comprises one or more of ester groups (for example, cleavable with an esterase).

12. The multifunctional nanoparticle of claim 11, wherein the linker comprises



group.

13. The multifunctional nanoparticle of any one of claims 3-12, wherein the linker comprises one or more of hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, or thioether groups, or a combination thereof, or other acid-labile groups that are hydrolyzable in the lysosome.

14. The multifunctional nanoparticle of any one of claims 3-13, wherein the linker comprises a disulfide group.

15. The multifunctional nanoparticle of any one of claims 3-14, wherein the linker excludes disulfide group or another group cleavable under reducing conditions.

16. The multifunctional nanoparticle of any one of claims 3-15, wherein the linker is cleavable by one intracellular or extracellular release agent.

17. The multifunctional nanoparticle of any one of claims 3-15, wherein the linker is cleavable by two or more intracellular or extracellular release agents (e.g., wherein the linker comprises two or more different chemical groups each cleavable by a different release agent).

18. The multifunctional nanoparticle claim 17, wherein the linker comprises at least two groups selected from an ester, hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, thioether, disulfide, and a peptide, wherein the peptide is at least two amino acids long, or at least three amino acids long, or at least four amino acids long; or the peptide is between two and twenty-five amino acids long.

19. The multifunctional nanoparticle of any one of claims 2-18, wherein the nucleic acid ligands are covalently attached to the hydrophilic groups of the amphiphiles through a thioether or alkenyl sulfide group.

20. The multifunctional nanoparticle of claim 19, wherein the thioether or alkenyl sulfide group attaching the nucleic acid ligands to the hydrophilic group of the amphiphile results from a reaction of alkyne or alkene moiety on the hydrophilic group of the amphiphile (e.g., on the ammonium group) and a thiol moiety (e.g., Cys) on the nucleic acid ligand.

22. The multifunctional nanoparticle of claim 20, wherein the alkyne or alkene moiety on the hydrophilic groups of the amphiphiles is prop-2-ynyl or prop-2-enyl, or prop-2-ynyl, or prop-2-enyl.

23. The multifunctional nanoparticle of claim 20, wherein the alkyne or alkene moiety on the hydrophilic group of the amphiphile is prop-2-yn-1-ylamino or allylamino.

24. The multifunctional nanoparticle of any of claims 2-23, wherein the nucleic acid ligands are capable of selectively binding to a cell surface antigen; and/or

the multifunctional nanoparticle of any one of claims 3-23 further comprising a targeting nucleic acid ligand (aptamer) attached to the exterior of the NAN, wherein the targeting nucleic acid ligands are capable of selectively binding to a cell surface antigen.

25. The multifunctional nanoparticle of any one of claims 2-23, wherein the nucleic acid ligand is capable of gene regulation, and wherein the nucleic acid is siRNA, DNAzyme, ribozyme, microRNA, or other therapeutic oligonucleotide.

26. The multifunctional nanoparticle of any one of claims 2-23, wherein the nucleic acid ligand is capable of selectively binding to a protein or a carbohydrate; and/or
the multifunctional nanoparticle of any one of claims 3-23 further comprising a targeting nucleic acid ligand (aptamer) attached to the exterior of the NAN, wherein the targeting nucleic acid ligands are capable of selectively binding to a protein or a carbohydrate.

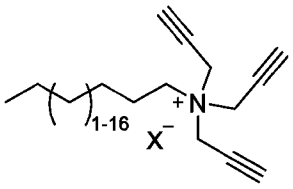
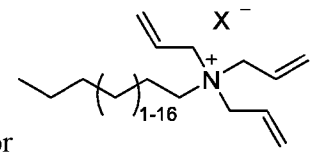
27. The multifunctional nanoparticle of claim 26, wherein the nucleic acid ligand and/or the targeting nucleic acid ligand is capable of selectively binding to a protein, wherein the protein is selected from the group consisting of tumor-markers, integrins, cell surface receptors, transmembrane proteins, ion channels, membrane transport protein, enzymes, antibodies, and chimeric proteins.

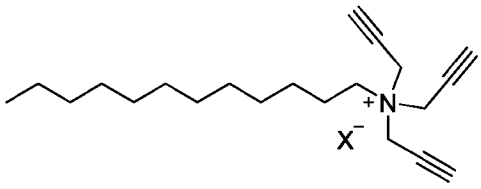
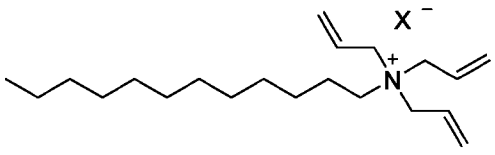
28. The multifunctional nanoparticle of claim 26, wherein the nucleic acid ligand and/or the targeting nucleic acid ligand is capable of selectively binding to a carbohydrate, wherein the carbohydrate is selected from the group consisting of glycoproteins, sugar residues, and glycocalyx.

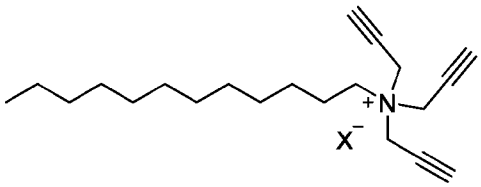
29. The multifunctional nanoparticle of claim 26, wherein the nucleic acid ligand and/or the targeting nucleic acid ligand is capable of selectively binding DNA, RNA, modified DNA, modified RNA, DNAzymes, ribozymes, mRNA, siRNA, microRNA, shRNA, and combinations thereof.

30. The multifunctional nanoparticle of any one of claims 2-23, wherein the nucleic acid ligand is capable of selectively binding to a cell during a specific developmental stage (e.g., stage having developmentally specific cell surface antigens) or to a cell in a specific disease state (e.g., a tumor cell that has tumor-associated antigens or tumor-specific antigens), and/or the

multifunctional nanoparticle of any one of claims 3-23, wherein the targeting nucleic acid ligand is capable of selectively binding to a cell during a specific developmental stage (e.g., stage having developmentally specific cell surface antigens) or to a cell in a specific disease state (e.g., a tumor cell that has tumor-associated antigens or tumor-specific antigens).

31. The multifunctional nanoparticle of any one of claims 3-30, wherein the non-polymeric amphiphiles are derived from  or  , wherein X is halogen.

32. The multifunctional nanoparticle of any one of claims 3-30, wherein the non-polymeric amphiphiles are derived from  or  , wherein X is halogen.

33. The multifunctional nanoparticle of any one of claims 3-30, wherein the non-polymeric amphiphile is  , wherein X is halogen.

34. The multifunctional nanoparticle of any one of claims 1-33, wherein the MOF comprises a plurality of metal ions and a plurality of organic ligands, wherein the metal ions and organic ligands are coordinated to form a porous, three-dimensional framework around the liposome.

35. The multifunctional nanoparticle of claim 34, wherein the metal ion comprises one or more of Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, Fe^{2+}, Ti^{4+}, Zr^{4+}, Hf^{4+}, Ni^{2+}, Ni^{+}, V^{4+}, V^{3+}, V^{2-}, and Al^{3+}.

36. The multifunctional nanoparticle of claim 35, wherein the metal ion is Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+}, or Fe^{2+}.

37. The multifunctional nanoparticle of claim 36, wherein the metal ion is Zn^{2+}.

38. The multifunctional nanoparticle of claim 34, wherein the organic ligand comprises one or more of 1-methylimidazole, 2-methylimidazole, 4-methyl-1H-imidazole, imidazole, benzimidazole, 1-methyl-1,2,4-triazole, 1-methyl-1H-tetrazole, or 2-imidazolcarbonitrile.

39. The multifunctional nanoparticle of claim 34, wherein the organic ligand comprises 1-methylimidazole, 2-methylimidazole, 4-methyl-1H-imidazole, imidazole, or benzimidazole.

40. The multifunctional nanoparticle of claim 39, wherein the organic ligand comprises 1-methylimidazole, or 2-methylimidazole.

41. The multifunctional nanoparticle of claim 39, wherein the organic ligand is 1-methylimidazole.

42. The multifunctional nanoparticle of claim 34, wherein the metal ion is Zn^{2+}, and the organic ligand is 1-methylimidazole.

43. The multifunctional nanoparticle of claim 42, wherein the MOF formed by Zn^{2+} and 1-methylimidazole is a Zeolitic Imidazolate Framework-8 (ZIF-8) MOF.

44. The multifunctional nanoparticle of any one of claim 1-43, wherein the liposome comprises one or more cationic lipids and a one or more non-cationic (helper) lipids.

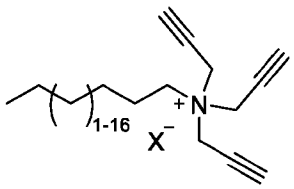
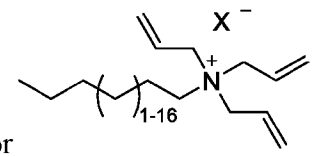
45. The multifunctional nanoparticle of claim 44, wherein the one or more cationic lipids is a cholesterol-based lipid.

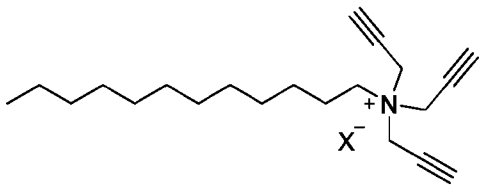
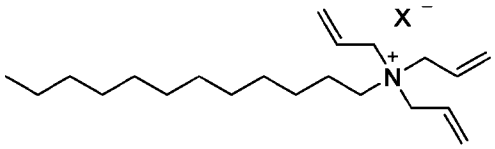
46. The multifunctional nanoparticle of claim 45, wherein the cholesterol-based lipid comprises 3ß-[N-(N',N'- dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol).

47. The multifunctional nanoparticle of any one of claims 44-46, wherein the one or more non-cationic (helper) lipids comprises a lipid that is neutral or zwitterionic at physiological pH.

48. The multifunctional nanoparticle of any one of claims 44-47, wherein the one or more non-cationic (helper) lipids comprises 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE).

49. The multifunctional nanoparticle of any one of claim 1-48, further comprising a DNA Surfactant Conjugate (DSC), a DNA-cholesterol conjugate, a DNA-tocopherol conjugate, or any combination thereof, incorporated into a bilayer of the liposome.

50. The multifunctional nanoparticle of claim 49, wherein the DSC comprises a non-polymeric amphiphile according to  or  , wherein X is halogen; and a thiolated oligonucleotide.

51. The multifunctional nanoparticle of claim 49, wherein the DSC comprises a non-polymeric amphiphile selected from  and  , wherein X is halogen; and a thiolated oligonucleotide.

52. The multifunctional nanoparticle of either of claim 50 or 51, wherein the thiolated oligonucleotide is between about 6 to 30 nucleotides in length, about 8 and 30 nucleotides in length, about 10 and 25 nucleotides in length, about 10 and 20 nucleotides in length, about 12 and 25 nucleotides in length, about 14 and 25 nucleotides in length, about 16 and 30 nucleotides in length, about 16 and 25 nucleotides in length, about 18 and 25 nucleotides in length, or about 18 and 20 nucleotides in length.

53. The multifunctional nanoparticle of any of claims 50-52, wherein the thiolated oligonucleotide is polyT_{20} DNA.

54. The multifunctional nanoparticle of any one of claim 50-53, wherein the thiolated oligonucleotide is reacted with the non-polymeric amphiphile via a thiolyne click chemistry reaction.

55. The multifunctional nanoparticle of claim 52, wherein the thiolated oligonucleotide is reacted with the non-polymeric amphiphile via a thiolyne click chemistry reaction.

56. The multifunctional nanoparticle of any one of claim 1-55, wherein the therapeutic agent comprises one or more of a therapeutic nucleic acid, a hydrophobic small molecule drug selected from group consisting of an anti-cancer agent, an antibiotic, an antiviral, an antiparasitic agent, an anticoagulant, an analgesic agent, an anesthetic agent, an ion channel potentiator, an ion channel inhibitor, an anti-inflammatory, a metallodrug, and any combination thereof.

57. The multifunctional nanoparticle of claim 56, wherein the therapeutic agent comprises camptothecin, doxorubicin, daunorubicin, vincristine, paclitaxel, neocarzinostatin, calicheamicin, cisplatin, carboplatin, oxaliplatin, satraplatin, picoplatin, lurtotecan, annamycin, docetaxel, tamoxifen, epirubicin, methotrexate, vinblastin, vincristin, topotecan, prednisone, prednisolone, abt-737, or any combination thereof.

58. The multifunctional nanoparticle of claim 56, wherein the therapeutic agent is a nucleic acid comprising one or more of Small Interfering RNA (siRNA), MicroRNA (miRNA), Antisense Oligonucleotides (ASOs), ribozymes, aptamers, mRNA, circularized mRNA, Short hairpin RNA (shRNA), plasmids, single guide RNA, Splice-Switching Oligonucleotides (SSOs), or any combination thereof.

59. The multifunctional nanoparticle of any one of claims 1-58, wherein the nanoparticle comprises a diagnostic agent, which is a fluorophore, a radiolabeled nucleotide, a radioisotope, biotin, tocopherol, cholesterol, a steroid, an electron dense tag and a metal chelator, or any combination thereof.

60. The multifunctional nanoparticle of any one of claims 1-59, wherein the nanoparticle is targeted to a specific cell receptor.

61. A composition for treating or preventing a disease, a disorder, or symptom in a subject, the composition comprising at least one of the multifunctional nanoparticle of any one of claims 1-60 or a pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable vehicle and/or excipient.

62. A method of treating a disease or disorder, comprising administering to a subject in need thereof an effective amount of the multifunctional nanoparticle of any one of claims 1-60 or the composition of claim 61, wherein the linker is cleavable by one or more intracellular or extracellular release agent present in the subject, thus releasing the therapeutic agent or diagnostic agent.

63. The method of claim 62, wherein no more than about 20%, no more than about 15%, no more than about 10%, no more than about 5%, no more than about 3%, or no more than about 1% of the linker is cleaved in an extracellular environment.

64. The method of claim 62 or 63, wherein no less than about 40%, no less than about 50%, no less than about 60%, no less than about 70%, no less than about 80%, or no less than about 90% of the linker is cleaved in an intracellular environment.

65. The method of claim 62, wherein the release mechanisms is an enzyme expressed by tumor cells.

66. The method of any of claims 62-65, wherein the release agent is a lysosome agent, endosome agent, and/or caveolae agent.

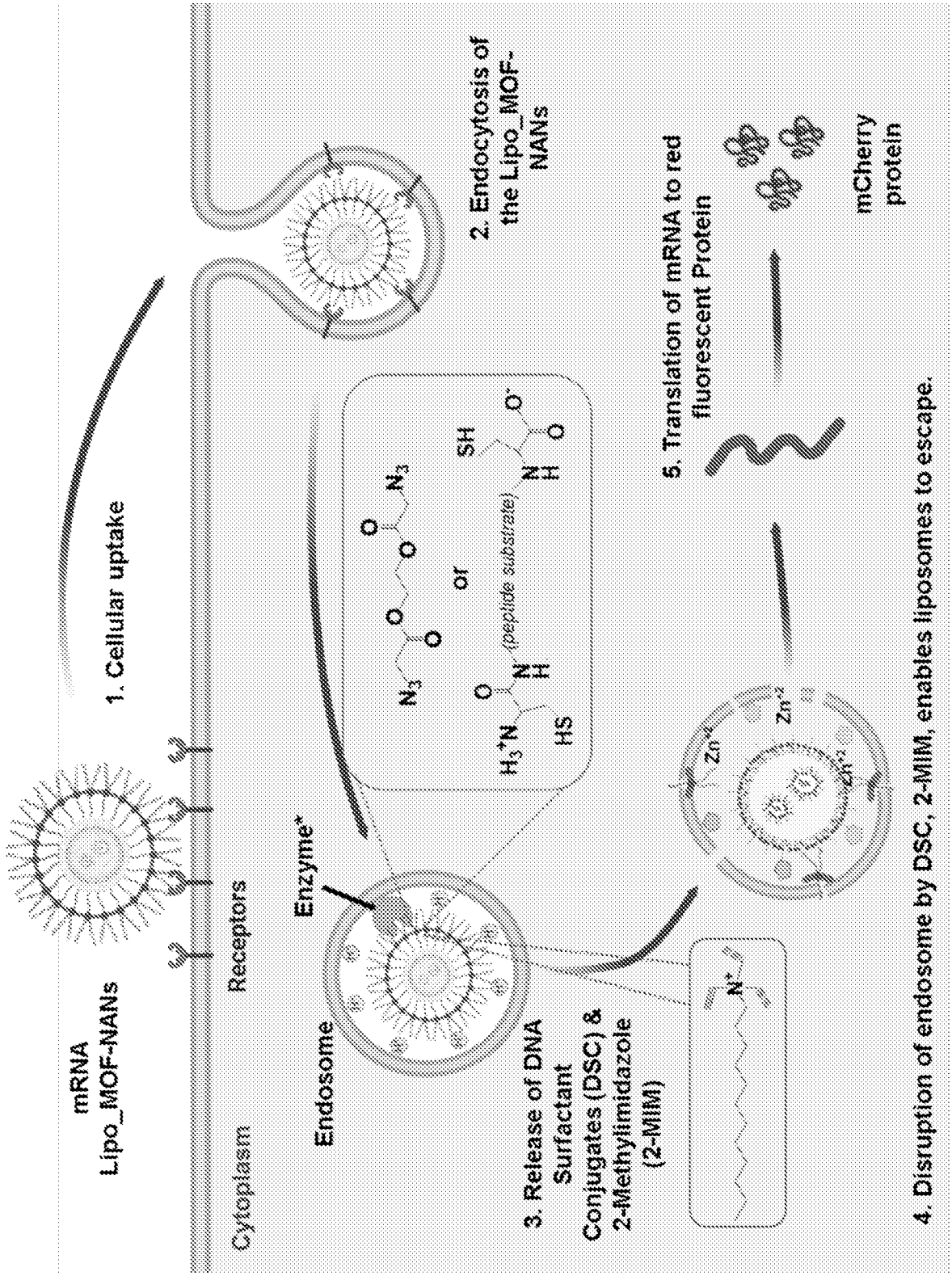

mRNA
Lipo_MOF-NANs
1. Cellular uptake
Receptors
Cytoplasm
Endosome
Enzyme*
2. Endocytosis of the Lipo_MOF-NANs
or
(peptide substrate)
3. Release of DNA Surfactant Conjugates (DSC) & 2-Methylimidazole (2-MIM)
5. Translation of mRNA to red fluorescent Protein
mCherry protein
4. Disruption of endosome by DSC, 2-MIM, enables liposomes to escape.

FIG. 1

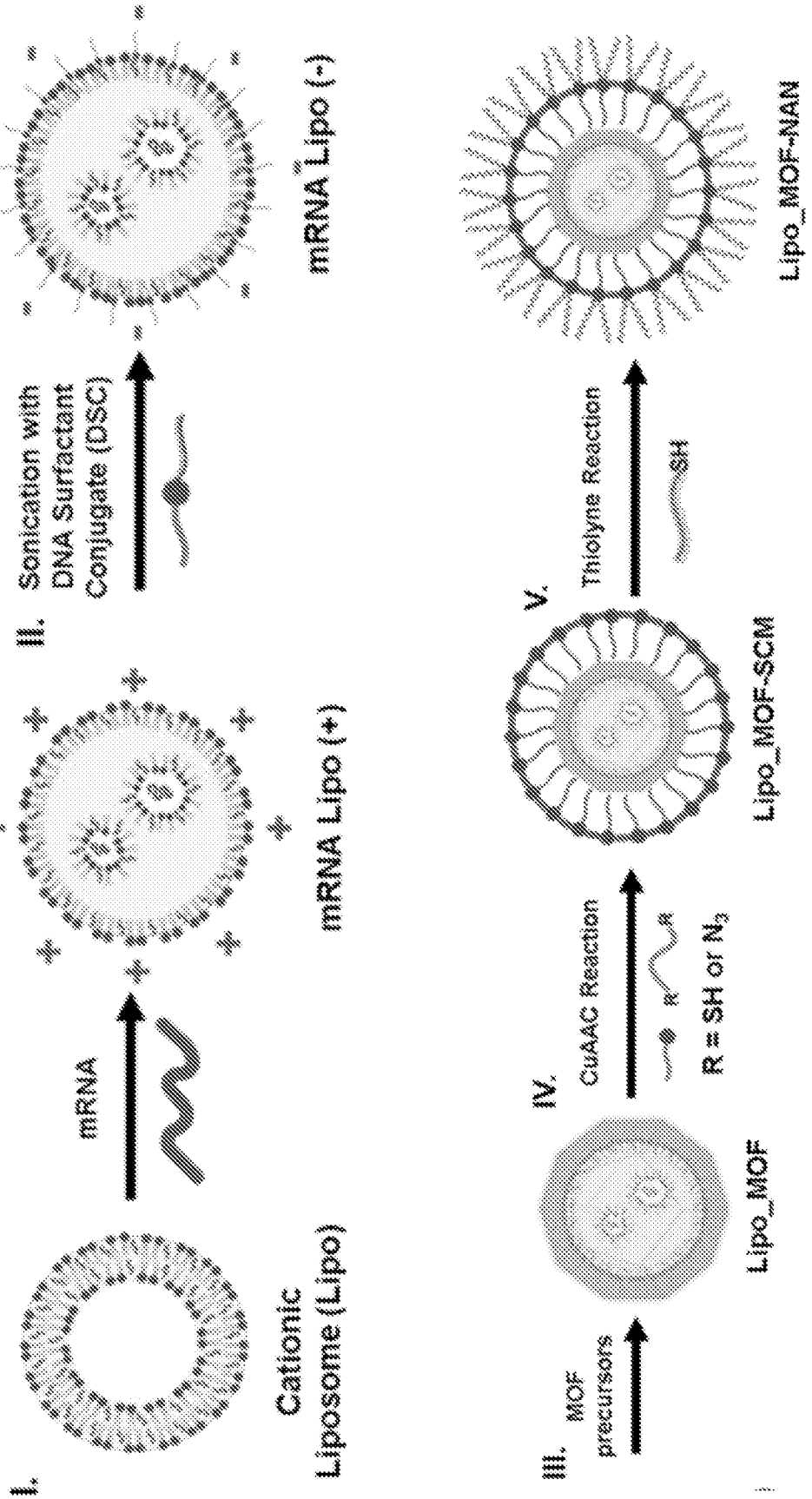

I.
Cationic
Liposome (Lipo)
mRNA
mRNA Lipo (+)
II.
Sonication with
DNA Surfactant
Conjugate (DSC)
mRNA Lipo (-)
III.
MOF
precursors
Lipo_MOF
IV.
CuAAC Reaction
R = SH or N3
Lipo_MOF-SCM
V.
Thiolyne Reaction
SH
Lipo_MOF-NAN

FIG. 2A

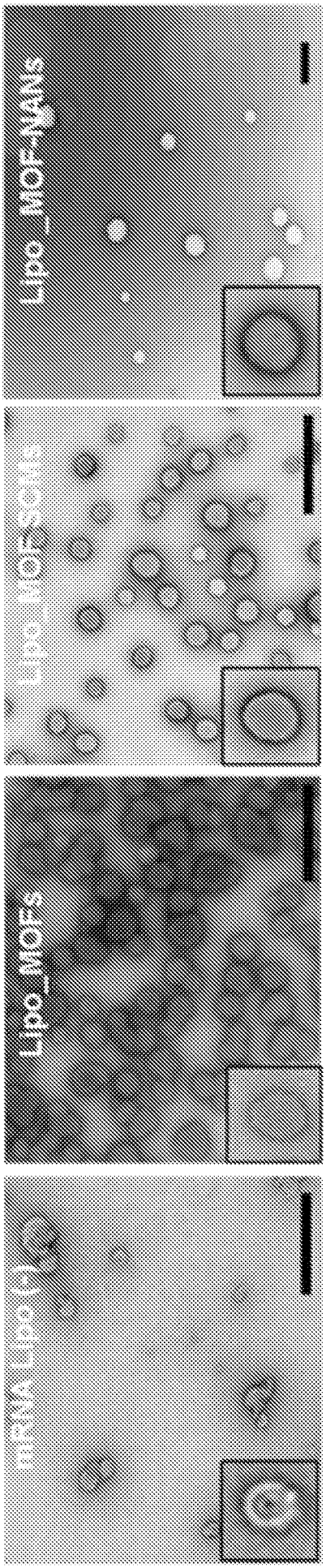

mRNA Lipo (L)
Lipo_MOFs
Lipo_MOF-SCMs
Lipo_MOF-NAMs

FIG. 2B

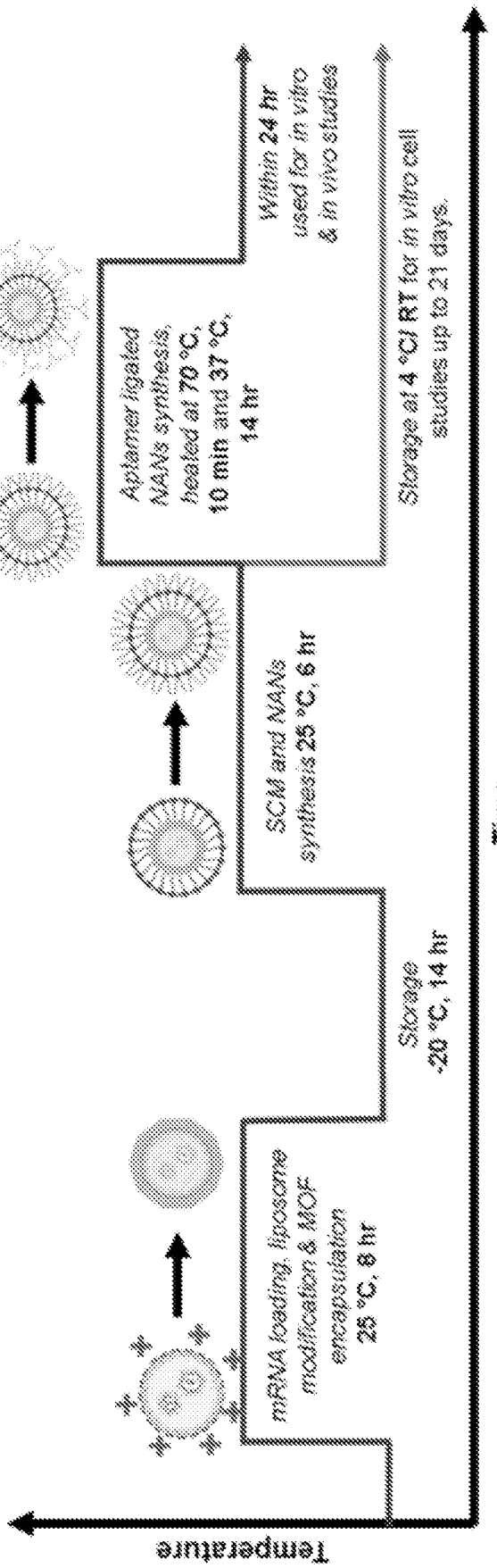

Temperature
Time
mRNA loading, liposome modification & MOF encapsulation 25 °C, 8 hr
Storage -20 °C, 14 hr
SCM and NANs synthesis 25 °C, 6 hr
Aptamer ligated NANs synthesis, heated at 70 °C, 10 min and 37 °C, 14 hr
Within 24 hr used for in vitro & in vivo studies
Storage at 4 °C/ RT for in vitro cell studies up to 21 days.

FIG. 2C

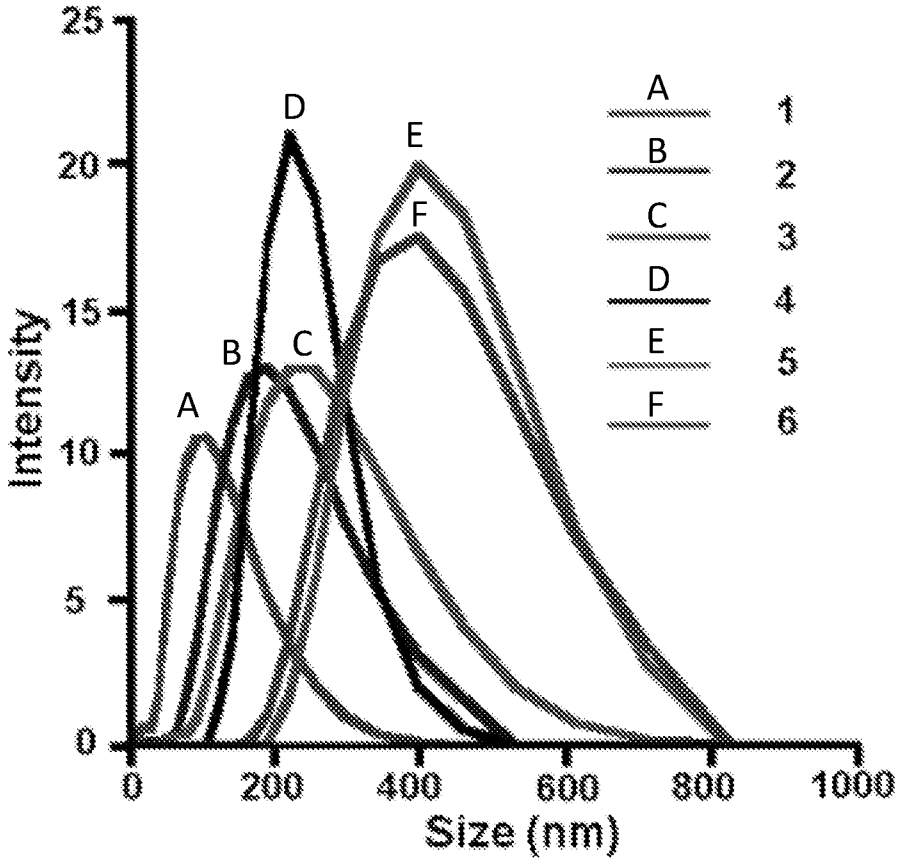

25
20
15
10
5
0
Intensity
0
200
400
600
800
1000
Size (nm)
A
B
C
D
E
F
A 1
B 2
C 3
D 4
E 5
F 6

FIG. 3A

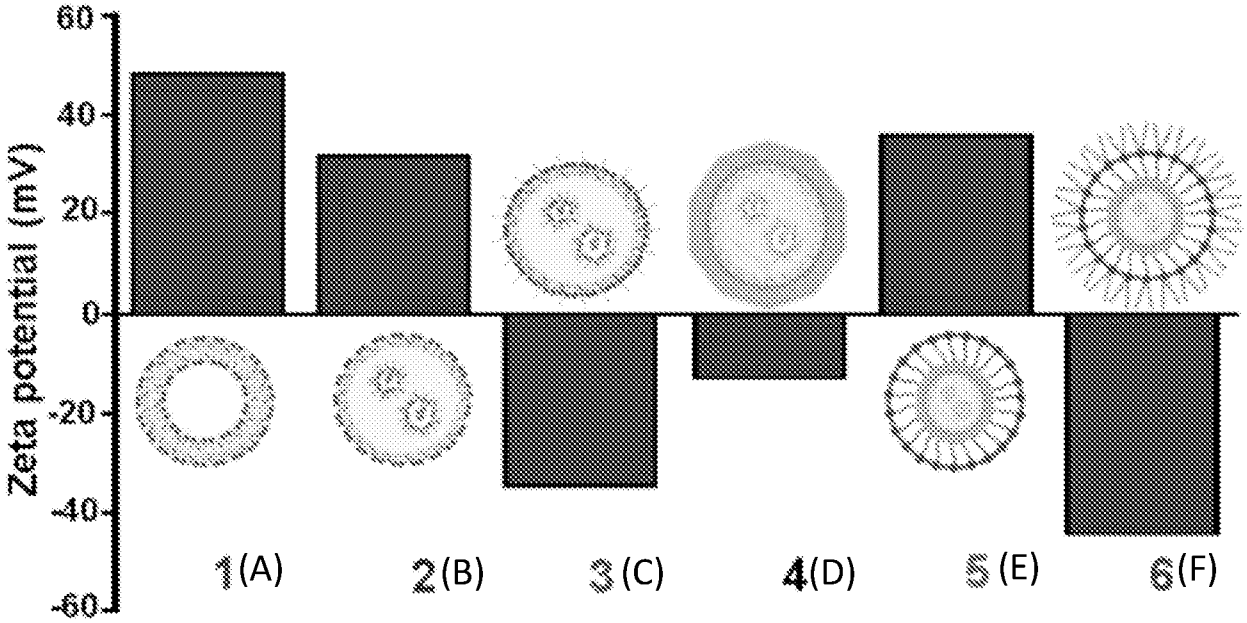

60
40
20
0
-20
-40
-60
Zeta potential (mV)
1(A)
2(B)
3(C)
4(D)
5(E)
6(F)

FIG. 3B

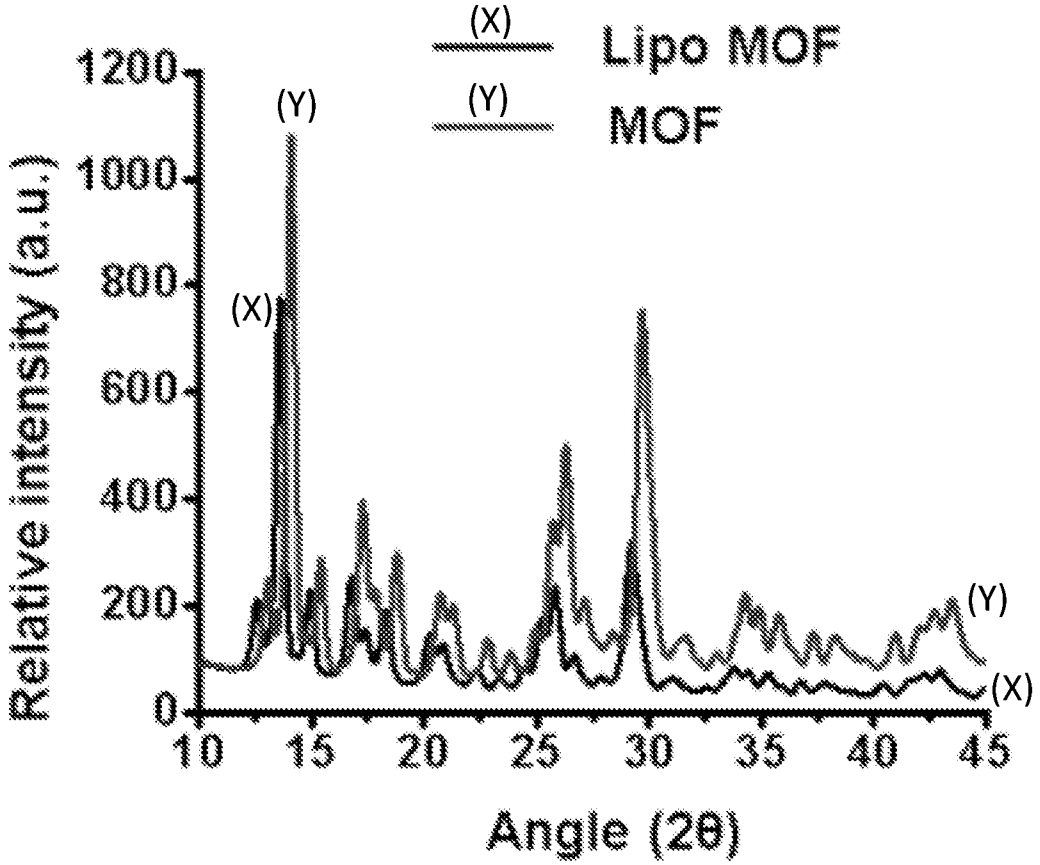

(X)
Lipo MOF
(Y)
MOF
Relative intensity (a.u.)
1200
1000
800
600
400
200
0
(Y)
(X)
(X)
(Y)
10
15
20
25
30
35
40
45
Angle (2θ)

FIG. 3C

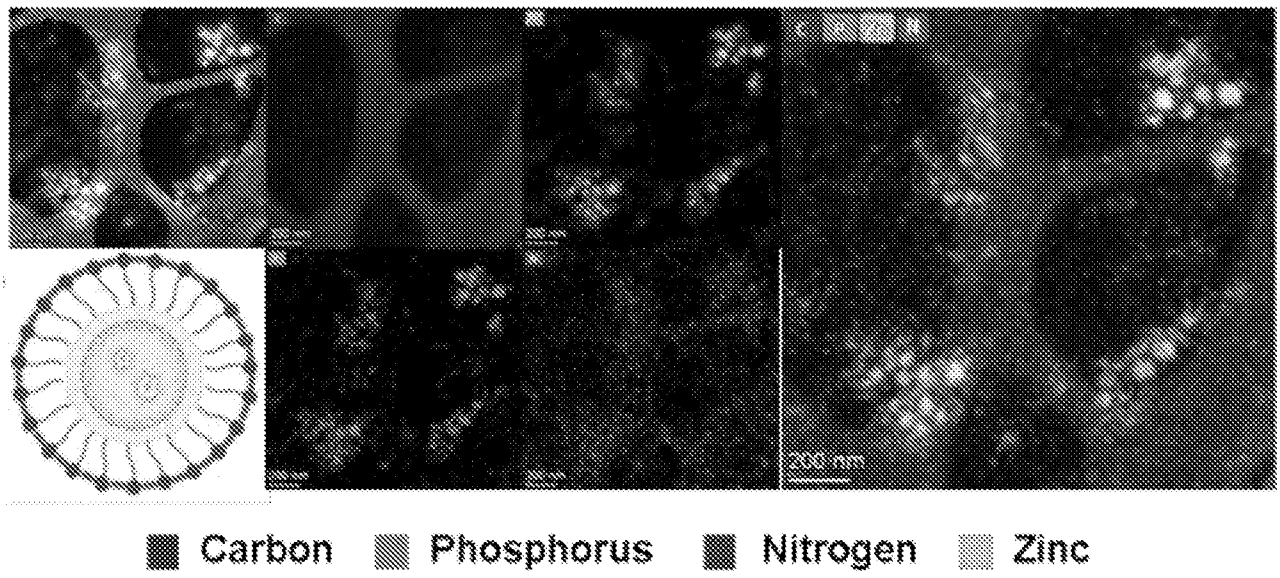

Carbon
Phosphorus
Nitrogen
Zinc

FIG. 3D

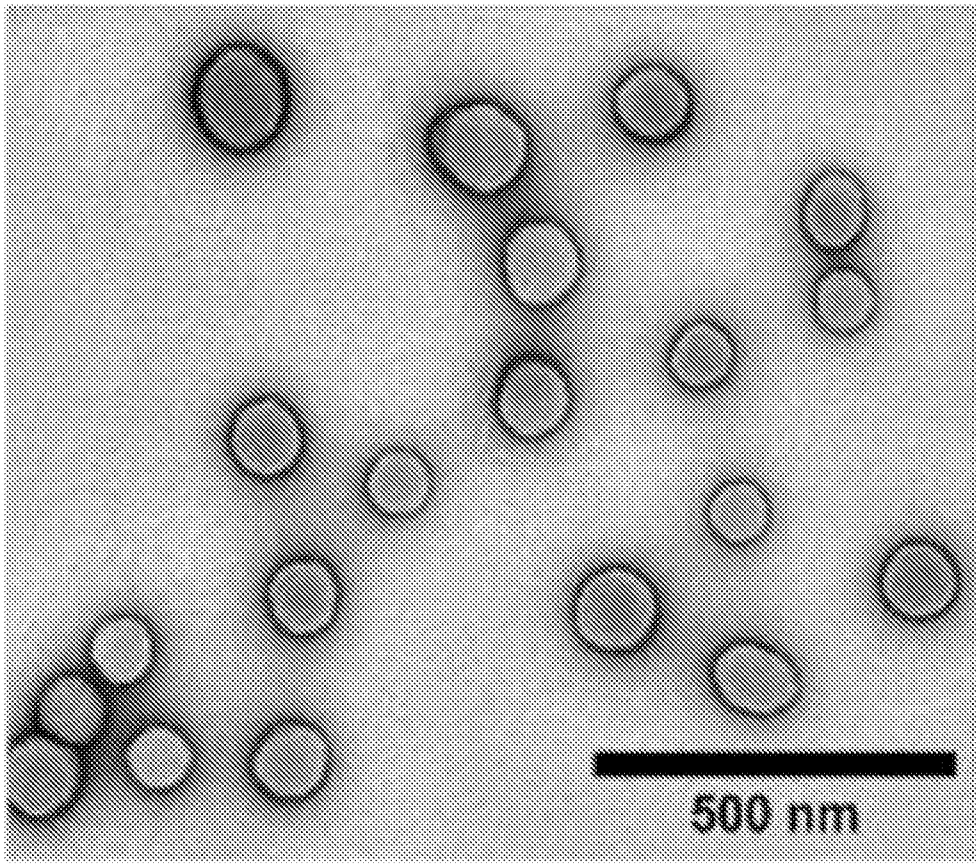


FIG. 3E

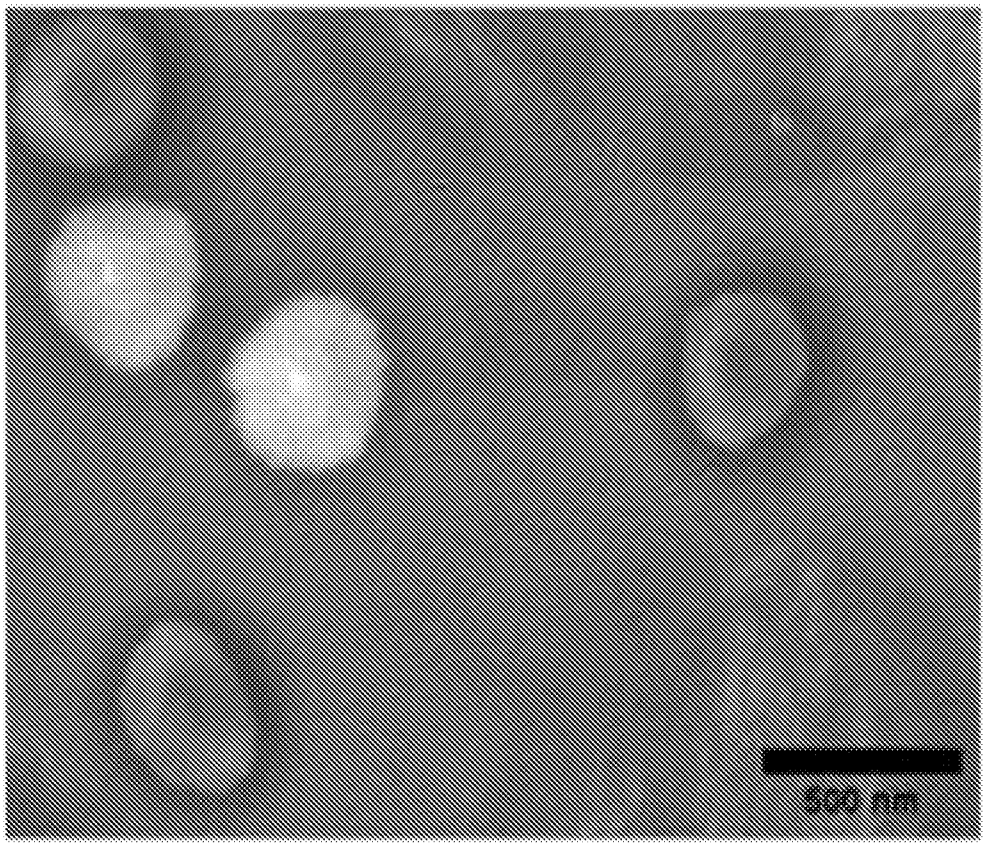


FIG. 3F

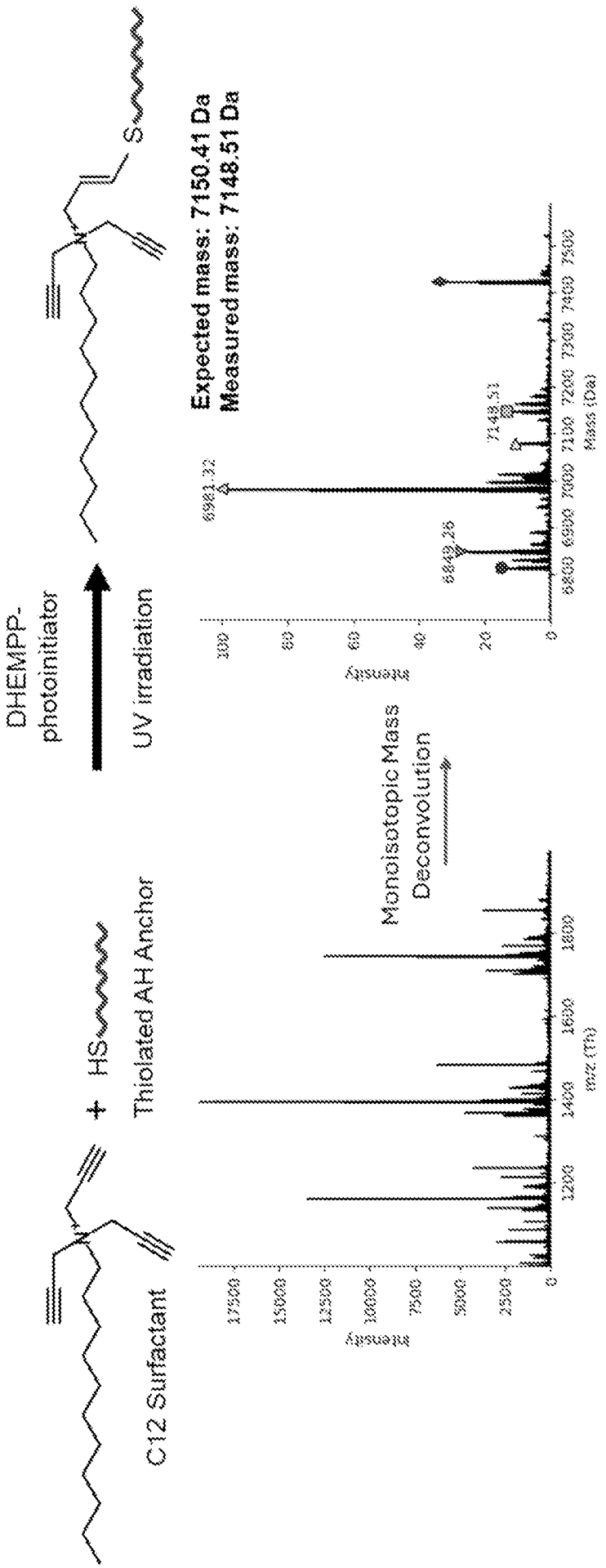

C12 Surfactant
+ HS
Thiolated AH Anchor
DHEMPP-photoinitiator
UV irradiation
Monoisotopic Mass Deconvolution
Expected mass: 7150.41 Da
Measured mass: 7148.51 Da
Intensity
m/z (Th)
Mass (Da)

FIG. 4

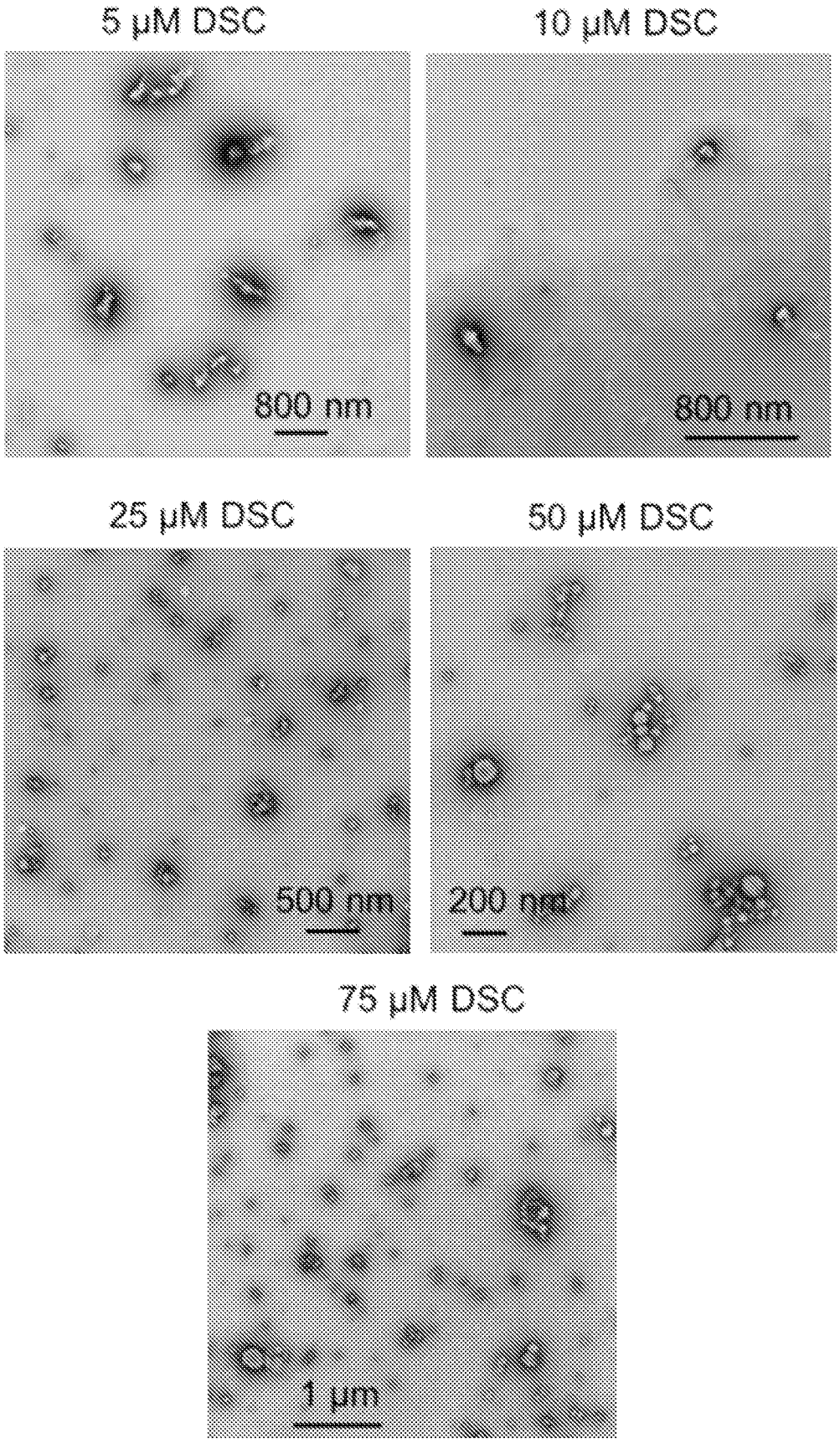

5 μM DSC
800 nm
10 μM DSC
800 nm
25 μM DSC
500 nm
50 μM DSC
200 nm
75 μM DSC
1 μm

FIG. 5

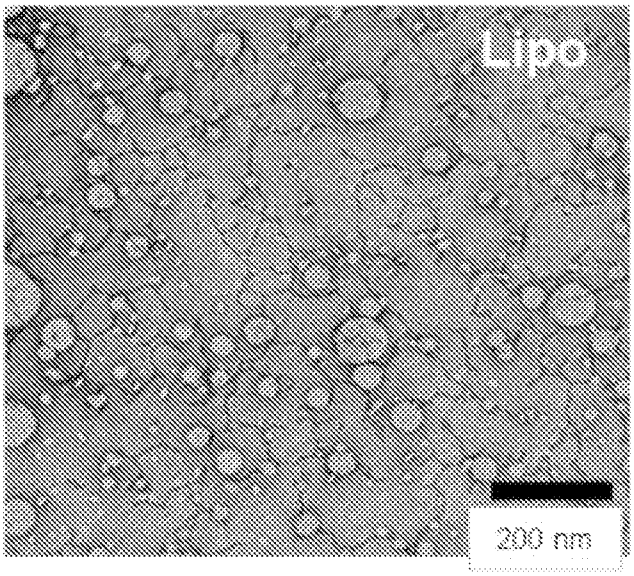


FIG. 6A

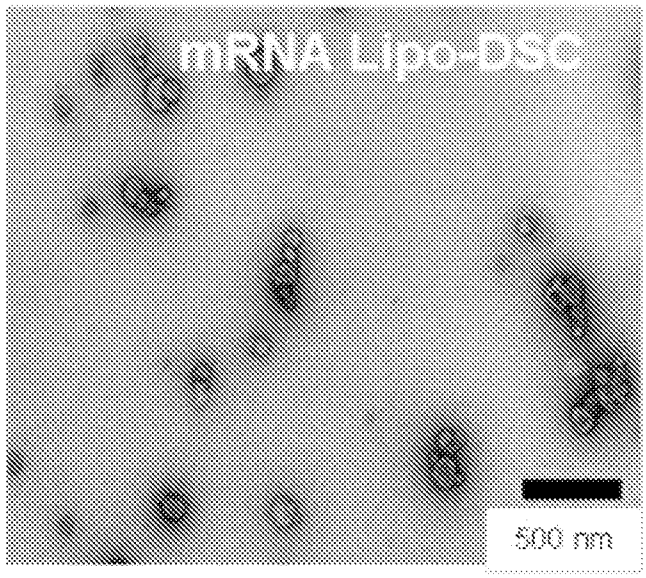


FIG. 6B

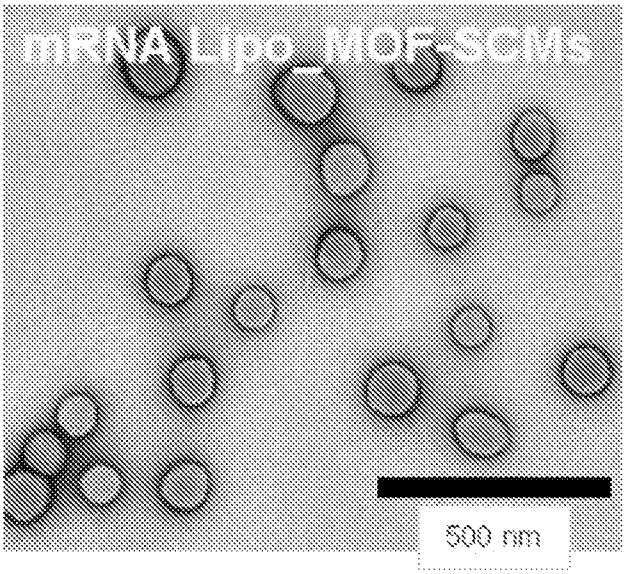


FIG. 6C

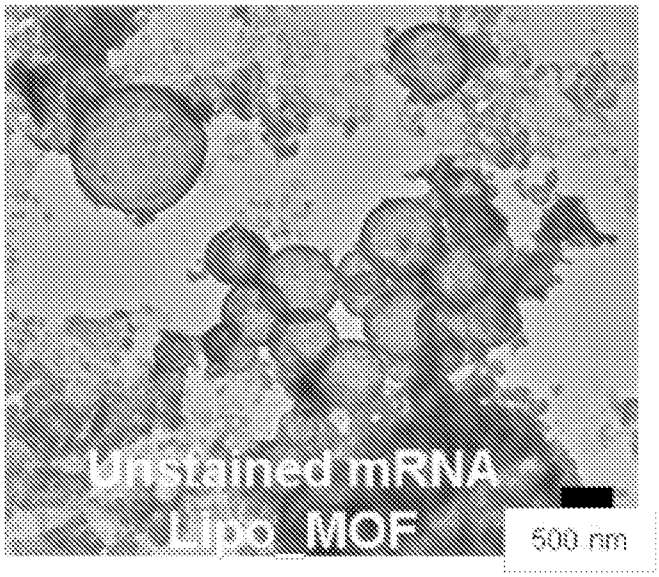


FIG. 6D

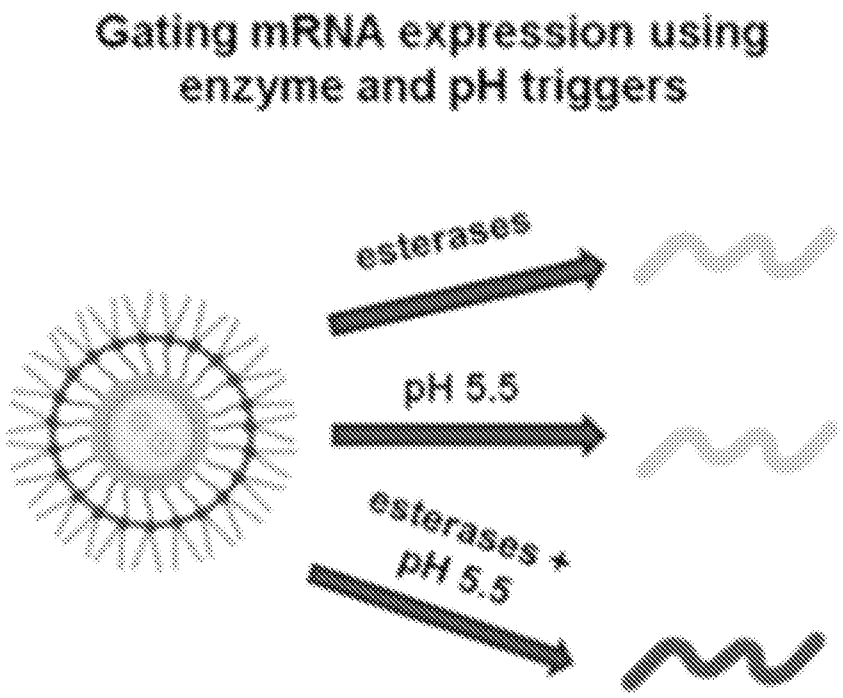

Gating mRNA expression using enzyme and pH triggers
esterases
pH 5.5
esterases + pH 5.5

FIG. 7A

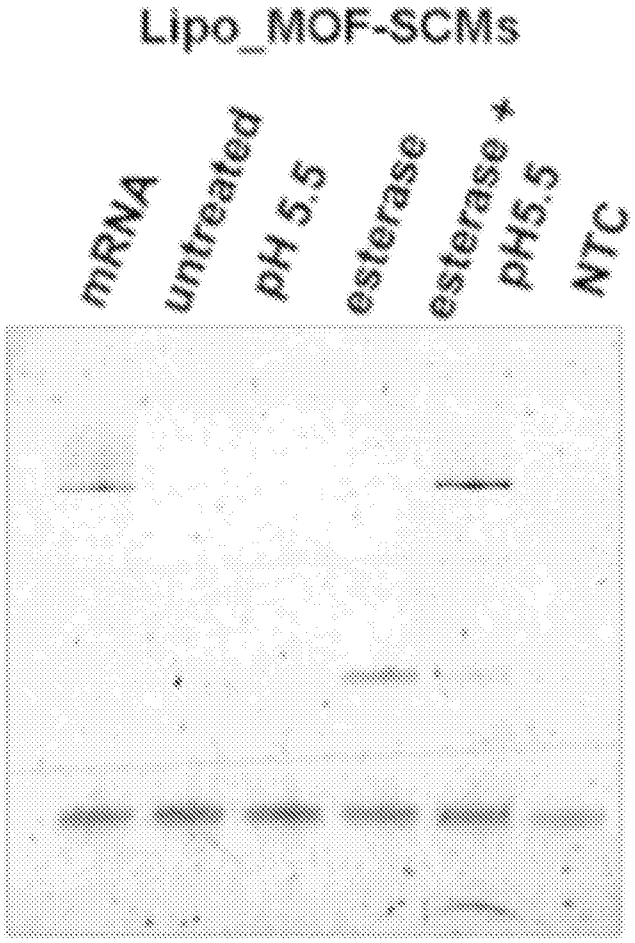

Lipo_MOF-SCMs
mRNA
untreated
pH 5.5
esterase
esterase + pH5.5
NTC

FIG. 7B

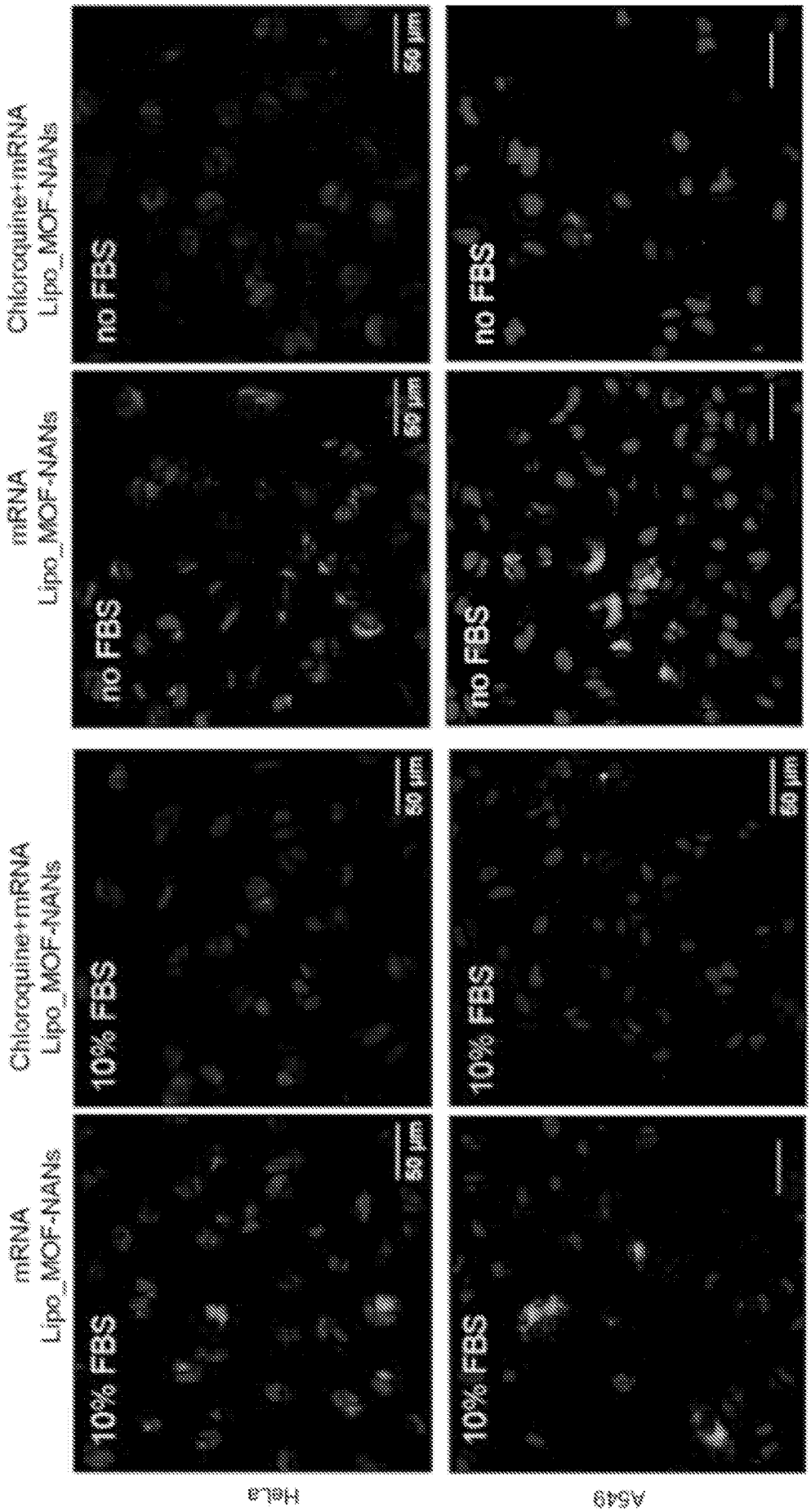

mRNA
Lipo_MOF-NANs
Chloroquine+mRNA
Lipo_MOF-NANs
mRNA
Lipo_MOF-NANs
Chloroquine+mRNA
Lipo_MOF-NANs
10% FBS
10% FBS
no FBS
no FBS
50 μm
HeLa
A549

FIG. 7C

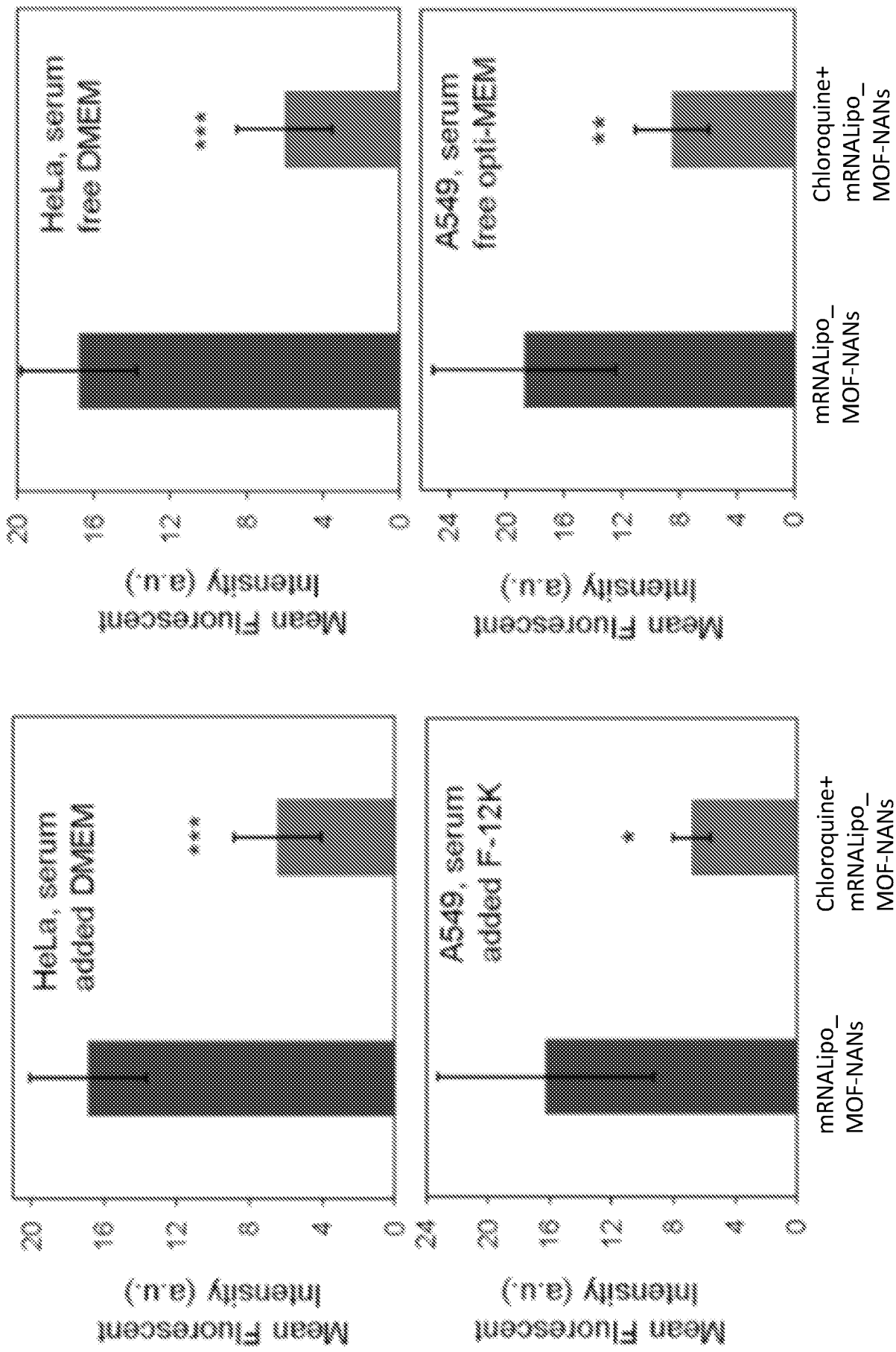
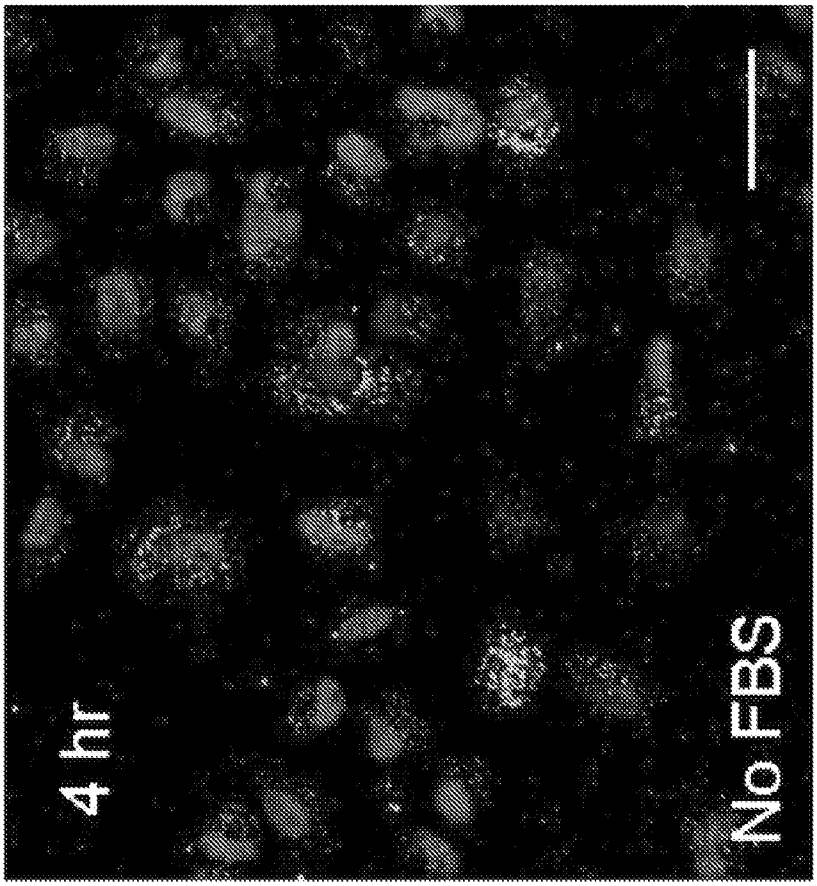

HeLa, serum added DMEM
A549, serum added F-12K
HeLa, serum free DMEM
A549, serum free opti-MEM
Mean Fluorescent Intensity (a.u.)
mRNALipo_ MOF-NANs
Chloroquine+ mRNALipo_ MOF-NANs
*
**
***

FIG. 7D



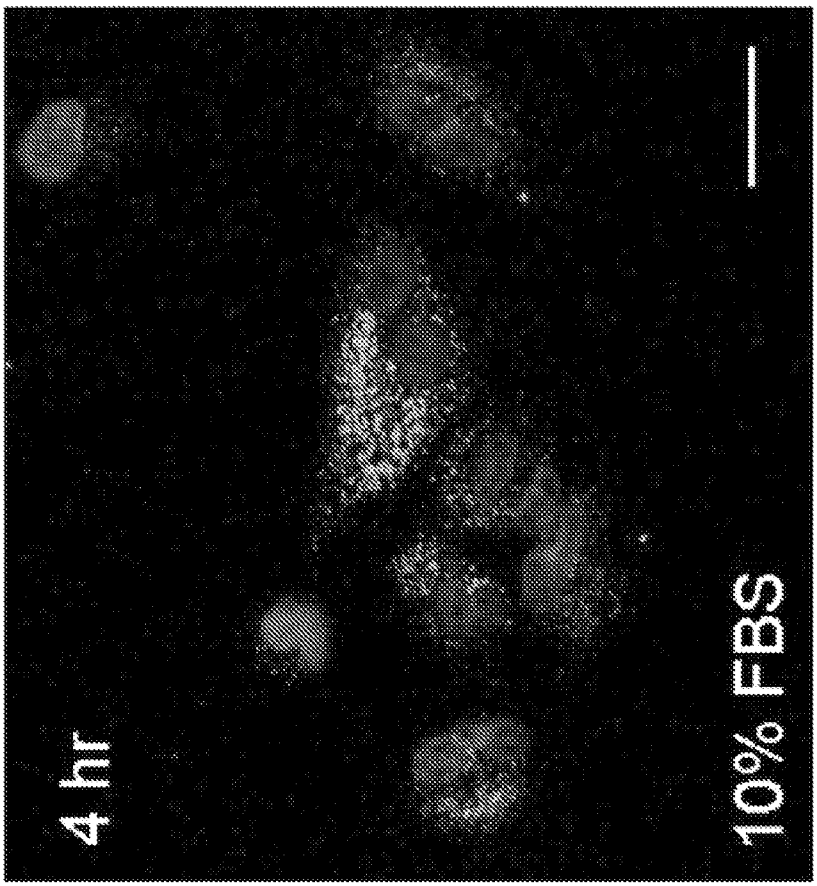


FIG. 8

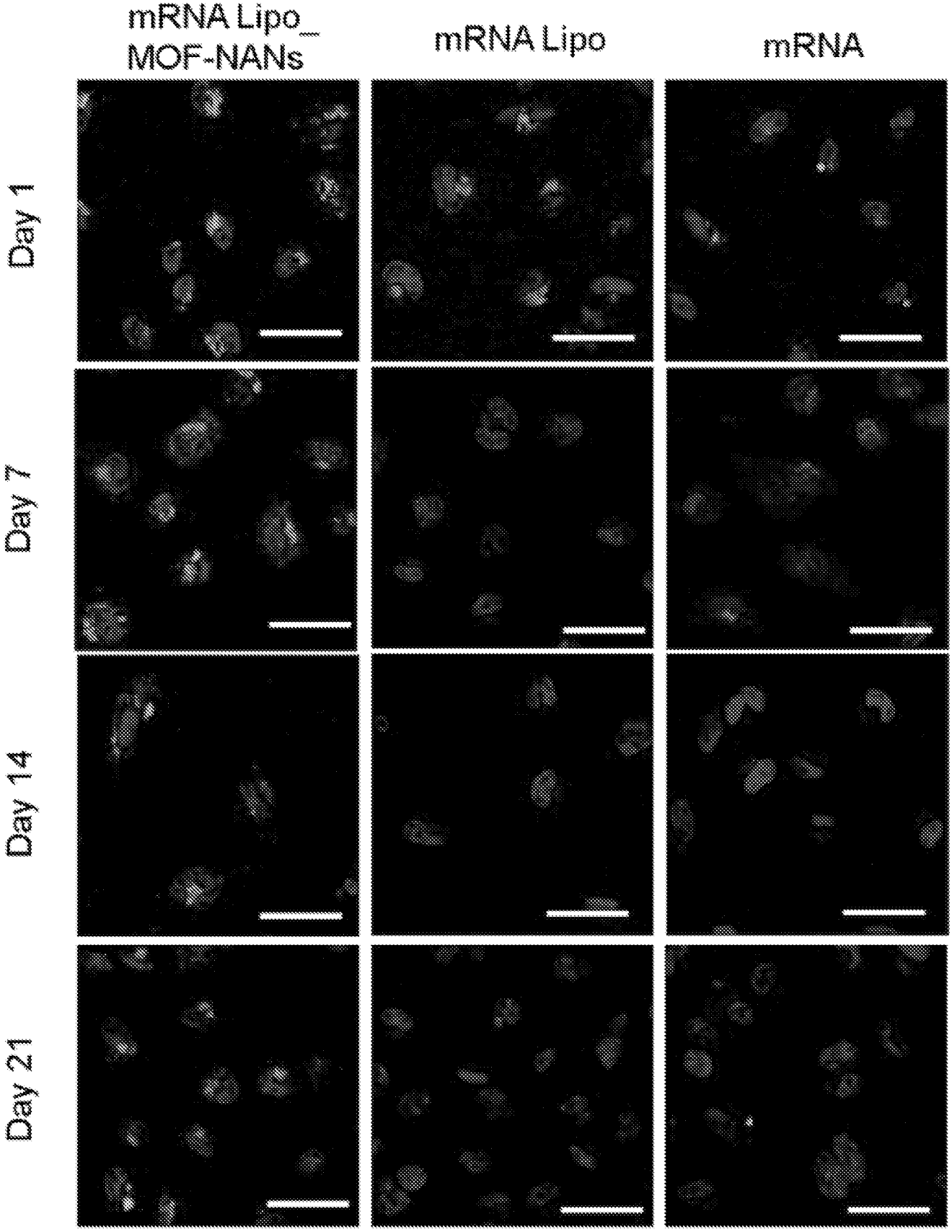


FIG. 9A

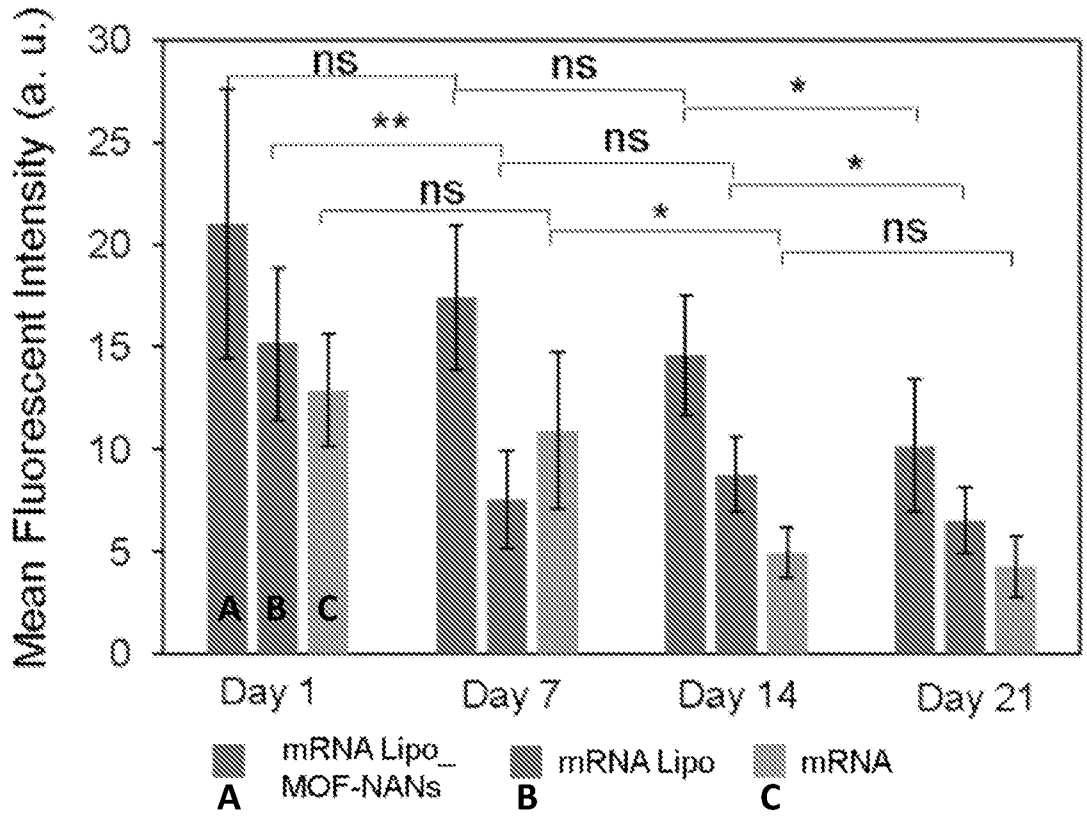

Mean Fluorescent Intensity (a. u.)
30
25
20
15
10
5
0
ns
ns
*
**
ns
*
ns
*
ns
A
B
C
Day 1
Day 7
Day 14
Day 21
mRNA Lipo_ MOF-NANs
A
mRNA Lipo
B
mRNA
C

FIG. 9B

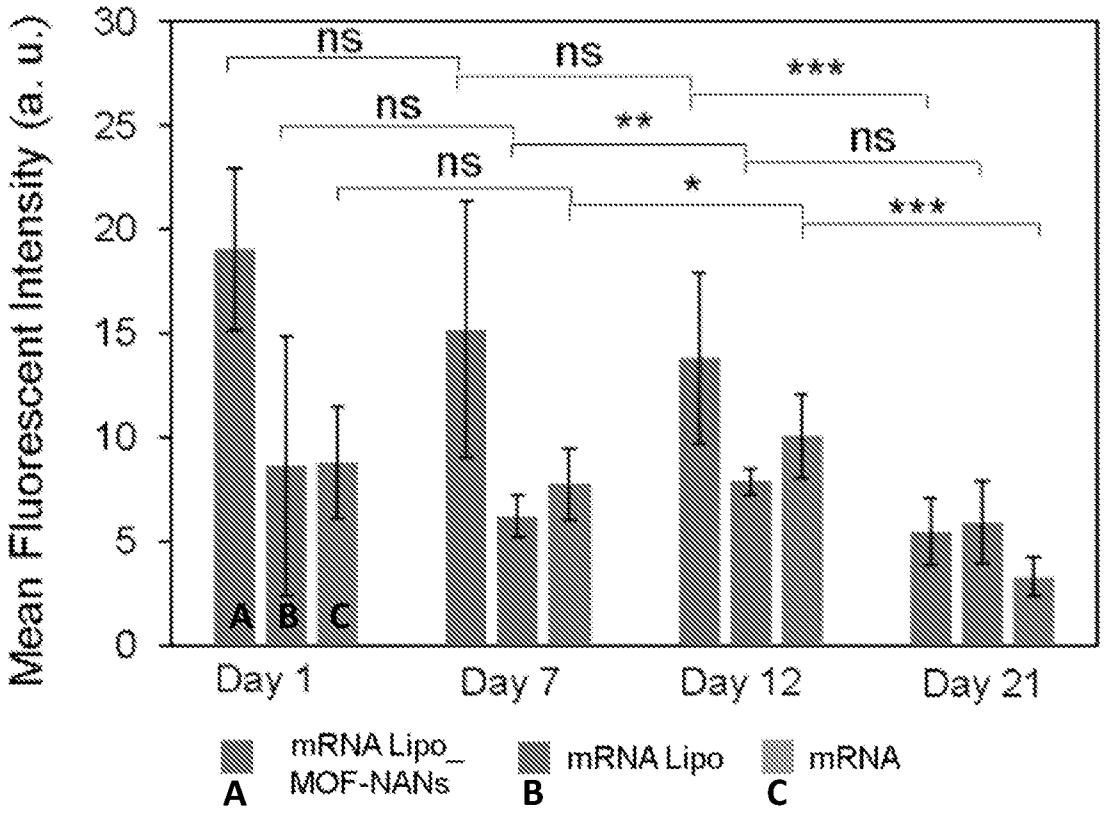

Mean Fluorescent Intensity (a. u.)
30
25
20
15
10
5
0
ns
ns
***
ns
**
ns
ns
*
***
A
B
C
Day 1
Day 7
Day 12
Day 21
mRNA Lipo_ MOF-NANs
A
mRNA Lipo
B
mRNA
C

FIG. 9C

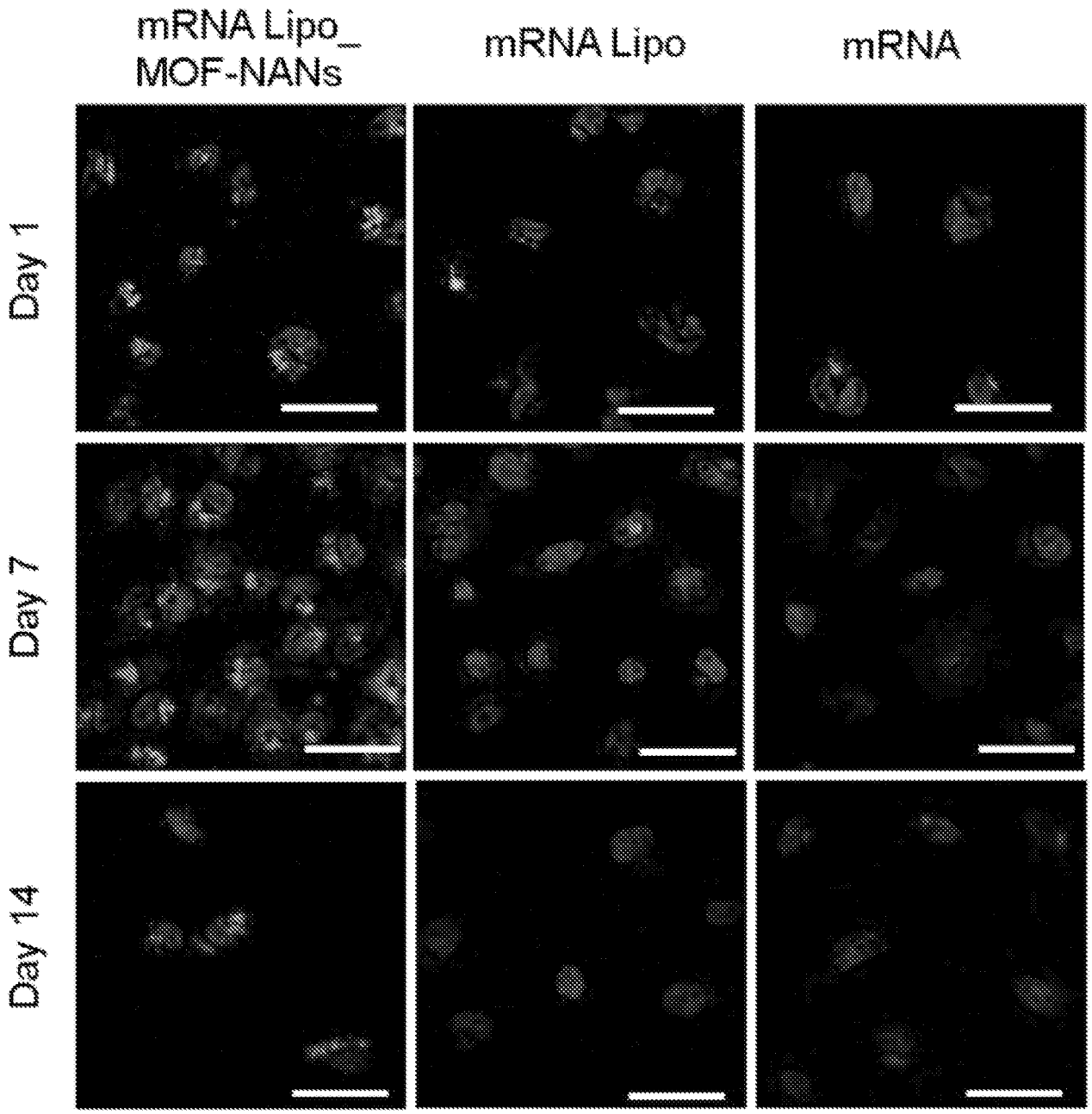


FIG. 10A

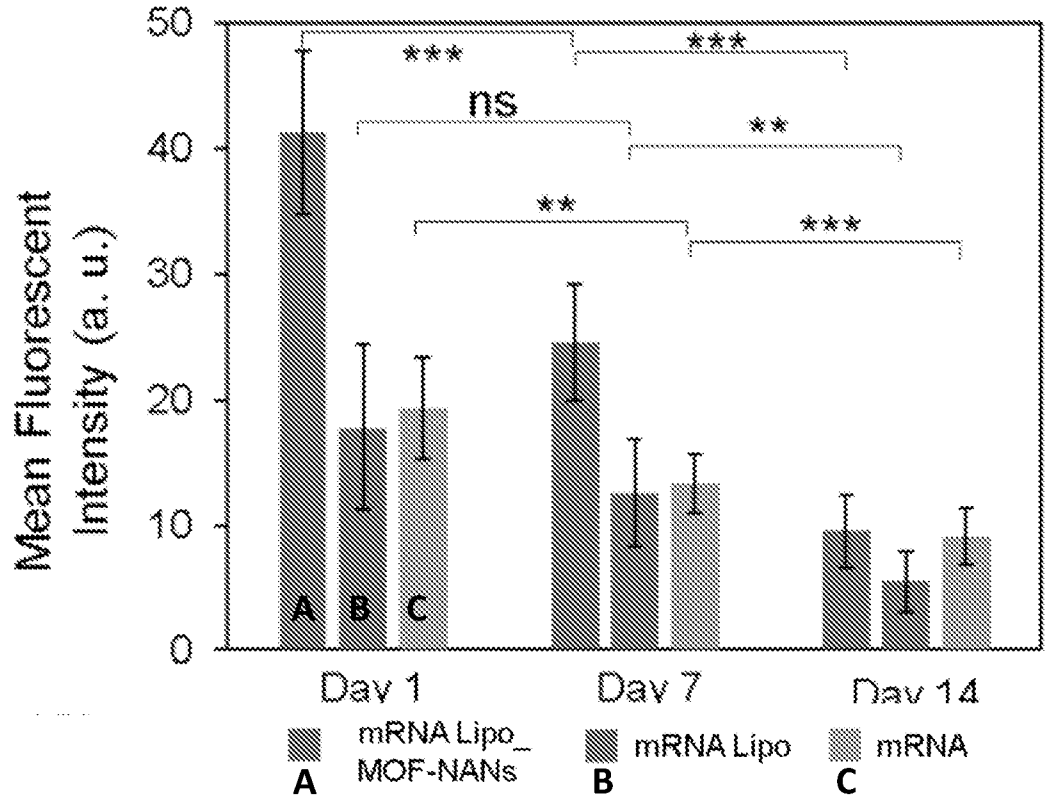


FIG. 10B

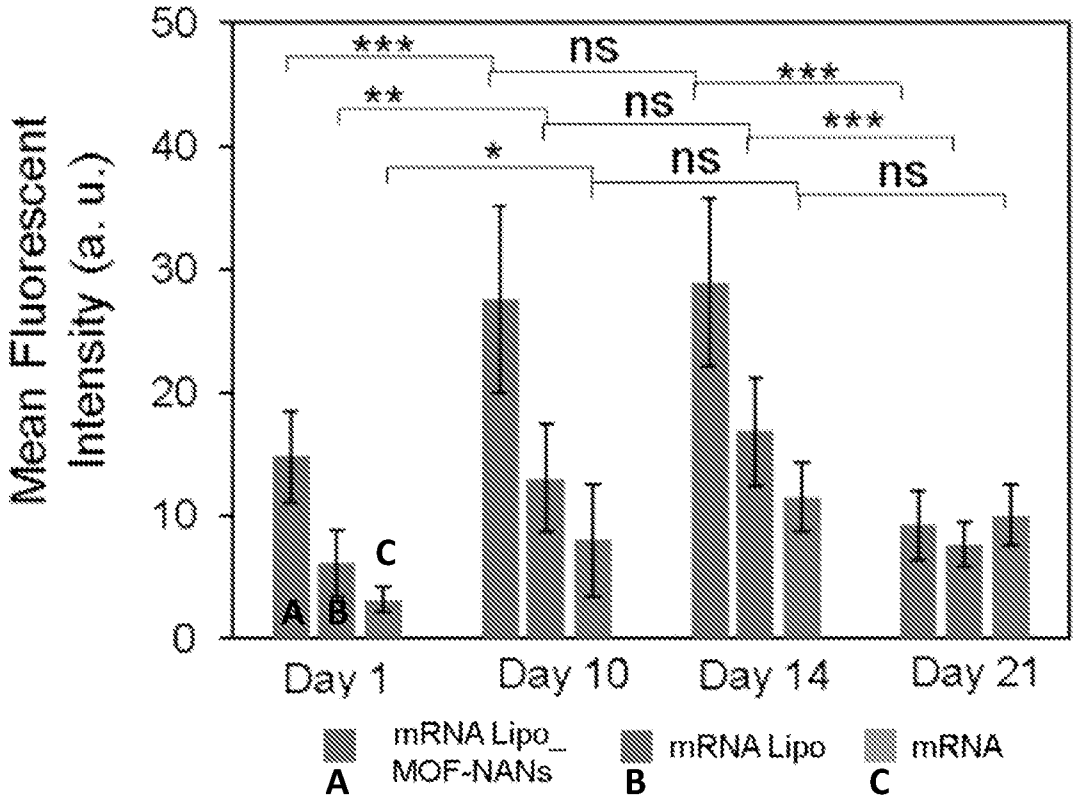


FIG. 10C

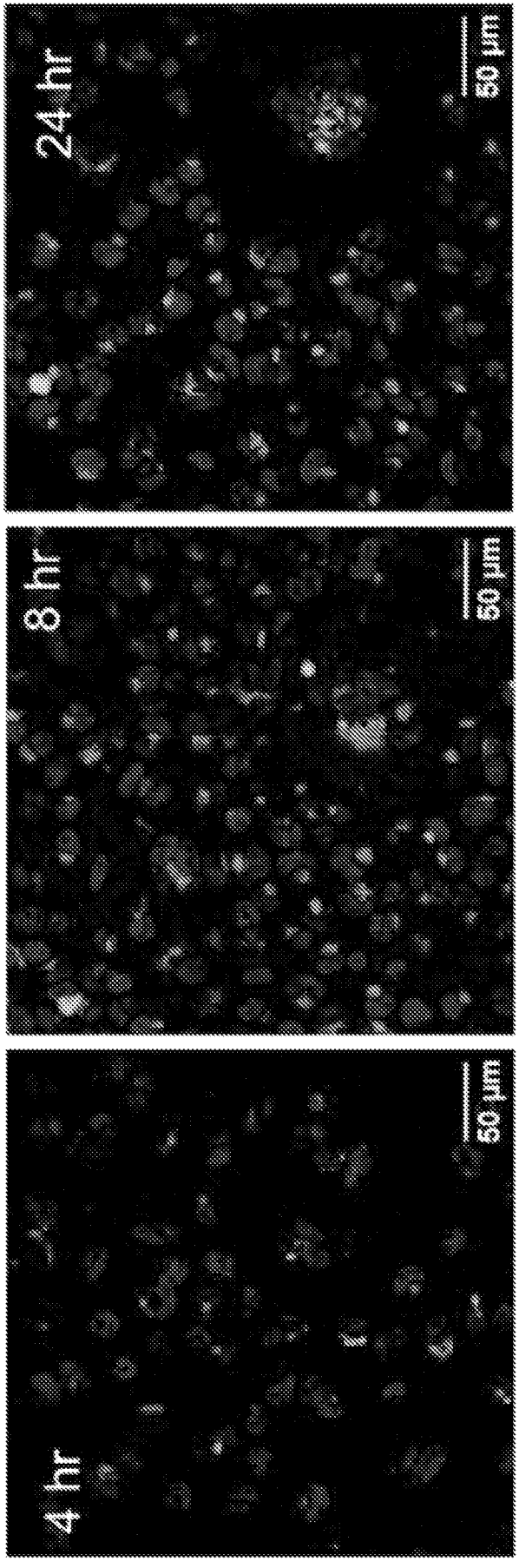


FIG. 11A

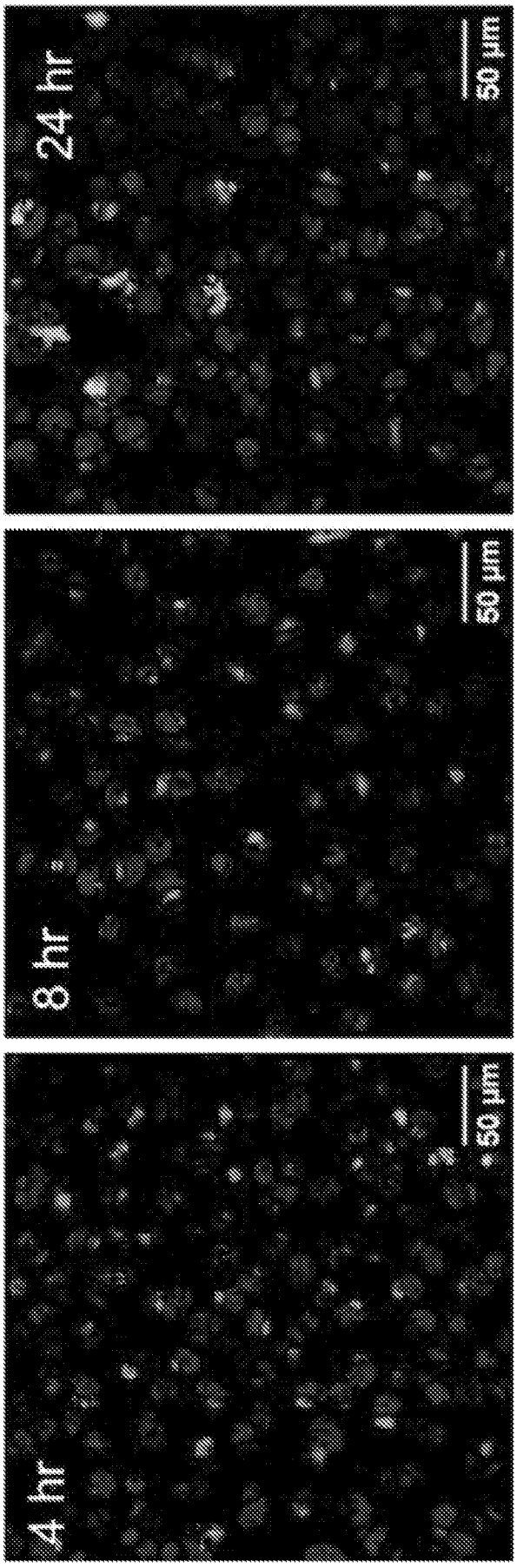


FIG. 11B

FIG. 11C

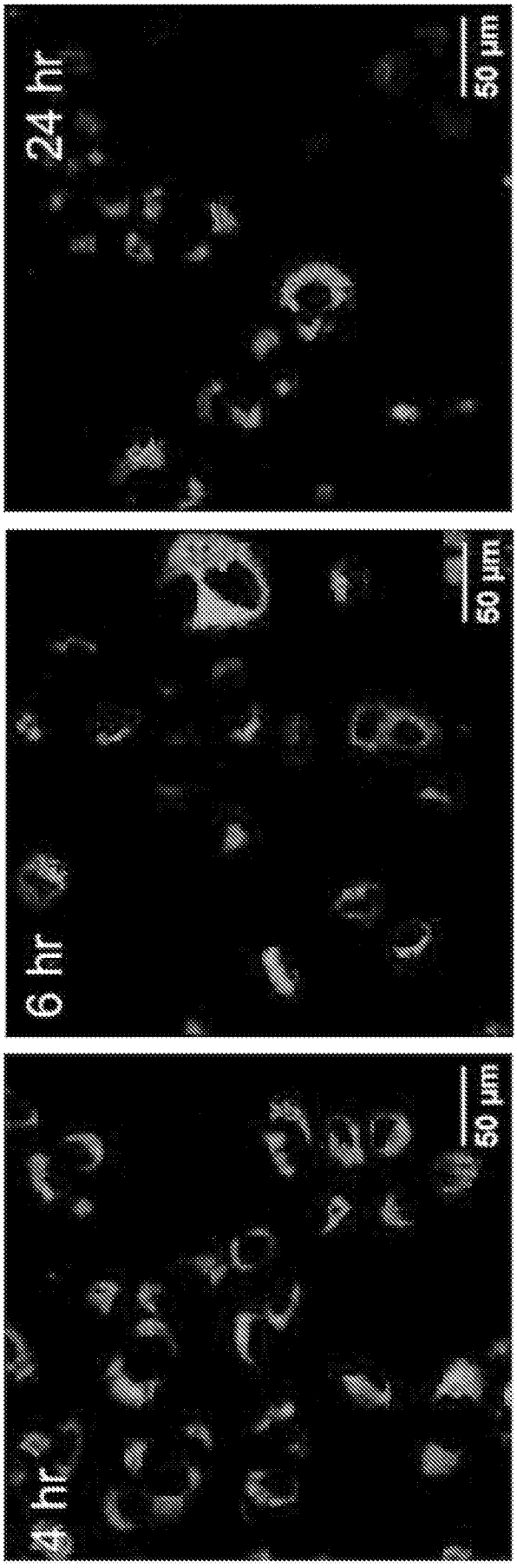


FIG. 11D

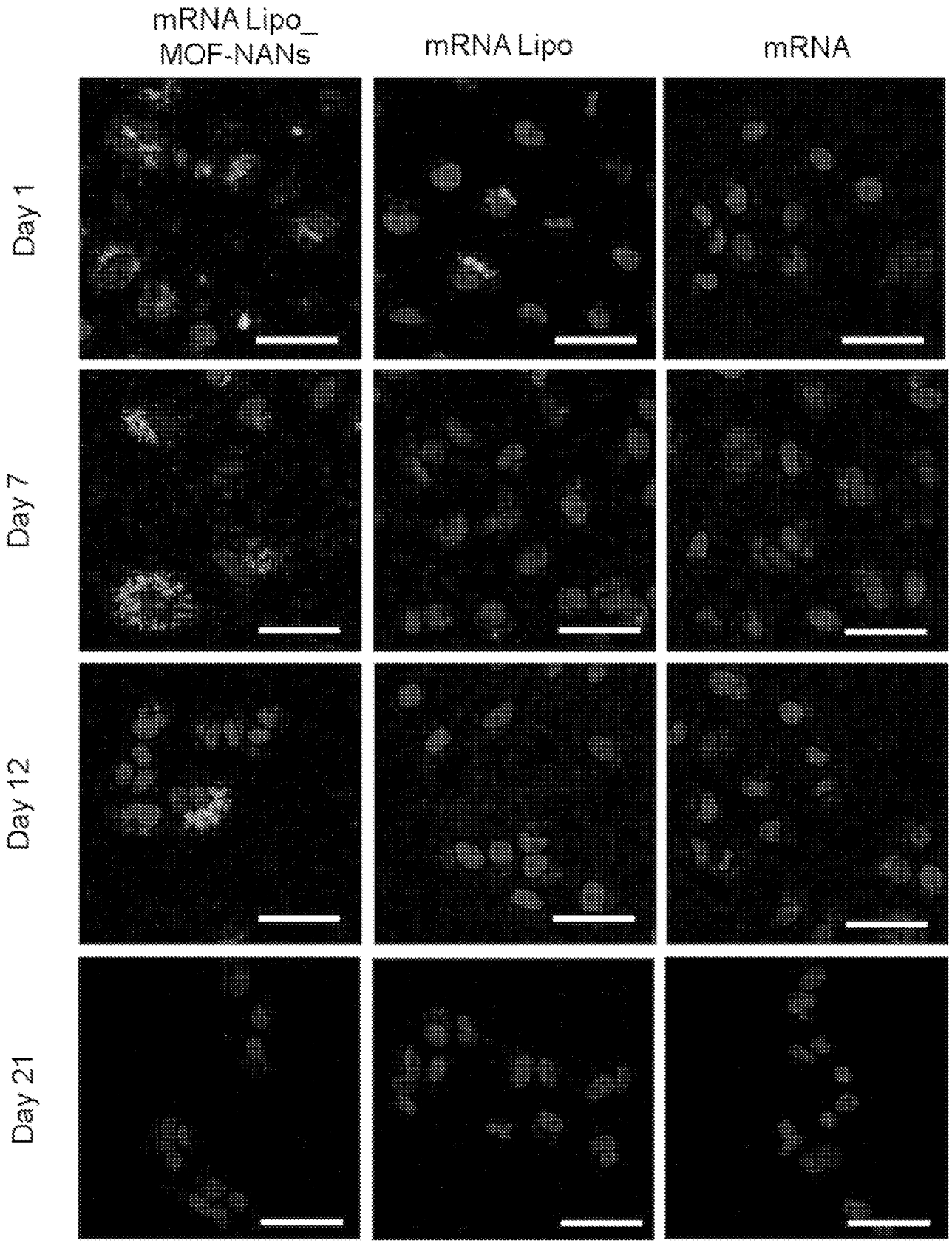


FIG. 12

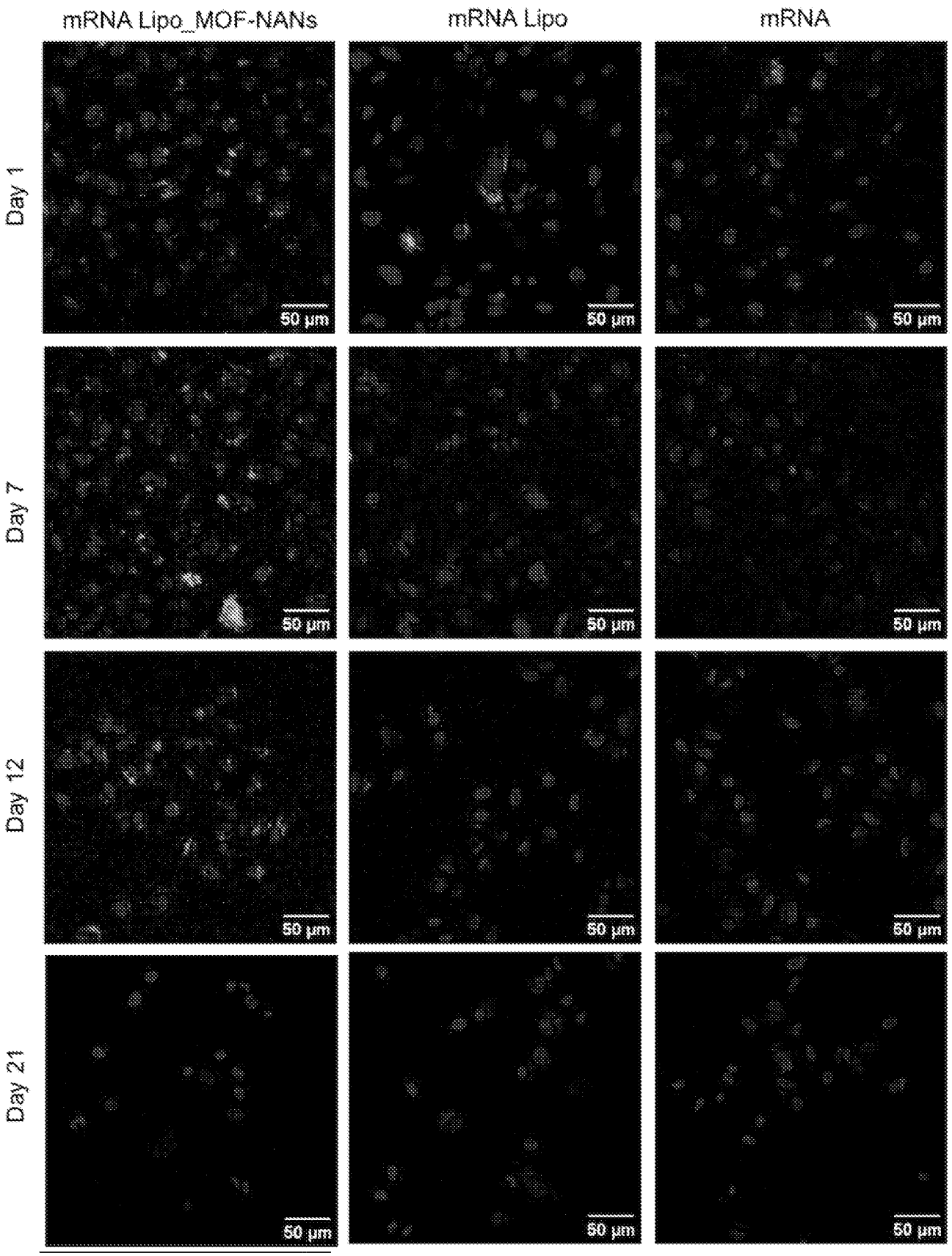


FIG. 13

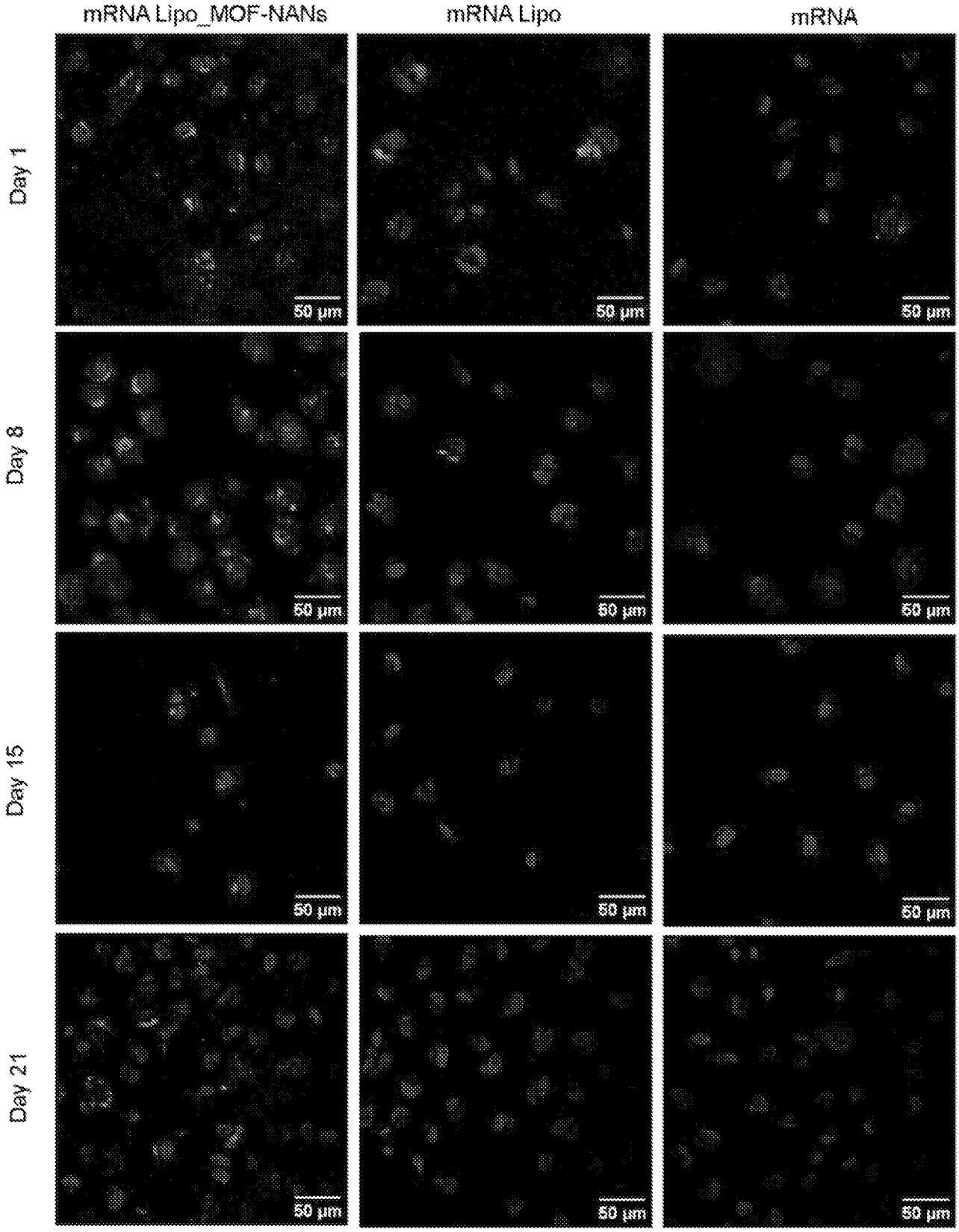


FIG. 14

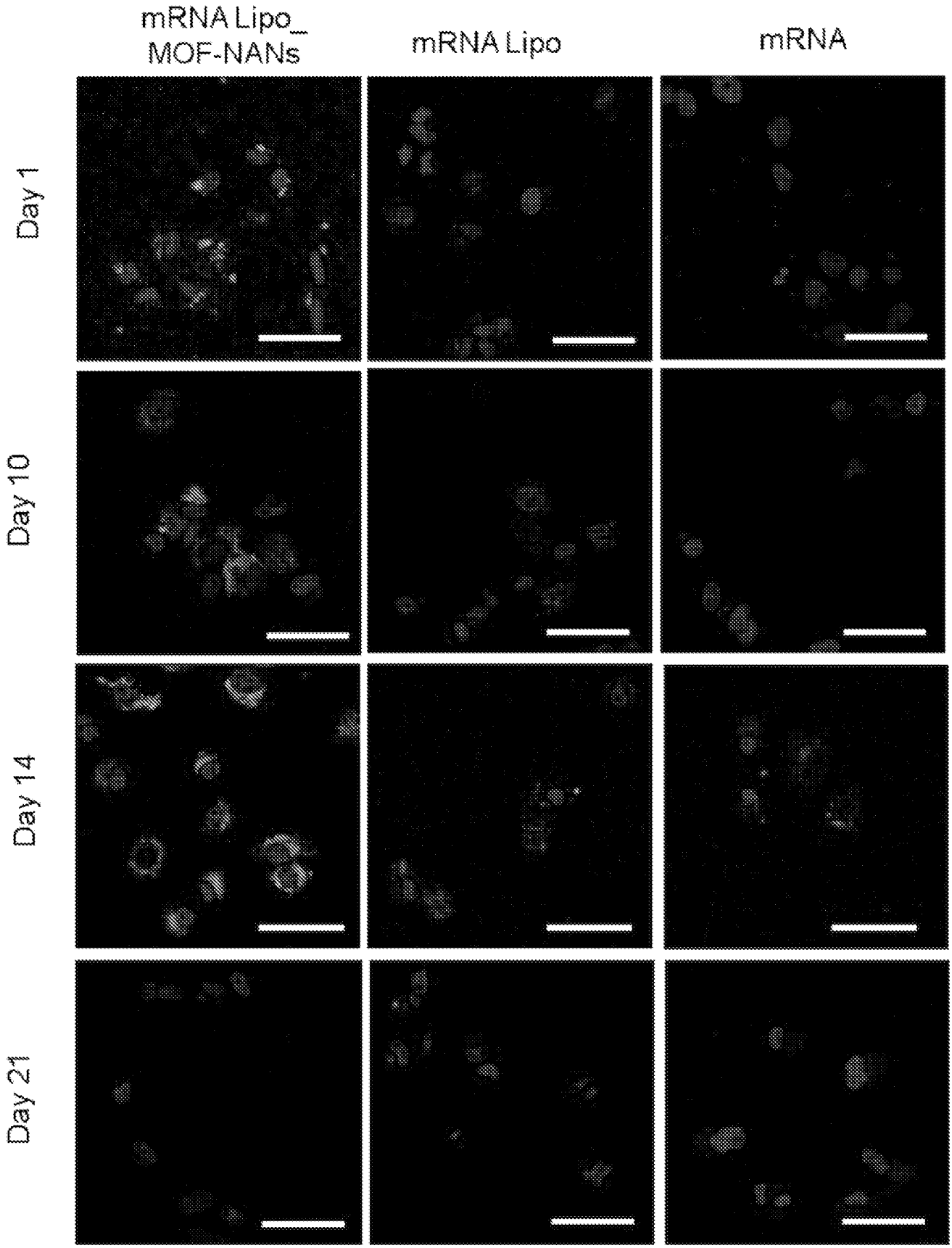


FIG. 15

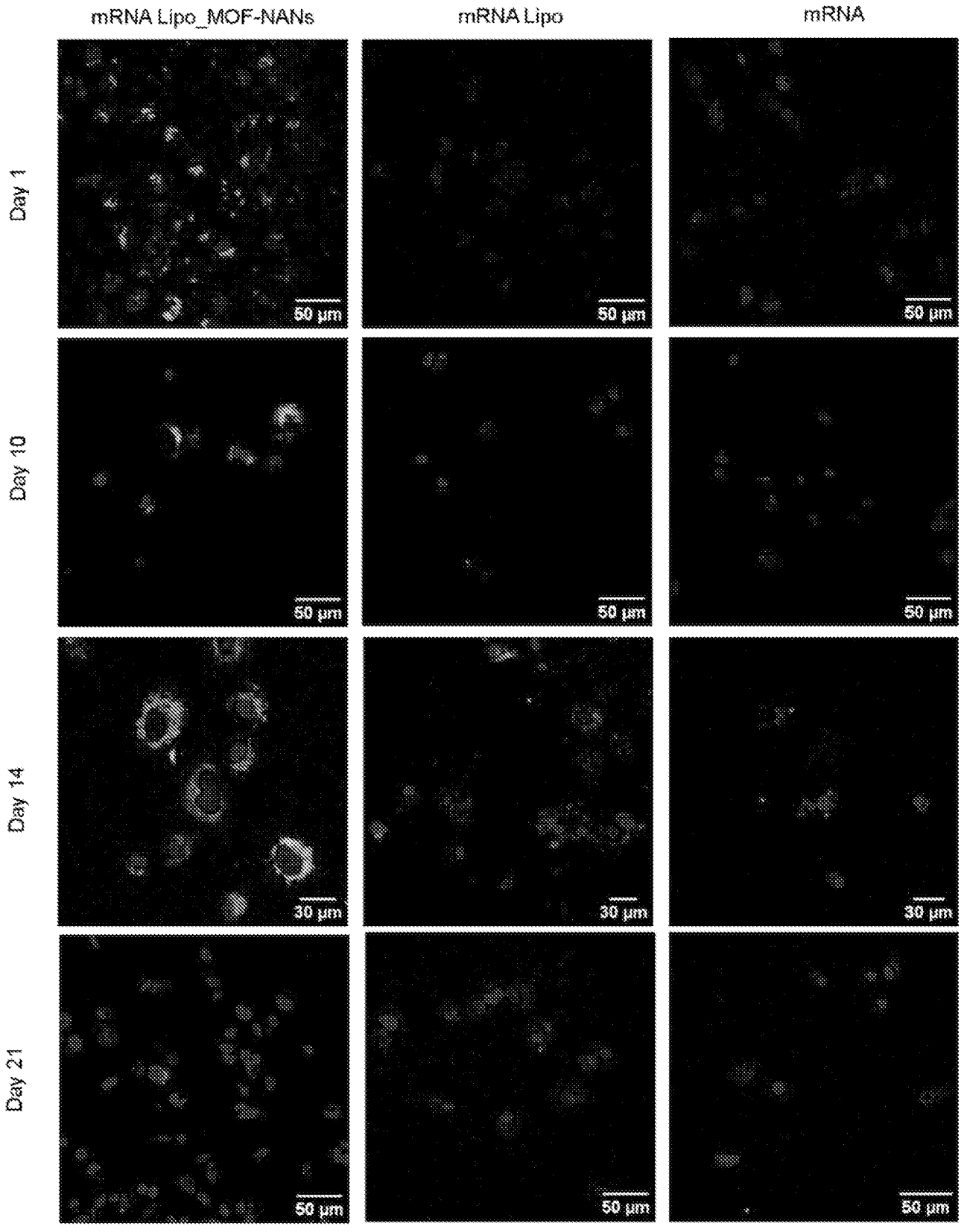

mRNA Lipo_MOF-NANs
mRNA Lipo
mRNA
Day 1
Day 10
Day 14
Day 21
50 μm
30 μm

FIG. 16

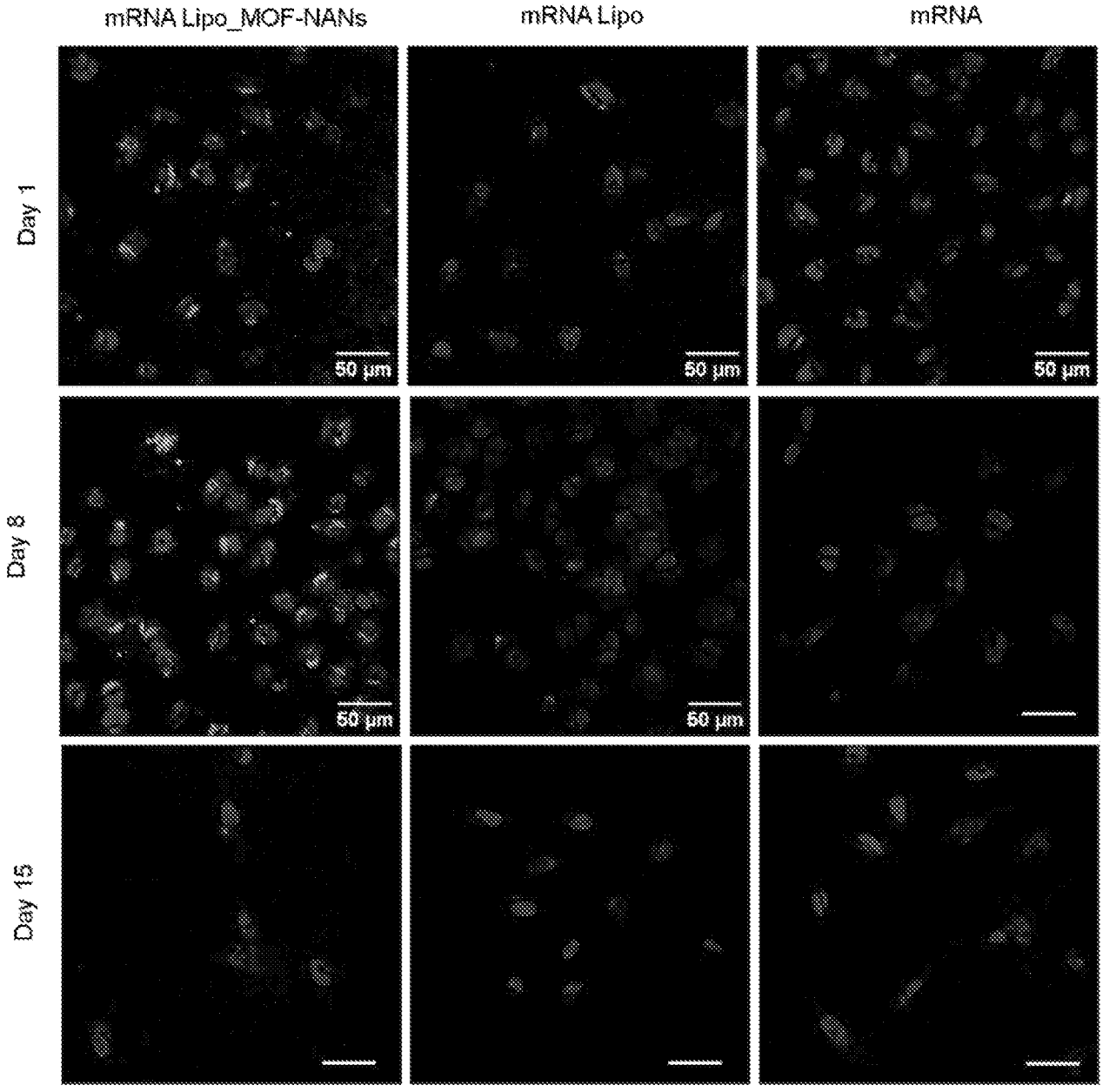

mRNA Lipo_MOF-NANs
mRNA Lipo
mRNA
Day 1
Day 8
Day 15
50 μm
50 μm
50 μm
50 μm
50 μm

FIG. 17

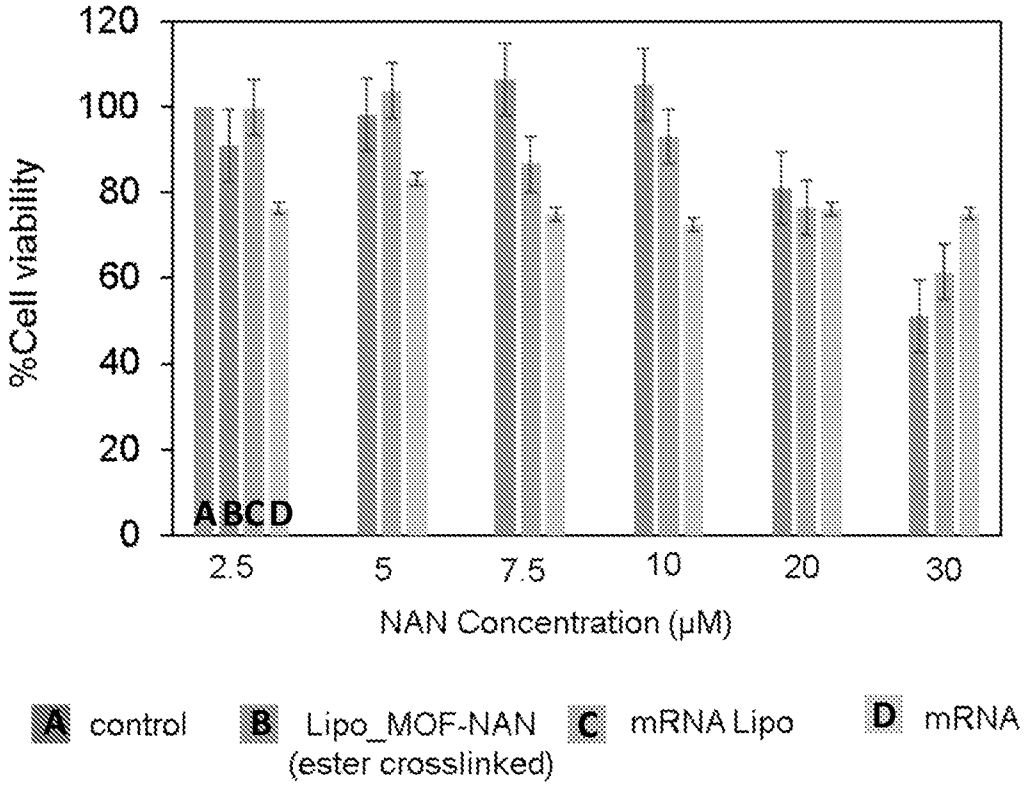


FIG. 18A

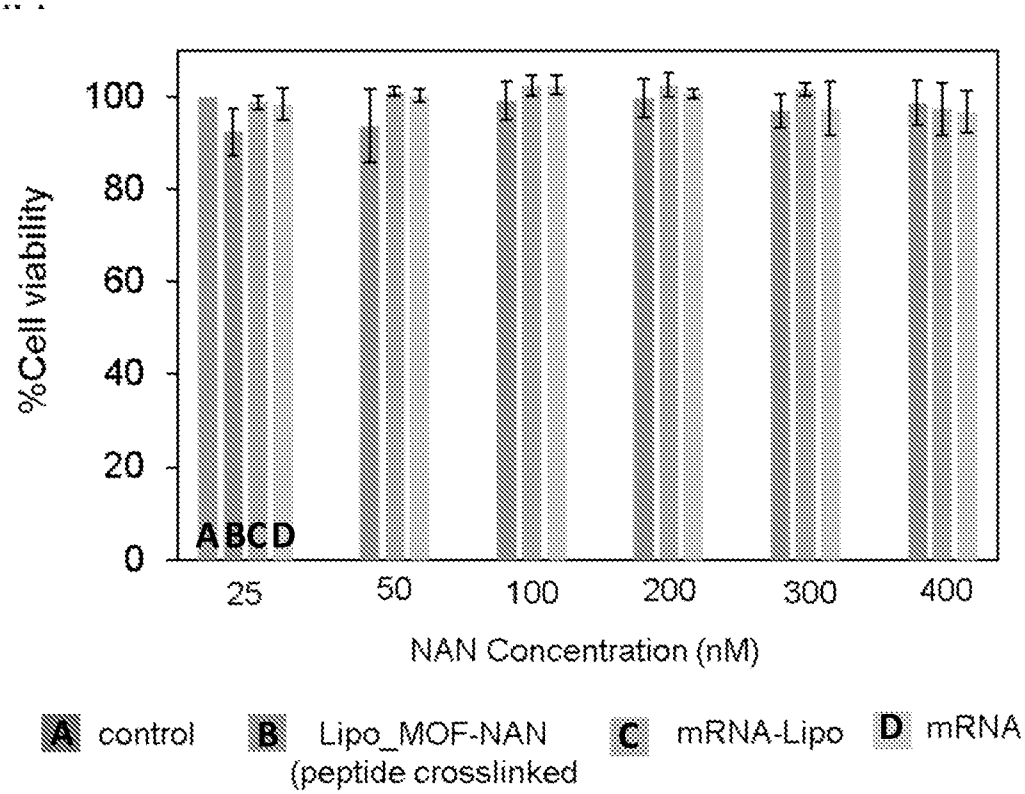
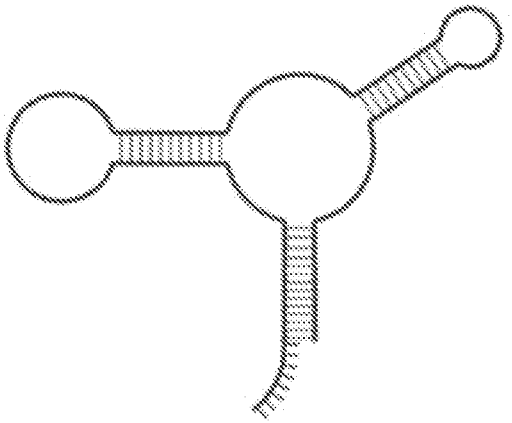


FIG. 18B



ACE-4 Aptamer

ACE4*: 5'-
GGGAGAUGAUCCGUUGA
UGCGAGGGAACGCAAGA
ACUGAGGCCAUGAGGCG
CCUUCCCUUGCUCAGGA
CGCAAGUCGUCGUUCGU
AGGCAGAAUC-3'
*Chemically modified for serum stability

FIG. 19A

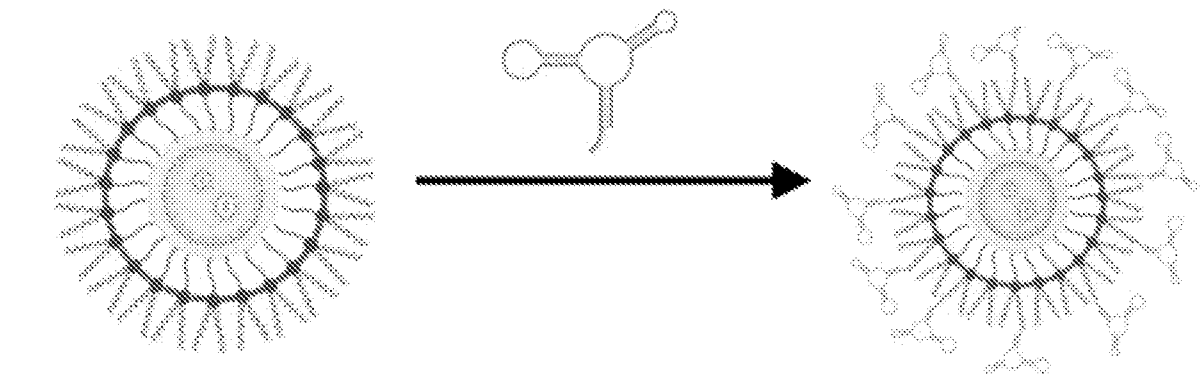


FIG. 19B

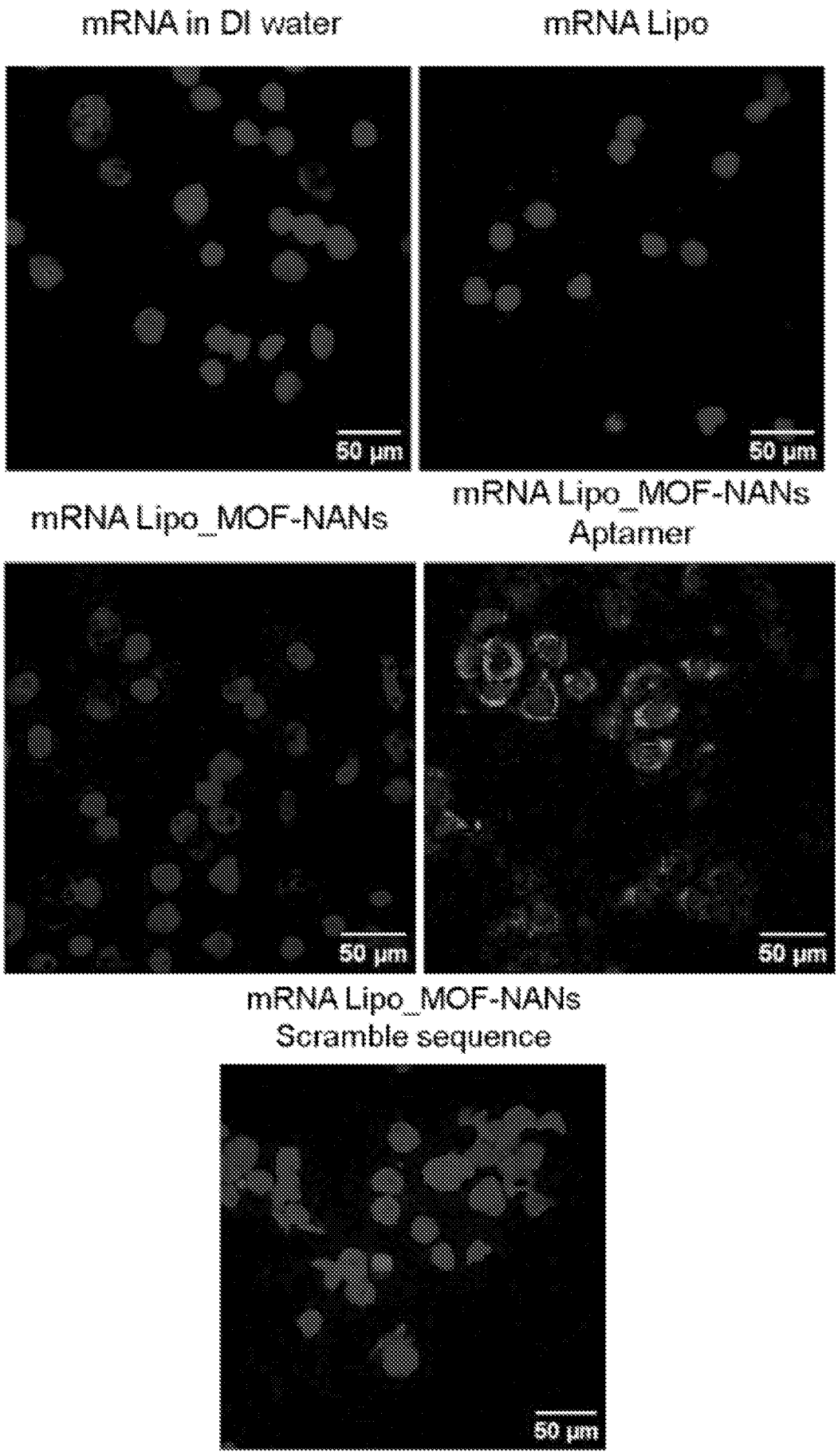
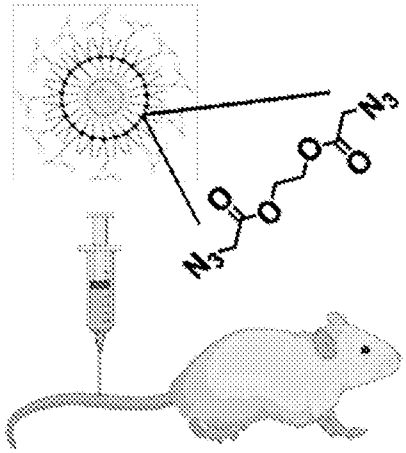
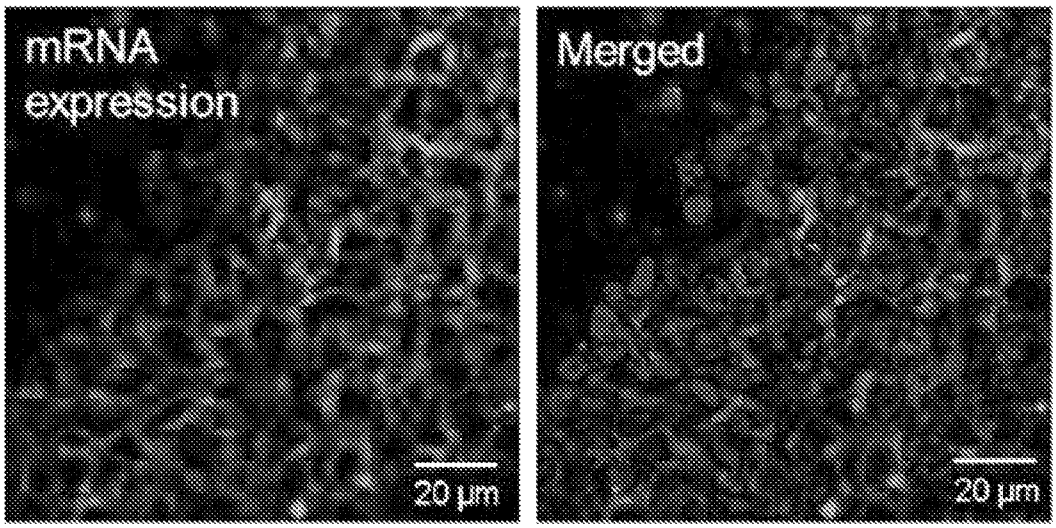

mRNA in DI water
mRNA Lipo
50 µm
50 µm
mRNA Lipo_MOF-NANs
mRNA Lipo_MOF-NANs
Aptamer
50 µm
50 µm
mRNA Lipo_MOF-NANs
Scramble sequence
50 µm

FIG. 19C



MCF7 tumor
bearing mice

mRNA Lipo_MOF-NANs Aptamer



mRNA Lipo_MOF-NANs Scramble sequence

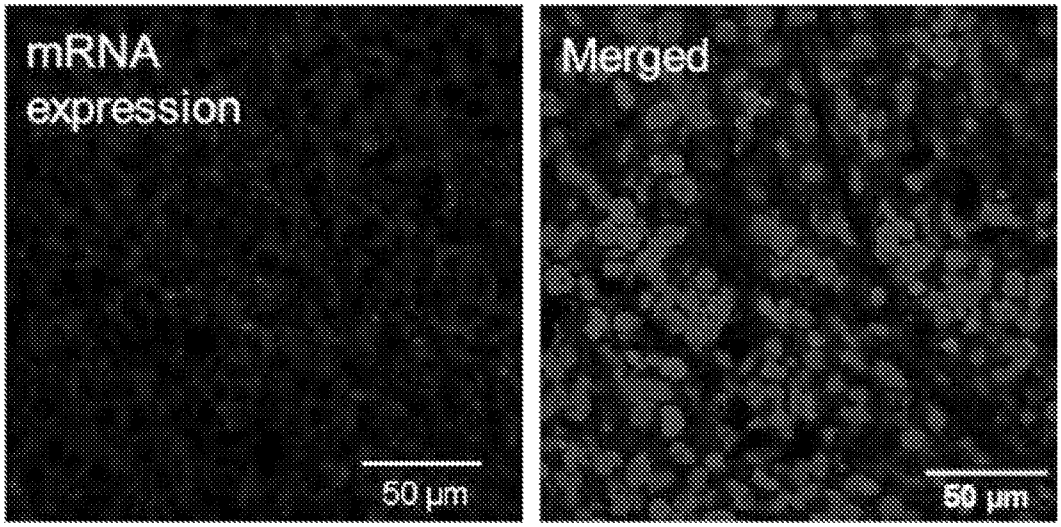


FIG. 19D

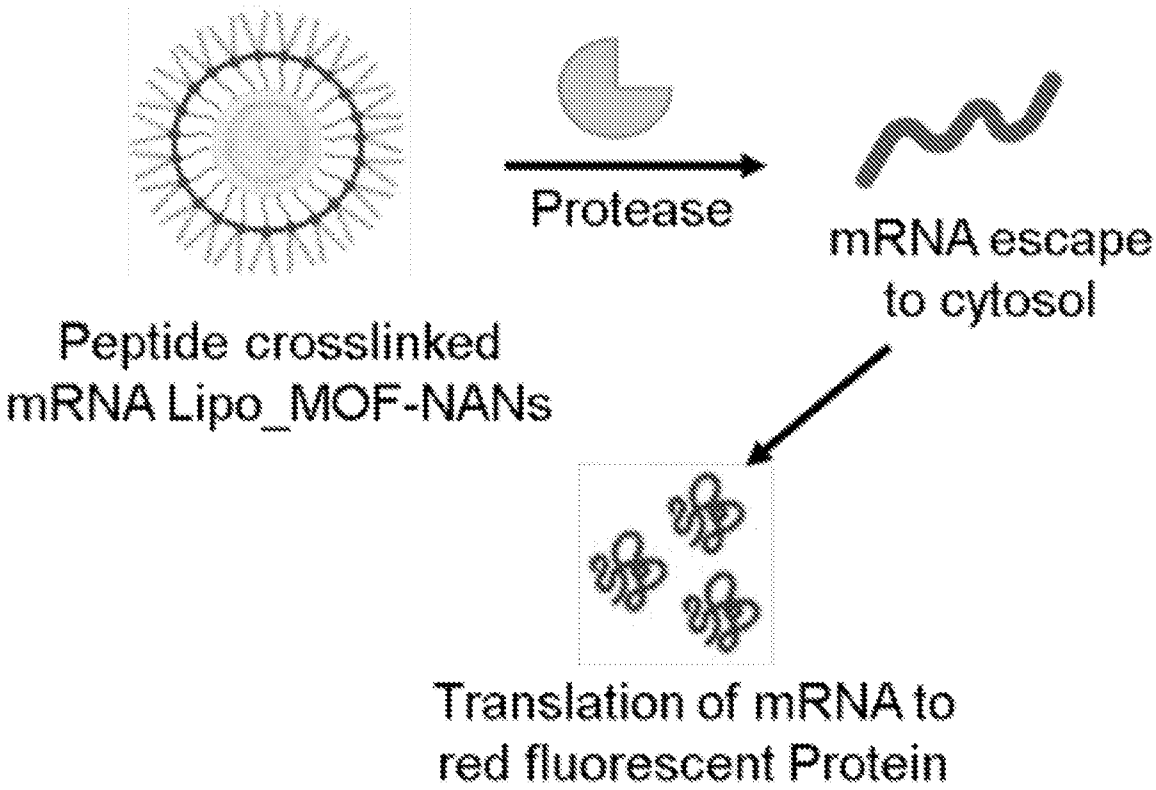

Protease
mRNA escape
to cytosol
Peptide crosslinked
mRNA Lipo_MOF-NANs
Translation of mRNA to
red fluorescent Protein

FIG. 20A

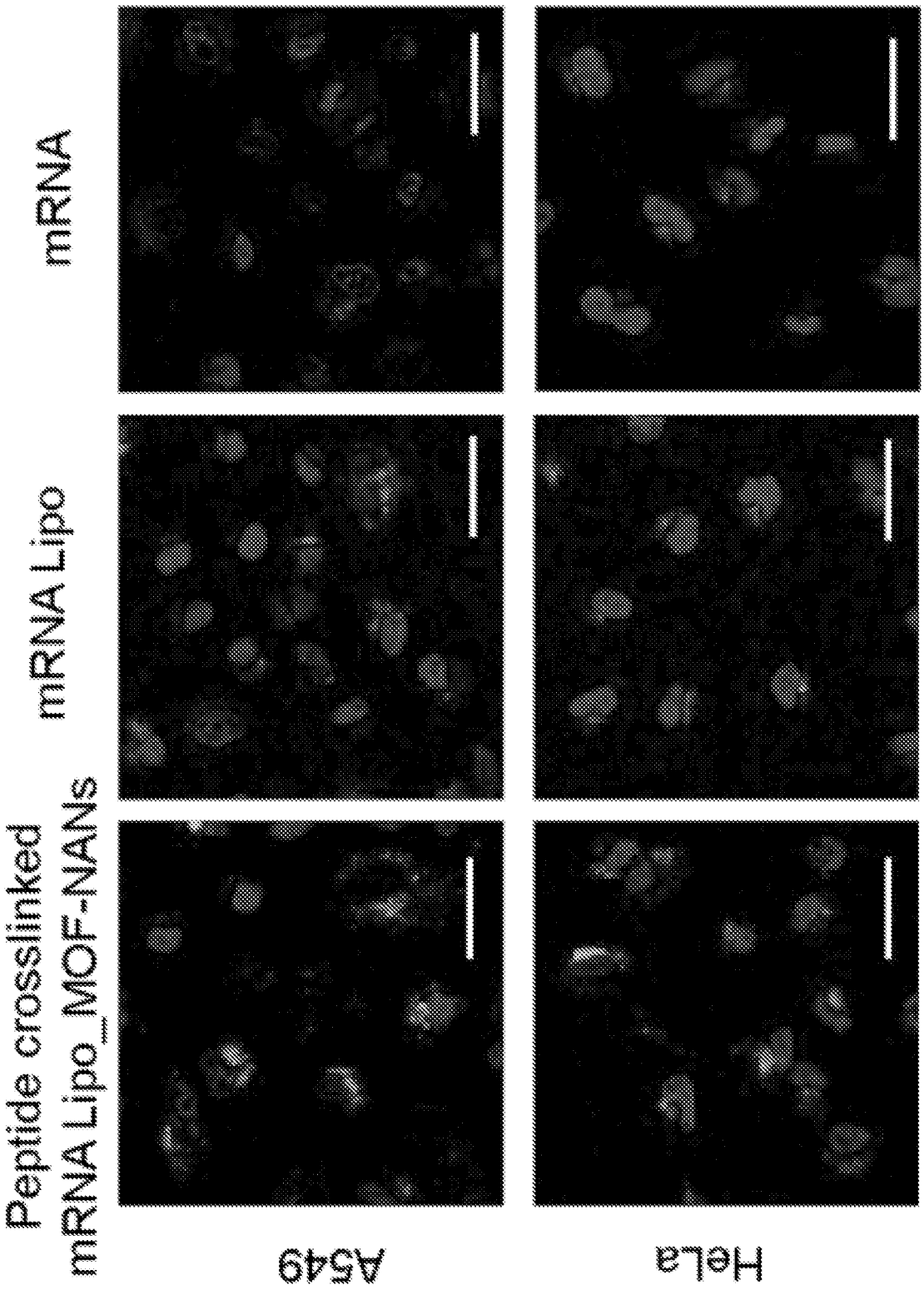

Peptide crosslinked
mRNA Lipo_MOF-NANs
mRNA Lipo
mRNA
A549
HeLa

FIG. 20B

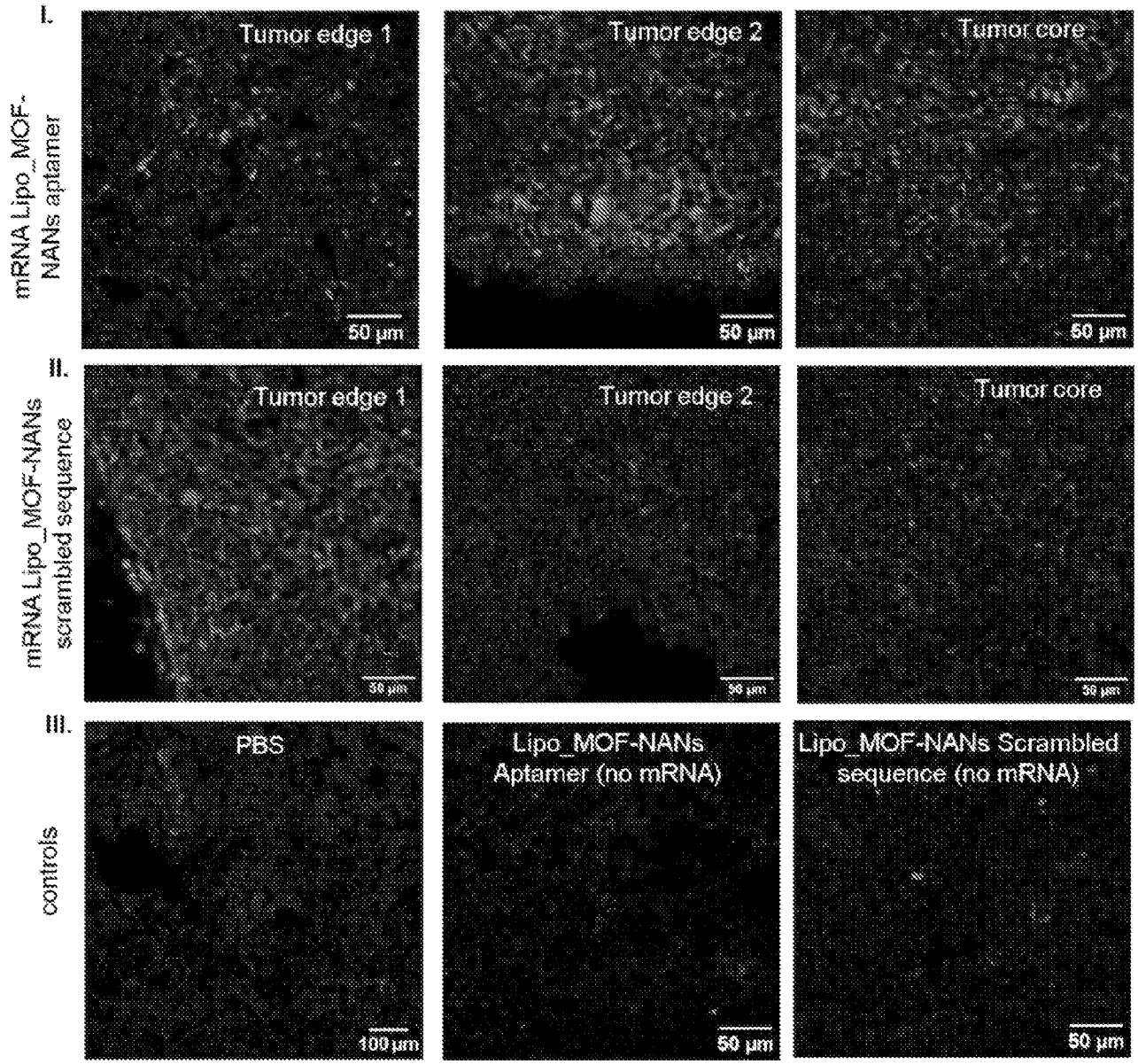
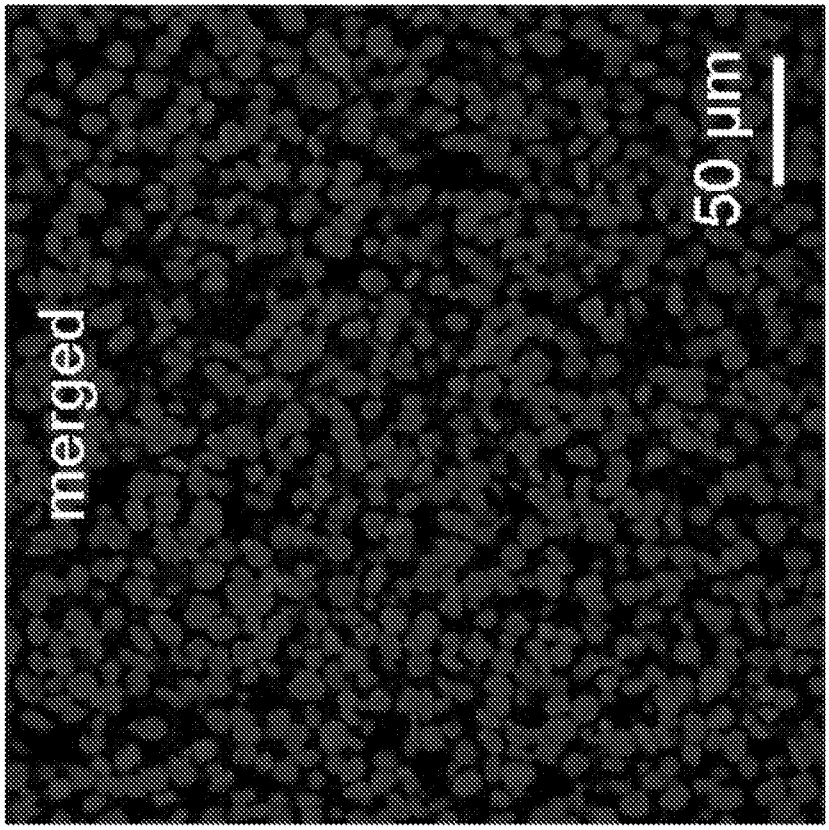

I.
mRNA Lipo_MOF-NANs aptamer
Tumor edge 1
50 µm
Tumor edge 2
50 µm
Tumor core
50 µm
II.
mRNA Lipo_MOF-NANs scrambled sequence
Tumor edge 1
Tumor edge 2
Tumor core
III.
controls
PBS
100 µm
Lipo_MOF-NANs Aptamer (no mRNA)
50 µm
Lipo_MOF-NANs Scrambled sequence (no mRNA)
50 µm

FIG. 20C



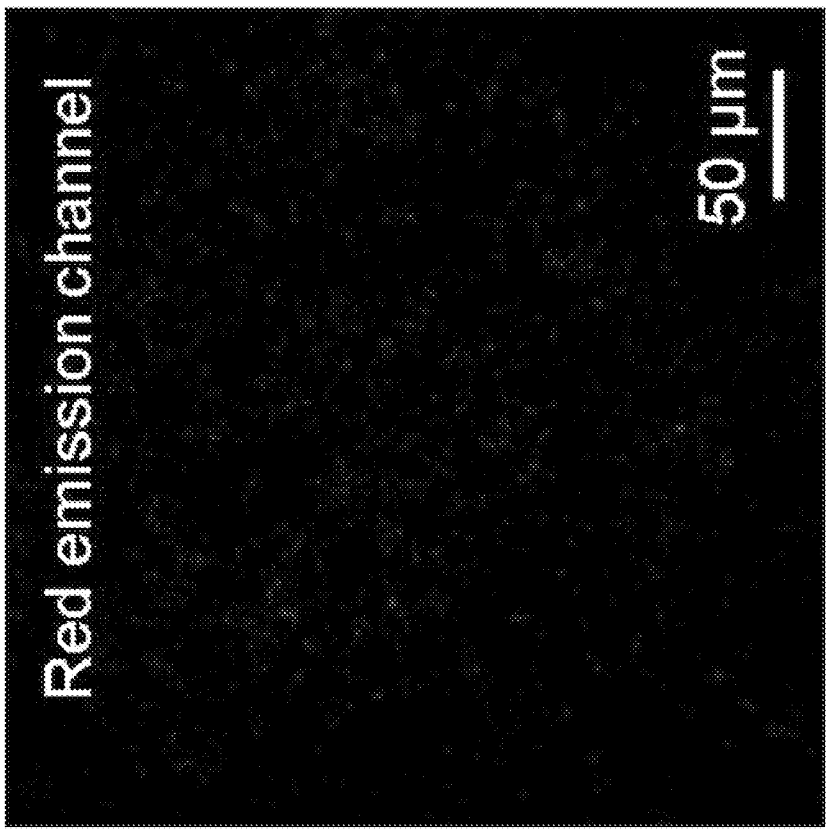


FIG. 21

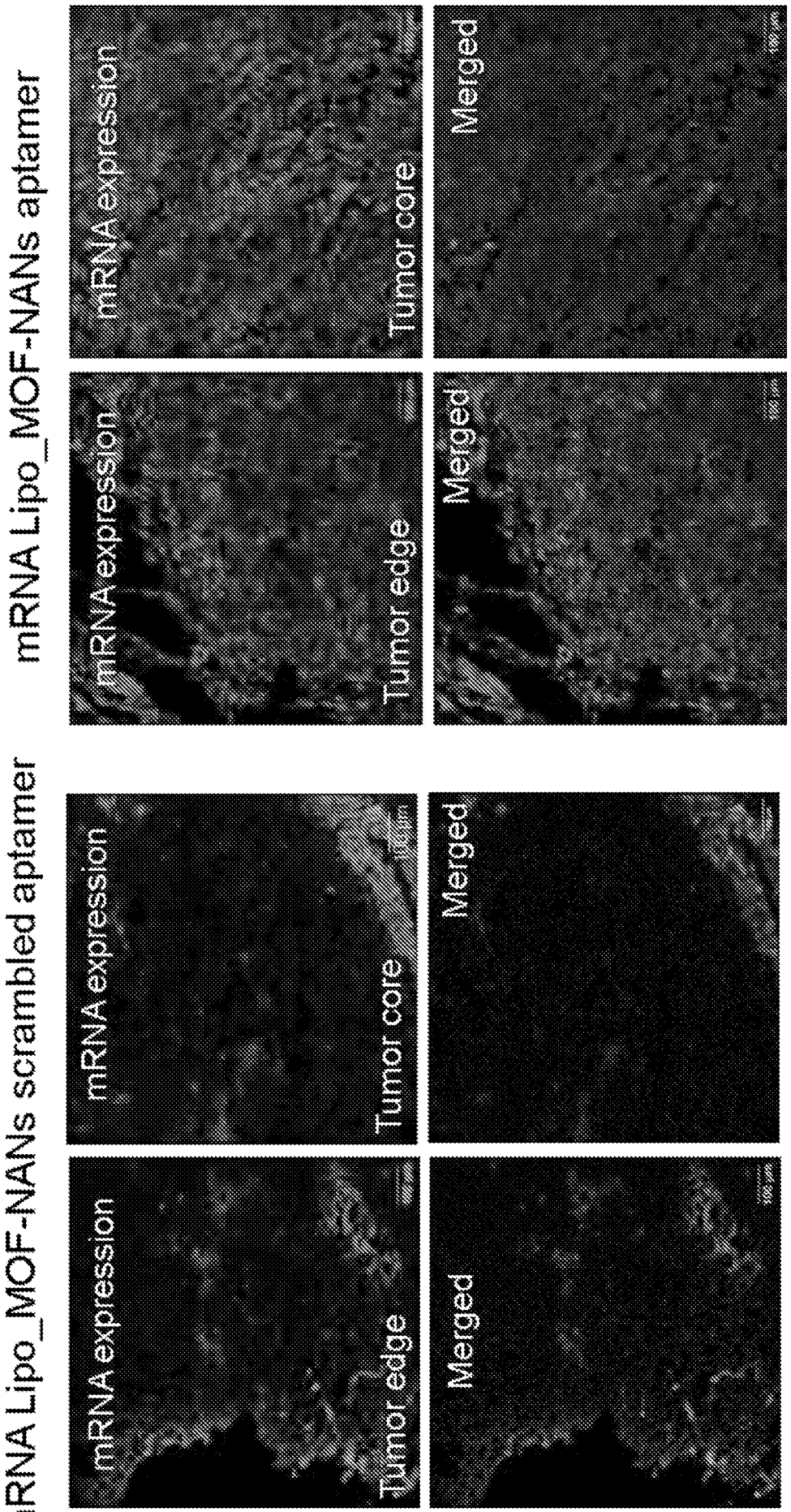
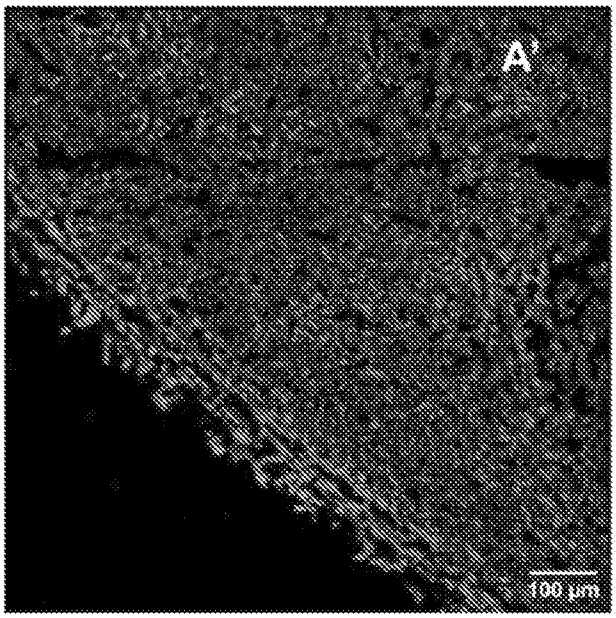

mRNA Lipo_MOF-NANs scrambled aptamer
mRNA expression
Tumor edge
mRNA expression
Tumor core
Merged
Merged
mRNA Lipo_MOF-NANs aptamer
mRNA expression
Tumor edge
mRNA expression
Tumor core
Merged
Merged

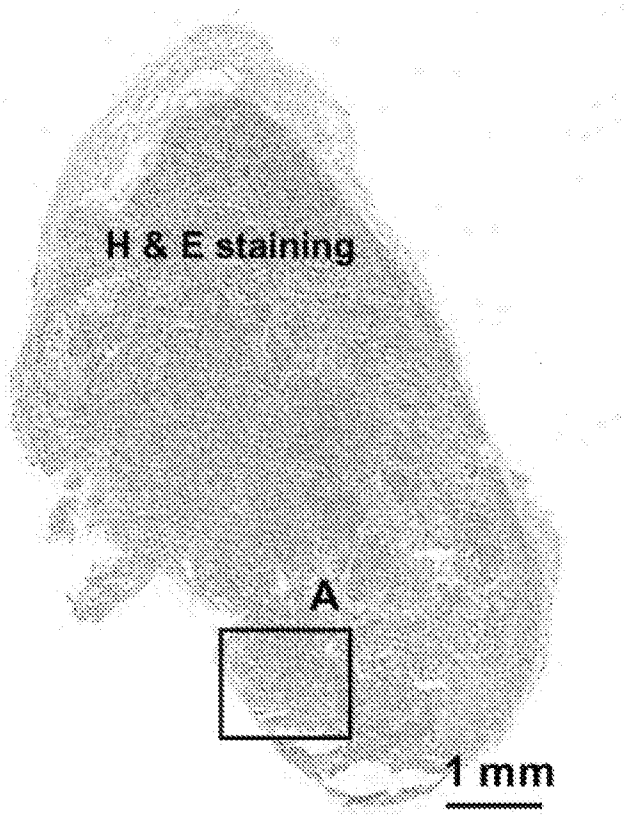
FIG. 22

mRNA Lipo_MOF-
NANs Aptamer
(tumor core)
13.6
12.7
13.8
13.7
100 μm
mRNA Lipo_MOF-
NANs Aptamer
(tumor edge)
13.8
13.6
12.6
13.3
100 μm
mRNA Lipo_MOF-
NANs scrambled
sequence
(tumor core)
8.0
6.8
17.9
11.4
13.2
17.9
100 μm
mRNA Lipo_MOF-
NANs scrambled
sequence
(tumor edge)
10.3
14.8
6.1
12.8
100 μm

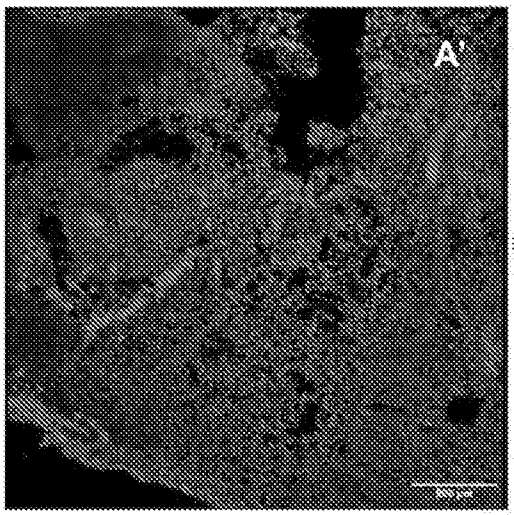
FIG. 23

(a) mRNA Lipo_MOF-NANs Aptamer treated mice tumor





(b) mRNA Lipo_MOF-NANs scrambled sequence treated mice tumor



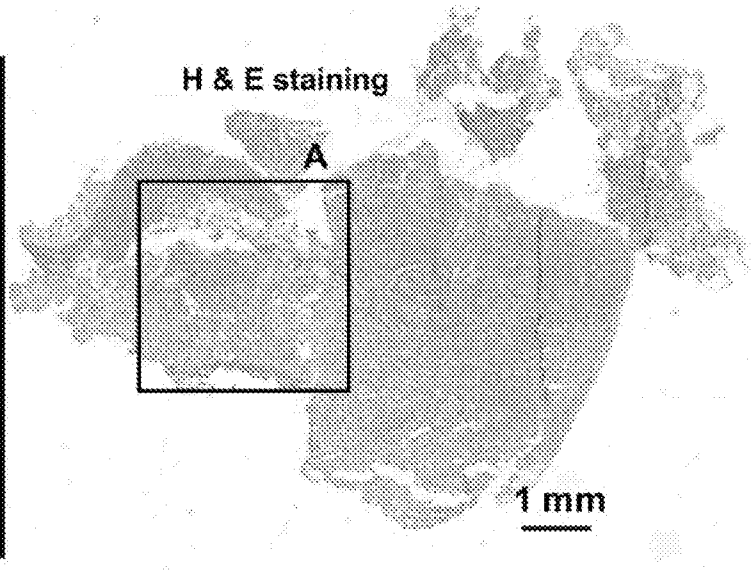


FIG. 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/034387

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***A61K 47/69*** (2024.01); ***A61K 9/51*** (2024.01); ***A61K 31/7088*** (2024.01); ***A61K 47/02*** (2024.01); ***C12N 15/115*** (2024.01); *B82B 1/00* (2024.01); *B82Y 5/00* (2024.01)

CPC: ***A61K 47/6929; A61K 47/02; A61K 9/51; A61K 31/7088; A61K 47/6911; C12N 15/115;*** *B82B 1/00; B82Y 5/00*

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)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2021/0038528 A1 (UNIVERSITY OF CONNECTICUT) 11 February 2021 (11.02.2021) entire document	1-3
A	US 2016/0271268 A1 (DANA-FARBER CANCER INSTITUTE INC.) 22 September 2016 (22.09.2016) entire document	1-3
A	US 2021/0236651 A1 (NORTHWESTERN UNIVERSITY) 05 August 2021 (05.08.2021) entire document	1-3
Y	HERBERT et al. "Stabilization of supramolecular membrane protein-lipid bilayer assemblies through immobilization in a crystalline exoskeleton." Nature Communications. 13 April 2021. Vol. 12:2022, Pgs. 1-13. entire document	1-3
Y	GAVITT et al. "A GATA3 Targeting Nucleic Acid Nanocapsule for In Vivo Gene Regulation in Asthma." ACS Nano. 27 July 2021, Vol. 15, Pgs. 11192-11201. entire document	3

☐ Further documents are listed in the continuation of Box C. ☐ 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
"D" document cited by the applicant in the international application
"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
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 August 2024 (10.08.2024)	**27 August 2024 (27.08.2024)**

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US **Commissioner for Patents** **P.O. Box 1450, Alexandria, VA 22313-1450**	**MATOS** **TAINA**
Facsimile No. **571-273-8300**	Telephone No. **571-272-4300**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/034387

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/034387

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☑ Claims Nos.: **4-20, 22-66**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).