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(54) Title: THERAPEUTIC EFFECTS OF CELLULAR DELIVERY OF SMALL MOLECULES AND MACROMOLECULES WITH LIPOSOMAL SPHERICAL NUCLEIC ACIDS

(57) Abstract: The present disclosure relates to liposomal particles, liposomal particles having encapsulated agents, methods of making the same, and uses thereof. Liposomal particles are useful in gene regulation and drug delivery.



THERAPEUTIC EFFECTS OF CELLULAR DELIVERY OF SMALL MOLECULES AND MACROMOLECULES WITH LIPOSOMAL SPHERICAL NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 62/396,733, filed September 19, 2016, the disclosure of which is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under HR0011-13-2-0018 awarded by the Defense Advanced Research Projects Agency (DARPA). The government has certain rights in the invention.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0003] This application contains, as a separate part of the disclosure, a Sequence Listing in computer-readable form which is incorporated by reference in its entirety and identified as follows: Filename: 2016-161_Seqlisting.txt; Size: 6,000 bytes, created : September 19, 2017.

FIELD OF THE INVENTION

[0004] The present disclosure relates to liposomal particles, liposomal particles having encapsulated agents, methods of making the same, and uses thereof. Liposomal particles are useful in gene regulation and drug delivery.

BACKGROUND

[0005] The concept of spherical nucleic acid (SNA) nanoparticle conjugates, structures typically synthesized from inorganic nanoparticle templates and shells of highly oriented nucleic acid ligands immobilized on the surface of such particles, was introduced in 1996 [Mirkin et al., Nature 1996, 382, 607]. Since then, SNAs have been prepared by many research groups in a variety of different forms [Cutler et al., J. Am. Chem. Soc. 2012, 134, 1376; Briley et al., In Nanomaterials for Biomedicine; Nagarajan R., editor. , Ed.; ACS Symposium Series 1119; American Chemical Society: Washington, DC, 2012; 1-20]. Core compositions including gold, silica [Young et al., Nano Lett. 2012, 12, 3867], iron oxide [Cutler et al., Nano Lett. 2010, 10, 1477], and Ag [Lee et al., Nano Lett. 2007, 7, 2112], with shell compositions consisting of DNA, RNA, LNA [Seferos et al., ChemBioChem 2007, 8, 1230], and PNA [Lytton-Jean et al., Adv. Mater. 2009, 21, 706], have all been prepared and explored. Hollow SNA structures consisting of cross-linked oligonucleotides [Cutler et al., J. Am. Chem. Soc. 2011, 133, 9254] along with DNA-block copolymer micelles [Li et al., Nano Lett. 2004, 4, 1055; Alemdaroglu et al., Adv. Mater. 2008, 20, 899; Liu et al., Chem.-Eur. J. 2010, 16, 3791; Chien et al., Chem. Commun. 2011, 47, 167] have been synthesized. Although there is now a tremendous structural and

compositional diversity among the known SNAs, they all share some common properties and features. Their polyvalent architectures allow them to cooperatively bind oligonucleotides and form duplex structures that exhibit very narrow melting transitions [Hurst et al., J. Am. Chem. Soc. 2008, 130, 12192]. These properties have been exploited in the development of high-sensitivity, high-selectivity, and massively multiplexed genomic detection systems [Alhasan et al., Anal. Chem. 2012, 84, 4153]. While linear nucleic acids do not enter cells well without polymer, peptide, or viral transfection agents, the three-dimensional SNA structure is recognized by Class A scavenger receptors [Patel et al., Bioconjugate Chem. 2010, 21, 2250; Choi et al., Proc. Natl. Acad. Sci. U.S.A. 2013, 110, 7625] and is rapidly taken up into over 60 different cell types without the need for an ancillary transfection agent [Zhang et al., Biomaterials 2010, 31, 1805; McAllister et al., J. Am. Chem. Soc. 2002, 124, 15198; Whitehead et al., Nat. Rev. Drug Discov. 2009, 8, 129]. This property has made such structures important elements in strategies for both intracellular detection [Zheng et al., Nano Lett. 2009, 9, 3258; Prigodich et al., ACS Nano 2009, 3, 2147] and gene regulation via antisense or siRNA pathways [Rosi et al., Science 2006, 312, 1027; Giljohann et al., J. Am. Chem. Soc. 2009, 131, 2072; Jensen et al., Sci. Transl. Med. 2013, 5, 209ra152].

[0006] The barrier to therapeutic use is high, however, especially when the structures are made from materials that have known problems with clearance or unknown biodistribution characteristics. Ideally, one would like an SNA structure that is made from readily available starting materials, can be synthesized at scale, and consists of components that have been a part of FDA-approved pharmaceuticals [Cutler et al., J. Am. Chem. Soc. 2012, 134, 1376; Farokhzad et al., Adv. Drug Delivery Rev. 2006, 58, 1456; Della Rocca et al., Nanomedicine 2012, 7, 303].

[0007] Toll-like receptors (TLRs) play a large role in the recognition of pathogens and modulation of the immune system.

SUMMARY OF THE INVENTION

[0008] Small unilamellar vesicles (SUVs) can be functionalized with polymers, peptides, DNA, and other molecules of interest by two distinct techniques. In a first approach, a modified molecule of interest is added to the mixture of lipids, lipid film or hydration buffer during the synthesis of liposome. This approach results in a liposomes containing a functional molecule of interest on both inner and outer layers of the liposomal membrane. Generally speaking, structures created by this method are not stable at a size smaller than 80 nanometers (nm). In an alternative approach, a SUV may be made by anchoring a substrate of interest into the lipid bilayer of the preformed vesicle (a "post modification technique"). This alternative approach

yields a liposomal nanoparticle containing a functional molecule of interest on the outer layer of the liposomal membrane. Importantly, this alternative post-modification approach allows the creation of liposome of any size, even less than 50 nanometers.

[0009] Applications of the technology disclosed herein include pre-treatment of transplantable organs prior to transplantation surgery, modulation of ischemia reperfusion injury in a transplant setting, and attenuation of inflammation as a result of TLR activation (autoimmune disease, sepsis). Advantages of the materials and methods disclosed herein include an increase in solubility of water insoluble small molecule drugs and biologics, and increase in circulation time/decrease in clearance rate of small molecules *in vivo*, and the ability to co-deliver multiple immunomodulatory molecules within a single entity.

[0010] Additional applications provided by the disclosure include the provision of selective and efficient TLR7/8 agonists; therapeutic cancer vaccines that can be tailored to a patient based on a tumor-associated antigen; and adjuvants of the immune system to be used on other types of vaccines. Further advantages of the disclosure include but are not limited to the provision of an advantage over free RNA agonists: increased activation of TLR7/8 in a model cell line, and high cellular uptake into many cell types (including immune cells); and the provision of an advantage over chemical modified RNA agonists: increased potency of activating TLR7/8, and a single-entity construct that can deliver an antigen and activate TLRs within the same cell.

[0011] Accordingly, in some aspects the disclosure provides a liposomal particle having a substantially spherical geometry and comprising a lipid bilayer comprising a plurality of lipid groups, a tumor antigen encapsulated therein, and an oligonucleotide on the surface of said liposomal particle. In some embodiments, the tumor antigen is a tumor lysate. In further embodiments, the oligonucleotide on the surface of said liposomal particle increases the activity of a toll-like receptor-9 (TLR-9).

[0012] In additional aspects of the disclosure, a liposomal particle is provided having a substantially spherical geometry and comprising a lipid bilayer comprising a plurality of lipid groups, a modulator of a first toll-like receptor (TLR) encapsulated in the liposomal particle, wherein said modulator is not an oligonucleotide, and an oligonucleotide on the surface of said liposomal particle that modulates the activity of a second toll-like receptor (TLR). In some embodiments, the first TLR is TLR-4 and the second TLR is TLR-9. In further embodiments, the first TLR is TLR-9 and the second TLR is TLR-4. In still further embodiments, the modulator of the first TLR is a small molecule. In related embodiments, the small molecule is TAK-242 (resatorvid), Oxidized 1-palmitoyl-2-arachidonyl-sn- glycerol-3-phosphorylcholine (OxPAPC),

polymyxin B or eritoran. In some embodiments, the second TLR is TLR-9 and the oligonucleotide on the surface of said liposomal particle increases the activity of TLR-9.

[0013] In further aspects, the disclosure provides a liposomal particle having a substantially spherical geometry and comprising a lipid bilayer comprising a plurality of lipid groups, an oligonucleotide on the surface of said liposomal particle that increases the activity of a first toll-like receptor (TLR), and an oligonucleotide on the surface of said liposomal particle that increases the activity of a second toll-like receptor (TLR). In some embodiments, the first TLR is TLR-7 and the second TLR is TLR-8. In further embodiments, the oligonucleotide comprises or consists of the sequence as set out in SEQ ID NO: 10. In still further embodiments, the oligonucleotide comprises or consists of the sequence as set out in SEQ ID NO: 11.

[0014] In additional aspects, the disclosure provides a liposomal particle having a substantially spherical geometry and comprising a lipid bilayer comprising a plurality of lipid groups, and an oligonucleotide on the surface of the liposomal particle comprising a modified backbone and a spacer. In some embodiments, the modification is a phosphorothioate backbone or a phosphodiester backbone. In further embodiments, the oligonucleotide comprises a phosphodiester homopolymeric sequence. In some embodiments, the homopolymer sequence comprises polyU. In still further embodiments, the oligonucleotide comprises UCA or CpG. In some embodiments, the spacer is an internal spacer.

[0015] In any of the aspects or embodiments of the disclosure, the lipid bilayer comprises 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 5% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(6-azidoheptanoyl) (DPPE-Azide). In some embodiments, the oligonucleotide is modified on its 5' end and/or 3' end with dibenzocyclooctyl (DBCO). In further embodiments, said plurality of lipid groups comprises a lipid selected from the group consisting of the phosphatidylcholine, phosphatidylglycerol, and phosphatidylethanolamine families of lipids. In still further embodiments, said lipid is selected from the group consisting of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-sn-phosphatidylcholine (DMPC), 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine (POPC), 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE).

[0016] In some embodiments, the oligonucleotide is an oligonucleotide-lipid conjugate containing a lipophilic tethered group, wherein said lipophilic tethered group is adsorbed into the lipid bilayer. In further embodiments, the lipophilic tethered group comprises tocopherol or

cholesterol. In related embodiments, tocopherol is chosen from the group consisting of a tocopherol derivative, alpha-tocopherol, beta-tocopherol, gamma-tocopherol and delta-tocopherol.

[0017] In any of the aspects or embodiments of the disclosure, said oligonucleotide comprises RNA or DNA. In some embodiments, said RNA is a non-coding RNA. In further embodiments, said non-coding RNA is an inhibitory RNA (RNAi). In still further embodiments, the RNAi is selected from the group consisting of a small inhibitory RNA (siRNA), a single-stranded RNA (ssRNA) that forms a triplex with double stranded DNA, and a ribozyme. In some embodiments, the RNA is a microRNA. In some embodiments, said DNA is antisense-DNA.

[0018] The disclosure also provides, in some embodiments, liposomal particles wherein diameter of said liposomal particle is less than or equal to about 50 nanometers. In some embodiments, the particle comprises from about 10 to about 80 oligonucleotides. In some embodiments, the particle comprises 70 oligonucleotides. In further embodiments, a liposomal particle of the disclosure comprises a modified oligonucleotide.

[0019] In some aspects, the disclosure provides a method of inhibiting expression of a gene comprising the step of hybridizing a polynucleotide encoding said gene product with one or more oligonucleotides complementary to all or a portion of said polynucleotide, said oligonucleotide being attached to the liposomal particle of the disclosure, wherein hybridizing between said polynucleotide and said oligonucleotide occurs over a length of said polynucleotide with a degree of complementarity sufficient to inhibit expression of said gene product. In some embodiments, expression of said gene product is inhibited *in vivo*. In further embodiments, expression of said gene product is inhibited *in vitro*. In some embodiments, said liposomal particle has a diameter about less than or equal to 50 nanometers. In further embodiments, said oligonucleotide comprises RNA or DNA. In yet further embodiments, said RNA is a non-coding RNA, and in some embodiments the non-coding RNA is an inhibitory RNA (RNAi). In related embodiments, the RNAi is selected from the group consisting of a small inhibitory RNA (siRNA), a single-stranded RNA (ssRNA) that forms a triplex with double stranded DNA, and a ribozyme. In some embodiments, said RNA is a microRNA. In some embodiments, said DNA is antisense-DNA.

[0020] In some aspects, a method for modulating activity of at least two toll-like receptors (TLRs) is provided, comprising contacting a cell having the toll-like receptors with a liposomal particle of the disclosure. In some embodiments, the modulating is up-regulating TLR activity or down-regulating TLR activity. In some embodiments, the method is performed *in vitro*. In further embodiments, the method is performed *in vivo*.

[0021] In some aspects, the disclosure provides a method of treating or preventing ischemia reperfusion injury (IRI) in a patient, comprising administering a liposomal particle of the disclosure to the patient, thereby treating or preventing IRI.

[0022] Additional aspects and embodiments of the disclosure are provided in the following enumerated paragraphs.

[0023] 1. A liposomal particle, the liposomal particle having a substantially spherical geometry and comprising: a lipid bilayer comprising a plurality of lipid groups; an oligonucleotide; and an agent encapsulated in the liposomal particle, wherein the molar ratio of lipid to encapsulated agent is from about 5:1 to about 100:1.

[0024] 2. The liposomal particle of paragraph 1, wherein the molar ratio of lipid to encapsulated agent is about 100:1, or about 100:3, or about 20:1, or about 25:4.

[0025] 3. The liposomal particle of paragraph 1 or paragraph 2, wherein the encapsulation efficiency is about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50% or higher.

[0026] 4. A liposomal particle, said liposomal particle having a substantially spherical geometry and comprising: a lipid bilayer comprising a plurality of lipid groups; and an oligonucleotide comprising a modified backbone.

[0027] 5. The liposomal particle of paragraph 4, wherein the modification is a phosphorothioate backbone or a phosphodiester backbone.

[0028] 6. The liposomal particle of paragraph 5, wherein the oligonucleotide comprises a phosphodiester homopolymeric sequence.

[0029] 7. The liposomal particle of paragraph 6, wherein the homopolymer sequence comprises polyU.

[0030] 8. The liposomal particle of paragraph 5, wherein the oligonucleotide comprises UCA or CpG.

[0031] 9. The liposomal particle of any one of paragraphs 4-8, wherein the oligonucleotide comprises a spacer.

[0032] 10. The liposomal particle of paragraph 9, wherein the spacer is an internal spacer.

BRIEF DESCRIPTION OF THE FIGURES

[0033] **Figure 1** depicts TLR signaling.

[0034] **Figure 2** provides a depiction of a LSNA containing TAK-242 and functionalized with a TLR9 antagonist oligonucleotide. The structure of TAK-242 is also shown.

[0035] **Figure 3** is a schematic of LSNA synthesis using a DOPC small unilamellar vesicle and 3'-tocopherol conjugated DNA. The final LSNA is approximately 60 nm in diameter.

[0036] **Figure 4** shows TLR activity as measured in HEK-Blue reporter cells using the secreted alkaline phosphatase (SEAP) assay.

[0037] **Figure 5** depicts Liquid Chromatography Mass Spectrometry (LC-MS) analysis. LC-MS was used to quantify TAK-242 encapsulated in liposomes. The standard curve for 100:1 (lipid:TAK) liposome was made using a standard addition approach to minimize matrix effects. Reserpine was the internal standard. Size and polydispersity indices were measured by DLS (Table 1).

[0038] **Figure 6** shows the uptake of dye-labeled LSNAs in mouse macrophages. Rhodamine-encapsulated liposomes were functionalized with Cy5-labeled DNA. Raw 264.7 were incubated with the dual fluorophore-labeled LSNAs at 100nM DNA for 30 minutes. Rhodamine channel shows the intracellular location of the liposome core (A), while Cy5 channel shows the intracellular location of the DNA (B). Overlaid image of Rhodamine and Cy5 channels (C) shows a high degree of colocalization, indicating that LSNAs enters the cells as a single entity and do not degrade within the transfection time.

[0039] **Figure 7** shows TLR inhibition by free INH-18 or TAK-242 is specific. RAW-Blue macrophages were stimulated with either 1826 CpG DNA (TLR9 ligand) or monophosphory lipid A (MPLA, TLR4 ligand). The SEAP assay was used to measure relative activation of cells. TLR9 inhibition is clearly specific to INH-18 oligonucleotide (A), but TLR4 inhibition, though mostly inhibited by TAK-242, is slightly inhibited by INH-18 (B). Crosstalk between TLR4 and TLR9 pathways could explain this phenomenon.

[0040] **Figure 8** shows potent TLR4 and TLR9 inhibition by LSNAs in mouse and primary human cells. Four different LSNAs were synthesized and RAW-Blue cells were treated with 500nM LSNAs to test for ability to modulate TLR activation (A-B). Only particles containing INH-18 inhibitory sequences are capable of suppressing TLR9 activity (A) and more potently than free INH-18 DNA, while particles containing TAK-242 suppress TLR4 activity (B) comparable to free TAK-242. CpG-induced human peripheral mononuclear blood cell (hPBMC) proliferation is inhibited in a dose dependent manner when treated with dual-targeting LSNAs (C). Changing the DNA backbone of INH-18 from phosphodiester (PO) to phosphorothioate

(PS) increases potency and is as effective as immunosuppressive glucocorticoid Dexamethasone (D).

[0041] **Figure 9** provides a depiction of the TLR signaling pathway.

[0042] **Figure 10** depicts functionalization of the LSNA surface with an inhibitory DNA sequence (INH-18).

[0043] **Figure 11** show potent TLR4 and TLR9 inhibition by LSNAs in engineered TLR reporter cells. Four different LSNAs were synthesized and HEK-Blue cells expressing mouse TLR4 or mouse TLR9 were treated with LSNAs (125nM by DNA, 75nM by TAK) to test for the ability to modulate TLR activation (A-B). Only particles containing INH-18 inhibitory sequences are capable of suppressing TLR9 activity (A), while particles containing TAK-242 suppress TLR4 activity (B).

[0044] **Figure 12** demonstrates the kinetic advantage of using LSNAs. The speed at which TLR9 activity is suppressed was assessed using HEK-Blue cells expressing mouse TLR9. Cells were treated for 4 hours simultaneously with agonist and antagonists (A) or with a 4 hour pulse of agonist followed by 8 hours with antagonist (B).

[0045] **Figure 13** depicts cytokine inhibition in TLR stimulated primary mouse splenocytes. primary mouse splenocytes were isolated and treated with 500nM TLR9 agonist (A-E) or 500ng/ml TLR4 agonist (F) to stimulate cytokine production. In general, dose dependence of antagonist was observed.

[0046] **Figure 14** shows the damage caused during liver ischemia reperfusion injury (IRI). A midline incision was made to expose the liver, and an atraumatic clamp was placed to cut off blood supply to the left lobe (A). Major histological changes (B) and cell death (C) were evident only after 90 minutes of ischemia and 4 hours of reperfusion (blue=DAPI, red=TUNEL).

[0047] **Figure 15** shows results of a liver function test and IL-6 levels following IRI. Ninety minutes of ischemia induced significant changes in the levels of alanine transaminase (ALT), albumin (ALB), alkaline phosphatase (ALP), total bilirubin (TBIL), cholesterol (CHOL) and pro-inflammatory cytokine IL-6.

[0048] **Figure 16** depicts LSNA core synthesis.

[0049] **Figure 17** provides final particle size and polydispersity index (PDI) of the liposomal cores as measured by DLS.

[0050] **Figure 18** shows TLR9 suppression in HEK-Blue cells expressing mouse TLR9. Experiment was run overnight.

[0051] **Figure 19** shows TLR4 suppression in HEK-Blue cells expressing mouse TLR4. Experiment was run overnight.

[0052] **Figure 20** shows TLR9 suppression in HEK-Blue cells expressing mouse TLR9. Experiment was run for 4 hours.

[0053] **Figure 21** depicts an encapsulated spherical nucleic acid (SNA) vaccine, as well as the average size and average antigen loading per particle.

[0054] **Figure 22** shows a blue clear spot for SNA which is from the Cy5. The graph shows the percentage of cells that were positive for Cy5 in the flow cytometer.

[0055] **Figure 23** demonstrates that IS-SNAs co-deliver immunostimulatory oligonucleotides and tumor-associated antigen. Y axis is the DNA and the X axis is the peptide. Each point is a cell and the four quadrants separate positive and negative cells. Upper right corner is the percentage of cells positive for both peptide and DNA, indicating a doubling of the co-delivery efficiency with the SNA formulation compared to a simple mixture of oligonucleotide and peptide.

[0056] **Figure 24** demonstrates that all of the cells were still being fully labeled, whereas on the right, multiple peaks to the right appear, indicating cells have divided. Each peak is one cell division so half of the cells have undergone multiple cell divisions with the peptide encapsulated SNA.

[0057] **Figure 25** shows that antigen-specific Killer T-cell activity was induced by IS-SNAs in a tumor model.

[0058] **Figure 26** shows the dose-dependent inhibition of CpG DNA-stimulated human PBMC proliferation by dual-targeting LSNAs measured by (³H)-labeled thymidine incorporation.

[0059] **Figure 27** shows data supporting the use of "top down" mass spectrometry analysis of intact proteins to identify unique protein signatures associated with IRI. The data show significant changes in proteoform expression between control and IRI-induced mouse kidneys.

[0060] **Figure 28** shows LSNAs that exhibit enhanced activation of TLR7/8 over free RNA, and RNA transfected with a cationic transfection agent.

[0061] **Figure 29** shows that the addition of LSNAs result in higher number of CD86+ receptors on dendritic cells than when they are introduced to RNA instead. These results are comparable to Resiquimod, a TLR7/8 agonist that was used as a positive control.

[0062] **Figure 30** shows that LSNAs induce the production of IL-6 (released in late endosomes) more so than free RNA, a control SNA, or untreated.

[0063] **Figure 31** is a schematic of the process for encapsulating proteins or peptides in a spherical nucleic acid (*e.g.*, a LSNA).

[0064] **Figure 32** shows that greater than 94% of the DOPC lipid is incorporated into liposomes as measured using a phosphotidyl choline assay kit.

[0065] **Figure 33** shows that encapsulation efficiencies for peptides was 11-17% of the starting material, and for labeled ovalbumin proteins, it was 29-31%. It was also observed that the encapsulation efficiency was independent of the starting concentration of the peptide or protein.

[0066] **Figure 34** shows (A) Scheme of LSNA functionalization: DOPC core functionalized with cholesterol-modified RNAs. (B) DLS of bare liposomes compared to LSNAs (in nm). (C) Gel electrophoresis of Cy3.5 labeled LSNAs compared to free 5'cholesterol ssRNA show an increase in size as determined by reduction in mobility.

[0067] **Figure 35** shows (A) immunostimulatory RNA-based LSNAs (IS-r-LSNAs) increase the NF- κ B activation of TLR 7/8 in Hek cells compared to free RNA or RNA transfected using a cationic transfection agent. Results are representative from more than 3 separate experiments; points represent mean $\pm$ SD, with n=3. (B) IS-r-LSNAs activate TLR7/8 in endosomal compartments, as no activity is present in the presence of chloroquine, an endosomal maturation blocker. Results are representative from three independent experiments; bars represent mean $\pm$ SD, with n=3. $P \leq 0.0001$ between all constructs with and without chloroquine treatment.

[0068] **Figure 36** shows (A) Confocal image shows high cellular uptake of Cy3.5 labeled SNAs into murine bone marrow derived dendritic cells (BMDCs) compared to free ssRNA or transfected ssRNA at 24 hours. Red denotes Cy3.5 labeled ssRNA and DAPI denotes a nuclear stain; scale bar indicates 5 μ m. (B) LSNAs are taken up by most cell types, including many immune cell types: pDC (B220⁺, CD11c^{med}, CD19⁻), cDC (CD11c⁺, CD19⁻, B220⁻) (CD19⁺, B220⁺), NK (NK1.1⁺, CD49b⁺, CD3⁻), macrophage (CD11b⁺, F4/80⁺), and granulocyte (Gr1⁺) as determined by using Cy3.5 labeled ssRNA. Constructs were incubated with mouse splenocytes for 8 hours (top) and 24 hours (bottom). Results reflect representative data from three independent experiments; bars indicate average mean fluorescence intensity from flow cytometry $\pm$ SD, with n=3.

[0069] **Figure 37** shows Z-stack of confocal images from Figure 36A were taken at 0.5 μ m slices; scale bar on right bottom indicates 5 μ m. (A) Untreated BMDCs, (B) BMDCs treated with free RNA, (C) BMDCs treated with LSNAs.

[0070] **Figure 38** shows (A) Primary murine immune cells secrete pro-inflammatory cytokines following a 24 hours incubation with IS-r-LSNAs. The dosage of all constructs are at 1 μ m by ssRNA concentration. Representative results from three separate experiments is shown; bars show mean pg/mL of each cytokine $\pm$ %CV (n=3). #: $p < 0.001$ between LSNA DR and all other constructs. (B) IS-r-LSNAs upregulate co-stimulatory receptor CD86 and MHCII in antigen presenting cells (pDC: PDCA⁺, CD11c^{med}, CD19⁻, B cells: CD19⁺, B220⁺, and macrophages: F4/80⁺) to a higher extent than free or cationically transfected ssRNA after a 24h incubation. In addition, IS-r-LSNAs also activate NK (NK1.1⁺, CD49b⁺, CD 3⁻), and T cells (CD3⁺). Representative results from three independent experiments are shown; bars indicate mean $\pm$ SD (n=3).

[0071] **Figure 39** shows (A) IS-r-LSNAs enhance antigen presentation rates on pDCs (PDCA⁺, CD11c^{med}, CD19⁻), B cells (CD19⁺, B220⁺), and macrophages (F4/80⁺), compared to that of free or cationically transfected ssRNA, after a 24 hour incubation, as quantified using flow cytometry. Interestingly, IS-r-LSNAs also increased antigen presentation rates of SIINFEKL SEQ ID NO: 12) on T cells (CD3⁺). Data is representative from three different experiments; bars indicate mean MFI $\pm$ SD with n=3. (B) IS-r-LSNA primed DCs (24 hours) induce greater OT-I CD8⁺ T cell proliferation than that of free or cationically transfected RNA after 72 hours. In addition, LSNA induce similar CD8⁺ T cell proliferation as resiquimod. The results are representative results from three independent experiments; bars indicate mean $\pm$ SD (n=3). **: $p < 0.01$ between LSNA DR and all groups except resiquimod, where there is no statistical significance between the two groups.

[0072] **Figure 40** shows that Bare DOPC SUVs have a smooth shell (left), unlike a functionalized IS-r-LSNA, which has a more "bumpy" surface due to the presence of RNA (right two). 5' cholesterol modified ssRNA can self-assemble or aggregate to form structures that make them visible under cryoEM (second from left). The clustering of RNA does not appear in the background in the LSNA sample, and instead, can be seen on the outside of the liposomal shell, confirming the successful functionalization of RNA onto liposomes. All scale bars are at 50nm.

[0073] **Figure 41** shows results of experiments probing structure/function relationships between TLRs7/8 and IS-r-LSNAs.

[0074] **Figure 42** depicts the viability of all constructs in Hek 293 cells expressing either TLR7 or 8 as determined using an Alamar Blue assay after a 24 hour incubation. While free RNA or rLSNAs do not decrease viability, the use of a cationic transfection agent LyoVec does

decrease cell viability. All data points were normalized to untreated (dotted line). Data is representative from three independent experiments, with $n=3 \pm \text{SD}$ shown.

[0075] **Figure 43** shows that overnight incubation of Hek-Blue cells with chloroquine does not decrease cell viability.

[0076] **Figure 44** shows cellular uptake of all constructs per cell type. Bars indicate the MFI of Cy-3.5 fluorescence for each sample at both 8 hours and 24 hours with mean $\pm \text{SD}$ ($n=3$; representative from three independent experiments).

[0077] **Figure 45** shows results of experiments to determine whether IS-r-LSNAs can specifically mature DCs, all constructs were incubated with positively selected (CD11c+) DC's for 24h, and analyzed using flow cytometry to look at the DC maturation through the upregulation of co-stimulatory and MHC-II receptors (Figure 39A). IS-r-LSNAs upregulate CD86 and MHCII receptors on pDCs (B220+ , CD11cmed) compared to control LSNAs, free RNA, and cationically transfected ssRNA, an important step in the DC maturation process. Bars represent mean $\pm \text{SD}$ ($n=3$; representative from three independent experiments).

[0078] **Figure 46** shows a comparison between ssRNA-DR as functionalized onto r-LSNAs or cationically transfected for cytokine secretion (A) and primary immune cell activation (B).

[0079] **Figure 47** depicts an immune-stimulating spherical nucleic acid vaccine as a treatment for triple negative breast cancer.

[0080] **Figure 48** is a schematic showing how SNAs are internalized by specialized immune cells, causing these cells to turn on the immune system. The activated cells then interact and communicate with T-cells, enabling the T-cells to recognize and kill tumor cells that express the antigens.

[0081] **Figure 49** shows that fluorescently labeled SNAs are rapidly internalized by macrophages (nucleus stained).

[0082] **Figure 50** shows that SNA vaccine treatment reduced the number of lung metastases in 4T1 tumor-bearing mice compared with untreated mice. Twenty-one days after treatment mouse lungs were cultured *ex vivo*, the 4T1 cells residing in these organs were stained (blue) and counted to quantify the number of metastases. One dot represents one metastasis.

[0083] **Figure 51** is a SDS-PAGE gel showing the generation of TNBC lysates through five free-thaw cycles of 4T1 cells. The SDS-Page gel shows the proteins in the lysates span molecular weights of 10-260 kDa. This demonstrates that the proteins loaded into the liposomes are a mixture of different proteins.

[0084] **Figure 52** depicts a schematic of how the lysate loaded liposomal SNAs were synthesized.

[0085] **Figure 53** demonstrates the high cellular uptake of TNBC lysate or CpG DNA into antigen presenting cells, including macrophages and dendritic cells, after one hour incubation time *in vitro*. Top/Bottom Left: Encapsulation of protein lysates into LSNAs enables higher cellular uptake of lysates into macrophages and dendritic cells compared with lysates encapsulated within bare liposome as demonstrated by confocal microscopy (brighter green signal inside cells) and flow cytometry (higher fluorescent intensity). Top/Bottom Right: Arranging DNA into a spherical architecture leads to greater cellular uptake compared with free DNA.

[0086] **Figure 54** demonstrates that co-delivery of TNBC antigen and CpG DNA to the same cell is enhanced when formulated as an LSNA. Co-delivery of lysate and DNA into the same cell is increased when formulated into a SNA. Cells were treated with three different combinations of samples: 1) Cy5-CpG SNA and AlexaFluor 488 (AF488)-labeled lysate encapsulated within bare liposomes, 2) free Cy5-labeled DNA and AF488-labeled lysate encapsulated within SNA, and 3) Dual labeled SNAs. The number of cells positive for both Cy5 and AF488 signals (upper right panel) was increased to 99% when the dual-labeled SNA was used compared with 30% for group 1 and 70% for group 2. This demonstrates that it is necessary to put both the lysate and DNA into the same SNA for maximal co-delivery into the same cell.

[0087] **Figure 55** provides a schematic showing the activation of dendritic cells (DCs).

[0088] **Figure 56** shows that TNBC LSNAs activate antigen presenting cells. Protein lysate LSNAs can activate mouse dendritic cells through toll-like receptor 9 signaling from the DNA, and also through delivery of the lysate. Protein lysates are known to activate dendritic cells through proteins that are released during necrotic cell death that are meant to signal to surrounding cells that there is danger in the area that caused this type of death. Activated dendritic cells increase their expression of co-stimulatory molecules (CD80, CD40, CD86, MHCII) and secretion of pro-inflammatory cytokines (TNF α , IL-12, IL-1 β , IL-6). Legend: Control – GpC DNA SNA (non-Toll-like receptor 9 activating DNA), TLR9 – CpG DNA SNA (Toll-like receptor 9 activating DNA), Empty – nothing inside the liposome, Lysate – liposomes with lysates encapsulated inside.

[0089] **Figure 57** is a schematic showing the vaccine protocol studies as conducted in mice. The figure also shows results of tumor growth monitoring in the mice. The tumor volume of 4T1 tumors in the right mammary gland measured overtime. Control LSNAs are liposomes

encapsulating lysates and GpC DNA. TLR9 LSNA are liposomes encapsulating lysates and CpG DNA. Treatment with TLR9 LSNA has a small effect on tumor volume; slowing growth at day 20 compared with untreated and control LSNA groups.

[0090] **Figure 58** shows that the percent of CD8+ T-cells within the tumor increased with TNBC LSNA treatment.

[0091] **Figure 59** shows that the percent of myeloid derived suppressive cells within the tumor decreased with TNBC LSNA treatment.

[0092] **Figure 60** provides data demonstrating that the TNBC LSNA vaccine reduced lung metastasis in a mouse model. Each dot represents one metastasis. * $p < 0.05$. The number of lung metastasis on day 21 was greatly reduced after TLR9 LSNA treatment compared with untreated or control LSNA groups. This was measured by counting the number of 4T1 cells that reached the lungs after implantation into the right mammary gland. Fewer 4T1 cells were able to colonize in the lungs in mice treated with TLR9 SNAs.

[0093] **Figure 61** shows the physical characterization of liposomal nanoparticles. Protein encapsulation was measured using bicinchoninic acid (BCA) assay DNA concentration measured using ultra-violet visible spectroscopy (A_{260}). It was determined that there was 1.1 (0.65) mg protein per μmol DNA (mean ($\pm$ standard deviation) of 3 batches). CryoEM images show the SNAs are spherical and approximately 50-100 nm in diameter after encapsulating a mixture of proteins. Agarose gel electrophoresis and dynamic light scattering both indicate an increase in size after functionalization with DNA. In the agarose gel, the SNA doesn't migrate as far in the gel due to its larger size compared with free DNA. The hydrodynamic diameter of bare liposomes is 73.4 nm and SNAs is 87.7 nm suggesting successful functionalization of DNA.

[0094] **Figure 62** demonstrates that IS-SNAs co-deliver immunostimulatory oligonucleotides and tumor-associated antigen.

DETAILED DESCRIPTION

[0095] Spherical Nucleic Acids (SNAs) are a class of nanoparticles functionalized with a dense layer of oligonucleotides surrounding an exchangeable core. This nucleic acid shell imparts several functionalities: the oligonucleotide coating forms a highly concentrated salt cloud that decreases endonuclease activity on the nanoparticle surface, and interacts with cell surface proteins, resulting in high cellular uptake in virtually all cell lines. The combination of these unique characteristics allows SNAs to behave as easily tailorable, single-entity agents that serve a variety of applications, such as cancer therapeutics.

[0096] Liposomes are spherical, self-closed structures in a varying size range consisting of one or several hydrophobic lipid bilayers with a hydrophilic core. The diameter of these lipid based carriers range from 0.15-1 micrometers, which is significantly higher than an effective therapeutic range of 20-100 nanometers. Liposomes termed small unilamellar vesicles (SUVs), can be synthesized in the 20-50 nanometer size range, but encounter challenges such as instability and aggregation leading to inter-particle fusion. This inter-particle fusion limits the use of SUVs in therapeutics.

[0097] In some aspects, the disclosure provides RNA based immunostimulatory SNAs (IS-SNAs). These IS-SNAs are shown herein to exhibit over 30-fold increase of secreted NF- κ B in murine macrophages compared to the free strand delivered using a cationic transfection agent (no activation was seen with just the free strand). In addition, treatment of mouse dendritic cells with RNA-based IS-SNAs in the presence of model tumor antigen improves the rate of antigen presentation, one important step in immunizing against cancer. This observation sets the foundation for the development of RNA-SNA based antitumor vaccines.

Terminology

[0098] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of skill in the art to which the invention pertains. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are described herein.

[0099] Certain terms are first defined. Additional terms are defined throughout the specification.

[0100] For the sake of brevity, a description of an embodiment of the disclosure in terms of a small unilamellar vesicle (SUV), a liposomal SNA (LSNA), IS-SNA, IS-r-LSNA, a liposomal particle, or a spherical nucleic acid (SNA) may also be applicable to an embodiment that uses any of the other foregoing terms. By way of example, a method of regulating gene expression using a liposomal SNA may also be described herein as a method of regulating gene expression using a liposomal particle. Small unilamellar vesicles (SUVs) are liposomal particles of sub-100 nanometer size and are used as the precursors to LSNAs. SUVs and LSNAs, as such, can be considered subclasses of liposomal particles.

[0101] Terms used herein are intended as "open" terms (for example, the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to").

[0102] Furthermore, in those instances where a convention analogous to "at least one of A, B and C, etc." is used, in general such a construction is intended in the sense of one having ordinary skill in the art would understand the convention (for example, "a system having at least one of A, B and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description or figures, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A or B" or "A and B."

[0103] All language such as "from," "to," "up to," "at least," "greater than," "less than," and the like include the number recited and refer to ranges which can subsequently be broken down into sub-ranges as discussed above.

[0104] A range includes each individual member. Thus, for example, a group having 1-3 members refers to groups having 1, 2, or 3 members. Similarly, a group having 6 members refers to groups having 1, 2, 3, 4, or 6 members, and so forth.

[0105] The modal verb "may" refers to the preferred use or selection of one or more options or choices among the several described embodiments or features contained within the same. Where no options or choices are disclosed regarding a particular embodiment or feature contained in the same, the modal verb "may" refers to an affirmative act regarding how to make or use an aspect of a described embodiment or feature contained in the same, or a definitive decision to use a specific skill regarding a described embodiment or feature contained in the same. In this latter context, the modal verb "may" has the same meaning and connotation as the auxiliary verb "can."

[0106] As used herein, the articles "a" and "an" refer to one or to more than one (for example, to at least one) of the grammatical object of the article.

[0107] "About" and "approximately" shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Exemplary degrees of error are within 20-25 percent (%), typically, within 10%, and more typically, within 5% of a given value or range of values.

[0108] The chemical structures described herein are named according to IUPAC nomenclature rules and include art-accepted common names and abbreviations where appropriate. The IUPAC nomenclature can be derived with chemical structure drawing software

programs, such as ChemDraw® (PerkinElmer, Inc.), ChemDoodle® (iChemLabs, LLC) and Marvin (ChemAxon Ltd.). The chemical structure controls in the disclosure to the extent that an IUPAC name is misnamed or otherwise conflicts with the chemical structure disclosed herein.

[0109] The present disclosure describes novel particles, termed liposomal particles, methods of making the same, and uses of these particles. The present liposomal particles are advantageous over other known liposomal based materials in that they are stable at a particle size that is smaller than other known liposomal particles, and the dense layer of DNA increases particle stability in a body, and therefore increases the circulation rate of liposomal vesicles, which improves bio-distribution of these particles inside the body.

[0110] General description of liposomal SNAs useful in the compositions and methods described herein is found in International Application Number PCT/US2014/068429, the disclosure of which is incorporated by reference herein in its entirety.

RNA-based Immunostimulatory Liposomal Spherical Nucleic Acids As Efficient TLR7/8 Receptor Modulators

[0111] At the core of vaccine development is the design of chemical and nanoscale architectures that can enter antigen-presenting cells (APCs) and bind to toll-like receptors (TLRs) in a selective and high affinity fashion. There are a variety of strategies in activating APCs and priming them against a specific antigen. One that is particularly promising involves attaching nucleic acids that are known to bind to TLRs in a radially outward fashion surrounding a nanoparticle core, forming a spherical nucleic acid (SNA). SNAs naturally enter cells due to their nucleic acid shell, and are high affinity and multi-avidity binders to membrane-associated proteins, such as TLRs, leading to more potent responses. This has been validated in DNA based structures aimed to target TLR9. RNA based structures hold promise in increased stability, immune cell uptake, and potency of TLR7/8 activation of RNA when compared to free or cationically transfected sequences. These immunostimulatory RNA-based LSNAs (IS-r-LSNAs) are also modular in design, allowing for the encapsulation of peptide antigens that can stimulate an antigen-specific response in CD8+ T cells. Further, these modular IS-r-LSNAs activate both arms of the immune system, which is useful for many applications including cancer immunology, autoimmune diseases or tolerance.

[0112] Spherical nucleic acids (SNAs), a class of nanoparticles composed of interchangeable cores functionalized with a dense oligonucleotide shell, can alter the way a cell sees nucleic acids [Cutler et al., J Am Chem Soc 2012, 134 (3), 1376-91; Mirkin et al., Nature 1996, 382 (6592), 607-9.]. On their own, nucleic acids have low cellular uptake in cells due to their negative charge; SNAs, due to their highly oriented shell of oligonucleotides, have increased

cell surface receptor interactions, resulting in high cellular uptake in virtually all cells, including immune cells [Rosi et al., Science 2006, 312 (5776), 1027-30; Giljohann et al., Nano Lett 2007, 7 (12), 3818-21]. In addition, the salt cloud surrounding SNAs reduces endonuclease activity on the nanoparticle surface, minimizing the concern of RNA instability [Seferos et al., Nano Lett 2009, 9 (1), 308-11; Patel et al., Mol Pharm 2011, 8 (4), 1285-91.]. SNAs are also non-cytotoxic [Massich et al., ACS Nano 2010, 4(10), 5641-6; Massich et al., Mol Pharm 2009, 6 (6), 1934-40], unless they are designed specifically to activate the immune system, allowing them to be used for a variety of biological applications. SNAs traffic through the endosomal pathway [Wu et al., J Am Chem Soc 2014, 136 (21), 7726-33], which makes them an ideal construct for immunomodulatory applications, such as the activation of endosomal toll-like receptors (TLR). TLRs are a class of pathogen recognition receptors (PRRs) that play an important role in our innate immune system by recognizing pathogen associated membrane patterns (PAMPs) [O'Neill et al., Nat Rev Immunol 2013, 13 (6), 453-60; Blasius et al., Immunity 2010, 32 (3), 305-15; Kawai et al., Nat Immunol 2010, 11 (5), 373-84; Rakoff-Nahoum et al., Nat Rev Cancer 2009, 9 (1), 57-63]. TLRs reside mostly in immune cells, and upon PAMP binding, initiate an innate immunity response resulting in the activation of many signaling transduction pathways relevant in inflammation, cytokine release, and adaptive immunity [Blasius et al., Immunity 2010, 32 (3), 305-15; Kawai et al., Nat Immunol 2010, 11 (5), 373-84; Rakoff-Nahoum et al., Nat Rev Cancer 2009, 9 (1), 57-63]. The use of TLR ligands to activate immune cells is advantageous over other types of adjuvants due to their well-characterized molecular structure, ligand-receptor specificity, and signaling pathways in a whole host of immune cells. One such family of TLRs are endosomal TLRs, which recognize nucleic acids of bacterial (TLR9) and viral (TLR7/8 and TLR3) origin [Blasius et al., Immunity 2010, 32 (3), 305-15; Junt et al., Nat Rev Immunol 2015, 15 (9), 529-44; Schon et al., Oncogene 2008, 27 (2), 190-9]. Broad expression of these TLRs in many immune cells justifies the increased interest in the development of efficient ligands for many therapeutic applications, such as cancer and autoimmune disorders (*i.e.*, rheumatoid arthritis, and systemic lupus erythematosus) [O'Neill et al., Pharmacol Rev 2009, 61 (2), 177-97]. In addition, the activation of these TLRs results in a rapid and potent dose of type I IFNs and pro-inflammatory cytokines, resulting in both innate and adaptive immunity [Reizis et al., Annual review of immunology 2011, 29, 163-83]. Although synthetic oligonucleotides could be used to modulate endosomal TLRs [Alter et al., J Immunol 2007, 178 (12), 7658-66; Diebold et al., Science 2004, 303 (5663), 1529-31; Heil et al., Science 2004, 303 (5663), 1526-9; Hornung et al., Nat Med 2005, 11 (3), 263-70], their clinical development is impeded by several factors associated with the inherent instability of linear oligonucleotides towards nucleases as well as inefficient cellular uptake of unmodified linear structures at clinically relevant

concentrations [Lan et al., Proc Natl Acad Sci USA 2007, 104 (34), 13750-5]. Although the delivery of DNA strands could be improved through special strand modifications, the delivery of RNA oligomers heavily relies on the use of cytotoxic transfection reagents [Bourquin et al., J Immunother 2010, 33 (9), 935-44; Nguyen et al., Proc Natl Acad Sci USA 2012, 109 (14), E797-803]. SNAs, due to their unique properties imparted by the oligonucleotide shell, can overcome instability and low uptake challenges faced by oligonucleotides. The use of DNA-based SNAs functionalized with CpG DNA has been successfully demonstrated previously, resulting in potent immunomodulation of TLR9 [Radovic-Moreno et al., Proc Natl Acad Sci USA 2015, 112 (13), 3892-7]. The success of these architectures was based on high uptake and fast delivery of TLR9 ligands into the endosomal compartments of immune cells. Based on this observation, we reasoned that one can design an SNA architecture targeting other endosomal receptors in a similar fashion. In this work, we have explored the development of novel SNA architectures targeting TLR7/8 receptors. We show that ssRNA TLR7/8 agonist functionalized liposomal SNAs (IS-r-LSNAs) are capable of efficiently delivering nucleic acid content, and activate TLR7/8 in APCs with a higher potency than that of free or cationically transfected ssRNAs. One possible application for these IS-r-LSNAs is as a cancer therapeutic, which entails the training of the immune system to recognize specific pathogens through simultaneous delivery of antigen and activation of APCs [Guo et al., Adv Cancer Res 2013, 119, 421-75; O'Hagan et al., Nat Rev Drug Discov 2003, 2 (9), 727-35]. Since the unique properties of the SNA is universal across many different cores, a liposomal [Banga et al., J Am Chem Soc 2014, 136 (28), 9866-9] core (composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine lipids, DOPC) was chosen for its biocompatibility and for its ability to encapsulate cargo within the core. The co-delivery of peptide antigen alongside a TLR 7/8 agonist via an IS-r-LSNA activates APCs and induces an antigen-specific CD8⁺ T cell response.

[0113] Currently, RNA oligonucleotides can be designed to activate toll-like receptors (TLRs) 7/8. Herein it is shown that orienting these RNA agonists in a SNA form can significantly increase their efficacy. This increase in activity can be attributed to the SNAs' rapid and high cellular uptake and their trafficking mechanism through the endocytic pathway. These potent TLR agonists are used to develop a therapeutic cancer vaccine that specifically induces cancer cell death through the co-delivery of an antigen delivered using SNA composed of a liposomal core (synthesized using FDA approved materials). The flexibility of the SNA platform allows for the development of new solutions for tackling various types of diseases (e.g., cancer) in a patient-specific manner.

[0114] Immuno-modulatory spherical nucleic acids (SNAs) have shown great promise in activating and suppressing toll-like receptor (TLR) 9 for applications in anti-tumor immunity and autoimmune diseases. In addition to TLR 9, there are three other endosomal TLRs (3, 7, and 8), which are responsible for recognizing double and single stranded RNA. Since various antigen-presenting cells (APCs) have differential TLR expression levels, targeting multiple TLRs can activate more types of immune cells, and also synergistically improve tumor eradication *in vivo*.

[0115] Currently, potent small molecule TLR 7/8 agonists (*e.g.*, resiquimod) are limited to topical applications due to significant toxicity caused by off-targeting effects and systemic immune activation. On the other hand, RNA-based TLR 7/8 agonists, while specific, can be easily degraded by nucleases and lack an efficient means of cellular uptake. This disclosure includes use of liposomal SNAs to activate TLR 3, 7 and 8 via RNA immuno-stimulatory oligonucleotides. Due to their dense oligonucleotide shell, SNAs exhibit increased interactions with cell surface proteins resulting in high cellular uptake in virtually all cell lines while localizing mainly in endosomal compartments, decreased activity of endonucleases on the nanoparticle surface due to a high local salt concentration, and also no cytotoxicity. As a result, SNAs are perfectly suited to act as single-entity RNA agonists for activating APCs.

[0116] While many TLR7/8 RNA agonists have been developed in the past, many suffer from stability and delivery challenges. This disclosure describes how insertion of single stranded TLR7/8 RNA agonists into a liposomal core to form liposomal spherical nucleic acids (LSNAs) offers improved transfection, internalization, and activation of TLR 7/8. Specifically, the 3D radial orientation of the nucleic acids increase the interaction of nucleic acids on LSNAs to cell surface proteins to yield higher uptake, while the high salt cloud on the LSNA offers stability against endonucleases. LSNAs activate TLR7/8 in a model cell line (human embryonic kidney 293 reporter cells expressing TLR7/8, Hek-Blue) with higher potency than free RNA, or RNA transfected with a cationic transfection agent, likely due to the multivalent binding of RNA to TLR7/8 in endosomal compartments. This increase in potency also translates into increased activation of dendritic cells, through the upregulation of co-stimulatory markers and increase in cytokine secretion, of LSNAs over their free RNA counterparts.

[0117] In some aspects, the present disclosure provides for the use of dual-targeting LSNAs for attenuating inflammation. Toll-like receptors (TLRs) are molecular first-responders of innate immune function that initiate host defense against diverse pathogens. Overstimulation of TLRs leads to dysregulation of downstream molecular targets and the development of inflammation-mediated diseases, such as autoimmune disorders, sepsis and ischemia reperfusion injury (IRI). Despite the pivotal role of TLRs in health and disease, to date, no therapeutic inhibitory system

has been developed that can restore normal function of TLRs following hyperactivation. Therefore, in some embodiments, the purpose of this disclosure was to synthesize and characterize dual TLR-inhibiting liposomal spherical nucleic acids (LSNAs). LSNAs are biodegradable nanoparticles synthesized from non-toxic, FDA-approved materials. The modularity of the LSNA construct permits encapsulation and surface functionalization of a number of small molecule drugs and biologically active molecules for tailoring the construct to a wide range of potential biomedical applications. This, combined with its chemical features and biological properties, such as rapid and efficient cellular uptake, make LSNAs a strong candidate for inhibiting TLR activation *in vivo*. In some embodiments, the LSNAs of the disclosure are designed to inhibit TLR4 and TLR9, which have both been implicated in inflammatory diseases. It is shown herein that, despite differences in the cellular location of TLR4 and TLR9, LSNAs are capable of suppressing the activity of both targets.

[0118] Based on the foregoing, the disclosure also provides, in some aspects, methods of reducing inflammation in a patient in need thereof, the method comprising the step of administering a LSNA of the disclosure to the patient in an amount sufficient to reduce inflammation. In some embodiments, such LSNA inhibit TLR-4 and/or TLR-9.

[0119] Thus, the LSNAs disclosed herein can be used to prevent or to treat subjects with inflammatory conditions. As used herein, an "inflammatory condition" means a disease, disorder or condition characterized by inflammation of body tissue or having an inflammatory component. These include local inflammatory responses and systemic inflammation. Examples of such inflammatory conditions include: transplant rejection, including skin graft rejection; chronic inflammatory conditions of the joints, including arthritis, rheumatoid arthritis, osteoarthritis and bone diseases associated with increased bone resorption; inflammatory bowel diseases such as ileitis, ulcerative colitis, Barrett's syndrome, and Crohn's disease; inflammatory lung disorders such as asthma, adult respiratory distress syndrome, and chronic obstructive airway disease; inflammatory disorders of the eye including corneal dystrophy, trachoma, onchocerciasis, uveitis, sympathetic ophthalmitis and endophthalmitis; chronic inflammatory conditions of the gums, including gingivitis and periodontitis; tuberculosis; leprosy; inflammatory diseases of the kidney including uremic complications, glomerulonephritis and nephrosis; inflammatory conditions of the skin including sclerodermatitis, psoriasis and eczema; inflammatory diseases of the central nervous system, including chronic demyelinating diseases of the nervous system, multiple sclerosis, AIDS-related neurodegeneration and Alzheimer's disease, infectious meningitis, encephalomyelitis, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and viral or autoimmune encephalitis; autoimmune disorders,

immune-complex vasculitis, systemic lupus and erythematoses; systemic lupus erythematosus (SLE); and inflammatory diseases of the heart such as cardiomyopathy, ischemic heart disease hypercholesterolemia, atherosclerosis); as well as various other diseases with significant inflammatory components, including preeclampsia; chronic liver failure, brain and spinal cord trauma, cancer). There may also be a systemic inflammation of the body, exemplified by gram-positive or gram negative shock, hemorrhagic or anaphylactic shock, or shock induced by cancer chemotherapy in response to pro-inflammatory cytokines, e.g., shock associated with pro-inflammatory cytokines. Such shock can be induced, e.g., by a chemotherapeutic agent used in cancer chemotherapy. "Treatment of an inflammatory condition" herein refers to administering a compound or a composition of the invention to a subject, who has an inflammatory condition, a symptom of such a condition or a predisposition towards such a condition, with the purpose to cure, relieve, alter, affect, or prevent the inflammatory condition, the symptom of it, or the predisposition towards it.

Ischemia Reperfusion Injury (IRI)

[0120] Toll-like receptors (TLRs) are central to the innate immune response, and their activation relies on specific recognition of conserved pathogenic motifs (Figure 2) (Schmidt et al., Nature biotechnology. 2007;25(8):825-6). TLRs also recognize certain damage-associated molecular patterns (DAMPs), such as denatured host DNA released from apoptotic cells after ischemic damage. TLR signaling plays a central role in the initiation and propagation of the pro-inflammatory response, ultimately converging upon NF- κ B and resulting in the production of cytokines and chemokines, immune cell activation, migration and proliferation, and the consequent shaping of the adaptive immune response. Persistent over-stimulation of TLRs leads to the development and progression of several diseases, such as bacterial and viral infections and sterile inflammatory disorders. TLR1-9 is highly conserved across humans and rodents. This high degree of conservation across species makes these receptors attractive therapeutic targets.

[0121] The critical involvement of TLRs in ischemia reperfusion injury (IRI) is widely documented (Howell et al., Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2014;20(3):270-80; Howell et al., Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2013;19(10):1099-107; Altemeier et al., PloS one. 2013;8(10):e77123; Arumugam et al., Shock. 2009;32(1):4-16; Chang et al., Journal of investigative surgery : the official journal of the Academy of Surgical Research. 2012;25(4):271-7; Frantz et al., Frontiers in bioscience : a

journal and virtual library. 2008;13:5772-9; Takeishi et al., Front Biosci (Landmark Ed). 2009;14:2553-8; Vilahur et al., Frontiers in physiology. 2014;5:496; Wang et al., Journal of neuroinflammation. 2015;12:80). Ischemia occurs when oxygen is cut off to an organ and the subsequent reperfusion injury initiates an inflammatory cascade upon reestablishment of blood flow. Clinically, IRI damages the organ's cellular parenchyma, releasing DAMPS that initiate the inflammatory cascade via TLR signaling and further compromise organ function due to resulting inflammation. Currently, static, cold preservation (e.g. University of Wisconsin solution) is the gold standard for organ preservation prior to transplantation (Lopukhin et al., The Annals of thoracic surgery. 1999;67(1):203-7; Lopukhin et al., Cryobiology. 1996;33(1):178-85; Belzer et al., Annals of surgery. 1992;215(6):579-83; Garcia et al., Transplantation proceedings. 2006;38(5):1236-7; Guarrera et al., Transplantation. 2004;77(8):1264-8; Parsons et al., Current opinion in organ transplantation. 2014;19(2):100-7; Lee et al., Organogenesis. 2009;5(3):105-12). The ischemia-reperfusion injury can be associated with ischemic and post-ischemic events in organs and tissues, including but not limited to thrombotic stroke; myocardial infarction; angina pectoris; embolic vascular occlusions; peripheral vascular insufficiency; splanchnic artery occlusion; arterial occlusion by thrombi or embolisms, arterial occlusion by non-occlusive processes such as following low mesenteric flow or sepsis; mesenteric arterial occlusion; mesenteric vein occlusion; ischemia-reperfusion injury to the mesenteric microcirculation; ischemic acute renal failure; ischemia-reperfusion injury to the cerebral tissue; intestinal intussusception; hemodynamic shock; tissue dysfunction; organ failure; restenosis; atherosclerosis; thrombosis; platelet aggregation. The ischemia-reperfusion injury can be alternatively following conditions including but not limited to procedures such as cardiac surgery; organ surgery; organ transplantation; angiography; cardiopulmonary and cerebral resuscitation.

[0122] Though not yet used clinically, preconditioning the organ graft with additional anti-inflammatory mediators in the preservation solution reduces oxidative stress and cellular damage by decreasing the release of DAMPS, consequently minimizing inflammation via decreased stimulation of Toll-like receptors (Farkas et al., Transplantation proceedings. 2005;37(1):435-8; Kato et al., Hepatology. 2003;38(2):364-73; Kirsch et al., The Annals of thoracic surgery. 1998;66(2):417-24; Wu et al., Kidney international. 2014;85(4):824-32). TLR4, located at the cell surface, is a central mediator of IRI following activation by endogenous, damage-associated, non-pathogenic ligands (Altemeier et al., PloS one. 2013;8(10):e77123; Chang et al., Journal of investigative surgery : the official journal of the Academy of Surgical Research. 2012;25(4):271-7; Ben et al., The Journal of surgical research. 2012;174(2):326-33; Ben-Ari et al., Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2012;30(2):489-98; Ajamieh et al., Journal

of gastroenterology and hepatology. 2012;27(8):1353-61; Kruger et al., Proceedings of the National Academy of Sciences of the United States of America. 2009;106(9):3390-5). TLR4 signals through two pathways, one dependent upon MyD88 and the other independent of this adapter protein (Figure 1). Activation of the TLR4 signal cascade culminates in NF- κ B and IFN γ activation and production of pro-inflammatory cytokines and chemokines, such as TNF- α , IL-6, IL-10, IFN γ and IL-1 β . TLR4 is expressed in nearly all cells of the body.

[0123] In contrast to TLR4, TLR9 is located in the endosome and recognizes unmethylated bacterial cytosine- phosphate-guanine (CpG) DNA, denatured host DNA, and a number of synthetic oligodeoxynucleotides that inhibit or activate TLR9 signaling. The ability of TLR9 to recognize endogenous DAMPs released by injured, apoptotic and necrotic cells indicates an involvement in the pro-inflammatory response associated with IRI (Chang et al., Journal of investigative surgery : the official journal of the Academy of Surgical Research. 2012;25(4):271-7; Bamboat et al., Hepatology. 2010;51(2):621-32; Huang et al., Hepatology. 2015;62(2):600-14). TLR9 is expressed in many cells that express TLR4, including monocytes and dendritic cells.

[0124] One approach to down-regulating overactivated TLRs is to competitively inhibit the receptor with soluble factors such as small molecules or antibodies. This approach is useful for targeting cell surface TLRs but is not effective against intracellular TLRs, such as TLR9. Moreover, the small size of TLR-inhibitors leads to their rapid clearance by the renal and reticuloendothelial system. Another approach to inhibit TLRs is through oligonucleotides, such as small interfering RNA (siRNA) or inhibitory DNA or RNA that bind endosomal TLRs. Despite the potency of oligonucleotide-based approaches, conventional delivery is impeded by the negative charge on oligonucleotides and requires cytotoxic transfection reagents. These challenges have led to a lack of therapeutic strategies to target these receptors (Hennessy et al., Nature reviews Drug discovery. 2010;9(4):293-307). The rational design of novel nanomaterials that harness specific chemical and material properties to enable molecular targeting while maintaining a strong pharmacological profile offers a strategy to overcome this challenge.

[0125] Liposomal spherical nucleic acids (LSNAs) are comprised of a small unilamellar liposomal core that is densely functionalized with highly oriented, radially pointing oligonucleotides bound to its surface (Figure 1) (Banga et al., Journal of the American Chemical Society. 2014;136(28):9866-9). The chemistry and geometry of LSNAs confers unique properties that are distinct from either the core or oligonucleotide ligands alone. Relative to free oligonucleotides, LSNAs have: 1) increased protection against nuclease degradation; 2) higher

binding affinity to complimentary oligonucleotides; 3) high cellular uptake without the use ancillary transfection reagents; and 4) are non-immunogenic (Cutler et al., Journal of the American Chemical Society. 2012;134(3):1376-91; Massich et al., ACS nano. 2010;4(10):5641-6; Massich et al., Molecular pharmaceutics. 2009;6(6):1934-40). These properties are governed by the chemistry of the oligonucleotide shell bound to the liposome surface. The shell confers stability at sub-50nm sizes; avoiding the tendency of small unilamellar liposomes to fuse together to reduce high surface energy. LSNAs are also highly modular; the core and the oligonucleotides are easily altered to carry fluorophores for imaging and diagnostic purposes, small molecules, peptides or proteins for therapeutic and/or targeting purposes, and the oligonucleotides themselves can be modified to function as antisense DNA, small interfering RNA (siRNA), or microRNAs for gene regulation (Giljohann et al., Journal of the American Chemical Society. 2009;131(6):2072-3; Halo et al., Proceedings of the National Academy of Sciences of the United States of America. 2014;111(48):17104-9; Mirkin et al., Oncotarget. 2014;5(1):9-10; Prigodich et al., Analytical chemistry. 2012;84(4):2062-6; Radovic-Moreno et al., Proceedings of the National Academy of Sciences of the United States of America. 2015;112(13):3892-7; Randeria et al., Cancer treatment and research. 2015;166:1-22; Rouge et al., ACS nano. 2014;8(9):8837-43; Young et al., Nano letters. 2012;12(7):3867-71; Zheng et al., Proceedings of the National Academy of Sciences of the United States of America. 2012;109(30):11975-80). Accordingly, the disclosure also provides, in some aspects, LSNAs and methods for treating or preventing IRI, the method comprising the step of administering a LSNA of the disclosure that inhibits TLR-4 and TLR-9.

Therapeutic cancer vaccines

[0126] The disclosure also provides, in various aspects, a spherical nucleic acid (SNA) vaccine designed to treat cancer. In some aspects, the cancer is triple negative breast cancer (TNBC) tumors. A SNA vaccine of the disclosure designed against TNBC reduces the overall size of the primary tumor and drastically reduces the number of lung metastases.

[0127] SNAs are a new class of molecules that can modulate the immune system through specific interactions with immune cells. Immune-stimulatory SNAs consist of a dense shell of nucleic acids that is radially oriented and attached to a liposomal nanoparticle core (Figure 47) [Banga et al., J. Am. Chem. Soc. 136, 9866–9869 (2014); Radovic-Moreno et al., Proc. Nat. Acad. Sci. USA. 112, 3892–3897 (2015)]. The nucleic acids are specifically designed to have multiple cytosine (C) and guanine (G) nucleotide repeats (CpG) within the linear sequence. These CpG motifs are recognized by toll-like receptor 9 (TLR9), which is found inside specialized immune cells [Krieg, Nat. Rev. Drug Discov. 5, 471–484 (2006)]. TNBC tumors do

not express epidermal growth factor-2 or estrogen and progesterone receptors. Immunotherapy is a potential treatment for TNBC because the number of tumor-infiltrating lymphocytes has been correlated to increased overall survival. Thus, as indicated above, certain aspects of the disclosure are directed to providing spherical nucleic acid vaccines (SNAs) for TNBC by delivering toll-like receptor 9 agonist DNA and TNBC-specific antigens to antigen presenting cells *in vivo*, and elicit an anti-tumor immune response against established TNBC tumors and metastasis. Protein cell lysates are being used as an antigen source for a LSNA cancer vaccine against triple negative breast cancer (TNBC) because TNBC does not have any known antigens or neoantigens due to a lack of molecular similarity between TNBC tumors. Therefore, whole cell lysates are being used as a large mixture of antigens that can direct an immune response.

[0128] In various aspects, the liposomal particle comprises tumor lysate. Tumor lysis can be effected using any of a number methods, such as repeated freezing and thawing of the tumor; physical disruption by homogenization or pulverization; or by chemically induction by, e.g., exposure to a hyper- or hypotonic solution or one or more non-ionic detergents. In various embodiments, the tumor lysate is obtained from a tumor associated with, e.g., breast cancer, colon cancer, lung cancer, prostate cancer, hepatocellular cancer, gastric cancer, liver cancer, pancreatic cancer, cervical cancer, ovarian cancer, bladder cancer, cancer of the urinary tract, thyroid cancer, renal cancer, carcinoma, melanoma, head and neck cancer, and brain cancer.

[0129] The liposomal core of the immune-stimulatory SNA is important as the core is used to encapsulate TNBC-specific antigens, which are a mixture of proteins expressed by TNBC cells and captured after cellular lysis. The SNA architecture enables co-delivery of the encapsulated antigens and CpG DNA to the same immune cells. The binding of the CpG-rich SNA to TLR9 leads to cellular activation and the immune system being turned on against the antigens that were delivered such that cytotoxic T-cells are primed to kill only the cells that express TNBC associated antigens (Figure 48).

[0130] SNAs are rapidly internalized into immune cells (Figure 49), with 100% of the SNAs being internalized after only two hours. The SNA architecture increased the co-delivery of DNA and antigens into the same cell by 50% compared with delivering the DNA and antigens as free entities. The delivered DNA activates immune cells through TLR9, resulting in increased secretion of pro-inflammatory cytokines, which are important biomolecules for driving a long-lasting immune response *in vivo*.

Liposomal particles

[0131] The present disclosure provides liposomal particles for use in methods including but not limited to regulation of gene expression and activation of TLRs. The liposomal particle has at least a substantially spherical geometry, an internal side and an external side, and comprises a lipid bilayer. The lipid bilayer is comprised of a first-lipid and a second-lipid. The first-lipid and second-lipid are, in some embodiments, the same. In further embodiments, the first-lipid and second-lipid are different.

[0132] The first-lipid is chosen from the phosphocholine family of lipids or the phosphoethanolamine family of lipids. While not meant to be limiting, the first-lipid is chosen from group consisting of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-sn-phosphatidylcholine (DMPC), 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine (POPC), 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE).

[0133] The second-lipid is chosen from the phosphocholine family of lipids or the phosphoethanolamine family of lipids. While not meant to be limiting, the second-lipid is chosen from group consisting of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-sn-phosphatidylcholine (DMPC), 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine (POPC), 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE).

[0134] In some embodiments, the liposomal particle further comprises a tocopherol modified oligonucleotide wherein the lipophilic end of the tocopherol modified oligonucleotide is absorbed into the lipid bilayer. The tocopherol is chosen from the group consisting of alpha-tocopherol, beta-tocopherol, gamma-tocopherol and delta-tocopherol. The non-lipophilic end of the tocopherol modified oligonucleotide is an oligonucleotide. This oligonucleotide is, in various embodiments, either RNA or DNA. The RNA can be an inhibitory RNA (RNAi) that performs a regulatory function, and in various embodiments is selected from the group consisting of a small inhibitory RNA (siRNA), an RNA that forms a triplex with double stranded DNA, and a ribozyme. Alternatively, and in further embodiments, the RNA is microRNA that performs a regulatory

function. The DNA is optionally an antisense-DNA. In still further embodiments, the RNA is a piwi-interacting RNA (piRNA).

[0135] Put another way, the disclosure provides a liposomal particle, said liposomal particle having a substantially spherical geometry, said liposomal particle comprising a lipid bilayer comprising a plurality of lipid groups; and an oligonucleotide. In various embodiments, the oligonucleotide is a modified oligonucleotide. In some embodiments, the plurality of lipid groups comprises a lipid selected from the group consisting of the phosphatidylcholine, phosphatidylglycerol, and phosphatidylethanolamine family of lipids. The oligonucleotide, in further embodiments is an oligonucleotide-lipid conjugate containing a lipophilic tethered group, wherein said lipophilic tethered group is adsorbed into the lipid bilayer. The lipophilic tethered group comprises, in various embodiments, tocopherol, palmitoyl, dipalmitoyl, stearyl, distearyl, or cholesterol.

[0136] With respect to the surface density of oligonucleotides on the surface of a liposomal particle of the disclosure, it is contemplated that a liposomal particle as described herein comprises from about 1 to about 400 oligonucleotides on its surface. In various embodiments, a liposomal particle comprises from about 10 to about 100, or from 10 to about 90, or from about 10 to about 80, or from about 10 to about 70, or from about 10 to about 60, or from about 10 to about 50, or from about 10 to about 40, or from about 10 to about 30, or from about 10 to about 20, or from about 50 to about 100, or from about 60 to about 100, or from about 70 to about 100, or from about 80 to about 100, or from about 90 to about 100 oligonucleotides on its surface. In further embodiments, a liposomal particle comprises at least about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, or 400 oligonucleotides on its surface.

Encapsulation

[0137] In some aspects of the disclosure it is contemplated that an agent is encapsulated within a LSNA. In some embodiments, a tumor antigen is encapsulated in a LSNA. In further embodiments, a modulator of a first TLR is encapsulated in a LSNA.

[0138] In still further aspects, a small molecule is encapsulated in a LSNA as disclosed herein. The term "small molecule," as used herein, refers to a chemical compound, for instance a chemotherapeutic or peptidomimetic that may optionally be derivatized, or any other low molecular weight organic compound, either natural or synthetic.

[0139] By "low molecular weight" is meant compounds having a molecular weight of less than 1000 Daltons, typically between 300 and 700 Daltons. Low molecular weight compounds, in various aspects, are about 100, about 150, about 200, about 250, about 300, about 350, about

400, about 450, about 500, about 550, about 600, about 650, about 700, about 750, about 800, about 850, about 900, or about 1000 Daltons.

[0140] Chemotherapeutic agents contemplated for use include, without limitation, alkylating agents including: nitrogen mustards, such as mechlor-ethamine, cyclophosphamide, ifosfamide, melphalan and chlorambucil; nitrosoureas, such as carmustine (BCNU), lomustine (CCNU), and semustine (methyl-CCNU); ethylenimines/methylmelamine such as triethylenemelamine (TEM), triethylene, thiophosphoramidate (thiotepa), hexamethylmelamine (HMM, altretamine); alkyl sulfonates such as busulfan; triazines such as dacarbazine (DTIC); antimetabolites including folic acid analogs such as methotrexate and trimetrexate, pyrimidine analogs such as 5-fluorouracil, fluorodeoxyuridine, gemcitabine, cytosine arabinoside (AraC, cytarabine), 5-azacytidine, 2,2'-difluorodeoxycytidine, purine analogs such as 6-mercaptopurine, 6-thioguanine, azathioprine, 2'-deoxycoformycin (pentostatin), erythrohydroxynonyladenine (EHNA), fludarabine phosphate, and 2-chlorodeoxyadenosine (cladribine, 2-CdA); natural products including antimitotic drugs such as paclitaxel, vinca alkaloids including vinblastine (VLB), vincristine, and vinorelbine, taxotere, estramustine, and estramustine phosphate; epipodophylotoxins such as etoposide and teniposide; antibiotics such as actinomycin D, daunomycin (rubidomycin), doxorubicin, mitoxantrone, idarubicin, bleomycins, plicamycin (mithramycin), mitomycinC, and actinomycin; enzymes such as L-asparaginase; biological response modifiers such as interferon-alpha, IL-2, G-CSF and GM-CSF; miscellaneous agents including platinum coordination complexes such as cisplatin, Pt(IV) and carboplatin, anthracenediones such as mitoxantrone, substituted urea such as hydroxyurea, methylhydrazine derivatives including N-methylhydrazine (MIH) and procarbazine, adrenocortical suppressants such as mitotane (o,p'-DDD) and aminoglutethimide; hormones and antagonists including adrenocorticosteroid antagonists such as prednisone and equivalents, dexamethasone and aminoglutethimide; progestin such as hydroxyprogesterone caproate, medroxyprogesterone acetate and megestrol acetate; estrogen such as diethylstilbestrol and ethinyl estradiol equivalents; antiestrogen such as tamoxifen; androgens including testosterone propionate and fluoxymesterone/equivalents; antiandrogens such as flutamide, gonadotropin-releasing hormone analogs and leuprolide; and non-steroidal antiandrogens such as flutamide.

Tocopherol Modified Oligonucleotides

[0141] In some aspects, an architecture comprising a tocopherol modified oligonucleotide is disclosed. In various embodiments, tocopherol is contemplated to be on the 5' end or the 3' end of an oligonucleotide or modified form thereof. A tocopherol-modified oligonucleotide comprises a lipophilic end and a non-lipophilic end. The lipophilic end comprises tocopherol, and may be

chosen from the group consisting of a tocopherol derivative, alpha-tocopherol, beta-tocopherol, gamma-tocopherol and delta-tocopherol. The lipophilic end, in further embodiments, comprises palmitoyl, dipalmitoyl, stearyl, or distearyl.

[0142] In further aspects, the disclosure contemplates that cholesterol or phospholipids are used instead of tocopherol. Cholesterol is attached in solid phase oligonucleotide synthesis, where it is mixed with the prepared liposomes to form SNAs. In some embodiments, liposomes composed of 95% 1,2-dioleoyl-*sn*-glycero-3 phosphatidylcholine (DOPC) and 5% 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(6-azidoheptanoyl) (DPPE-Azide) are prepared as described below. Then DBCO-modified oligonucleotides are added, which react with the azide lipid to functionalize the surface.

[0143] In still further aspects, a phospholipid conjugated oligonucleotide is prepared as follows: First, a phosphatidylethanolamine lipid, such as DOPE, is reacted with succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) by mixing 25 mg/mL lipid, 1 equivalent SMPB and 1 equivalent of *N,N*-Diisopropylethylamine in chloroform. The mixture is reacted overnight. Next, the product is purified by flash chromatography using silica column (solvent A: dichloromethane, solvent B: methanol). The thiol-modified oligonucleotide (3' or 5' end modified) is reduced with 0.2M DTT and 0.1 M phosphate buffer (pH 8) at 40°C for 2 hours. The oligonucleotide is then purified in a size exclusion column using water. The phosphatidylethanolamine-SMPB lipid is dried over nitrogen gas and dissolved in ethanol in the same volume as the oligonucleotide. The oligonucleotide is then mixed with the lipid such that the reaction is 50:50 water and ethanol. This mixture is reacted overnight, and the excess lipid is extracted by washing the reaction mixture with chloroform three times. Next, the aqueous phase and the interface are dried and dissolved in water. All lipid-conjugated oligonucleotides as disclosed herein are contemplated to be used interchangeably in the preparation of LSNA.

[0144] The non-lipophilic end of the tocopherol-modified oligonucleotide is an oligonucleotide. The oligonucleotide is either RNA or DNA. The RNA can be an inhibitory RNA (RNAi) that performs a regulatory function, and is chosen from the group consisting of a small RNAi that is selected from the group consisting of a small inhibitory RNA (siRNA), an RNA that forms a triplex with double stranded DNA, and a ribozyme. Alternatively, the RNA is microRNA that performs a regulatory function. In still further embodiments, the RNA is a piwi-interacting RNA (piRNA). The DNA is, in some embodiments, an antisense-DNA.

[0145] Oligonucleotides contemplated for use according to the disclosure are from about 5 to about 100 nucleotides in length. Methods and compositions are also contemplated wherein the oligonucleotide is about 5 to about 90 nucleotides in length, about 5 to about 80 nucleotides in

length, about 5 to about 70 nucleotides in length, about 5 to about 60 nucleotides in length, about 5 to about 50 nucleotides in length about 5 to about 45 nucleotides in length, about 5 to about 40 nucleotides in length, about 5 to about 35 nucleotides in length, about 5 to about 30 nucleotides in length, about 5 to about 25 nucleotides in length, about 5 to about 20 nucleotides in length, about 5 to about 15 nucleotides in length, about 5 to about 10 nucleotides in length, and all oligonucleotides intermediate in length of the sizes specifically disclosed to the extent that the oligonucleotide is able to achieve the desired result. Accordingly, oligonucleotides of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, and 100 nucleotides in length are contemplated.

Modified Oligonucleotides

[0146] Specific examples of oligonucleotides include those containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. Modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone are considered to be within the meaning of "oligonucleotide."

[0147] Modified oligonucleotide backbones containing a phosphorus atom include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Also contemplated are oligonucleotides having inverted polarity comprising a single 3' to 3' linkage at the 3'-most internucleotide linkage, *i.e.* a single inverted nucleoside residue which may be abasic (the nucleotide is missing or has a hydroxyl group in place thereof). Salts, mixed salts and free acid forms are also contemplated. Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253;

5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, the disclosures of which are incorporated by reference herein.

[0148] Modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages; siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts. See, for example, U.S. Patent Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, the disclosures of which are incorporated herein by reference in their entireties.

[0149] In still other embodiments, oligonucleotide mimetics wherein both one or more sugar and/or one or more internucleotide linkage of the nucleotide units are replaced with "non-naturally occurring" groups. In one aspect, this embodiment contemplates a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone. See, for example US Patent Nos. 5,539,082; 5,714,331; and 5,719,262, and Nielsen *et al.*, 1991, *Science* **254**: 1497-1500, the disclosures of which are herein incorporated by reference.

[0150] In still other embodiments, oligonucleotides are provided with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and including —CH₂—NH—O—CH₂—, —CH₂—N(CH₃)—O—CH₂—, —CH₂—O—N(CH₃)—CH₂—, —CH₂—N(CH₃)—N(CH₃)—CH₂— and —O—N(CH₃)—CH₂—CH₂— described in US Patent Nos. 5,489,677, and 5,602,240. Also contemplated are oligonucleotides with morpholino backbone structures described in US Patent No. 5,034,506.

[0151] In various forms, the linkage between two successive monomers in the oligonucleotide consists of 2 to 4, desirably 3, groups/atoms selected from —CH₂—, —O—, —S—, —NR^H—, >C=O, >C=NR^H, >C=S, —Si(R'')₂—, —SO—, —S(O)₂—, —P(O)₂—, —PO(BH₃)—, —P(O,S)—, —P(S)₂—, —PO(R'')—, —PO(OCH₃)—, and —PO(NHR^H)—, where RH is selected from hydrogen and C₁₋₄-alkyl, and R'' is selected from C₁₋₆-alkyl and phenyl. Illustrative examples of

such linkages are $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{CO}-\text{CH}_2-$, $-\text{CH}_2-\text{CHOH}-\text{CH}_2-$, $-\text{O}-\text{CH}_2-\text{O}-$, $-\text{O}-\text{CH}_2-\text{CH}_2-$, $-\text{O}-\text{CH}_2-\text{CH}=(\text{including } R^5 \text{ when used as a linkage to a succeeding monomer})$, $-\text{CH}_2-\text{CH}_2-\text{O}-$, $-\text{NR}^H-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{CH}_2-\text{NR}^H-$, $-\text{CH}_2-\text{NR}^H-\text{CH}_2-$, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{NR}^H-$, $-\text{NR}^H-\text{CO}-\text{O}-$, $-\text{NR}^H-\text{CO}-\text{NR}^H-$, $-\text{NR}^H-\text{CS}-\text{NR}^H-$, $-\text{NR}^H-\text{C}(=\text{NR}^H)-\text{NR}^H-$, $-\text{NR}^H-\text{CO}-\text{CH}_2-\text{NR}^H-\text{O}-\text{CO}-\text{O}-$, $-\text{O}-\text{CO}-\text{CH}_2-\text{O}-$, $-\text{O}-\text{CH}_2-\text{CO}-\text{O}-$, $-\text{CH}_2-\text{CO}-\text{NR}^H-$, $-\text{O}-\text{CO}-\text{NR}^H-$, $-\text{NR}^H-\text{CO}-\text{CH}_2-$, $-\text{O}-\text{CH}_2-\text{CO}-\text{NR}^H-$, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{NR}^H-$, $-\text{CH}=\text{N}-\text{O}-$, $-\text{CH}_2-\text{NR}^H-\text{O}-$, $-\text{CH}_2-\text{O}-\text{N}=(\text{including } R^5 \text{ when used as a linkage to a succeeding monomer})$, $-\text{CH}_2-\text{O}-\text{NR}^H-$, $-\text{CO}-\text{NR}^H-\text{CH}_2-$, $-\text{CH}_2-\text{NR}^H-\text{O}-$, $-\text{CH}_2-\text{NR}^H-\text{CO}-$, $-\text{O}-\text{NR}^H-\text{CH}_2-$, $-\text{O}-\text{NR}^H-$, $-\text{O}-\text{CH}_2-\text{S}-$, $-\text{S}-\text{CH}_2-\text{O}-$, $-\text{CH}_2-\text{CH}_2-\text{S}-$, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{S}-$, $-\text{S}-\text{CH}_2-\text{CH}=(\text{including } R^5 \text{ when used as a linkage to a succeeding monomer})$, $-\text{S}-\text{CH}_2-\text{CH}_2-$, $-\text{S}-\text{CH}_2-\text{CH}_2-\text{O}-$, $-\text{S}-\text{CH}_2-\text{CH}_2-\text{S}-$, $-\text{CH}_2-\text{S}-\text{CH}_2-$, $-\text{CH}_2-\text{SO}-\text{CH}_2-$, $-\text{CH}_2-\text{SO}_2-\text{CH}_2-$, $-\text{O}-\text{SO}-\text{O}-$, $-\text{O}-\text{S}(\text{O})_2-\text{O}-$, $-\text{O}-\text{S}(\text{O})_2-\text{CH}_2-$, $-\text{O}-\text{S}(\text{O})_2-\text{NR}^H-$, $-\text{NR}^H-\text{S}(\text{O})_2-\text{CH}_2-$; $-\text{O}-\text{S}(\text{O})_2-\text{CH}_2-$, $-\text{O}-\text{P}(\text{O})_2-\text{O}-$, $-\text{O}-\text{P}(\text{O},\text{S})-\text{O}-$, $-\text{O}-\text{P}(\text{S})_2-\text{O}-$, $-\text{S}-\text{P}(\text{O})_2-\text{O}-$, $-\text{S}-\text{P}(\text{O},\text{S})-\text{O}-$, $-\text{S}-\text{P}(\text{S})_2-\text{O}-$, $-\text{O}-\text{P}(\text{O})_2-\text{S}-$, $-\text{O}-\text{P}(\text{O},\text{S})-\text{S}-$, $-\text{O}-\text{P}(\text{S})_2-\text{S}-$, $-\text{S}-\text{P}(\text{O})_2-\text{S}-$, $-\text{S}-\text{P}(\text{O},\text{S})-\text{S}-$, $-\text{S}-\text{P}(\text{S})_2-\text{S}-$, $-\text{O}-\text{PO}(\text{R}'')-\text{O}-$, $-\text{O}-\text{PO}(\text{OCH}_3)-\text{O}-$, $-\text{O}-\text{PO}(\text{OCH}_2\text{CH}_3)-\text{O}-$, $-\text{O}-\text{PO}(\text{OCH}_2\text{CH}_2\text{S}-\text{R})-\text{O}-$, $-\text{O}-\text{PO}(\text{BH}_3)-\text{O}-$, $-\text{O}-\text{PO}(\text{NHR}^N)-\text{O}-$, $-\text{O}-\text{P}(\text{O})_2-\text{NR}^H-\text{H}-$, $-\text{NR}^H-\text{P}(\text{O})_2-\text{O}-$, $-\text{O}-\text{P}(\text{O},\text{NR}^H)-\text{O}-$, $-\text{CH}_2-\text{P}(\text{O})_2-\text{O}-$, $-\text{O}-\text{P}(\text{O})_2-\text{CH}_2-$, and $-\text{O}-\text{Si}(\text{R}'')_2-\text{O}-$; among which $-\text{CH}_2-\text{CO}-\text{NR}^H-$, $-\text{CH}_2-\text{NR}^H-\text{O}-$, $-\text{S}-\text{CH}_2-\text{O}-$, $-\text{O}-\text{P}(\text{O})_2-\text{O}-\text{O}-\text{P}(\text{O},\text{S})-\text{O}-$, $-\text{O}-\text{P}(\text{S})_2-\text{O}-$, $-\text{NR}^H-\text{P}(\text{O})_2-\text{O}-$, $-\text{O}-\text{P}(\text{O},\text{NR}^H)-\text{O}-$, $-\text{O}-\text{PO}(\text{R}'')-\text{O}-$, $-\text{O}-\text{PO}(\text{CH}_3)-\text{O}-$, and $-\text{O}-\text{PO}(\text{NHR}^N)-\text{O}-$, where RH is selected from hydrogen and C_{1-4} -alkyl, and R'' is selected from C_{1-6} -alkyl and phenyl, are contemplated. Further illustrative examples are given in Mesmaeker *et al.*, 1995, *Current Opinion in Structural Biology*, **5**: 343-355 and Susan M. Freier and Karl-Heinz Altmann, 1997, *Nucleic Acids Research*, vol **25**: pp 4429-4443.

[0152] Still other modified forms of oligonucleotides are described in detail in U.S. Patent Application No. 20040219565, the disclosure of which is incorporated by reference herein in its entirety.

[0153] Modified oligonucleotides may also contain one or more substituted sugar moieties. In certain aspects, oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Other embodiments include $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$, $\text{O}(\text{CH}_2)_n\text{OCH}_3$, $\text{O}(\text{CH}_2)_n\text{NH}_2$, $\text{O}(\text{CH}_2)_n\text{CH}_3$,

$O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_mCH_3]_2$, where n and m are from 1 to about 10. Other oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3 , OCN, Cl, Br, CN, CF_3 , OCF_3 , $SOCH_3$, SO_2CH_3 , ONO_2 , NO_2 , N_3 , NH_2 , heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. In one aspect, a modification includes 2'-methoxyethoxy (2'-O- $CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, 1995, *Helv. Chim. Acta*, **78**: 486-504) *i.e.*, an alkoxyalkoxy group. Other modifications include 2'-dimethylaminoethoxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), *i.e.*, 2'-O- CH_2 -O- CH_2 - $N(CH_3)_2$, also described in examples herein below.

[0154] Still other modifications include 2'-methoxy (2'-O- CH_3), 2'-aminopropoxy (2'-O- $CH_2CH_2CH_2NH_2$), 2'-allyl (2'- CH_2 - $CH=CH_2$), 2'-O-allyl (2'-O- CH_2 - $CH=CH_2$) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. In one aspect, a 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, for example, at the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. See, for example, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, the disclosures of which are incorporated herein by reference in their entireties.

[0155] In one aspect, a modification of the sugar includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring, thereby forming a bicyclic sugar moiety. The linkage in certain aspects is a methylene ($-CH_2-$) $_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

[0156] Oligonucleotides may also include base modifications or substitutions. As used herein, "unmodified" or "natural" bases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified bases include other

synthetic and natural bases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified bases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified bases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further bases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, 1991, *Angewandte Chemie*, International Edition, **30**: 613, and those disclosed by Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press, 1993. Certain of these bases are useful for increasing the binding affinity and include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C. and are, in certain aspects combined with 2'-O-methoxyethyl sugar modifications. See, U.S. Pat. Nos. 3,687,808, U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; 5,750,692 and 5,681,941, the disclosures of which are incorporated herein by reference.

[0157] A "modified base" or other similar term refers to a composition which can pair with a natural base (e.g., adenine, guanine, cytosine, uracil, and/or thymine) and/or can pair with a non-naturally occurring base. In certain aspects, the modified base provides a T_m differential of 15, 12, 10, 8, 6, 4, or 2°C. or less. Exemplary modified bases are described in EP 1 072 679 and WO 97/12896.

[0158] By "nucleobase" is meant the naturally occurring nucleobases adenine (A), guanine (G), cytosine (C), thymine (T) and uracil (U) as well as non-naturally occurring nucleobases such as xanthine, diaminopurine, 8-oxo-N⁶-methyladenine, 7-deazaxanthine, 7-deazaguanine, N⁴,N⁴-ethanocytosine, N',N'-ethano-2,6-diaminopurine, 5-methylcytosine (mC), 5-(C³—C⁶)-alkynyl-cytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridin, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner *et al.*, U.S. Pat. No. 5,432,272 and Susan M. Freier and Karl-Heinz Altmann, 1997, *Nucleic Acids Research*, vol. **25**: pp 4429-4443. The term "nucleobase" thus includes not only the known purine and pyrimidine heterocycles, but also heterocyclic analogues and tautomers thereof. Further naturally and non-naturally occurring nucleobases include those disclosed in U.S. Pat. No. 3,687,808 (Merigan, *et al.*), in Chapter 15 by Sanghvi, in *Antisense Research and Application*, Ed. S. T. Crooke and B. Lebleu, CRC Press, 1993, in Englisch *et al.*, 1991, *Angewandte Chemie*, International Edition, **30**: 613-722 (see especially pages 622 and 623, and in the Concise Encyclopedia of Polymer Science and Engineering, J. I. Kroschwitz Ed., John Wiley & Sons, 1990, pages 858-859, Cook, *Anti-Cancer Drug Design* 1991, 6, 585-607, each of which are hereby incorporated by reference in their entirety). The term "nucleosidic base" or "base unit" is further intended to include compounds such as heterocyclic compounds that can serve like nucleobases including certain "universal bases" that are not nucleosidic bases in the most classical sense but serve as nucleosidic bases. Especially mentioned as universal bases are 3-nitropyrrole, optionally substituted indoles (*e.g.*, 5-nitroindole), and optionally substituted hypoxanthine. Other desirable universal bases include, pyrrole, diazole or triazole derivatives, including those universal bases known in the art.

Methods of making tocopherol-modified oligonucleotides

[0159] Methods of making tocopherol oligonucleotides are disclosed herein. First, an oligonucleotide and phosphoramidite-modified- tocopherol are provided. Then, the oligonucleotide is exposed to the phosphoramidite-modified- tocopherol to create the tocopherol modified oligonucleotide. While not meant to be limiting, any chemistry to one of skill in the art can be used to attach the tocopherol to the oligonucleotide, including amide linking or click chemistry.

Methods of making liposomal particles

[0160] The disclosure also provides methods of making liposomal particles. In some embodiments, a phospholipid, solvent, and a tocopherol modified oligonucleotide are provided. Then, the phospholipid is added to the solvent to form a first mixture comprising liposomes. The

size of the liposomes in the first mixture is between about 100 nanometers and about 150 nanometers.

[0161] Next, the liposomes are disrupted to create a second mixture comprising liposomes and small unilamellar vesicles (SUV). The size of the liposomes and SUVs in the second mixture is between about 20 nanometers and about 150 nanometers.

[0162] Next, the SUVs having a particle size between about 20 nanometers and about 50 nanometers are isolated from the second mixture. Finally, the tocopherol modified oligonucleotide is added to the isolated SUVs to make a liposomal particle.

[0163] The particle size of the liposomal particles created by a method of the disclosure is less than or equal to about 50 nanometers. In some embodiments, a plurality of liposomal particles is produced and the particles in the plurality have a mean diameter of less than or equal to about 50 nanometers (*e.g.*, about 5 nanometers to about 50 nanometers, or about 5 nanometers to about 40 nanometers, or about 5 nanometers to about 30 nanometers, or about 5 nanometers to about 20 nanometers, or about 10 nanometers to about 50 nanometers, or about 10 nanometers to about 40 nanometers, or about 10 nanometers to about 30 nanometers, or about 10 nanometers to about 20 nanometers). In further embodiments, the particles in the plurality of liposomal particles created by a method of the disclosure have a mean diameter of less than or equal to about 20 nanometers, or less than or equal to about 25 nanometers, or less than or equal to about 30 nanometers, or less than or equal to about 35 nanometers, or less than or equal to about 40 nanometers, or less than or equal to about 45 nanometers.

[0164] In some aspects, the method comprises: (1) adding 1X PBS to dry lipids to a final concentration of 1-25 mg/mL (thus, in various embodiments, the final concentration is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 mg/ml); (2) freezing rapidly in liquid nitrogen and thawing in a bath sonicator 3 times; (3) extruding through 200, 100, 80, 50 and 30 nm filters. Double filters are used and typically passed 2-10 times through each filter. In some embodiments, the process is stopped at 50 nm, but if 30 nm structures are desired, then the 30 nm filter is additionally added. In further aspects, when 30 nm liposomes are desired, one probe sonicates after step (2). Next, the liposomes are centrifuged at 21000 x g for 10 minutes to remove metal shavings that come off in sonication and the mixture is extruded through a 30 nm filter as described in step (3).

[0165] Thus, in some aspects the disclosure provides a method of making a liposomal particle, comprising adding a phospholipid to a solvent to form a first mixture, said first mixture comprising a plurality of liposomes; disrupting said plurality of liposomes to create a second

mixture, said second mixture comprising a liposome and a small unilamellar vesicle (SUV); isolating said SUV from said second mixture, said SUV having a particle size between about 20 nanometers and 50 nanometers; and adding an oligonucleotide to the isolated SUV to make the liposomal particle.

Gene regulation

[0166] In some aspects of the disclosure, an oligonucleotide associated with a LSNA inhibits the expression of a gene. Methods for inhibiting gene product expression provided herein include those wherein expression of the target gene product is inhibited by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% compared to gene product expression in the absence of a LSNA. In other words, methods provided embrace those which results in essentially any degree of inhibition of expression of a target gene product.

[0167] The degree of inhibition is determined *in vivo* from a body fluid sample or from a biopsy sample or by imaging techniques well known in the art. Alternatively, the degree of inhibition is determined in a cell culture assay, generally as a predictable measure of a degree of inhibition that can be expected *in vivo* resulting from use of a specific type of liposomal SNA and a specific oligonucleotide.

[0168] In some aspects of the disclosure, it is contemplated that a LSNA performs both a gene inhibitory function as well as an agent delivery function. In such aspects, an agent is encapsulated in a LSNA as disclosed herein and the particle is additionally functionalized with one or more oligonucleotides designed to effect inhibition of target gene expression.

[0169] In various aspects, the methods include use of an oligonucleotide which is 100% complementary to the target polynucleotide, *i.e.*, a perfect match, while in other aspects, the oligonucleotide is at least (meaning greater than or equal to) about 95% complementary to the polynucleotide over the length of the oligonucleotide, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, at least about 50%, at least about 45%, at least about 40%, at least about 35%, at least about 30%, at least about 25%, at least about 20% complementary to the polynucleotide over the length of the oligonucleotide to the extent that the oligonucleotide is able to achieve the desired degree of inhibition of a target gene product.

[0170] It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. Moreover, an oligonucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). The percent complementarity is determined over the length of the oligonucleotide. For example, given an antisense compound in which 18 of 20 nucleotides of the antisense compound are complementary to a 20 nucleotide region in a target polynucleotide of 100 nucleotides total length, the oligonucleotide would be 90 percent complementary. In this example, the remaining noncomplementary nucleotides may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleotides. Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656).

[0171] Accordingly, the disclosure provides methods of utilizing LSNAs in gene regulation therapy. This method comprises the step of hybridizing a polynucleotide encoding said gene product with one or more oligonucleotides complementary to all or a portion of said polynucleotide, said oligonucleotide being attached to a liposomal particle, wherein hybridizing between said polynucleotide and said oligonucleotide occurs over a length of said polynucleotide with a degree of complementarity sufficient to inhibit expression of said gene product. The liposomal particle has a diameter that is about less than or equal to 50 nanometers. The inhibition of gene expression may occur *in vivo* or *in vitro*.

[0172] The oligonucleotide utilized in this method is either RNA or DNA. The RNA can be an inhibitory RNA (RNAi) that performs a regulatory function, and in various embodiments is selected from the group consisting of a small inhibitory RNA (siRNA), a single-stranded RNA (ssRNA), and a ribozyme. Alternatively, the RNA is microRNA that performs a regulatory function. The DNA is, in some embodiments, an antisense-DNA.

EXAMPLES

Example 1

[0173] Example 1 discloses the synthesis of a single liposomal spherical nucleic acid (LSNA) nanoparticle capable of dual inhibition of TLRs 4 and 9 (Figure 1). TLR4 inhibition will occur by encapsulation of TAK242, a small molecule inhibitor of TLR4. TLR9 inhibition will occur by functionalization of the LSNA surface with an inhibitory CpG (iCpG) sequence called INH-18 (sequence: 5'-CCTGGATGGGAACTTACCGCTGCA-3'; SEQ ID NO: 1) (Figure 2).

Materials and methods

[0174] Lipid monomer 1,3-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and TAK-242 were dissolved in chloroform, combined in defined ratios and dehydrated to create a thin film. Lipids were rehydrated with a Tris buffer solution containing NaCl to create liposomes. Liposomes were probe-sonicated and membrane extruded to produce small unilamellar vesicles (SUVs). SUVs were passed through a sepharose size exclusion column, then particle size was measured by dynamic light scattering (DLS). To make the SUV an LSNA, tocopherol-anchored DNA was incubated with SUVs with agitation. Liposomes were surface functionalized with oligonucleotides that bind the lipid membrane through a hydrophobic anchor (tocopherol or cholesterol). The size and polydispersity index of these LSNAs are small as determined by dynamic light scattering (DLS) (**Table 1**, PDI=0.0 is a perfectly monodisperse solution; <0.200 is considered a relatively homogenous preparation). A size shift from approximately 35nm to approximately 60nm indicates surface functionalization with oligonucleotides (Figure 3). The modularity and versatility of LSNA chemistry make this construct a desirable platform for delivery of therapeutic agents. Therefore, LSNAs have been designed for dual TLR inhibition by taking advantage of the properties of the liposome core and oligonucleotide shell. The combination of dual TLR inhibitory motifs within a single nanoparticle construct is more effective than single targeting due to the co-stimulation of specific TLRs and pathway crosstalk in certain conditions and diseases (Abdollahi-Roodsaz et al., The Journal of clinical investigation. 2008;118(1):205-16; Doyle et al., Immunity. 2002;17(3):251-63; Holle et al., Rheumatology. 2013;52(7):1183-9; Lee et al., Immunobiology. 2013;218(2):192-200). The LSNAs were specifically designed to display TLR9-inhibitory DNA at the surface of the particle and encapsulate the small molecule TLR4-inhibitor, TAK-242 (ethyl(6R)-6-[N-(2-chloro-4-fluorophenyl) sulfamoyl]cyclohex-1-ene-1-carboxylate [(R)-(+)-5n], within the core membrane. A previously reported TLR9-inhibitor DNA sequence (INH-18) was chosen for its specificity for both mouse and human TLR9 (Lenert et al., Arthritis research & therapy. 2009;11(3):R79). TAK-242 was chosen as a TLR4 inhibitor because it binds the intracellular domain of TLR4 and interferes with binding of adaptor proteins, preventing signal transduction (li et al., Molecular pharmacology. 2006;69(4):1288-95; Kawamoto et al., European journal of pharmacology. 2008;584(1):40-8; Matsunaga et al., Molecular pharmacology. 2011;79(1):34-41). LSNAs are recognized by Scavenger Receptor A on the cell surface, which mediates endocytosis of the particle (Choi et al., Proceedings of the National Academy of Sciences of the United States of America. 2013;110(19):7625-30; Patel et al., Bioconjugate chemistry. 2010;21(12):2250-6; Wu et al., Journal of the American Chemical Society. 2014;136(21):7726-33). Preliminary studies showed that within the endosome, the oligonucleotide shell binds to and inhibits TLR9, and that

eventual degradation of the nanoparticle releases TAK-242 to inhibit the intracellular portion of TLR4. Two approaches for measuring TAK-242 encapsulation within the lipid bilayer of the LSNA were employed: liquid chromatography mass spectrometry (LC-MS) and inductively-coupled plasma optical emission spectroscopy (ICP-OES). ICP-OES, an elemental analysis tool, measures the sulfur content of the LSNA, which reflects the sulfamoyl moiety in TAK242. This method is favored due to ease of sample preparation and speed of data acquisition. LC-MS (and MS2) measures the presence of whole TAK-242 (MW=361) and a fragment of TAK-242 (MW=179). These methods were used to provide independent corroborative data. Particles made from different molar ratios of lipid to TAK-242 have been shown to retain their small particle size and low polydispersity (**Table 1**). Once the particles are characterized, 3'-tocopherol is added to TLR9-inhibitory DNA, which anchors it into the liposomal bilayer.

***In vitro* validation of LSNA inhibitory properties**

[0175] Preliminary studies using an NF- κ B reporter mouse cell line (RAW-Blue) measured TLR9 and TLR4 inhibition by LSNA. RAW-Blue cells are engineered mouse macrophages that produce secreted embryonic alkaline phosphatase (SEAP) when NF- κ B is activated. Secretion of SEAP into the media leads to a colorimetric change from pink to blue/purple, and the absorbance correlated with TLR/NF- κ B activation. The activity of free TAK-242 against CpG (TLR9)- or MPLA (TLR4)-stimulated RAW-Blue cells indicated that inhibition is specific to TLR4 activation by monophosphoryl lipidA (MPLA), and free INH-18 leads to inhibition of CpG-activated cells in a dose-dependent manner (Figures 7 and 8). Notably, tests of free INH-18 activity showed a limited inhibition of MPLA activation (Figure 7B) while exhibiting a high degree of inhibition of CpG-stimulated cells.

[0176] Importantly, initial validation studies of LSNA inhibition using four different types of LSNA with varying bioactive components in RAW-Blue cells (Table 2) show that LSNA bearing the INH-18 oligonucleotide sequence exhibited greater inhibition of CpG activation (TLR9) than free INH-18 alone (Figure 8A: Free INH-18 vs. LSNA 2 +INH/-(no)TAK-242 or LSNA 4 +INH/+TAK-242). Furthermore, due to the presentation of INH-18 on the LSNA surface, activity against MPLA-induced activation (Figure 8B: LSNA2 +INH/-(no)TAK-242) remains at baseline without inhibition, indicating greater specificity of the oligonucleotide (INH-18) when used in this delivery system. Finally, the inhibitory effect of LSNA carrying TAK-242 was equivalent to free TAK-242 (Figure 8B: Free TAK-242 vs. LSNA3 +T40/+TAK-242 or LSNA-4 +INH/+TAK-242) in MPLA-activated cells, indicating that encapsulated TAK-242 was effective at reaching and inhibiting the intracellular domain of TLR4.

[0177] TAK-242 encapsulation and quantity was measured by sulfur analysis using inductively coupled plasma optical emission spectrometry (ICP-OES) and confirmed by liquid chromatography mass spectrometry (LC-MS) (Figures 3 and 5).

[0178] TLR activity was measured in HEK-Blue reporter cells using the secreted alkaline phosphatase (SEAP) assay. Briefly, cells engineered to express human or mouse TLRs are stimulated in response to their appropriate ligands. TLR activation leads to downstream activation of NF- κ B by alkaline phosphatase activation. AP1 is secreted into the media and converts it from pink to purple/blue (Figure 4).

Results

[0179] Table 1 shows the composition and characterization of TAK-encapsulated liposomes.

Table 1

Ratio lipid:TAK	Size (nm)	PDI	TAK encap. (nmol/nm ³)
100:1	34 $\pm$ 9	0.134	1.83 $\times$ 10 ⁻²⁴
100:3	35 $\pm$ 10	0.094	2.88 $\times$ 10 ⁻²⁴
20:1	38 $\pm$ 10	0.174	7.33 $\times$ 10 ⁻²⁴
25:4	32 $\pm$ 9	0.162	1.46 $\times$ 10 ⁻²³

[0180] Figure 6 shows the uptake of dye-labeled LSNAs in mouse macrophages. Figure 7 shows TLR inhibition by free INH-18 or TAK-242 is specific. RAW-Blue macrophages were stimulated with either 1826 CpG DNA (TLR9 ligand) or monophosphory lipid A (MPLA, TLR4 ligand). The SEAP assay was used to measure relative activation of cells. TLR9 inhibition is clearly specific to INH-18 oligonucleotide (A), but TLR4 inhibition, though mostly inhibited by TAK-242, is slightly inhibited by INH-18 (B). Crosstalk between TLR4 and TLR9 pathways could explain this phenomenon.

Table 2

Name	DNA	Core
LSNA-1	T40	-TAK
LSNA-2	INH-18	-TAK
LSNA-3	T40	+TAK
LSNA-4	INH-18	+TAK

[0181] Figure 8 shows potent TLR4 and TLR9 inhibition by LSNAs in mouse and primary human cells. Four different LSNAs were synthesized and RAW-Blue cells were treated with 500nM LSNAs to test for ability to modulate TLR activation (A-B). Only particles containing INH-18 inhibitory sequences are capable of suppressing TLR9 activity (A) and more potently than free INH-18 DNA, while particles containing TAK-242 suppress TLR4 activity (B) comparable to free TAK-242. CpG-induced human peripheral mononuclear blood cell (hPBMC) proliferation is inhibited in a dose dependent manner when treated with dual-targeting LSNAs (C). Changing the DNA backbone of INH-18 from phosphodiester (PO) to phosphorothioate (PS) increases potency and is as effective as immunosuppressive glucocorticoid Dexamethasone (D).

Conclusions

[0182] This example demonstrates successful synthesis and *in vitro* characterization of a liposomal nanoparticle that can encapsulate a hydrophobic small molecule inhibitor of TLR4 and features TLR9 inhibiting oligonucleotides on the surface. This construct was rapidly taken up by murine macrophages and effectively suppressed downstream activation of NF- κ B. It also diminished human lymphocyte proliferation. Finally, the construct was tunable for TLR9 suppression by altering the backbone of the oligonucleotides from phosphodiester to phosphorothioate.

[0183] Multiple TLR9 inhibiting sequences will be screened and the backbone chemistry will be varied. The dose and time dependent response of RAW-Blue and HEK-Blue reporter cells induced with MPLA and CpG DNA simultaneously to dual TLR-targeting LSNAs will also be examined. Further, downstream inhibition of cytokines will be measured using human PBMCs.

Ultimately, this dual-targeting particle will be tested in an ischemia reperfusion/transplantation mouse model.

References

1. Banga RJ, Chernyak N, et al. Journal of the American Chemical Society. 2014.
2. Radovic-Moreno AF, Chernyak N, et al. Proceedings of the National Academy of Sciences. 2015.

Example 2

[0184] Toll-like receptors (TLRs) play a large role in the recognition of pathogens and modulation of the immune system. Overstimulated TLRs lead to dysregulation of downstream molecular targets and the development of inflammation mediated diseases, such as autoimmune disorders, sepsis and ischemia reperfusion injury (IRI). To date, no therapeutic inhibitory platform has been developed that can restore normal function of TLRs after overstimulation. Suppression of these TLRs requires an efficient and targeted approach, and nanoparticle-mediated delivery of TLR inhibitors offers a path towards a potential therapeutic.

[0185] Like example 1, the purpose of this example was to synthesize and further characterize a single liposomal spherical nucleic acid (LSNA) nanoparticle capable of dual inhibition of TLRs 4 and 9 (Figure 9). TLR4 inhibition occurs by encapsulation of a small molecule (TAK-242). TLR9 inhibition occurs by functionalization of the LSNA surface with an inhibitory DNA sequence (INH-18) (Figure 10).

[0186] Lipid monomer 1,3-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and TAK-242 were dissolved in chloroform, combined in defined ratios and dehydrated to create a thin film. Lipids were rehydrated with a Tris buffer solution containing NaCl to create liposomes. Liposomes were probe sonicated and membrane extruded to produce small unilamellar vesicles (SUVs). SUVs were passed through a sepharose size exclusion column, then particle size was measured by dynamic light scattering (DLS). To make the SUV into an LSNA, cholesterol-anchored DNA was incubated with SUVs at 37°C with agitation.

[0187] The amount of TAK-242 encapsulated was measured by sulfur analysis using inductively coupled plasma optical emission spectrometry (ICP-OES) and confirmed by liquid chromatography Mass spectrometry (LC-MS). TLR activity was measured in HEK-Blue reporter cells using the secreted alkaline phosphatase (SEAP) assay. Briefly, human cells engineered to express mouse TLRs were stimulated with their appropriate ligands. TLR activation leads to downstream activation of NF- κ B and subsequent alkaline phosphatase translation. AP1 is secreted into the media and converts the media from pink to purple/blue. See Figures 3 and 4.

[0188] For *ex vivo* and *in vivo* studies, male C57BL/6 mice were used. Flow cytometry was used to measure cytokine production from splenocytes *ex vivo* as well as in serum from animals. Blood and liver sections were analyzed for liver function markers, histological changes and cell death following ischemia reperfusion injury *in vivo*.

[0189] Figure 11 shows potent TLR4 and TLR9 inhibition by LSNAs in engineered TLR reporter cells. Four different LSNAs were synthesized and HEK-Blue cells expressing mouse TLR4 or mouse TLR9 were treated with LSNAs (125nM by DNA, 75nM by TAK) to test for the ability to modulate TLR activation (A-B). Only particles containing INH-18 inhibitory sequences were capable of suppressing TLR9 activity (A), while particles containing TAK-242 suppressed TLR4 activity (B).

[0190] Figure 12 demonstrates the kinetic advantage of using LSNAs. The speed at which TLR9 activity was suppressed was assessed using HEK-Blue cells expressing mouse TLR9. Cells were treated for 4 hours simultaneously with agonist and antagonists (A) or with a 4 hour pulse of agonist followed by 8 hours with antagonist (B).

[0191] Figure 13 demonstrates that LSNAs are potent inhibitors of pro-inflammatory cytokine production in primary mouse cells. Primary mouse splenocytes were isolated and treated with 500nM TLR9 agonist (Figure 13A-E) or 500ng/ml TLR4 agonist (Figure 13F) to stimulate cytokine production. In general, dose dependence of antagonist was observed.

Ischemia Reperfusion Injury (IRI) Model

[0192] Figure 14 shows the damage caused during liver IRI. A midline incision was made to expose the liver, and an atraumatic clamp was placed to cut off blood supply to the left lobe (A). Major histological changes (B) and cell death (C) were evident only after 90 minutes of ischemia and 4 hours of reperfusion (blue=DAPI, red=TUNEL).

[0193] Figure 15 shows results of a liver function test and IL-6 levels following IRI. Ninety minutes of ischemia induced significant changes in the levels of alanine transaminase (ALT), albumin (ALB), alkaline phosphatase (ALP), total bilirubin (TBIL), cholesterol (CHOL) and pro-inflammatory cytokine IL-6.

Conclusions

[0194] This example demonstrated the successful synthesis and characterization of a single entity liposomal nanoparticle that encapsulated a hydrophobic small molecule inhibitor of TLR4 and displays TLR9 inhibiting oligonucleotides on the particle surface. This construct was rapidly taken up by engineered and primary mouse cells and effectively suppressed downstream activation of NF- κ B and effector molecules of this pathway.

Example 3

[0195] The nanoparticle core, which is used to encapsulate the TLR4 antagonizing small molecule drug TAK-242 (*i.e.*, Resatorvid; CLI-095), is comprised to lipid monomers. DOPC is the lipid monomer most commonly used due to it being an FDA approved material, but other lipids may be incorporated to impart chemical features and/or biological effects.

[0196] The outer surface of the lipid core is decorated with synthesized DNA that has a hydrophobic moiety conjugated to it. The hydrophobic moiety inserts into the lipid bilayer of the nanoparticle core and anchors the DNA to the outer surface. The DNA sequence chosen is ODN INH-18, which is a TLR9 inhibitor specific to mouse and human TLR9. Other TLR9 targeting sequences were screened, but INH-18 was chosen for its potency.

Core Synthesis

[0197] The core containing TLR4 inhibiting small molecule drug (TAK-242 (*i.e.*, Resatorvid) is made by dissolving the TAK-242 in chloroform. This is mixed with lipid monomers that come dissolved in chloroform, and the solvent is evaporated under nitrogen. The lipid films are dried overnight using lyophilization. The dried lipid films are rehydrated with buffer and probe sonicated, ultracentrifuged, and extruded to obtain uniformly sized liposomes containing TAK. Any unencapsulated material is removed using size exclusion. See Figure 16.

Core Characterization

[0198] Following core synthesis, the size and polydispersity index (PDI) are determined using dynamic light scattering (DLS). To determine the amount of TAK-242 encapsulated within the core, inductively coupled plasma-optical emission spectrometry (ICP-OES) is used to determine the phosphorous content (lipid concentration) and sulfur content (TAK-242 concentration). See Table 3.

Table 3. Core characterization.

RATIO OF LIPID:TAK	SIZE (nm)	PDI
100:1	34 ± 9	0.134
100:3	35 ± 10	0.094
20:1	38 ± 10	0.174
25:4	32 ± 9	0.162

LSNA Synthesis

[0199] Once the concentration of the nanoparticles and encapsulated TAK was determined, 1.3mM of lipid nanoparticles were mixed with 15uM TLR9-targeting DNA. This mixture was

heated to 37°C and allowed to shake on an orbital shaker for 4 hours. The final particle size and PDI was again measured by DLS. See Figure 17.

LSNA *in vitro* and *ex vivo* testing

[0200] To determine the function of the newly synthesized particles, engineered RAW 264.7 and HEK293 cells expressing mouse TLRs 4 and 9 were generated. Briefly, activation of a TLR pathway results in the secretion of an enzyme that leads to a measurable colorimetric change in the media. In these experiments, cells expressing either mouse TLR4 and/or mouse TLR9 were stimulated with only one of the appropriate agonists (at a time) while simultaneously being treated overnight with various LSNA.

[0201] In a time course study to tease out the advantage to using a nanoparticle system over the free components, it was observed that in cells expressing activated mouse TLR9, the LSNA platforms faster. In other words, the LSNA more quickly suppressed TLR9 activation.

[0202] The ability of cells to suppress TLR activation in primary mouse splenocytes will be conducted. This is expected to be a better model to test LSNA activity than the engineered cells because it is a more clinically relevant model.

[0203] Figure 18 shows TLR9 suppression in HEK-Blue cells expressing mouse TLR9. Experiment was run overnight.

[0204] Figure 19 shows TLR4 suppression in HEK-Blue cells expressing mouse TLR4. Experiment was run overnight.

[0205] Figure 20 shows TLR9 suppression in HEK-Blue cells expressing mouse TLR9. Experiment was run for 4 hours.

Example 4

[0206] This example demonstrates that immuno-stimulatory SNAs (IS-SNAs) are taken up by dendritic cells in lymph nodes. More specifically, this example demonstrates the use of encapsulated spherical nucleic acid (SNA) vaccines. In this example, the encapsulated payload was tumor-specific antigen. See Figure 21.

[0207] Mice were injected with SNAs and the uptake of labeled oligonucleotide was measured in the draining lymph node. Figure 22 shows a blue clear spot for SNA which is from the Cy5. The graph shows the percentage of cells that were positive for Cy5 in the flow cytometer.

[0208] Next, the uptake of DNA and peptide together into dendritic cells was measured. This experiment demonstrated that IS-SNAs co-deliver immunostimulatory oligonucleotides and

tumor-associated antigen. The constructs shown in Figures 23 and 62 were incubated with primary dendritic cells in a petri dish. After the incubation, the cells were run through a flow cytometer. The Y axis of Figures 23 and 62 is the DNA and the X axis is the peptide. Each point is a cell and the four quadrants separate positive and negative cells. The upper right corner is the percentage of cells positive for both peptide and DNA, indicating a doubling of the co-delivery efficiency with the SNA formulation compared to a simple mixture of oligonucleotide and peptide.

[0209] In the next experiment, it was demonstrated that dendritic cells treated with IS-SNAs showed enhanced ability to prime T-cells. SNAs were incubated with dendritic cells and killer T cells and T cell division was measured. When T cells are activated, they divide, which is used as a measure of activation. The cells were labeled with a dye on the surface and as the cells divided, the dye got diluted. The left plot in Figure 24 demonstrates that all of the cells were still being fully labeled, whereas on the right, multiple peaks to the right appear, indicating cells have divided. Each peak is one cell division so half of the cells have undergone multiple cell divisions with the peptide encapsulated SNA.

[0210] The next experiment showed that antigen-specific Killer T-cell activity was induced by IS-SNAs in a tumor model. Wild-type mice were immunized and injected with a 50:50 mixture of cells with target (OVA1) and control (gp100) peptides. The target peptide containing cells got killed by the immune system upon vaccination, and the ratio is used to determine specific killing. The SNA outperformed the mixture of oligonucleotide and antigen by 3-fold. This was a 3-day experiment. See Figure 25.

Example 5

Define the impact and molecular mechanism of chemical modifications to TLR9-targeting oligonucleotides and the resulting influence on the properties and potency of dual inhibitory LSNAs.

[0211] Modifying the backbone and sequence of the TLR9-targeting oligonucleotide enables molecular tuning of LSNAs and influences production of downstream TLR mediators and cytokines in MPLA- and CpG DNA-stimulated cells.

[0212] Based on data (Example 1) showing that LSNAs attenuate the stimulatory effects of MPLA and CpG, it is believed that chemical modification of the synthetic oligonucleotide bound to the liposome core will influence the receptor-ligand interaction and will control signaling cascades downstream of TLR. Thus, both oligonucleotide sequence and backbone chemistry influence potency of TLR9 suppression by the LSNA construct are investigated. The capacity and specificity of non-interacting (negative control), single inhibitory (e.g., against TLR4 or

TLR9) and dual TLR-inhibitory LSNAs are compared based on various oligonucleotide constructs to inhibit activation of TLR4 and/or TLR9. It is expected that the dual inhibitory approach will result in a synergistic interaction between TLR4 and TLR9 inhibitors that modulates crosstalk between TLR4 and TLR9 receptors and downstream pathways. Engineered reporter cell lines as well as primary cells are used as tools to measure NF- κ B activation, cell proliferation, and cytokine production as a readout of TLR activity. Results from these studies will provide insight and link oligonucleotide chemistry to the cellular response elicited by LSNAs and the synergistic effect of combining two TLR-inhibitory strategies in a single nanoparticle.

Evaluation of LSNA oligonucleotide sequence and backbone chemistry on TLR inhibitory response.

[0213] These experiments will determine the benefits of LSNA modularity by measuring the impact of varying the sequence and backbone chemistry of the TLR9-targeting oligonucleotide on the degree of TLR9 inhibition. To probe this effect, various TLR9-inhibiting sequences that differ in length and composition (Table 4) are screened in RAW-Blue cells. How changing the DNA backbone from phosphodiester (PO) to phosphorothioate (PS) influences LSNA potency using RAW-Blue cells as an initial screen will also be investigated. DNA phosphorothioate modification results from the substitution of sulfur for the non-bridging oxygen of the sugar-phosphate backbone. Although this does not change the charge of the oligonucleotide, this modification renders the internucleotide linkage resistant to nuclease degradation (Gan et al., *Sci Rep.* 2014;4:6642; Uhlmann et al., *Antisense & nucleic acid drug development.* 1997;7(4):345-50). Additionally, substitution of oxygen for sulfur may alter the binding affinity, and thus, the potency of TLR9 inhibition (Beltinger et al., *The Journal of clinical investigation.* 1995;95(4):1814-23; Beck et al., *Pharmaceutical research.* 1996;13(7):1028-37; Ge et al., *RNA.* 2010;16(1):118-30; Roberts et al., *Molecular immunology.* 2011;48(8):1027-34; Sester et al., *Journal of immunology.* 2000;165(8):4165-73; Wang et al., *Nucleic acid therapeutics.* 2013;23(4):253-63; Zhang et al., *Cancer immunology, immunotherapy : CII.* 2014;63(4):357-67). The data provided by these experiments will provide insight into specific oligonucleotide-TLR9 interactions and how stereochemistry and electrostatic interactions influence TLR9 activation and inhibition. A more thorough understanding of receptor-activator-inhibitor interactions and a more detailed knowledge of the ligand binding site will aid not only in the construction of finely tuned LSNAs but may also lead to new insights into targeting this critical cellular pathway.

Table 4

Oligonucleotide	Specificity	Sequence 5' → 3'	SEQ ID NO
INH-18	human/mous	CCTGGATGGGAACCTTACCGCTGCA	1
4084-F	human/mous	CCTGGATGGGAA	2
INH-1	human/mous	CCTGGATGGGAATTCCCATCCAGG	3

Determination of the effective concentration (EC₅₀) of LSNAs *in vitro* using human embryonic kidney (HEK)-Blue cells.

[0214] Though many studies suggest that TLRs are highly conserved and homologous across species, it is important to verify LSNA effects due to chemistry and sequence in human cells. TLR9 expression varies between mouse and human cells, and this may result in differences in potency of TLR9 inhibition by LSNAs (Fitzner et al., International immunopharmacology. 2011;11(11):1789-95; Haining et al., Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(17):5626-34; Peter et al., Immunology. 2008;123(1):118-28). These experiments are designed to determine the potency of LSNAs using engineered human cells (HEK-Blue) in preparation for later studies with primary human cells. Like RAW-Blue cells, HEK-Blue cells secrete alkaline phosphatase when NF-κB is activated. HEK-Blue cells, however, are designed to respond to only one TLR receptor ligand, which will inform experiments aimed at delineating the specificity of LSNA inhibition via inhibitory oligonucleotides or TAK-242. Additionally, although mouse and human TLR4 and TLR9 are conserved, the sensitivity of these engineered cell lines to TLR stimulation and inhibition may vary. EC₅₀ data from these HEK-blue studies will be used for testing LSNAs in human primary cells.

Elucidate the effect of LSNA treatment on human Peripheral Blood Mononuclear Cells (PBMCs) stimulated with MPLA and/or CpG.

[0215] PBMCs play a role in the propagation of the inflammatory response and the cell-based immunologic response to IRI. These cells are recruited to the reperfused, transplanted organ, proliferate, and facilitate death of cells within the tissue and release inflammatory signals. To assess the inhibitory effect of LSNAs on PBMC proliferation, tritium (3H)-labeled thymidine incorporation is used as a measure of LSNA inhibitory activity against activated PBMCs. Cells are stimulated with the appropriate ligands for TLR4 or TLR9 (MPLA or stimulatory CpG DNA, respectively) or with polyhemagglutinin, a plant mitogen, as a control. A dose response test using the best performing LSNAs is performed. Data supporting these studies showed that proliferation of CpG DNA-stimulated hPBMCs is inhibited by 20-40% in a dose-dependent manner across varying concentrations (5- 500nM) of inhibitory LSNAs containing both TAK-242 and INH-18 compared to control, non-targeting LSNAs (Figure 26). The results of this study

allow us to correlate potency of response (inhibition of proliferation) to LSNA backbone chemistry and oligonucleotide sequence.

Delineate the downstream effect of dual-targeting LSNAs on mRNA and protein expression of classical pro-inflammatory markers and molecular mediators of TLR signal transduction.

[0216] TLR signaling pathways involve multiple molecular mediators that ultimately result in the production of cytokines and chemokines. Classical mediators of a pro-inflammatory response in stimulated PBMCs include IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-12, TNF α , IFN γ , and GM-CSF. To look beyond the classical markers, complete cytokine and chemokine profiles released into the media will be obtained during the proliferation experiments discussed above using the Luminex system, which is capable of multi-analyte (30+ analytes simultaneously) detection in very small sample volumes. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and Western blot are used to analyze TLR4 and TLR9 mRNA and protein expression and mRNA and protein expression and phosphorylation levels of TLR signal transduction mediators; such as MyD88, TRIF and TRAF3 (MyD88 independent pathway), JNK and p38 MAPK, and NF- κ B.

Expected outcomes

[0217] Based on *in vitro* results obtained in engineered mouse macrophages (Figure 8), it is expected that dual targeting of TLRs will have an additive or synergistic effect in blunting production of downstream molecular mediators of the pro-inflammatory response and will lead to decreased proliferative capacity of hPBMCs. It is likely that the potency of LSNAs will vary depending on the targeting oligonucleotide sequence and backbone of the DNA. Comparison of the secondary structures of PO and PT-DNA based oligonucleotides suggest that the PT backbone DNA will confer higher potency than PO DNA due to closer interaction with the TLR9 binding site and will result in faster LSNA uptake into cells and ultimately, increased resistance to nucleases.

[0218] If it is found that LSNAs with altered oligonucleotide sequences and backbone chemistry are less potent than the original formulation (Figure 8A-B), then how the chemistry of the particle core affects TLR inhibition is examined. There are alternative lipid monomers that may be substituted for DOPC or used in combination that may alter stability of the core, change its capacity to encapsulate TAK-242, and influence degradation of the core within the endosome and the release kinetics of TAK-242. Incorporation of cholesterol, for example, will increase core rigidity and may increase TAK-242 encapsulation and prevent drug leaching from the core. Incorporation of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) may increase release of

TAK-242 and entry into the cytoplasm. DOPE, like DOPC, is a neutral lipid (as opposed to cationic or anionic), but DOPE is pH-sensitive. At lower pH, as found in the endosome, DOPE undergoes a conformational change that may facilitate core fusion with the endosome and increased endosomal escape of TAK-242 (Bergstrand et al., Biophysical chemistry. 2003;104(1):361-79; Du et al., Sci Rep. 2014;4:7107; Ferreira Ddos et al., Therapeutic delivery. 2013;4(9):1099-123; Yotsumoto et al., Journal of immunology. 2008;180(2):809-16). These strategies are explored if increased TLR4 inhibition is required.

Examine the ability and efficacy of LSNAs to reduce ischemia reperfusion injury in a mouse model of liver transplantation.

[0219] It is believed that treatment with LSNAs will reduce the impact of IRI after organ transplantation, with the most effective treatment time occurring prior to organ removal from the donor animal. Toll-like receptors have been implicated as one of the primary mediators injury-induced inflammation associated with ischemia reperfusion injury (IRI) of the liver (Huang et al., Hepatology. 2015;62(2):600-14; Wang et al., Chinese medical journal. 2007;120(6):509-14). The goal is to determine the efficacy of dual function LSNAs in reducing IRI and minimizing hepatocellular damage after organ harvest and preservation. Specifically, the optimal time course and approach for LSNA administration in a liver transplantation model is determined by comparing the effects of pretreatment of the donor animal prior to liver harvest to pretreatment of the liver while it is in cold preservation solution containing LSNAs, and to intravenous treatment of the recipient animal at the time of transplantation. The results of this study will inform on the timing of treatment when transplantation IRI damage is most responsive to LSNA treatment. The expectation is that treatment with dual-targeting LSNAs will reduce IRI-induced damage to a transplantable organ with the greatest efficacy of treatment obtained by administering LSNAs prior to the harvest of the donor liver.

Assess effects of LSNA treatment on organ function and pro-inflammatory cytokine levels following graft reperfusion.

[0220] Using a murine liver transplant model, the effects of 30 minute pretreatment are compared with control (e.g., non-interacting) and dual-targeting LSNAs on liver markers. LSNAs are administered to donor mice 30 minutes prior liver removal, in cold University of Wisconsin preservation solution for an hour at 4° C, or intravenously to recipient syngeneic mice at the time of transplantation. Blood samples are collected at 30 minutes, 24 hours, and 72 hours following transplantation or sham surgery (control). These samples are tested for markers of liver damage: ALT (alanine aminotransferase), AST (aspartate aminotransferase) and AlkPhos (alkaline phosphatase). Bilirubin levels will also be compared between treated transplant recipient animals and control animals given non-targeting, inert LSNAs (Scheig,

Primary care. 1996;23(3):551-60). In addition, the Luminex system is used to quantify the production of classical pro-inflammatory markers, specifically IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-12, TNF α , IFN γ , and GM-CSF, in collected blood samples.

[0221] Alternatively, IRI can be induced by hilar clamping of the portal vein and hepatic artery that supplies blood to the liver, thereby eliminating the complexity of the liver transplantation model and reducing the number of animals needed. However, hilar clamping does not recapitulate the clinical scenario of liver transplantation and the effects of the cold ischemia component on IRI. Moreover, it removes the ability to evaluate *ex vivo* treatment of the liver graft, which is an attractive route for direct treatment of the graft and avoids the pharmacological challenges associated with systemic LSNA administration.

Measure proteoform (protein isoform) changes in response to IRI and LSNA treatment within the liver graft.

[0222] To corroborate the response of liver enzyme levels and cytokine production to LSNA treatment, alternative biomarkers related to IRI severity and treatment response are examined. This approach deploys "top down" mass spectrometry analysis of intact proteins to identify unique protein signatures associated with cellular injury (Jiang et al., The Journal of biological chemistry. 2007;282(38):27923-34; Siuti et al., Nature methods. 2007;4(10):817-21; Kellie et al., Molecular bioSystems. 2010;6(9):1532-9; Tran et al., Nature. 2011;480(7376):254-8; Savaryn et al., Genome medicine. 2013;5(6):53; Ye et al., PloS one. 2014;9(4):e92831). This method is adapted to examine the impact of treatment with dual targeting LSNAs on liver IRI. This strategy is highly sensitive and specific for distinguishing variably modified protein isoforms (proteoforms) (Smith et al., Nature methods. 2013;10(3):186-7). Preliminary data supporting the use of this assay in IRI showed significant changes in proteoform expression between control and IRI-induced mouse kidneys (Figure 27). Histone modifications were noticeably altered by IRI: there is a significant increase in single or doubly modified H2A2A, H2AJ, and HMGN1 at 2 hours. Mouse blood, from the study outlined above, and liver graft tissue recovered from necropsy at 72 hours will be analyzed for proteoform frequency, which will be correlated with liver function (ALT, AST), recipient survival and LSNA treatment regimen. The goal is to develop an initial biomarker signature to correlate severity of liver IRI and response to LSNA treatment.

Expected outcomes

[0223] It is expected that pretreating donor animals with LSNAs prior to organ recovery and cold preservation will have the greatest impact on minimizing IRI because the cells of the donor organ will actively take-up LSNAs within endosomes; thereby 'priming' the graft. While direct

treatment of the donor organ may be technically easier and avoid potential degradation by nucleases in blood, the low temperature of the preservation solution may decrease cellular activity and limit LSNA endocytosis. Alternatively, systemic treatment of the recipient may attenuate recipient leukocytes that infiltrate the donor graft. Taken as a whole, the findings will aid in identifying a treatment strategy that confers the most protection from IRI and will offer insight into the etiology of the target cell population (*e.g.*, infiltrating recipient leukocytes or passenger immune cells within the donor graft, *e.g.*, Kupffer cells). However, as an alternative strategy to further define the etiology (donor vs. recipient) of the target cell population, TLR4 or TLR9 knockout mice (available from The Jackson Laboratory) are used as either donors or recipients along with wild-type animals in studies using mono-inhibitory LSNAs. As only one population of animals (donor or recipient) will express the wild-type TLRs, any inhibitory effects seen from LSNA treatment are attributed to cells originating from the wild-type animal.

Example 6

Designing a potent TLR 7/8 LSNA agonist

[0224] SNAs are synthesized by adding single-stranded RNA to liposomes for 4 hours at 37°C while shaking. Liposomes are constructed from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), a biocompatible FDA approved material, using sonication and extrusion extruded through a 30 nm membrane. Concentration of liposomes are quantified using an ICP-OES for phosphorous. Next, single-stranded RNA containing a hydrophobic anchor (tocopherol or cholesterol) was synthesized using an RNA synthesizer, processed using recommended procedures on the Glen Research website and purified using reverse phase HPLC (see below for more details pertaining to sequence). The ratio of lipid monomer to RNA upon mixing is 100 to yield approximately 70 strands of RNA/LSNA. The resulting LSNA is around 30-50 nm in diameter (see, *e.g.*, Figure 3).

[0225] To ensure that the RNA was stable in biological conditions, LSNAs were incubated at 37°C for 14 hours. RNA was extracted from the liposomal using a chloroform extraction, heated, denatured using 8M urea, and analyzed using a 12% denaturing PAGE gel. The results indicated that RNA (both with a phosphothiolate and a phosphodiester backbone and modified with a hydrophobic anchor) was intact while free RNA contained several degradation peaks. Further stability studies will be conducted after treatment with fetal bovine serum (FBS) and analyzed using HPLC.

[0226] The mechanism of LSNAs' mode of entry is through endocytosis by caveolin-mediated lipid rafts, and traffic through the endocytic pathway. Confocal results indicate that LSNAs co-localize with late endosomal and lysosomal markers within 45 minutes of transfection.

[0227] To test if the LSNAs bind and activate TLR 7/8, they were transfected into TLR7/8 expressing reporter cell lines (human embryonic kidney 293 engineered cells (Hek-Blue) that are engineered to contain either TLR7 or TLR8 and an inducible secreted alkaline phosphatase (SEAP) upon NF- κ B activation) for 24 hours at 37°C (40,000 cells seeded/well). Relative activity of TLR7/8 activation was quantified using a QuantiBlue Assay (through the formation of NBD diformazan and an indigo dimer). Figure 28 shows that LSNAs exhibit enhanced activation of TLR7/8 over free RNA, and RNA transfected with a cationic transfection agent.

[0228] In order to obtain the most potent activator of TLR7/8, various sequences of single stranded RNA agonists and sequences modifications were tested using Hek-Blue cells and the QuantiBlue assay. The following sequences were explored (listed from 5' to 3'):

Sequence Name	Sequence (5'→3')	SEQ ID NO
SSRNA 40	GCC CGU CUG UUG UGU GAC UC	4
SSRNA 41 (Control RNA)	GCC CGA CAG AAG AGA GAC AC	5
SSRNA DR	UGU CCU UCA AUG UCC UUC AA	6
ORN 02	UUA UUA UUA UUA UUA UUA UU	7
ORN 06	UUG UUG UUG UUG UUG UUG UU	8
SIMRA9	UNC URC UUC UN (N=7-DEAZA-GUANINE)	9

[0229] The following modifications were also explored:

1. Anchor type and sequence orientation (5' and 3' anchors for both tocopherol and cholesterol)
2. RNA Backbone: phosphothiolate and phosphodiester backbones, where phosphothiolate backbone is expected to increase the stability and perhaps the uptake of LSNAs into cells
3. Sequence length (increasing and decreasing the length of the RNA sequence by increasing the number or repeats in the sequence)
4. Internal modifications: presence and absence of PEG spacers were thought to alter the flexibility and therefore the binding and activity of RNAs to TLRs 7/8
5. Sequences specific to increasing the activation of either TLR 7 or 8: a phosphodiester "UUU" was added to the ends of sequences to enhance TLR8 activity while "UCA" sequences were synthesized with a phosphodiester backbone to increase TLR7 activity

[0230] The most potent sequence tested for activating TLR7 was "cholesterol - UGU CCU UCA AUG UCC UUC AA" (SEQ ID NO: 10) while the most potent sequence tested for activating TLR8 was "cholesterol - PEG spacer - UGU CCU UCA AUG UCC UUC AA" (SEQ ID NO: 11),

all with a phosphothiolate backbone. Further studies will explore the role of oligonucleotide density in determining TLR7/8 activity in Hek-Blue reporter cells.

In vitro and Ex vivo Validation of LSNAs

[0231] LSNAs induce dendritic cell maturation by upregulating co-stimulatory molecules CD80 and CD86 (CD86+, CD11c+ shown below) on C57BL/7 dendritic cell after a 24 hour incubation at 37°C at a cell density of 400,000 cells/mL. After incubation, cells were stained with fluorescent antibodies against CD80, CD86, and CD11c, and fixed in 0.5% paraformaldehyde. Samples were then analyzed using an LSR II flow cytometer. Results indicated that the addition of LSNAs result in higher number of CD86+ receptors on dendritic cells than when they are introduced to RNA instead. These results are comparable to Resiquimod, a TLR7/8 agonist that was used as a positive control (Figure 29). The upregulation of co-stimulatory markers is crucial for dendritic cells to activate T-cells.

[0232] To determine if LSNAs activate antigen presenting cells, they were introduced to dendritic cells (1,000,000 cells/mL for 48 hours at 37°C), B cells (1,000,000 cells/mL for 24 hours 37°C), and splenocytes (1,000,000 cells/mL 37°C) from C57BL/6 mice. Dendritic cells were directly purchased, while B cells were isolated using an enrichment kit from C57BL/7 splenocytes. After the incubation period, cell supernatants were isolated and analyzed using magnetic bead immunoassays and quantified using the Luminex. Results indicate that LSNAs induce the production of IL-6 (released in late endosomes) more so than free RNA, a control SNA, or untreated (Figure 30). This coincides with the localization of LSNAs (in the endolytic pathway). In addition, levels of IL-2 and IFN-γ were elevated in splenocytes treated with LSNAs compared to untreated, free RNA or control LSNAs.

[0233] The next step in this study is to co-deliver an antigen alongside the RNA agonists on the same nanoparticle and study antigen presentation in dendritic cells. The rate of antigen presentation (peptides expressed on MHC class II receptors) is analyzed using flow cytometry while cytokine activity after antigen presenting cells transfected with LSNA cancer vaccines is quantified.

[0234] To prove that these LSNAs function as effective therapeutic cancer vaccines, they must (1) bind and activate TLR7/8 selectively, (2) activate dendritic cells, (3) present tumor antigen on cell surfaces, and (4) induce cytotoxic T-cell mediated apoptosis of tumor cells. Thus far, it is shown herein that LSNAs are selective in binding and activating TLR7/8 in HEK-Blue reporter cell lines that stably express TLR7 and TLR8 (A). They have also been shown to mature dendritic cell through the upregulation of CD80/CD86 co-stimulatory markers as

analyzed by flow cytometry (B). In addition, LSNAs induce cytokine secretion (IL-6 upregulation in C57BL/6 B cells, dendritic cells splenocytes as well as IL-2 and IFN- γ secretion C75BL/6 splenocytes (C). The present disclosure demonstrates that LSNAs can activate dendritic cells, which is an important step in immunizing against cancer. As explained above, this observation sets the foundation for the development of RNA-LSNA based antitumor vaccines.

[0235] Herein it is shown that LSNA TLR7/8 agonists can be used to activate and mature dendritic cells in a potent and selective manner. Experiments elucidating the mechanism of immune cell activation by studying downstream activation and maturation of T-cells, B cells, and NK cells are being explored. In addition, tumor-associated antigens are being encapsulated in the liposomal core of the LSNA to probe antigen presentation on dendritic cells and cytotoxic T-cell mediated cell death in tumor cells. Lastly, these LSNA cancer vaccine formulation are being tested in vivo to confirm their tumor cell lysis efficacy.

Example 7

Encapsulation of peptides or proteins in a LSNA

[0236] **Peptides Used:**

- Gp100
- OVA-I (SIINFEKL (SEQ ID NO: 12))
- Modified OVA-I Peptides: SIINFEKLC (SEQ ID NO: 13), CSIINFEKL (SEQ ID NO: 14), Alexa Fluor 488 and FITC labeled OVA-I
- E7 peptide (RAHYNIVTF) (SEQ ID NO: 22)

[0237] **Protein Used:**

- Ovalbumin protein labeled with Alexa Fluor 488

[0238] See Figure 31.

[0239] The method used for preparing the liposomes was as described herein above. For encapsulation, the peptide or protein was added into the 1X PBS that was used for rehydrating the lipids. After freeze/thaw cycles and extrusion, excess lipid was removed by dialyzing against 1X PBS with a suitable dialysis membrane (typically 10K for peptides, 100K for proteins).

Quantification of DOPC in the liposomes

[0240] A phosphatidyl choline assay kit from Sigma Aldrich (CAT# MAK049) was used per manufacturer's instructions. Figure 32 shows that greater than 94% of the DOPC lipid is incorporated into liposomes.

Quantification of the encapsulated peptides and proteins

[0241] For fluorescent proteins, liposomes were lysed with SDS and the fluorescence of the proteins and peptides encapsulated therein was measured. The encapsulation efficiency was quantified compared to a standard curve of known amount of fluorescent protein or peptides. For unlabeled proteins, a peptide quantification kit from Pierce was used. Figure 33 shows that encapsulation efficiencies for peptides was 11-17% of the starting material, and for labeled ovalbumin proteins, it was 29-31%. It was also observed that the encapsulation efficiency was independent of the starting concentration of the peptide or protein.

Example 8

Methods

Oligonucleotide Synthesis

[0242] RNA was synthesized on a MerMade 12 using phosphoramidites from ChemGenes: ssRNA DR (5'-gcc cga cag aag aga gac ac-3' (SEQ ID NO: 5) and ssRNA-41 (5'-ugu ccu uca aug ucc uuc aa-3' (SEQ ID NO: 6)), all with a phosphorothiolate backbone. More detailed sequence information for RNA used in this paper are located in the Table 5. Cy-3.5 modified sequences were used for all uptake experiments (Figure 36); functional experiments were conducted with non-dye-labeled oligonucleotides. Standard oligonucleotide downstream processing of oligonucleotide sequences was conducted according to Glen Research's recommended protocols.

Table 5

NAME	SEQUENCE
SSRNA SEQUENCES:	
5' CHL DR (SEQ ID NO: 15)	5'CHOLESTEROL-GCC CGA CAG AAG AGA GAC AC-3'
5' CHL 41 (SEQ ID NO: 10)	5'CHOLESTEROL- UGU CCU UCA AUG UCC UUC AA -3'
FREE DR (SEQ ID NO: 5)	5'-GCC CGA CAG AAG AGA GAC AC-3'

FREE 41 (SEQ ID NO: 6)	5'-UGU CCU UCA AUG UCC UUC AA-3'
5'CHL CY3.5 DR (SEQ ID NO: 16)	5'-CHOLESTEROL-CY-3.5-GCC CGA CAG AAG AGA GAC AC-3
5'CHL CY3.5 41 (SEQ ID NO: 17)	5'-CHOLESTEROL-CY-3.5- UGU CCU UCA AUG UCC UUC AA -3'
CY3.5 FREE DR (SEQ ID NO: 18)	5'-DTDT-CY-3.5-GCC CGA CAG AAG AGA GAC AC-3'
CY3.5 FREE 41 (SEQ ID NO: 19)	5'-DTDT-CY-3.5- UGU CCU UCA AUG UCC UUC AA -3'

Cy-3.5 labeled cholesterol-modified RNA contain an internal Cy-3.5 adjacent to the cholesterol, while Cy3.5 labeled RNAs without cholesterol contain a dTdT (phosphate backbone) 5' modification to prevent the assembly of micelles.

Nanoparticle Synthesis

[0243] 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, dried into a thin film, and hydrated using a solution containing a model peptide antigen, SIINFEKL (SEQ ID NO: 12) (1mg/mL) dissolved in 30mM HEPES buffer and 0.15M NaCl. Next, liposomes were underwent freeze/thawed in LiqN₂/RT and manually extruded through a 200, 100, 80, 50 and 30 nm filter for 10 passes each. The liposomes were then purified using tangential flow filtration module (30k pore size) with 20x excess HEPES/Saline buffer to remove the excess peptides that were not encapsulated in the liposomes. The concentration of liposomes in solution was determined by relating the phosphate content as determined using ICP-OES [Banga et al., J Am Chem Soc 2014, 136 (28), 9866-9]. Finally, liposomes were functionalized with RNA at a ratio of 1:80 (RNA to lipid monomer) and shaken overnight at room temperature.

Nanoparticle Characterization

[0244] Size and charge of LSNAs were measured using DLS (Malvern). To determine the functionalization efficiency of LSNAs, 10pmol of Cy3.5 labeled RNA and LSNA was loaded onto an agarose gel (1%) and run at 80V for 45 minutes on ice and imaged. The percentage of free oligonucleotides was determined using band intensity analysis on ImageJ, resulting in approximately 12% free cholesterol and Cy3.5 labeled RNA. Nanoparticle loading (approximately 350 strands/65nm liposome was determined by dividing number of liposomes in solution (see previously established methods [Banga et al., J Am Chem Soc 2014, 136 (28), 9866-9]) by the number of strands added to the liposome, while taking into account the percentage of free, non-functionalized strands. Peptide encapsulation efficiency was

determined through using a Pierce Quantitative Fluorometric Peptide Assay (about 16/liposome at approximately 2% efficiency).

[0245] Approximately 3 uL were applied on a 400 × 100 mesh Quantifoil® S7/2 holey carbon film on copper grids (Quantifoil Micro Tools GmbH, Jena, Germany). Excess liquid was removed from the grid with filter paper, and then it was immediately shock- frozen by injecting it into liquid ethane. These sample preparation steps were done in a climate-controlled room using a CryoBox 340719 (Carl Zeiss, Oberkochen, Germany). The subsequent fixation of the grid on the sample rod (626-DH, Gatan, Warrendale, USA) and transfer of the rod into the transmission electron micro- scope (Leo 912 -mega®, Leo, Oberkochen, Germany) was performed under a nitrogen atmosphere at a temperature of 90K (-183°C). The instrument was operated at 120 kV and pictures with a 6300- to 12,500-fold magnification were taken from 7–21 different positions on the grid.

Cell culture

[0246] Reporter cells engineered to express TLR7/8 (Hek-Blue mTLR7 and Hek-Blue hTLR8) were purchased from Invivogen and cultured according to the manufacturer's protocols. Primary mouse splenocytes (both C57/BL6 WT and OT-1) were gifted from the Bin Zhang Laboratory. Human PBMCs were purchased from Zen-Bio. All primary cells were cultured in RPMI containing 10% FBS and 1% Pen-Strep.

Reporter Cell Line Experiments

[0247] To test the NF-κB activation of LSNAs in reporter Hek-Blue cells containing either mTLR7 or hTLR8, 40k cells were seeded per well in a 96 well plate, and treated with the constructs for 18 hours. 50uL of cell supernatants were then incubated with 150uL of QuantiBlue (Invivogen) for 1 hour to detect secreted embryonic alkaline phosphatase (SEAP), a measure of NF-κB activation. To block endosomal maturation, 50uM of chloroquine was incubated with Hek-Blue cells for 18 hours. There was no observed decrease in viability as a result of chloroquine incubation.

Confocal Microscopy Experiments

[0248] Bone marrow derived dendritic cells were incubated with Cy-3.5 labeled ssRNA in free, cationically transfected, or LSNA form at a concentration of 1 um at 24 hours. Cells were washed with PBS and fixed with 3.7% paraformaldehyde. Cells were then resuspended in VectaShield Mounting Medium with DAPI and fixed on a slide. All images were taken on a Zeiss LSM800 using a 63x oil objective.

Primary Murine Splenocyte Uptake Experiments

[0249] Murine splenocytes (seeded at 1E6 cells/mL) were incubated with Cy-3.5 labeled ssRNAs in free, cationically transfected, or LSNA form for both 8 hours and 24 hours. After the incubation, cells were washed with PBS, and stained at 4° C for 20min with the following cell surface markers: pDC (B220⁺, CD11c^{med}, CD19⁻), cDC (CD11c⁺, CD19⁻, B220⁻) (CD19⁺, B220⁺), NK (NK1.1⁺, CD49b⁺, CD3⁻), macrophage (CD11b⁺, F4/80⁺), and granulocyte (Gr1⁺). Next, cells were fixed using 3.7% paraformaldehyde and analyzed using flow cytometry. All antibodies were purchased from either BD Biosciences, eBioscience, or Biolegend.

Cytokine Secretion Quantification

[0250] Murine splenocytes were seeded at a density of 5x10⁶ cells/well and treated with constructs containing 1uM of RNA (and 1uM for resiquimod). Cytokine release was quantified using magnetic bead assays kits from either eBioscience, R&D Biosystems, or Millipore and analyzed on a Luminex 200, all according to each manufacturer's instructions. Various dilutions of cell culture supernatants (from no dilution to 10x dilution) were used to ensure the measurement was within range of instrument detection.

Primary Murine Splenocyte Activation Experiments

[0251] To determine the activation potential of IS-r-LSNAs on other types of immune cells, murine splenocytes were seeded at a concentration of 1x10⁶ cells/mL and treated with constructs (1uM by RNA) for 24 hours. Cells were then washed and stained with the following antibodies for lineage: pDCs (B220⁺, PDCA⁺, CD11c^{med}), B cell (B220⁺), macrophages (F4/80⁺, CD11b⁺), NK (NK1.1⁺, CD49b⁺, CD3⁻) and T cells (CD3⁺). Cells were also stained with CD86, MHCII, and CD69 to measure activation and H-2kb/SIINFEKL (SEQ ID NO: 12) for antigen presentation. All samples were analyzed using an LSRII. All antibodies were purchased from either BD Biosciences, eBioscience, or Biolegend.

CD8+ T cell Proliferation

[0252] DC's primed with all constructs (1uM by RNA, 1uM resiquimod) were seeded at 0.5E6 cells/mL for 24 hours before they were washed and co-cultured with positively selected CD8+ OT-1 T cells (EasySep™ Mouse CD8+ T Cell Isolation Kit from STEMCELL Technologies) at a 1:1 ratio (50k cells DC's : 50k OT-1 T cells to reach a total density of 1E6 cells/mL) for 72 hours. Next, cells were washed and stained using the antibodies against CD8 and CD90.1. The membrane was then permeated with a FOXP3 permeation kit and stained with KI-67. All samples were analyzed using LSRII. All antibodies were purchased from either BD Biosciences, eBioscience, or Biolegend.

[0253] Abbreviations used herein: CCR2, CC chemokine receptor 2; CCL2, CC chemokine ligand 2; CCR5, CC chemokine receptor 5; TLC, thin layer chromatography.

IS-r-LSNA Characterization

[0254] IS-r-LSNAs functionalized with ssRNA range from 70-75 nm in diameter (Figure 34B), with a size increase of approximately 10nm over bare liposomes, as determined using DLS. The functionalization of RNA to bare liposomes can be also be visualized via agarose gel, where bands denoting Cyanine 3.5 (Cy3.5) labeled RNA functionalized LSNAs have lower mobility compared 5'cholesterol and Cy3.5 modified RNA (Figure 34C). An increase in RNA stability can also be seen on IS-r-LSNAs compared to that of free RNA, where degradation products of free RNA appear after a 1-hour incubation in 10% FBS, while RNA isolated from LSNAs remain intact. CryoEM images were also captured (Figure 40).

Determining the Potency of LSNAs in Activating NF- κ B

[0255] To test the efficiency of the IS-r-LSNAs in binding and activating TLR7/8, r-LSNA DR (ssRNA LSNA TLR7/8 agonist), r-LSNA 41 (control ssRNA LSNA), free DR (ssRNA TLR7/8 agonist only), and LyoVec DR (ssRNA TLR7/8 agonist admixed with cationic transfection agent) were added to Hek-Blue Reporter cell lines expressing either mouse TLR7 or human TLR8. Activation of NF- κ B as a result of TLR7/8 binding was determined using a QUANTI-Blue assay (Figure 35A). The results indicate IS-r-LSNAs are 3x more potent in activating TLR7 than free RNA or cationic transfected RNA, while it is 12x and 3x more potent in activating TLR8 than free RNA or cationic transfected RNA, respectively.

[0256] Figure 41 shows data obtained during the probing of the structure/function relationship between TLRs 7/8 and IS-r-LSNAs. Figure 41A shows that several sequences were screened to identify the optimal sequence that was most potent in activating TLR7/8. The potency of five different sequences (free or cationically transfected RNA) was screened by quantifying NF- κ B activity in Raw Blue cells (Invivogen). The most potent sequence (ssRNA-DR) was chosen for all further downstream experiments. To determine the optimal backbone composition for ssRNA-DR, free, cationically transfected or LSNA DR were synthesized with either a phosphodiester (PO) or phosphothiolate (PS) backbone and tested using Raw Blue cells. All further studies were conducted using a phosphothiolate backbone (Figure 41B). To probe the structure-function relationship between ssRNA-DR and its TLR7/8 receptor, either the 5' or 3' end were blocked off with a cholesterol group, and functionalized onto an LSNA (Figure 41C).

[0257] LSNAs functionalized with a 3'cholesterol modified ssRNA-DR significantly decreased its potency, as compared to a 5'cholesterol modification, suggesting that that TLR7/8 of ssRNA-

DR occurs at the 3' end. Interestingly, this is the opposite for other sequences, such as ssRNA-40, which binds TLR7/8 at the 5' end (a 5' cholesterol completely abrogates all activity). All ssRNA-DR were functionalized with a 5' cholesterol for the rest of the studies (Figure 41C). To determine the hydrophobic moiety that offers the best potency for ssRNA functionalized onto the LSNA, a comparison between 5' cholesterol and 5' tocopherol was conducted in both Hek-Blue mTLR7 and hTLR8 cells. It was found that 5' cholesterol demonstrated the best activity, and was thus used for all of the experiments (Figure 41D). Figure 41E shows a comparison of LSNAs functionalized with 5' cholesterol modified ssRNA-DR with and without 2 PEG spacer units (hexaethylene glycol), which offers the RNA additional flexibility when binding to the TLR7/8 receptor. However, this additional flexibility does not offer increased potency in binding TLR7/8, and as such, ssRNAs were not modified with 2 PEG spacer units at the 5' end. All experiments were conducted 3 times, with representative data shown with $n=3 \pm \text{SD}$.

[0258] To prove that IS-r-LSNAs are binding TLR7/8 in endosomal compartments, chloroquine, a chemical blocker that inhibits endosomal maturation, a necessary step in endosomal TLR activation, was added to Hek-Blue mTLR7 and hTLR8 cells treated with each construct (1 μM by RNA or 1 μM resiquimod). A decline to near-baseline NF- κB activity for all constructs confirms that all observed activity is occurring in endosomal compartments (Figure 35B).

Uptake of IS-r-LSNAs in Various Immune Cells

[0259] Since SNAs have shown to have high cellular uptake in various cell types [Rosi et al., Science 2006, 312 (5776), 1027-30; Giljohann et al., Nano Lett 2007, 7 (12), 3818-21], it was important to access the uptake of IS-r-LSNAs in various immune cells as well, as higher particle uptake would suggest higher activation of TLR7/8. To determine if LSNAs can enter primary bone marrow derived dendritic cells (BMDCs), ssRNAs were modified with an internal Cy-3.5 in free or LSNA form 24hrs and imaged using confocal microscopy (Figure 36A). The punctate dots of Cy3.5-labeled RNA (in red) surrounding the nucleus (DAPI stain) suggest localization in endosomes and endolytic trafficking. Due to enhanced LSNA-cell surface receptor interactions, we expect an increase in cellular uptake for IS-r-LSNAs compared to free RNA. As expected, BMDCs take more LSNAs compared to free RNA, as determined by the increase in fluorescence intensity of Cy3.5 dye. The appreciable uptake of Cy-3.5-labeled free RNA can be attributed to either to the phosphothiolate backbone, which allows it to enter cells [Overhoff et al., EMBO Rep 2005, 6 (12), 1176-81; Koller et al., Nucleic Acids Res 2011, 39 (11), 4795-807; Moseman et al., J Immunol 2013, 191 (11), 5615-24] or via non-specific phagocytosis by the BMDCs.

[0260] To determine how other types of primary immune cells take up LSNAs, mouse splenocytes were treated with ssRNA in free, cationically transfected, and LSNA form (Figure 36B). Various immune cell populations were separated using flow cytometry analysis through their cell surface phenotypic markers: pDC (B220⁺, CD11c^{med}, CD19⁻), cDC (CD11c⁺, CD19⁻, B220⁻) (CD19⁺, B220⁺), NK (NK1.1⁺, CD49b⁺, CD3⁻), macrophage (CD11b⁺, F4/80⁺), and granulocyte (Gr1⁺). The selected immune cell types were chosen since they all express TLR7, with the exception of cDCs [Blasius et al., *Immunity* 2010, 32 (3), 305-15; Hayashi et al., *Blood* 2003, 102 (7), 2660-9; Song et al., *AIDS Res Hum Retroviruses* 2009, 25 (12), 1287-95; Adib-Conquy et al., *Immunol Cell Biol* 2014, 92 (3), 256-62]. At 8 hours and 24 hours, LSNAs exhibit higher uptake into cells than free RNA or cationically transfected RNA in pDC's, macrophages, and NK cells. In addition, uptake of LSNAs in pDCs at 8h is the highest compared to any other cell type, and is 2-5x higher than that of free ssRNA or cationically transfected ssRNA. Interestingly, the uptake of LSNAs in pDCs does not differ significantly in pDCs between 8 hours and 24 hours (Figure 37). A similar saturation effect is seen for the uptake of free and cationically transfected RNA into the various types of primary immune cells, but is not the case for LSNAs. In fact, uptake of LSNAs in cDCs, B cells, macrophages, granulocytes, and NK cells steadily increases between 8hrs and 24 hours. This suggests that the uptake of LSNAs into pDCs is rapid and becomes saturated as early as 8h while uptake into other immune cells is slower and sustained. The rapid uptake of LS-r-LSNAs into DCs, and in particular pDCs, is ideal since DCs are "professional" antigen presenting cells, TLR7 is expressed in pDCs, and ssRNA agonists have been shown to be potent activators of pDCs [Diebold et al., *Science* 2004, 303 (5663), 1529-31; Hornung et al., *Nat Med* 2005, 11 (3), 263-70; Bekeredjian-Ding et al., *J Immunol* 2005, 174 (7), 4043-50]. Thus preferential uptake into those pDCs might result in higher activation. High uptake in other APCs, such as macrophages and B cells, or other lymphocytes, such as NK and T cells, are also important, as increased uptake could lead to increased TLR7 activation, as well as crosstalk between cells. Figure 44 shows uptake of all constructs separated by cell type.

[0261] Figure 42 shows results of studies examining the viability of all constructs in Hek 293 cells expressing either TLR7 or 8 as determined using an Alamar Blue assay after a 24 hour incubation. While free RNA or rLSNAs do not decrease viability, the use of a cationic transfection agent LyoVec does decrease cell viability. Figure 43 shows that overnight incubation of Hek-Blue cells with chloroquine does not decrease cell viability.

Stimulatory Activity of IS-r-LSNAs in Primary Immune Cells

[0262] It is widely accepted that in order for APC to drive antigen-specific CD8⁺ T cells response, they must undergo three major phenotypic changes: (1) release of cytokines and chemokines, (2) increase in co-stimulatory molecule expression, and (3) increase in antigen presentation on major histocompatibility complex (MHC) on APC cell surfaces [Reizis et al., Annual review of immunology 2011, 29, 163-83; Desmet et al., Nat Rev Immunol 2012, 12 (7), 479-91; Kambayashi et al., Nat Rev Immunol 2014, 14 (11), 719-30; Tan et al., J Leukoc Biol 2005, 78 (2), 319-24; Trinchieri et al., Nat Rev Immunol 2007, 7 (3), 179-90]. To determine if IS-r-LSNAs induce cytokine secretion in murine splenocytes, all constructs were incubated with murine splenocytes for 24 hours, and cell supernatants were processed to determine cytokine, chemokine and IFN release (Figure 38A). IS-r-LSNAs induced significantly higher levels type I IFNs (mostly secreted from plasmacytoid dendritic cells, as they can secrete 100x more type I IFNs as compared to other APCs upon TLR7 activation [Reizis et al., Annual review of immunology 2011, 29, 163-83; Hornung et al., Nat Med 2005, 11 (3), 263-70; Gary-Gouy et al., J Interferon Cytokine Res 2002, 22 (6), 653-9; Gilliet et al., Nat Rev Immunol 2008, 8 (8), 594-606]) than that of free RNA. While the secreted levels of IFN. are significantly higher than any of the other cytokines detected, as expected for TLR7/8 agonists, IS-r-LSNAs also induce other pro-inflammatory cytokines, such as IL-6, IL10, IP-10, IL-12p40, and TNF α , which are secreted from many immune cell types: DCs, B cells, macrophages, T cells, monocytes, mast cells, and granulocytes [Lacy et al., Blood 2011, 118 (1), 9-18; Stanley et al., Physiology (Bethesda) 2010, 25 (4), 218-29; Couper et al., J Immunol 2008, 180 (9), 5771-7; Liu et al., Cytokine Growth Factor Rev 2011, 22 (3), 121-30; Hamza et al., Int J Mol Sci 2010, 11 (3), 789-806], all of which are higher than free RNA and control LSNAs. Thus, while IS-r-LSNAs can activate pDCs, as evidenced by high IFN secretion, the secretion of other pro-inflammatory cytokines indicate that many other types of immune cells are also activated. Figure 46 shows the results of a comparison between ssRNA-DR as functionalized onto r-LSNAs or cationically transfected for cytokine secretion.

[0263] To determine whether IS-r-LSNAs can specifically mature DCs, all constructs were incubated with positively selected (CD11c⁺) DC's for 24 hours, and analyzed using flow cytometry to look at the DC maturation through the upregulation of co-stimulatory and MHC-II receptors (Figure 39A). IS-r-LSNAs upregulate CD86 and MHCII receptors on pDCs (B220⁺, CD11c^{med}) compared to control LSNAs, free RNA, and cationically transfected ssRNA, an important step in the DC maturation process (Figure 45).

[0264] To pinpoint which cell types IS-r-LSNAs can activate, all constructs were incubated with a mixed pool of primary immune cells (murine splenocytes) for 24 hours. Flow cytometry was used to identify various immune cell types and to evaluate each cell's ability to induce a second signal of APC activation: upregulation of co-stimulatory and MHC-II receptors (Figure 38B). As expected, IS-r-LSNAs show increased CD86 and MHCII receptor expression on pDCs (PDCA⁺, CD11c^{med}, B220⁺) compared to free ssRNA and on-par if not better than cationically transfected ssRNA. IS-r-LSNAs also induce maturation two other types of APCs: B cells (B220⁺) and macrophages (F4/80⁺, CD11b⁺) via CD86 and MHCII receptor upregulation compared to free ssRNA or cationically transfected ssRNA. Interestingly, IS-r-LSNAs also activate NK (NK1.1⁺, CD49b⁺, CD3⁻) and T cells (CD3⁺), as indicated by upregulation of CD69 when compared to free ssRNA.

Antigen Presentation on APCs

[0265] Since the LSNA architecture allowed for the encapsulation of a model peptide (SIINFEKL (SEQ ID NO: 12)) within the liposome core, IS-r-LSNAs are able to activate many types of immune cells, including APCs while precisely co-delivering antigen to the same cell. This is key, as the delivery of antigen without the without presence of an immunogenic adjuvant can induce tolerance [O'Neill et al., Nat Rev Immunol 2013, 13 (6), 453-60; Mahnke et al., Immunol Cell Biol 2002, 80 (5), 477-83; Dudek et al., Front Immunol 2013, 4, 438]. The combined introduction of antigen and TLR7/8 agonists within the IS-r-LSNA was expected to enhance antigen presentation rates of SIINFEKL(SEQ ID NO: 12) as compared to admixed free or cationically transfected ssRNA and SIINFEKL(SEQ ID NO: 12) peptide. To test this, murine splenocytes were incubated with all constructs, containing both a TLR7/8 agonist and SIINFEKL(SEQ ID NO: 12) peptide, for 24 hours. The presentation of SIINFEKL(SEQ ID NO: 12) on MHC-I receptors for each immune cell was determined using an antibody that binds the SIINFEKL(SEQ ID NO: 12)-MHC-I complex (H-2Kb-SIINFEKL (SEQ ID NO: 12), eBioscience), quantified using flow cytometry (Figure 39A). As predicted, IS-r-LSNAs increase antigen presentation rates in APCs, particularly in DCs, which see the highest expression of MHC-I bound SIINFEKL(SEQ ID NO: 12) after treatment by IS-r-LSNAs compared to free or cationically transfected ssRNA.

[0266] To test the efficiency of antigen cross-presentation, we pre-incubated DCs with IS-r-LSNAs encapsulating ovalbumin (257-264) peptide (SIINFEKL(SEQ ID NO: 12)) for 24 hours and washed before co-culturing them with CD8⁺ OT-1 T cells for 72 hours. All cells were then stained with an antibody against Ki-67, a marker for proliferation, and CD8 before they were analyzed using flow cytometry (Figure 39B). IS-r-LSNAs increased T-cell proliferation

compared to free or cationically transfected ssRNA admixed with SIINFEKL (SEQ ID NO: 12), and on par with that of resiquimod. The increased T cell proliferation for IS-r-LSNAs was expected since pDCs rapidly took up IS-r-LSNAs, secreted high levels of IFN., upregulated co-stimulatory, MHC II and MHC-I bound SIINFEKL (SEQ ID NO: 12) receptors, all at higher levels than free or cationically transfected RNA.

Conclusions

[0267] A new class of immunomodulatory spherical nucleic acids, IS-r-LSNAs, is disclosed herein. The IS-r-SNAs consist of a 65nm liposomal core decorated with TLR7/8 agonist RNA strands. These constructs demonstrate high cellular uptake and potent modulation of endosomal TLR7/8 in multiple reporter cell lines and primary cells *in vitro*. In addition, IS-r-LSNAs induce activation of APCs and also NK and T cells, through increased cytokine secretion and co-stimulatory receptor upregulation. The modular nature of IS-r-LSNAs also allowed the co-delivery of a peptide antigen within the same construct, thus increasing antigen presentation rates in APCs, resulting in an enhanced antigen-specific CD8+ T cell response in OT-1 cells when compared to free or cationically transfected ssRNA. These results indicate the strength of the SNA platform for modulating the immune system, while the versatile nature of IS-r-LSNAs prompts further applications not only as cancer therapeutics but also for other types of immune-related diseases.

Example 9

Spherical Nucleic Acids to Treat Triple Negative Breast Cancer (TNBC)

Introduction

[0268] SNAs were tested in the 4T1 syngeneic tumor mouse model of TNBC for their ability to activate the immune system and initiate an antitumor response against established TNBC mammary tumors. Mice were vaccinated twice, five days apart, after the tumors had already started growing to mimic the clinical situation where a patient would begin treatment after the tumors are detected. This treatment regime enabled a 30% reduction in the final tumor volume compared with untreated tumor-bearing animals. There was also double the number of T-cells in the tumor microenvironment suggesting the SNA vaccine was successful in turning on the immune system and recruiting active T-cells to the tumor site. Additionally, the SNA vaccine treatment greatly reduced the number of lung metastases (Figure 50), which is highly significant as metastatic tumors are notoriously more difficult to treat than the primary breast tumor [Zeichner et al., Breast Cancer: Basic Clin. Res. 10, 25–36 (2016)], and are the leading cause of

cancer-related deaths [Mehlen et al., Nat. Rev. Cancer 6, 449–458 (2006)]. The ability to reduce metastasis is an important advancement in the fight against TNBC.

[0269] Additional strategies contemplated by the disclosure include increasing the vaccination dose, targeting different TLRs, or combining SNAs with other immunotherapies such as checkpoint inhibitors [Postow et al., J. Clin. Oncol. 33, 1974–1982 (2015)].

Methods

[0270] **Generation and characterization of cell lysates:** 4T1 cells were grown in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. All lysates were prepared from cells under passage number six. Cells were collected and resuspended at 10E6 cells/mL in phosphate buffered saline then subjected to five freeze-thaw cycles in liquid nitrogen and a 37°C water bath. Cellular debris was removed by centrifugation and the supernatant was collected as the protein lysate. The total protein concentration was measured using the bicinchoninic acid (BCA) assay and protein content was characterized using SDS-Page electrophoresis and loading 1 mg/mL total protein.

[0271] **Synthesis and characterization of lysate liposomal SNAs:** The protein lysate was encapsulated within DOPC liposomes using the thin-layer rehydration method. A 1mg/mL protein solution in phosphate buffered saline was used to rehydrate 5 mM DOPC lipids. Liposomes were formed by five freeze-thaw cycles freeze-thaw cycles in liquid nitrogen and a 37°C water bath followed by ten rounds of high-pressure extrusion through polycarbonate filters with pore sizes of 200, 100, 80, and 50 nm. Tangential flow filtration with a pore size of 500 kDa was used to remove any non-encapsulated proteins and the sample was repeatedly washed with phosphate buffered saline until no protein was detected in the flow-through, as monitored by measuring the absorption of the flow-through at 280 nm with ultraviolet-visible spectroscopy. The amount of protein encapsulated within the liposomes was measured using the BCA assay after disruption of the liposome with 1% SDS to release the encapsulated protein. The phospholipid concentration was measured using a phosphatidylcholine quantification kit. Cholesterol terminated oligonucleotides were attached to the surface of the liposomes by mixing 20 µM oligonucleotides with a solution of liposomes at 1.63 mM lipid at 25°C overnight. The oligonucleotide concentration was determined by measuring the absorption at 260 nm with ultraviolet-visible spectroscopy. This average ratio of protein to DNA was determined to be 1.1 (0.65) mg protein per µmol DNA for three independent batches of protein lysate encapsulated LSNAs.

[0272] Lysate liposomal SNAs were characterized using transmission electron microscopy, gel electrophoresis, and dynamic light scattering.

[0273] CryoEM samples were prepared by FEI Vitrobot Mark III by dropping 4 μ L on a 200-mesh copper TEM grid with lacey carbon film, blotted for 5 seconds, and then plunged into liquid ethane before transferred to a cryo holder and stored in liquid nitrogen prior to imaging. CryoEM imaging was performed using Hitachi HT7700 transmission electron microscope with Gatan cryo-transfer holder under 120kV accelerating voltage, and images were taken with a Gatan imaging camera at a magnification of 30000x.

[0274] 50 pmol of Cy5-labeled cholesterol oligonucleotides and cy5-labeled liposomal SNAs were loaded into a 1% agarose gel on ice and ran at 100 V for 45 minutes.

[0275] The hydrodynamic diameter of bare liposomes and liposomal SNAs was measured by dynamic light scattering.

[0276] **Cellular uptake of lysate liposomal SNAs:** SNAs were prepared by encapsulating AlexaFluor488-labeled lysate and functionalizing cy5-labeled DNA using the same methods described above. Fluorescently labeled SNAs were used to measure the uptake of these particles in mouse macrophages (RAW blue) and mouse bone marrow-derived dendritic cells (BMDCs). RAW blue macrophages were plated at 20,000 cells per well in 24-well plates in DMEM supplemented with 10% fetal bovine serum, 100 μ g/mL normocin, and 200 μ g/mL zeocin. Twenty-four hours later, cells were treated in triplicated with 1 μ M cy5-labeled DNA, alexafluor488-labeled lysate, or double labeled SNAs for 1 hour. Cells were washed, fixed with 4% paraformaldehyde, the nucleus was stained with DAPI, and imaged with confocal microscopy (Zeiss LSM 800) using a 60x objective.

[0277] Bone marrow was isolated from femurs of C57BL/6 mice and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, and 20 ng/mL GM-CSF for six days. BMDCs were added to 24-well plates at 500,000 cells per well, and immediately treated with samples for 1 hour, washed, fixed with 4% paraformaldehyde, and resuspended in phosphate buffered saline for flow cytometry (BD LSRFortessa).

[0278] **Activation of bone marrow derived dendritic cells:** BMDCs were cultured as described above and 500,000 cells were treated with samples at 100 nM DNA for 24 hours. Cells and the supernatant were collected for analysis. Cells were incubated with antibodies for CD40 (PE) and CD80 (Brilliant Violet 421) for 30 minutes at 4°C, washed and resuspended in phosphate buffered saline for flow cytometry (BD LSRFortessa). The supernatants were used to measure the concentration of cytokines (TNF α , IL-12) using Luminex.

[0279] **Tumor model and metastasis quantification:** 500,000 4T1 cells were injected (100 μ L) into the right mammary gland of 6-week old female BALB/c mice (n = 10). On days 6 and

11, 200 μL of 50 μM SNAs were injected subcutaneously near the tumors. Tumor volumes were calculated by measuring the length and width with calipers and applying the formula $V = L \times W \times W/2$. On day 21, five mice were euthanized and the lungs were dissected for metastasis quantification. Lungs were cut into small pieces and digested in 1 mg/mL collagenase type 4 and 6 units/mL elastase for 1 hour at 4°C. Samples were then filtered through 70 μM nylon strainers and washed with phosphate buffered saline. The cells were then plated in 10-cm tissue culture plates in RPMI media supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, and 60 μM thioguanine for 10 days. The remaining cell colonies were fixed with methanol and stained with 0.03% methylene blue and then counted.

Synthesis and Characterization of TNBC LSNAs

[0280] Protein lysates are generated using 4T1 cells, a mouse TNBC cell line, by subjecting these cells to repeated freeze-thaw cycles to cause necrotic cell death and continued until no live cells are remaining. The cellular debris is then purified away from the soluble lysate mixture using centrifugation. The protein content is characterized using SDS-Page electrophoresis and the total protein concentration is measuring using the bicinchoninic acid (BCA) assay. Here, TNBC lysates containing antigens (Figure 51) were encapsulated within DOPC liposomes. Cholesterol terminated DNA was then adsorbed into the lipid bilayer to form liposomal SNAs (Figure 52). The LSNAs were 73.4 (2.6) nm in diameter with 1-10 proteins/SNA. The protein lysate is encapsulated within DOPC liposomes using the thin-layer rehydration method. A 1 mg/mL protein solution in phosphate buffered saline is used to rehydrate 5 mM DOPC lipids. Liposomes are formed by five freeze-thaw cycles and 10 rounds of high-pressure extrusion through polycarbonate filters with pore sizes of 200, 100, 80, and 50 nm. Tangential flow filtration with a pore size of 500 kDa is used to remove any non-encapsulated proteins and the sample is repeatedly washed until no protein can be detected in the flow-through. The amount of protein encapsulated within the liposomes is measured using the BCA assay after disruption of the liposome to release encapsulated protein. DNA is functionalized to the liposome surface through hydrophobic interactions between a cholesterol end group and the lipid bilayer. The average ratio of protein to DNA is 1.1 (0.65) mg protein per μmol DNA for three independent batches of protein lysate encapsulated LSNAs.

[0281] Encapsulation of protein lysates into LSNAs enables higher cellular uptake of lysates into macrophages and dendritic cells compared with lysates encapsulated within bare liposome. Co-delivery of the lysates and DNA into the same cell is increased to 99% of cells compared with when the two components are simply mixed where approximately 70% of cells take up both protein and DNA. Protein lysate LSNAs can activate mouse dendritic cells through toll-like

receptor 9 signaling from the DNA, and also through delivery of the lysate. Protein lysates are known to activate dendritic cells through proteins that are released during necrotic cell death that are meant to signal to surrounding cells that there is danger in the area that caused this type of death. Activated dendritic cells increase their expression of co-stimulatory molecules (CD80, CD40, CD86, MHCII) and secretion of pro-inflammatory cytokines (TNF α , IL-12). Vaccination of mice bearing mammary gland 4T1-tumors with protein lysate LSNA (200 nmol DNA, 22 μ g protein) results in slower tumor growth and a large reduction in lung metastasis.

[0282] The oligonucleotide sequences used were as follows:

OLIGONUCLEOTIDE NAME	SEQUENCE (5'→3')	SEQ ID NO:
TL9 DNA	5'-TCCATGACGTTCTGACGTT(SP18)2-CHOL-3'	20
CONTROL	5'-TCCATGAGCTTCTGAGCTT(SP18)2-CHOL-3'	21

[0283] Physical characterization of the prepared LSNA confirmed both protein and DNA loading into the liposomal nanoparticles. Protein encapsulation was measured using Bicinchoninic Acid (BCA) Assay, while DNA Concentration Measured using Ultra-Violet Visible Spectroscopy (A_{260}) (Figure 61).

LSNAs Enable Greater Cellular Delivery of TNBC Antigens and CpG DNA Compared with Free Antigens or DNA

[0284] The LSNA allowed for high cellular uptake of TNBC lysate or CpG DNA into antigen presenting cells, including macrophages and dendritic cells, after one hour incubation time *in vitro*. See Figure 53. Lysate was labeled with Alexa fluor 488 Protein Labeling Kit (Thermo Fisher), encapsulated in liposomes. DNA was Cy-5 labeled. The confocal images represent mouse macrophages treated for 1 hour with 1 μ M DNA.

Co-delivery of TNBC Antigen and CpG DNA to the Same Cell is Enhanced when Formulated as an LSNA

[0285] Co-delivery of adjuvants and antigens into the same cell is important for proper activation and antigen presentation. The number of cells that take up both CpG DNA and TNBC lysate is increased when these two components are formulated into the same SNA (Figure 54). The different combinations shown were incubated with 500,000 mouse dendritic cells for 1 hour based on 1 μ M DNA or the equivalent amount of lysate liposome.

TNBC LSNAs Activate Antigen Presenting Cells

[0286] A schematic showing the activation of dendritic cells (DCs) is shown in Figure 55. Treatment of bone marrow derived dendritic cells (BMDCs) with CpG LSNAs increased the expression of co-stimulatory molecules and the amount of cytokines secreted (Figure 56). Delivered lysate also activated BMDCs without CpG DNA (Figure 56). Expression of co-stimulatory molecules was determined by flow cytometry by staining with fluorescent antibodies. In the experiment, 500,000 dendritic cells were treated for 24 hours with 100 nM DNA. Cytokines were measured with Luminex after the same treatment.

TNBC LSNA Vaccine Slows 4T1 Primary Mammary Tumor Growth

[0287] Here, 4T1 mouse breast cancer cells were implanted into the mammary gland of Balb/C mice (see Figure 57 for protocol). 4T1 tumors were created on day 0. LSNAs were injected on day 6, 11 after tumor implantation. Tumor volume was measured by calipers up to 28 days. The control LSNA is lysate encapsulated with non-CpG DNA on the outside (scrambled GC DNA). 4T1 tumors began to slow in growth 10 days after the final vaccine treatment (Figure 57).

Immune Cell Infiltration into the Primary Tumor is Altered with TNBC LSNA Treatment

[0288] The percent of CD8+ T-cells within the tumor increased with TNBC LSNA treatment (Figure 58). The percent of myeloid derived suppressive cells within the tumor decreased with TNBC LSNA treatment (Figure 59).

TNBC LSNA Vaccine Reduces Lung Metastasis

[0289] It is known that 4T1 tumors rapidly metastasize to the lungs. Thus, excised lung cells were cultured *ex vivo* on day 21 after tumor initiation and it was found that TNBC LSNA treatment reduced the number of 4T1 colonies (Figure 60). Lung tissue were removed from mice on day 21. The lungs were digested to single cells then culture in media supplemented with 6-thioguanine to which 4T1 cells are resistant. All healthy cells die and only 4T1 cells survive and form colonies which are stained blue after 10 days in culture. Each blue dot was counted and represents one metastasis (Figure 60). The treatment was the same as that described above.

Conclusions

[0290] A spherical nucleic acid TNBC vaccine was developed by combining CpG DNA and cell lysates to activate the immune system against TNBC specific antigens. The TNBC LSNA vaccine is readily taken up by immune cells and activates antigen presenting cells in vitro.

Treatment of the 4T1 breast cancer model with the TNBC LSNA vaccine resulted in slower growth of the primary tumor and a reduction in lung metastasis.

[0291] This example demonstrated that the lysates encapsulated into immunostimulatory SNAs allow the use of SNAs against cancers where no established cancer antigens exists.

WHAT IS CLAIMED IS:

1. A liposomal particle having a substantially spherical geometry and comprising:
a lipid bilayer comprising a plurality of lipid groups;
a tumor antigen encapsulated therein; and
an oligonucleotide on the surface of said liposomal particle.
2. The liposomal particle of claim 1, wherein the tumor antigen is a tumor lysate.
3. The liposomal particle of claim 1 or claim 2, wherein the oligonucleotide on the surface of said liposomal particle increases the activity of a toll-like receptor-9 (TLR-9).
4. A liposomal particle having a substantially spherical geometry and comprising:
a lipid bilayer comprising a plurality of lipid groups;
a modulator of a first toll-like receptor (TLR) encapsulated in the liposomal particle,
wherein said modulator is not an oligonucleotide; and
an oligonucleotide on the surface of said liposomal particle that modulates the activity of a second toll-like receptor (TLR).
5. The liposomal particle of claim 4, wherein the first TLR is TLR-4 and the second TLR is TLR-9.
6. The liposomal particle of claim 4, wherein the first TLR is TLR-9 and the second TLR is TLR-4.
7. The liposomal particle of claim 4 or claim 5, wherein the modulator of the first TLR is a small molecule.
8. The liposomal particle of claim 7, wherein the small molecule is TAK-242 (resatorvid), Oxidized 1-palmitoyl-2-arachidonyl-sn- glycerol-3-phosphorylcholine (OxPAPC), polymyxin B or eritoran.
9. The liposomal particle of any one of claims 4-8, wherein the second TLR is TLR-9 and the oligonucleotide on the surface of said liposomal particle increases the activity of TLR-9.
10. A liposomal particle having a substantially spherical geometry and comprising:
a lipid bilayer comprising a plurality of lipid groups;
an oligonucleotide on the surface of said liposomal particle that increases the activity of a first toll-like receptor (TLR); and

an oligonucleotide on the surface of said liposomal particle that increases the activity of a second toll-like receptor (TLR).

11. The liposomal particle of claim 10, wherein the first TLR is TLR-7 and the second TLR is TLR-8.

12. The liposomal particle of claim 11, wherein the oligonucleotide comprises or consists of the sequence as set out in SEQ ID NO: 10.

13. The liposomal particle of claim 11 or claim 12, wherein the oligonucleotide comprises or consists of the sequence as set out in SEQ ID NO: 11.

14. A liposomal particle having a substantially spherical geometry and comprising:
a lipid bilayer comprising a plurality of lipid groups; and
an oligonucleotide on the surface of the liposomal particle comprising a modified backbone and a spacer.

15. The liposomal particle of claim 14, wherein the modification is a phosphorothioate backbone or a phosphodiester backbone.

16. The liposomal particle of claim 15, wherein the oligonucleotide comprises a phosphodiester homopolymeric sequence.

17. The liposomal particle of claim 16, wherein the homopolymer sequence comprises polyU.

18. The liposomal particle of claim 15, wherein the oligonucleotide comprises UCA or CpG.

19. The liposomal particle of claim 14, wherein the spacer is an internal spacer.

20. The liposomal particle of claim any one of claims 1-19, wherein the lipid bilayer comprises 1,2-dioleoyl-*sn*-glycero-3 phosphatidylcholine (DOPC) and 5% 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(6-azidohexanoyl) (DPPE-Azide).

21. The liposomal particle of claim 20, wherein the oligonucleotide is modified on its 5' end and/or 3' end with dibenzocyclooctyl (DBCO).

22. The liposomal particle of any one of claims 1-19, wherein said plurality of lipid groups comprises a lipid selected from the group consisting of the phosphatidylcholine, phosphatidylglycerol, and phosphatidylethanolamine families of lipids.

23. The liposomal particle of claim 22, wherein said lipid is selected from the group consisting of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-sn-phosphatidylcholine (DMPC), 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine (POPC), 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE).

24. The liposomal particle of any one of claims 1-19 or 22-23, wherein the oligonucleotide is an oligonucleotide-lipid conjugate containing a lipophilic tethered group, wherein said lipophilic tethered group is adsorbed into the lipid bilayer.

25. The liposomal particle of claim 24 wherein the lipophilic tethered group comprises tocopherol or cholesterol.

26. The liposomal particle of claim 25 wherein tocopherol is chosen from the group consisting of a tocopherol derivative, alpha-tocopherol, beta-tocopherol, gamma-tocopherol and delta-tocopherol.

27. The liposomal particle of any one of claims 1-26 wherein said oligonucleotide comprises RNA or DNA.

28. The liposomal particle of claim 27 wherein said RNA is a non-coding RNA.

29. The liposomal particle of claim 28 wherein said non-coding RNA is an inhibitory RNA (RNAi).

30. The liposomal particle of claim 28 or claim 29 wherein the RNAi is selected from the group consisting of a small inhibitory RNA (siRNA), a single-stranded RNA (ssRNA) that forms a triplex with double stranded DNA, and a ribozyme.

31. The liposomal particle of claim 28 or claim 29 wherein the RNA is a microRNA.

32. The liposomal particle of claim 27 wherein said DNA is antisense-DNA.

33. The liposomal particle of any one of claims 1-32 wherein diameter of said liposomal particle is less than or equal to about 50 nanometers.

34. The liposomal particle of any one of claims 1-33 wherein the particle comprises from about 10 to about 80 oligonucleotides.

35. The liposomal particle of claim 34 wherein the particle comprises 70 oligonucleotides.

36. The liposomal particle of any one of claims 1-13 or 19-35, wherein the oligonucleotide is a modified oligonucleotide.

37. A method of inhibiting expression of a gene comprising the step of hybridizing a polynucleotide encoding said gene product with one or more oligonucleotides complementary to all or a portion of said polynucleotide, said oligonucleotide being attached to the liposomal particle of any one of claims 1-36, wherein hybridizing between said polynucleotide and said oligonucleotide occurs over a length of said polynucleotide with a degree of complementarity sufficient to inhibit expression of said gene product.

38. The method of claim 37 wherein expression of said gene product is inhibited *in vivo*.

39. The method of claim 37 wherein expression of said gene product is inhibited *in vitro*.

40. The method of any one of claims 37-39 wherein said liposomal particle has a diameter about less than or equal to 50 nanometers.

41. The method of any one of claims 37-40 wherein said oligonucleotide comprises RNA or DNA.

42. The method of claim 41 wherein said RNA is a non-coding RNA.

43. The method of any one of claims 42 wherein said non-coding RNA is an inhibitory RNA (RNAi).

44. The method of claim 43 wherein the RNAi is selected from the group consisting of a small inhibitory RNA (siRNA), a single-stranded RNA (ssRNA) that forms a triplex with double stranded DNA, and a ribozyme.

45. The method of claim 41 wherein said RNA is a microRNA.

46. The method of claim 41 wherein said DNA is antisense-DNA.

47. A method for modulating activity of at least two toll-like receptors (TLRs) comprising contacting a cell having the toll-like receptors with a liposomal particle of any one of claims 4-13 or 22-36.

48. The method of claim 47 wherein the modulating is up-regulating TLR activity or down-regulating TLR activity.

- 49. The method of claim 47 or claim 48 which is performed *in vitro*.
- 50. The method of claim 47 or claim 48 which is performed *in vivo*.
- 51. A method of treating or preventing ischemia reperfusion injury (IRI) in a patient, comprising administering a liposomal particle of any one of claims 4-8 or 22-36 to the patient, thereby treating or preventing IRI.

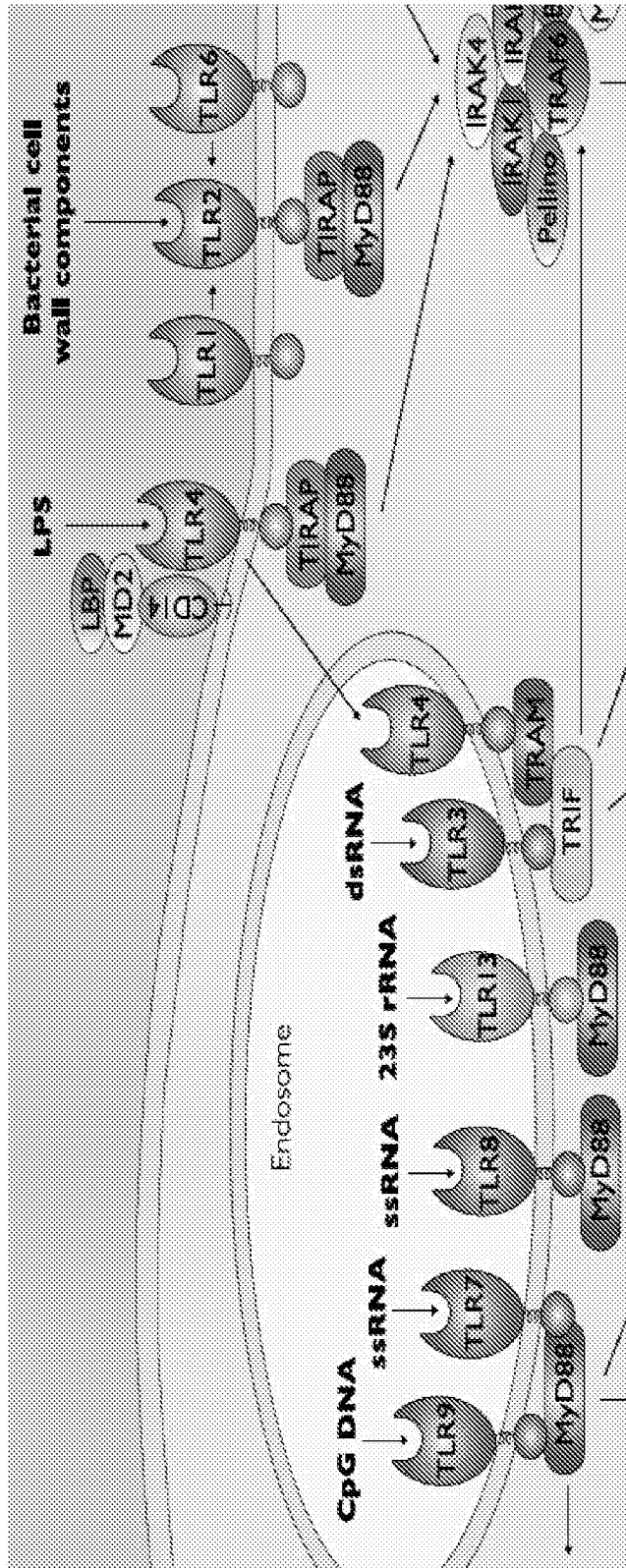
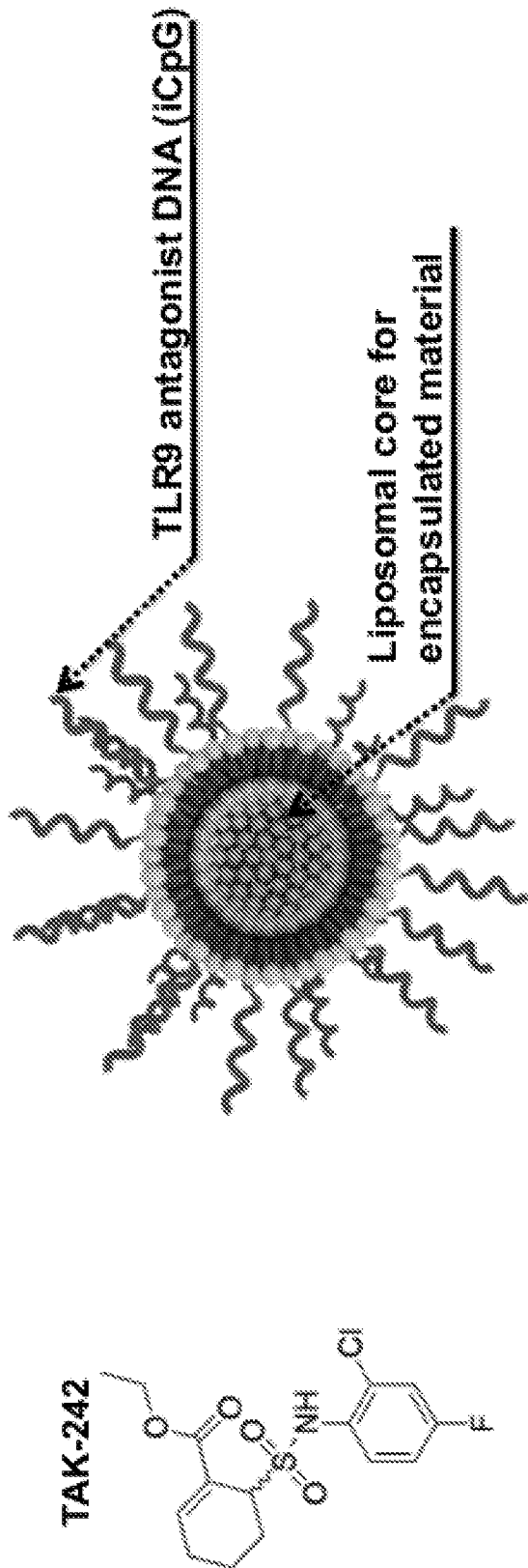


Figure 1

**Figure 2**

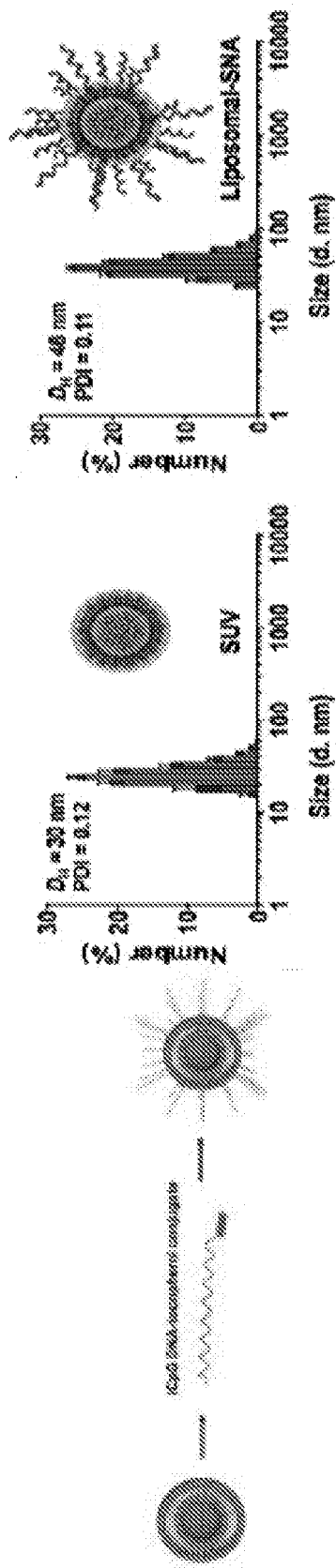


Figure 3

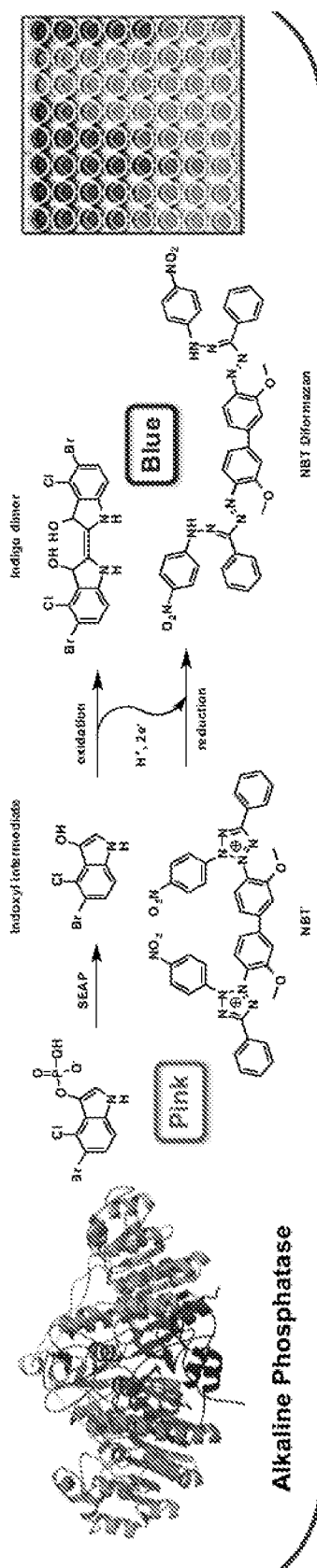
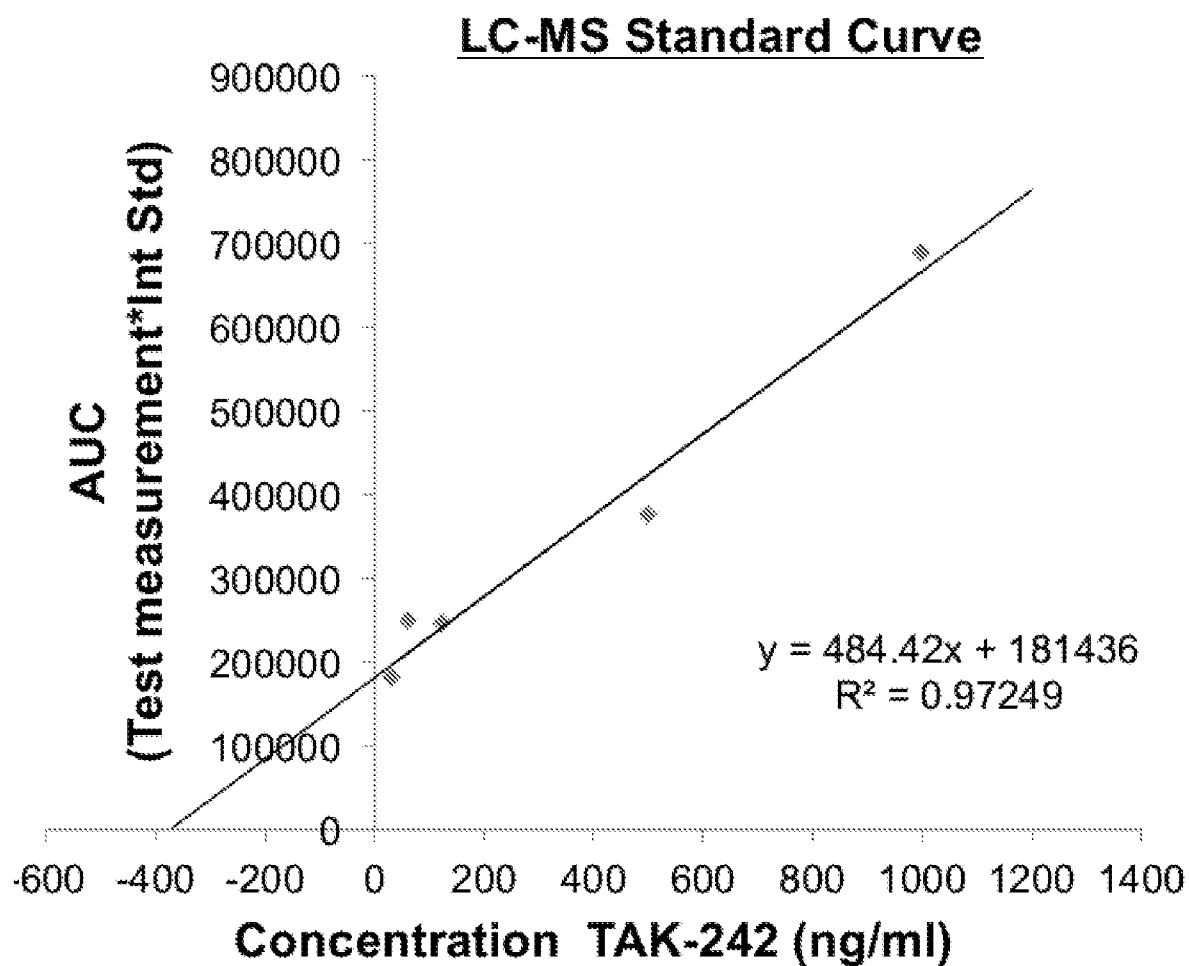


Figure 4

**Figure 5**

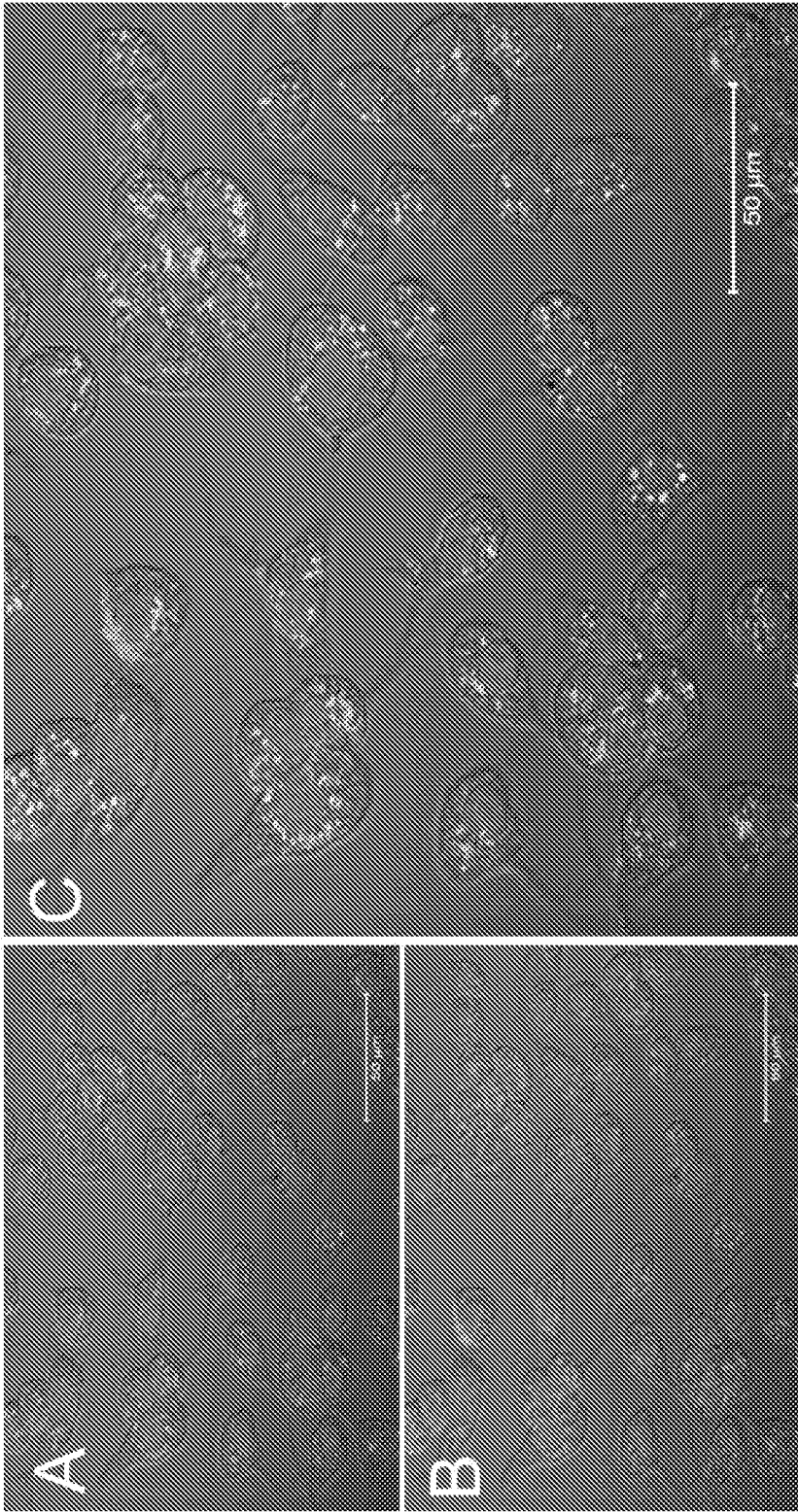


Figure 6

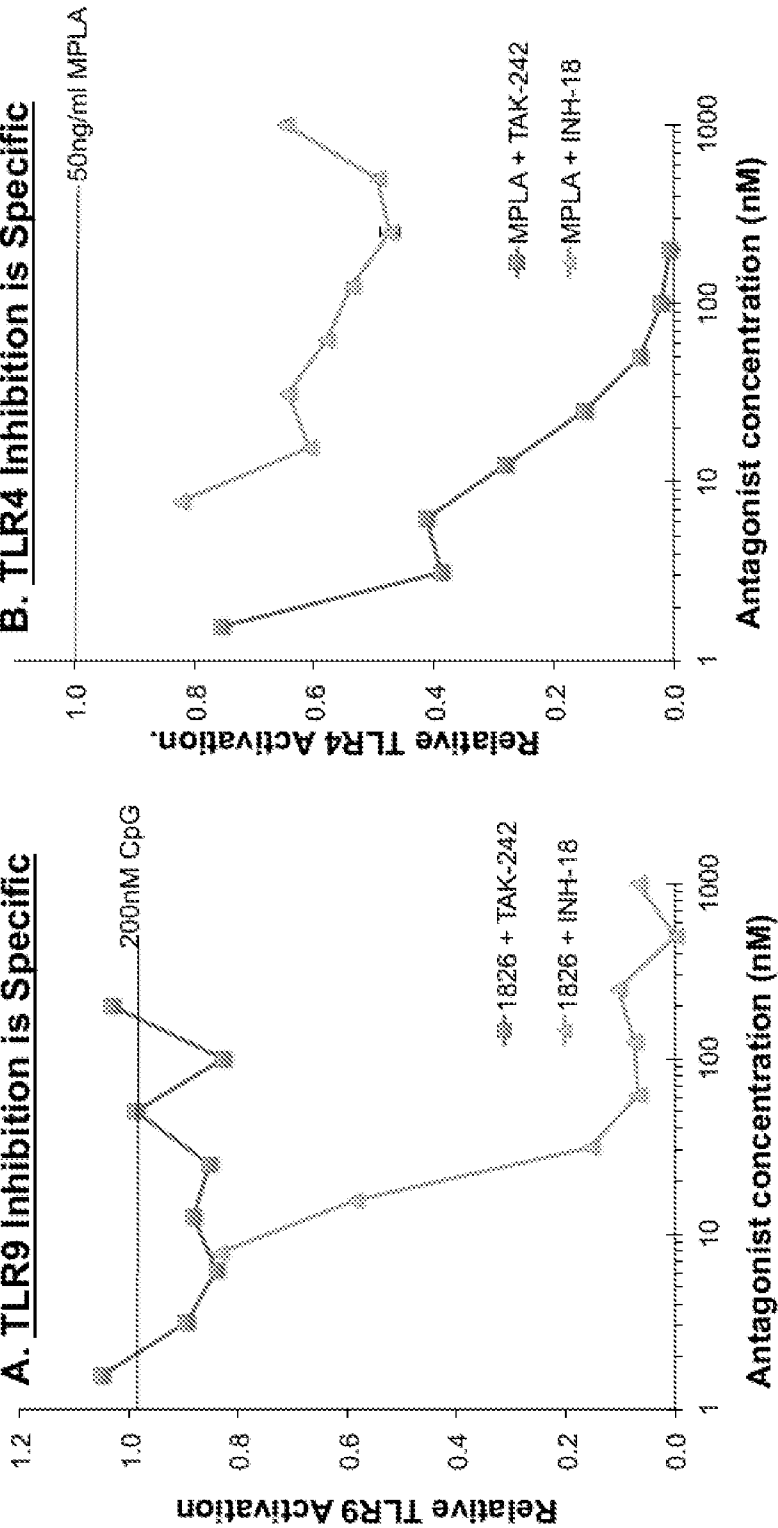


Figure 7

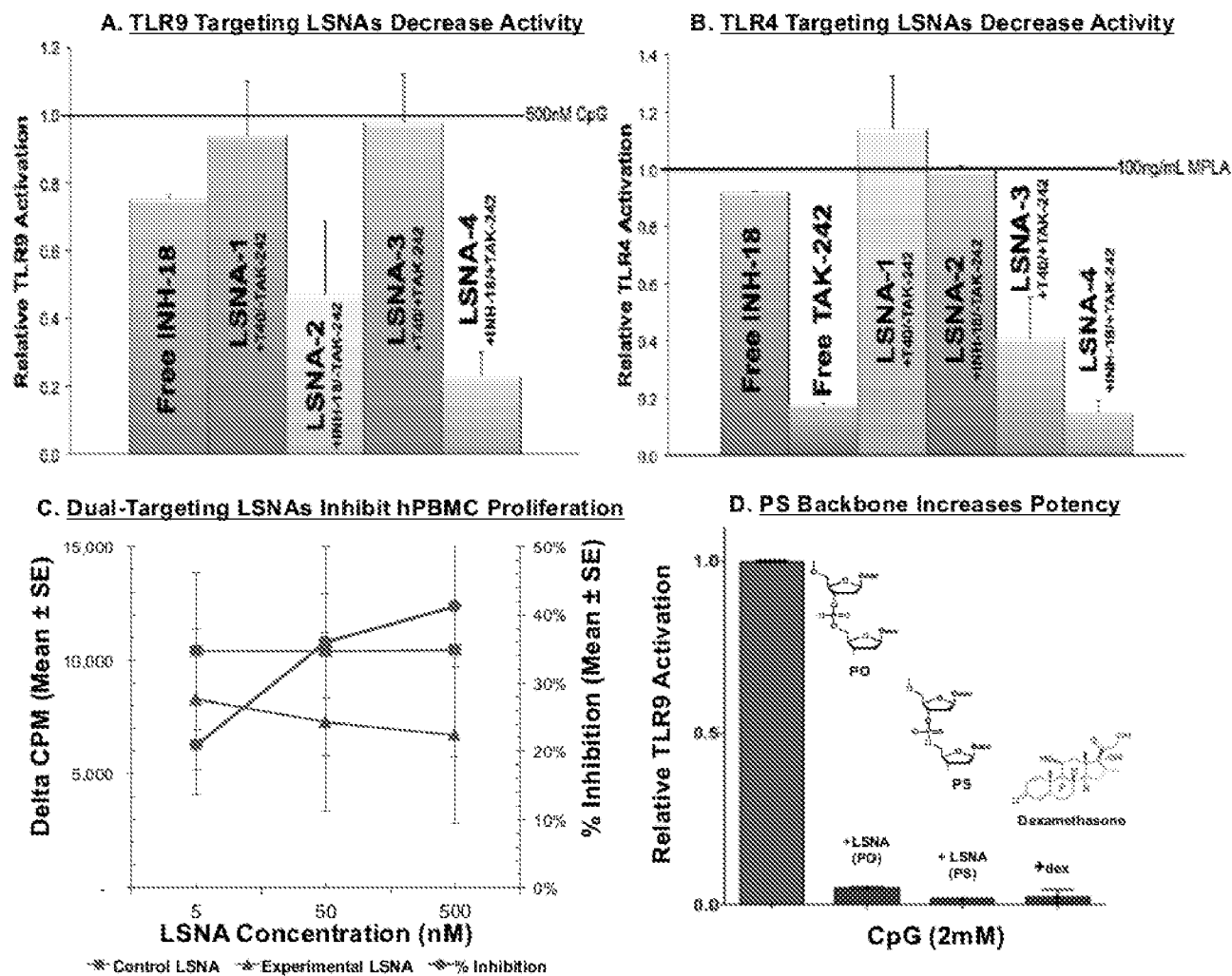
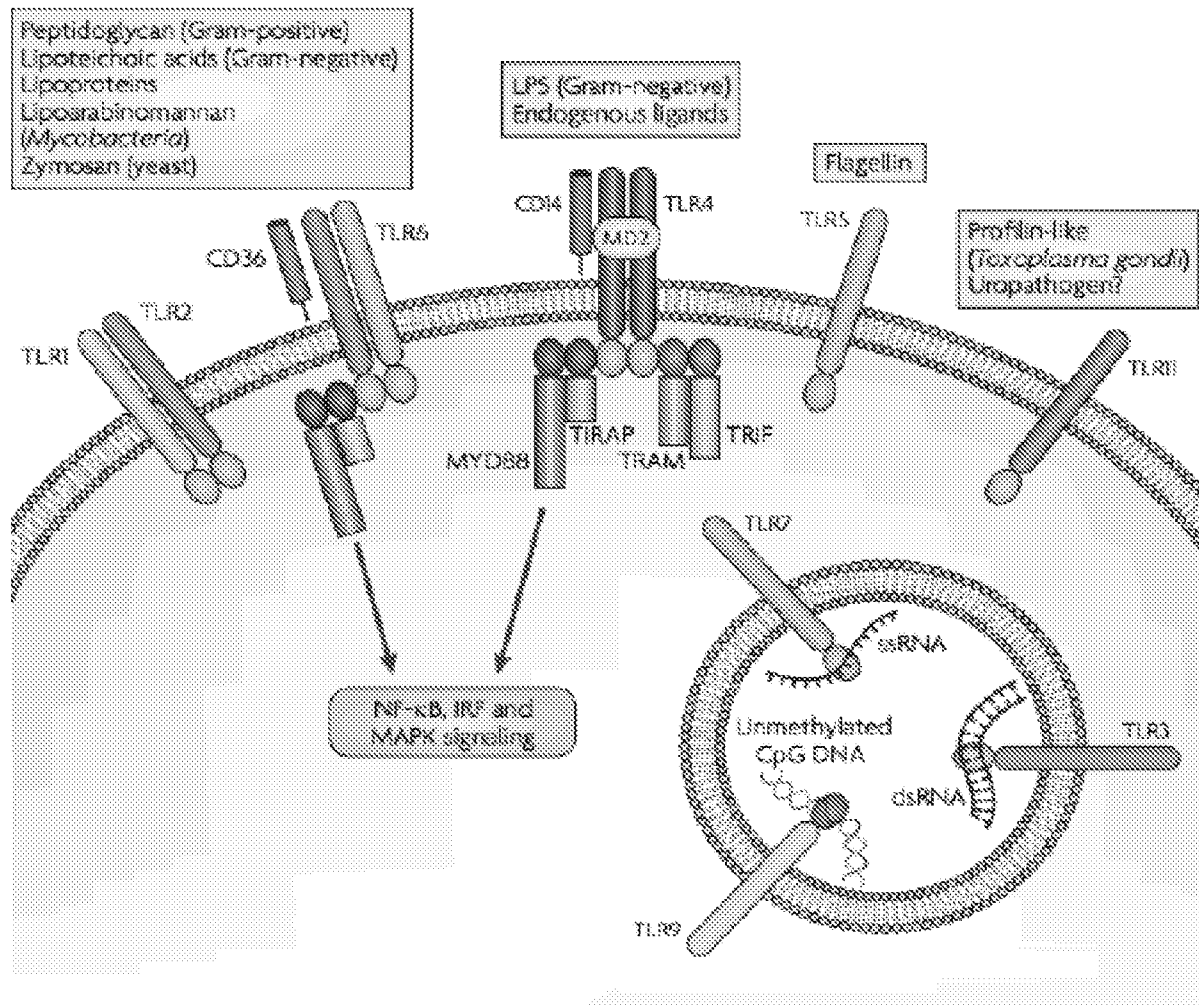
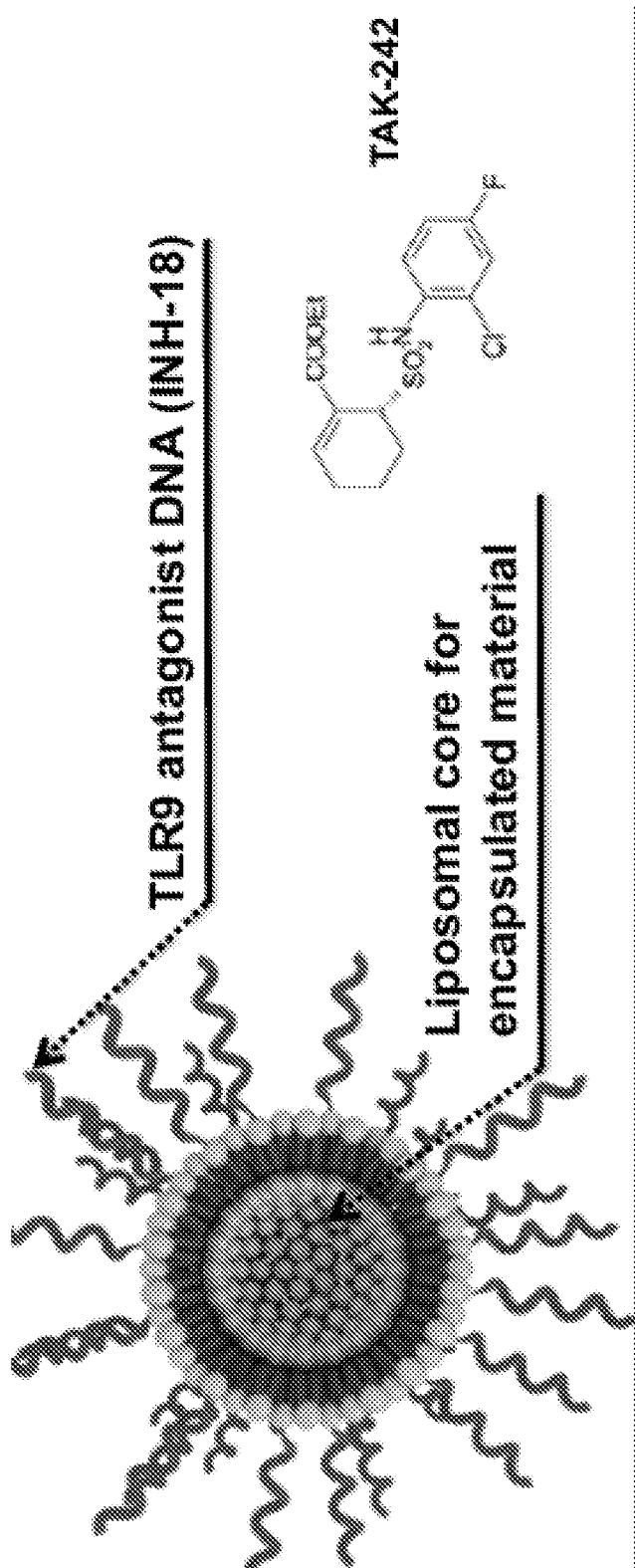
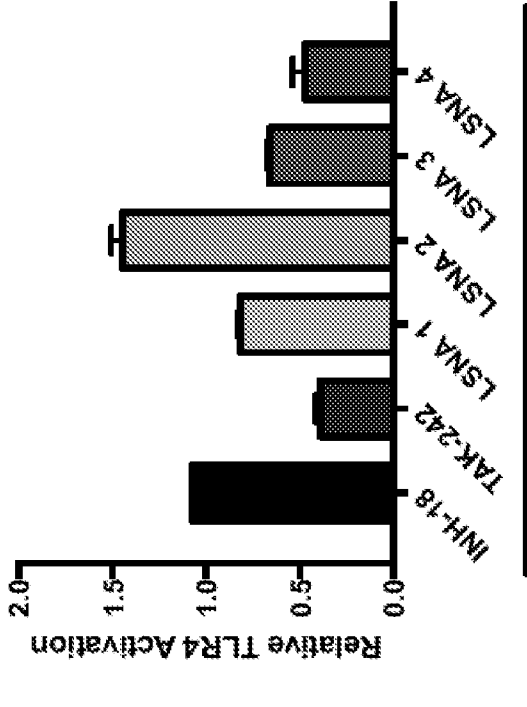


Figure 8

**Figure 9**

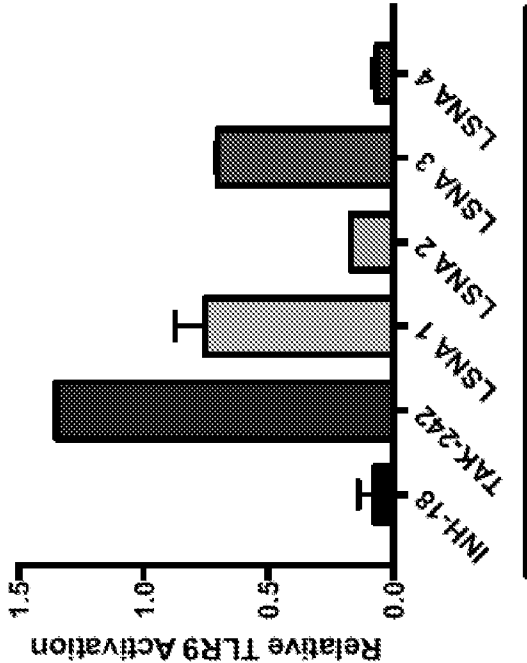
**Figure 10**

B. TLR4 Targeting LSNAs Decrease Activity



TLR9 inhib.	+	-	+
TLR4 inhib.	-	-	+

A. TLR9 Targeting LSNAs Decrease Activity



TLR9 inhib.	+	-	+
TLR4 inhib.	-	+	-

Figure 11

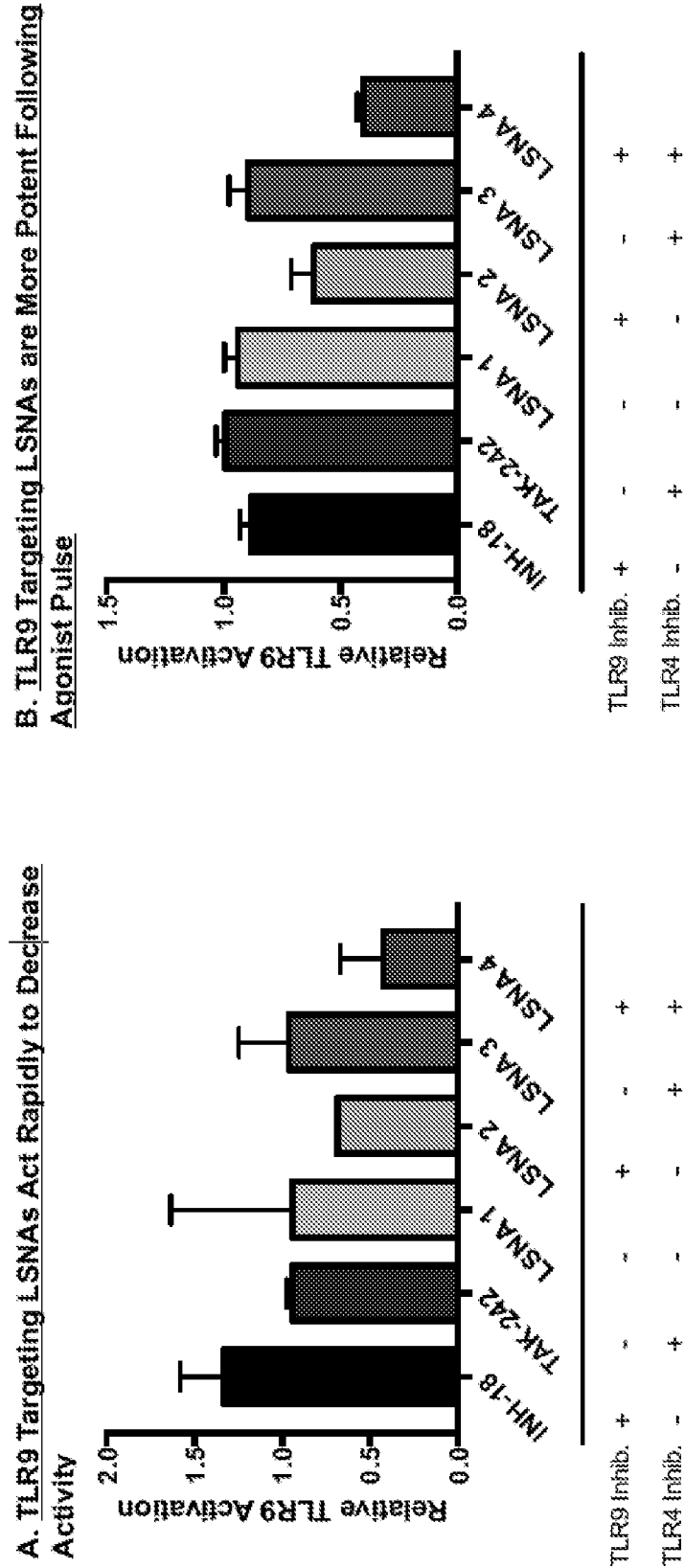


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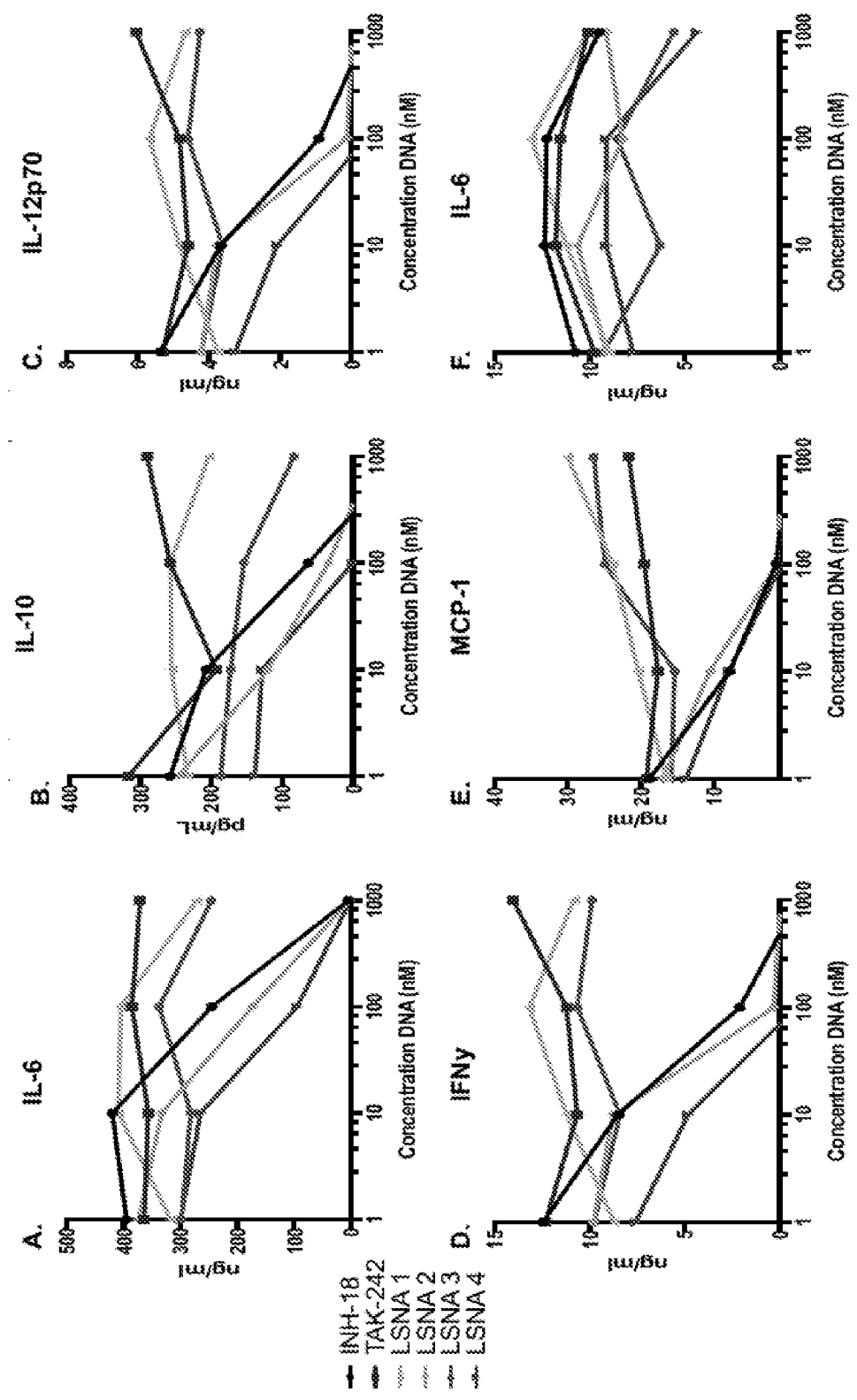


Figure 13

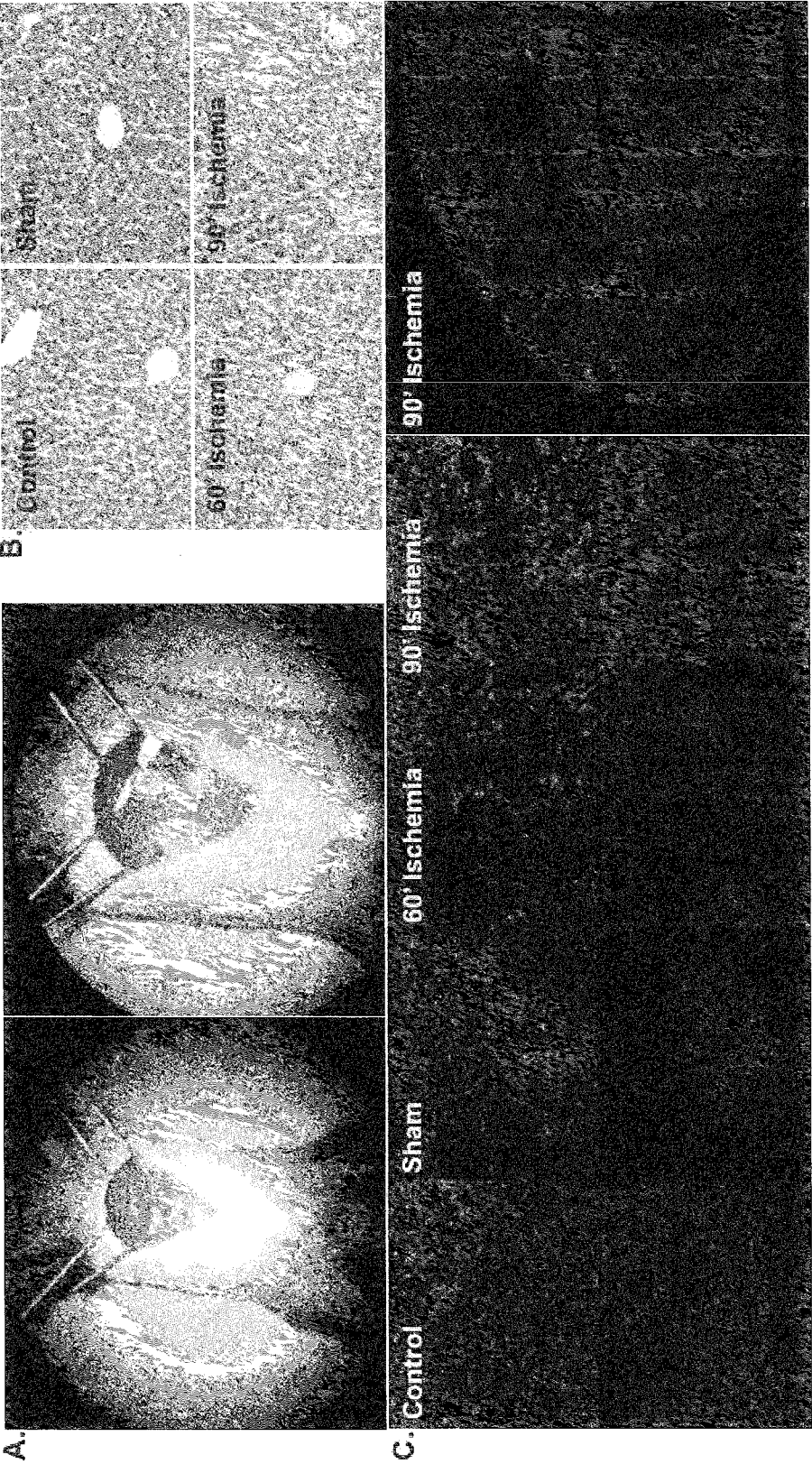


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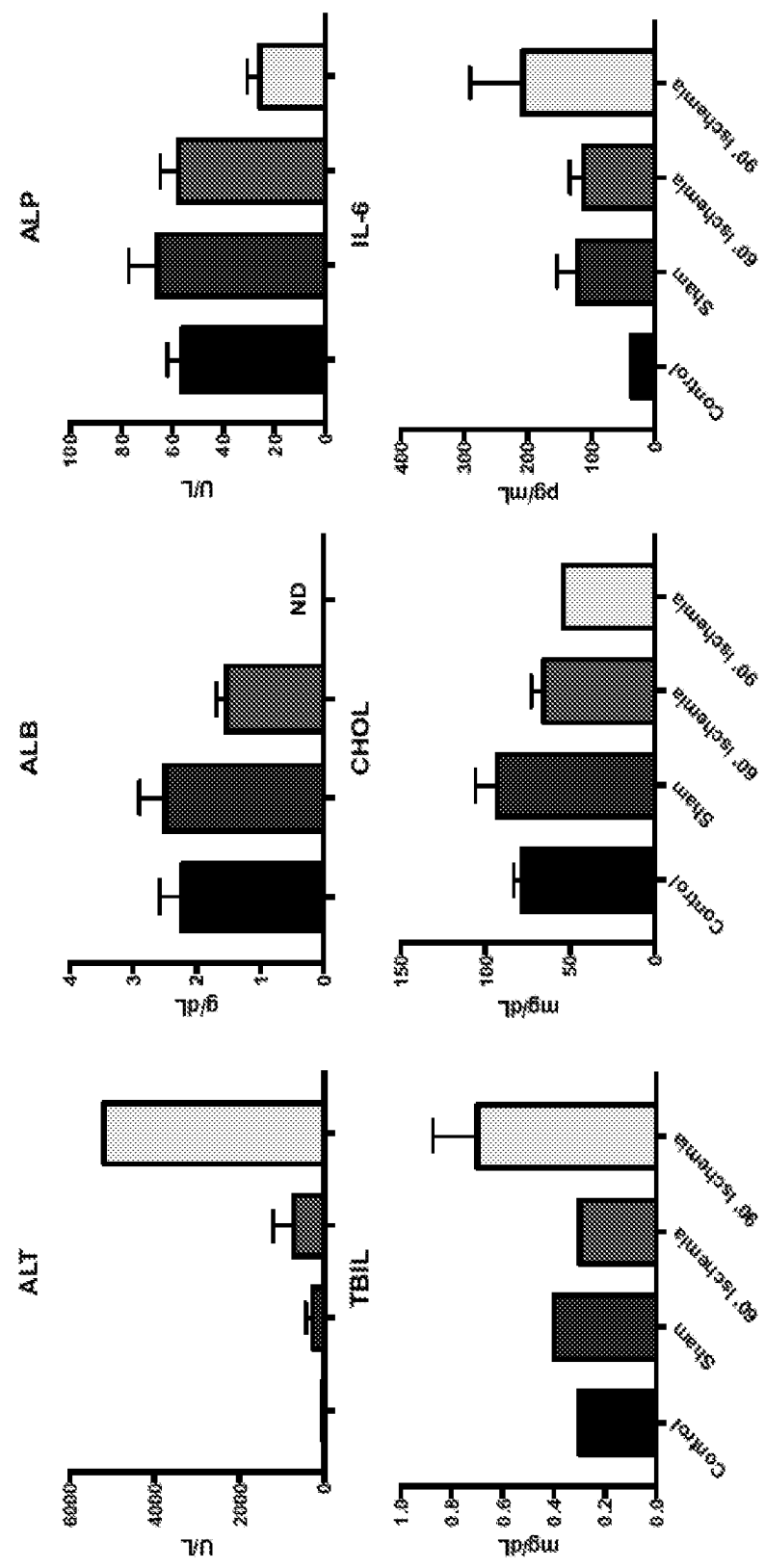


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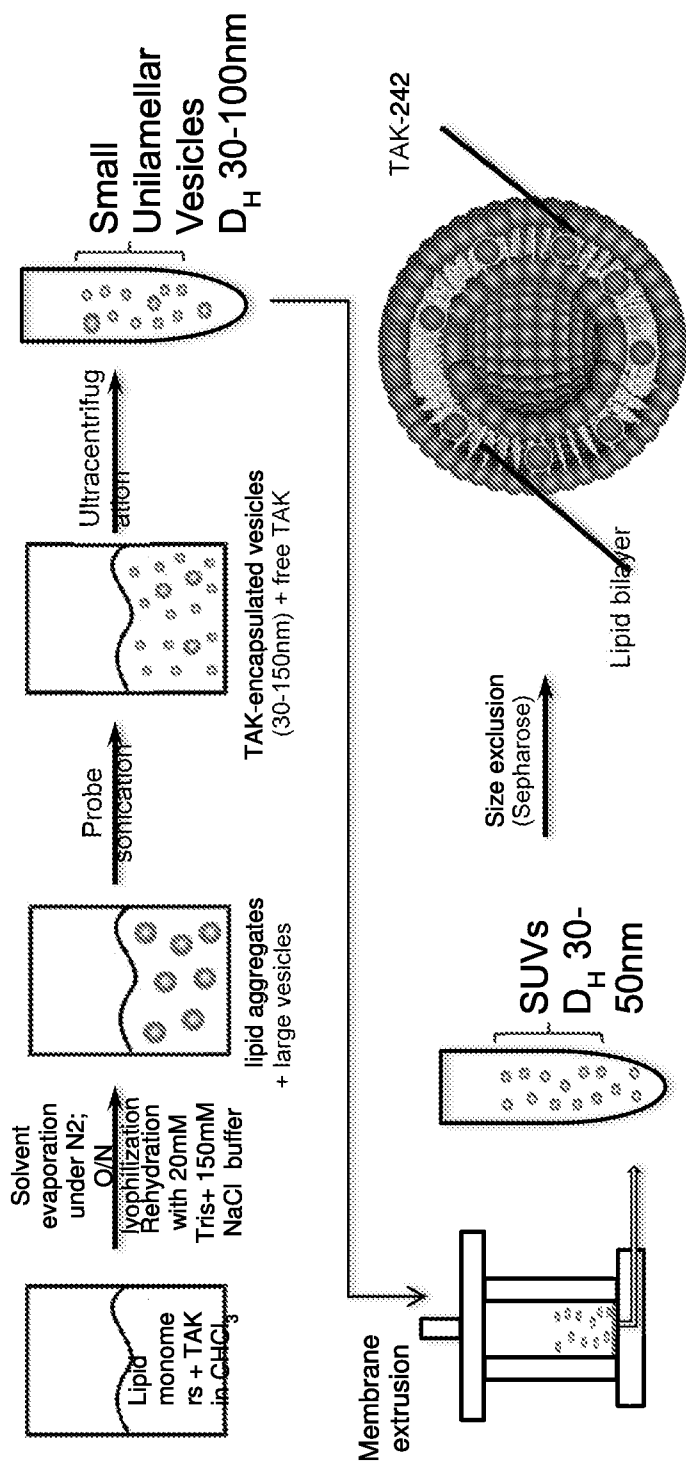


Figure 16

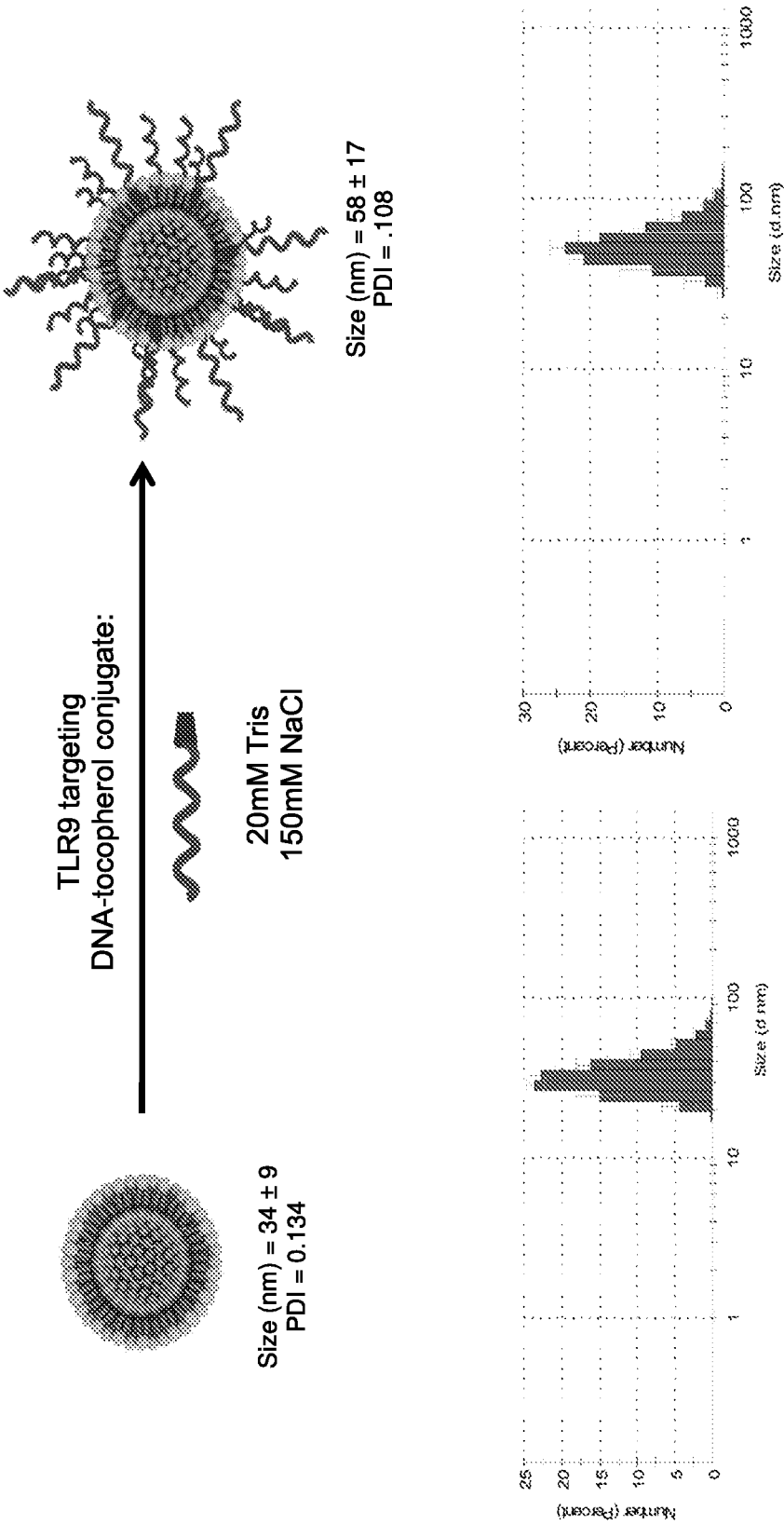


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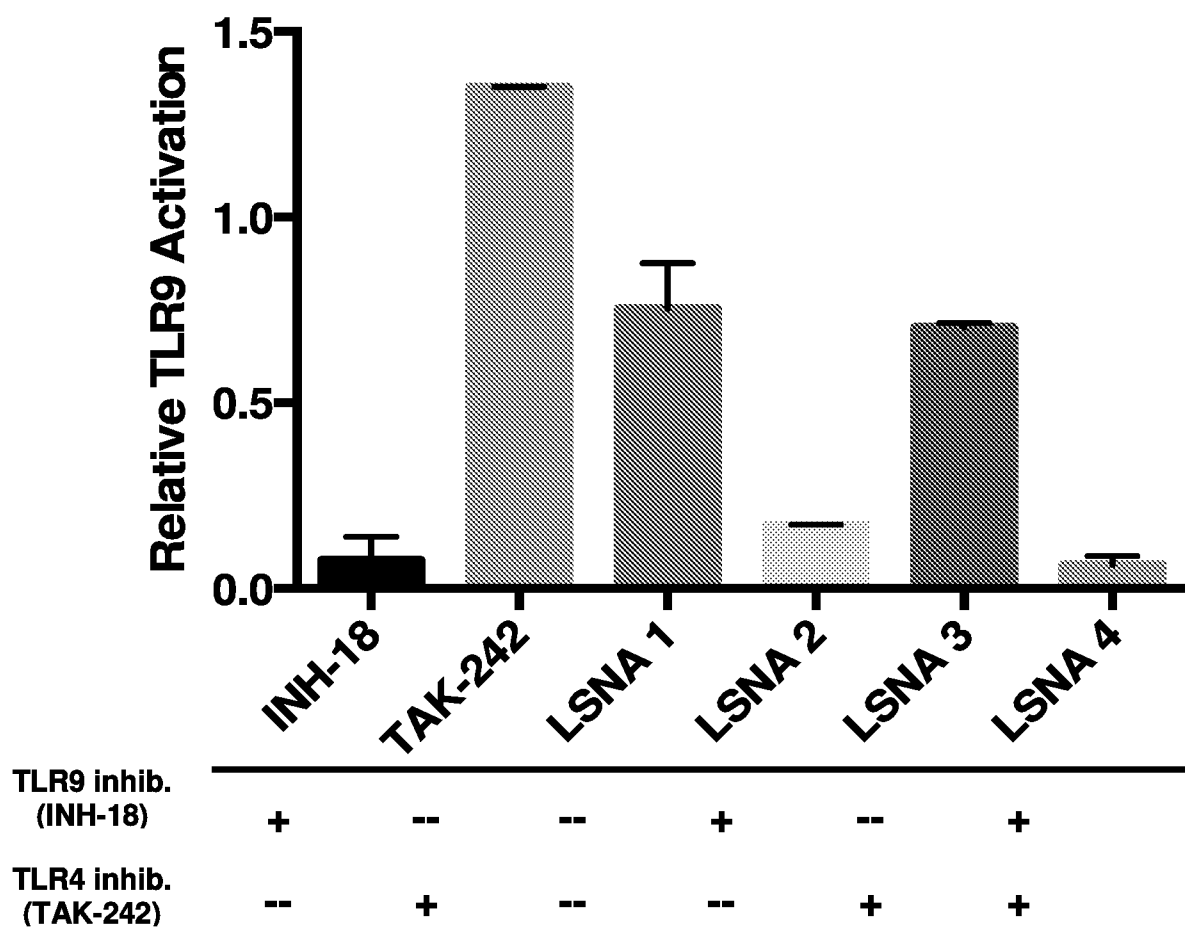


Figure 18

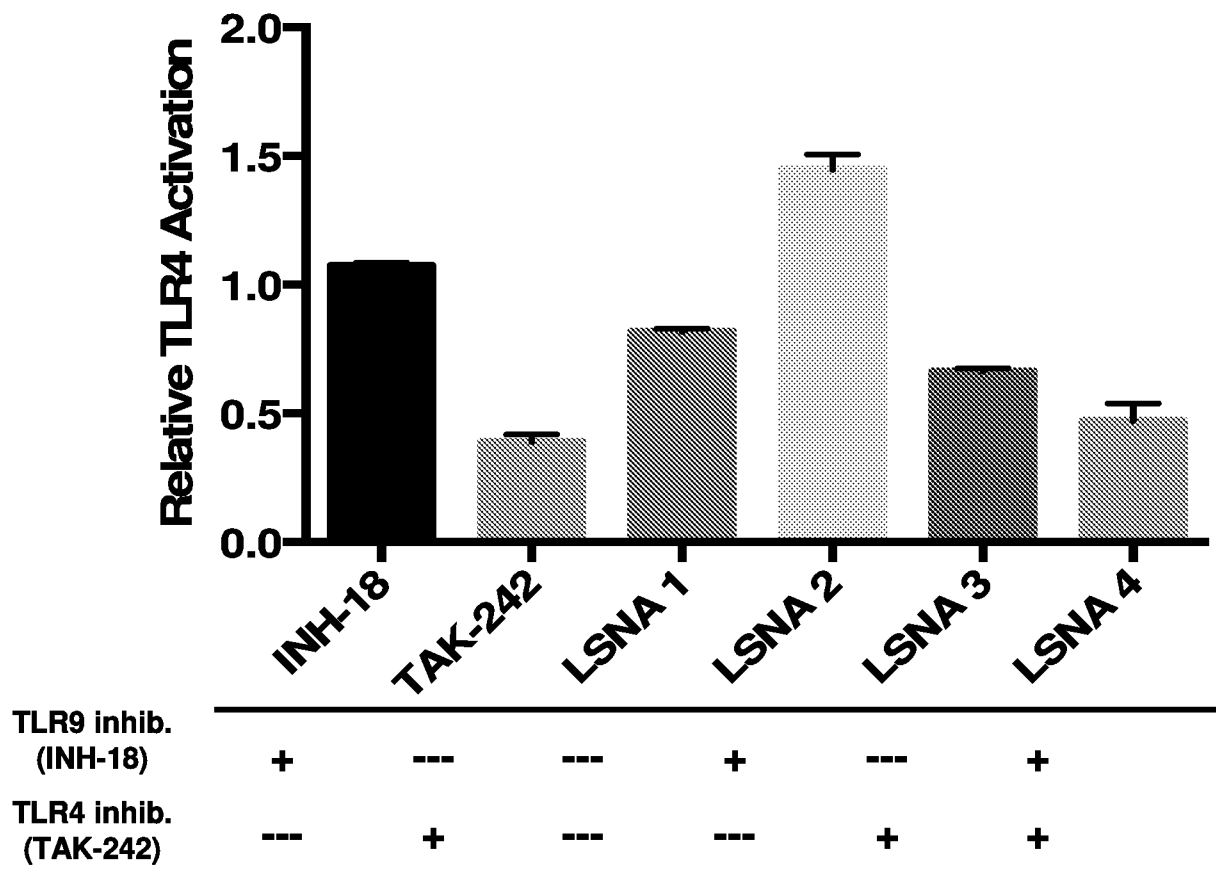


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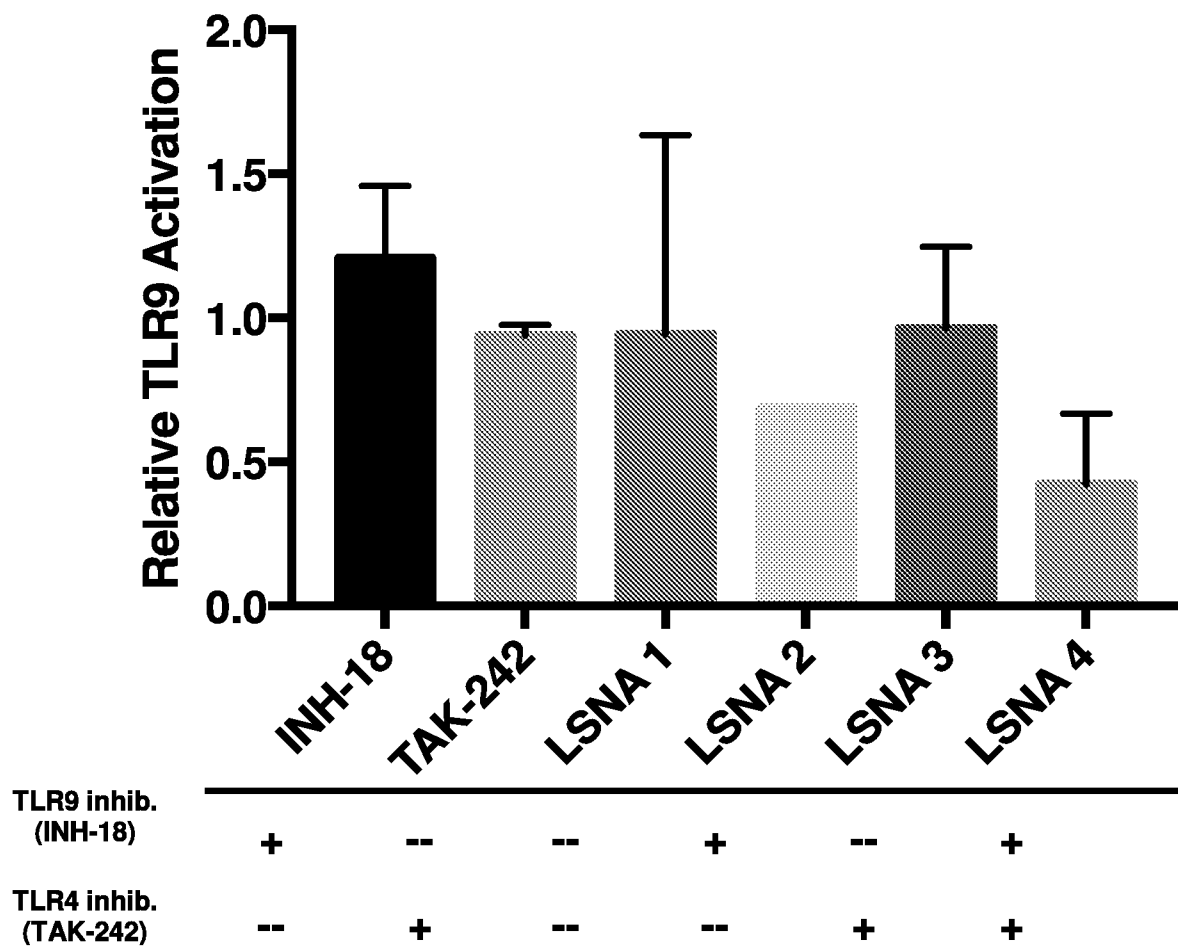
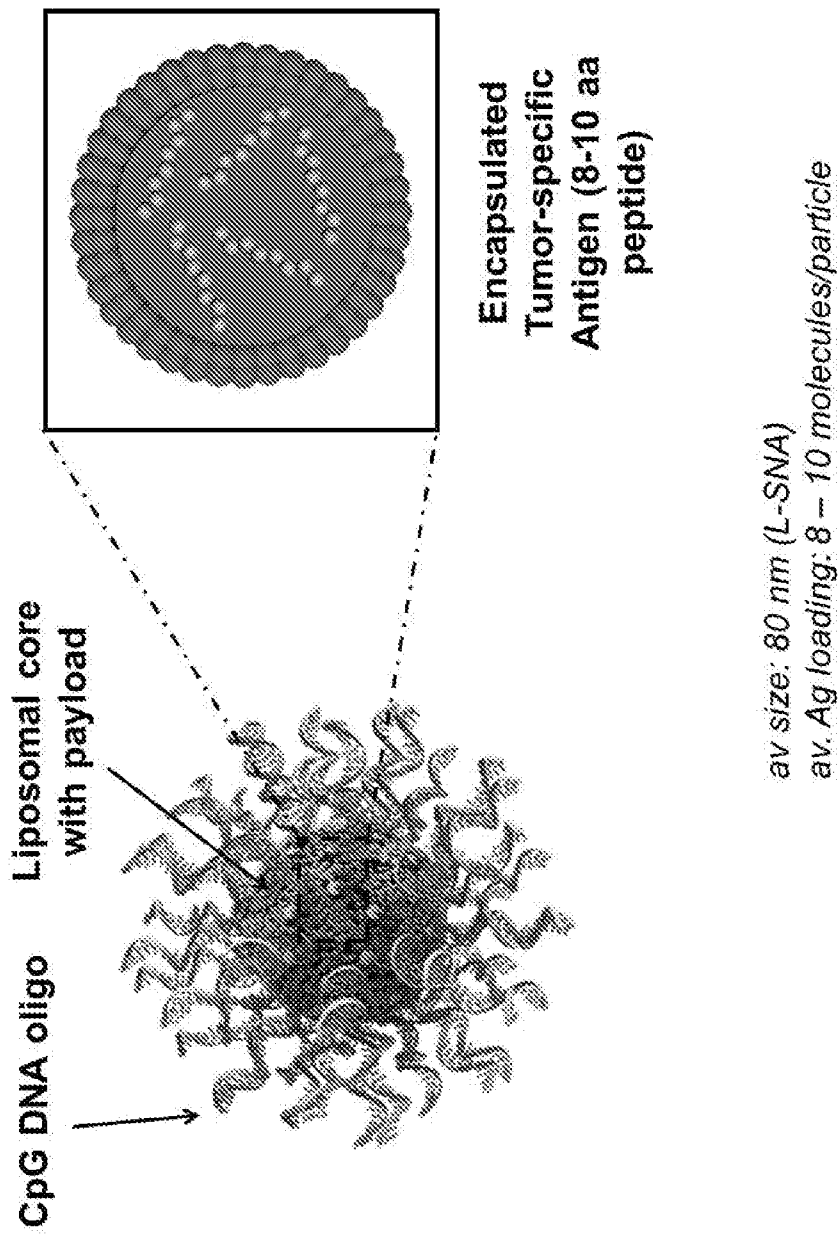


Figure 20

**Figure 21**

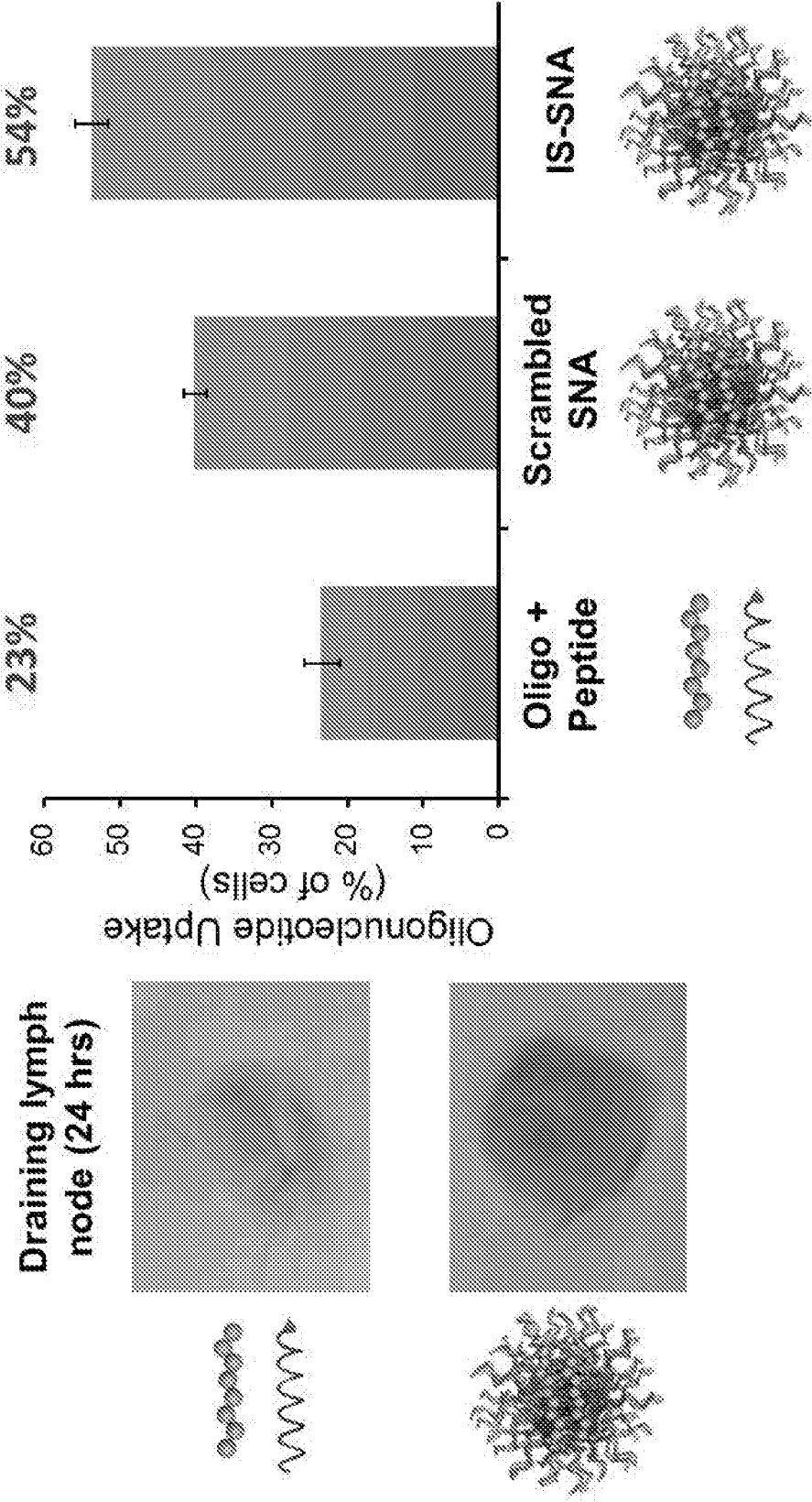


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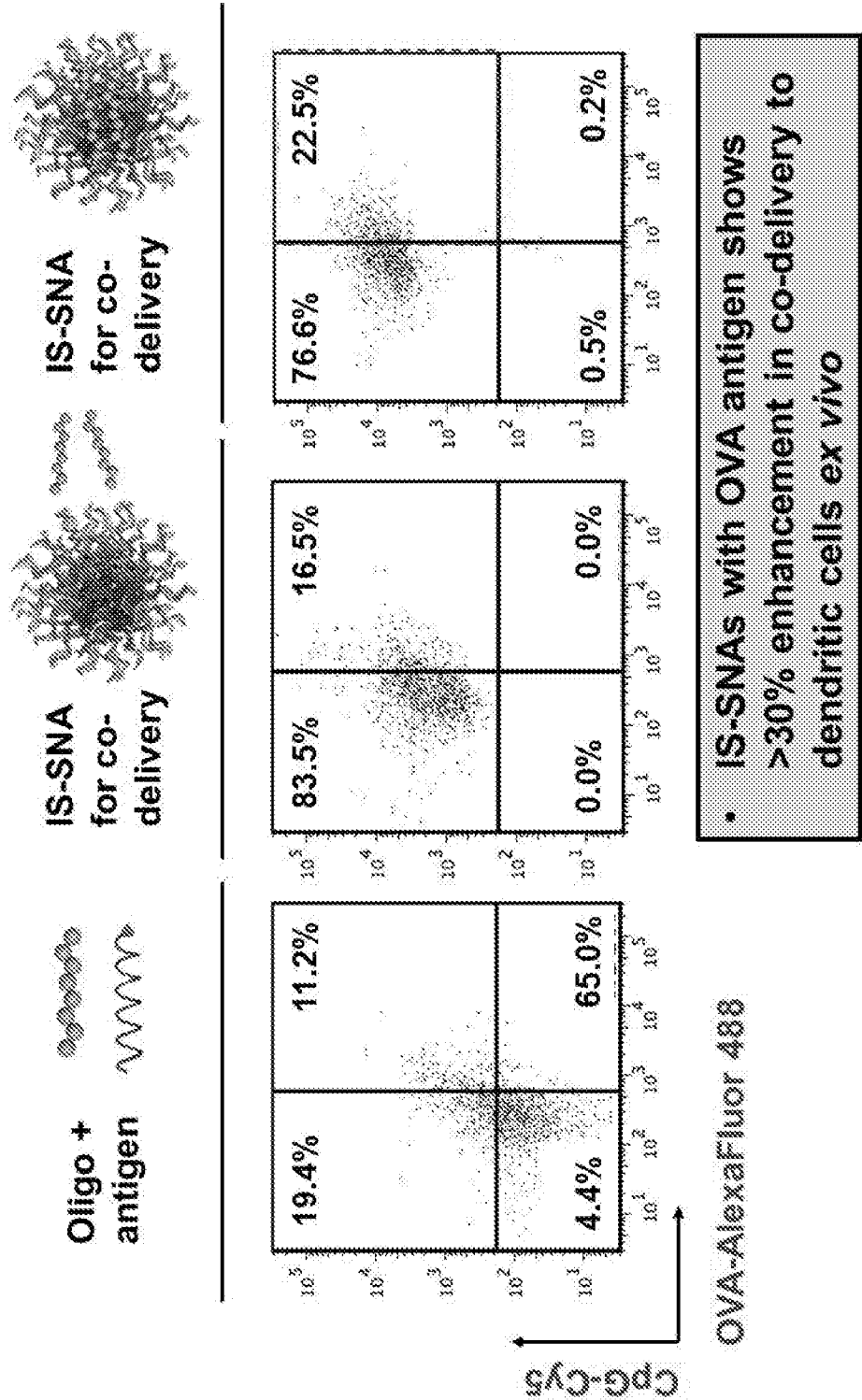


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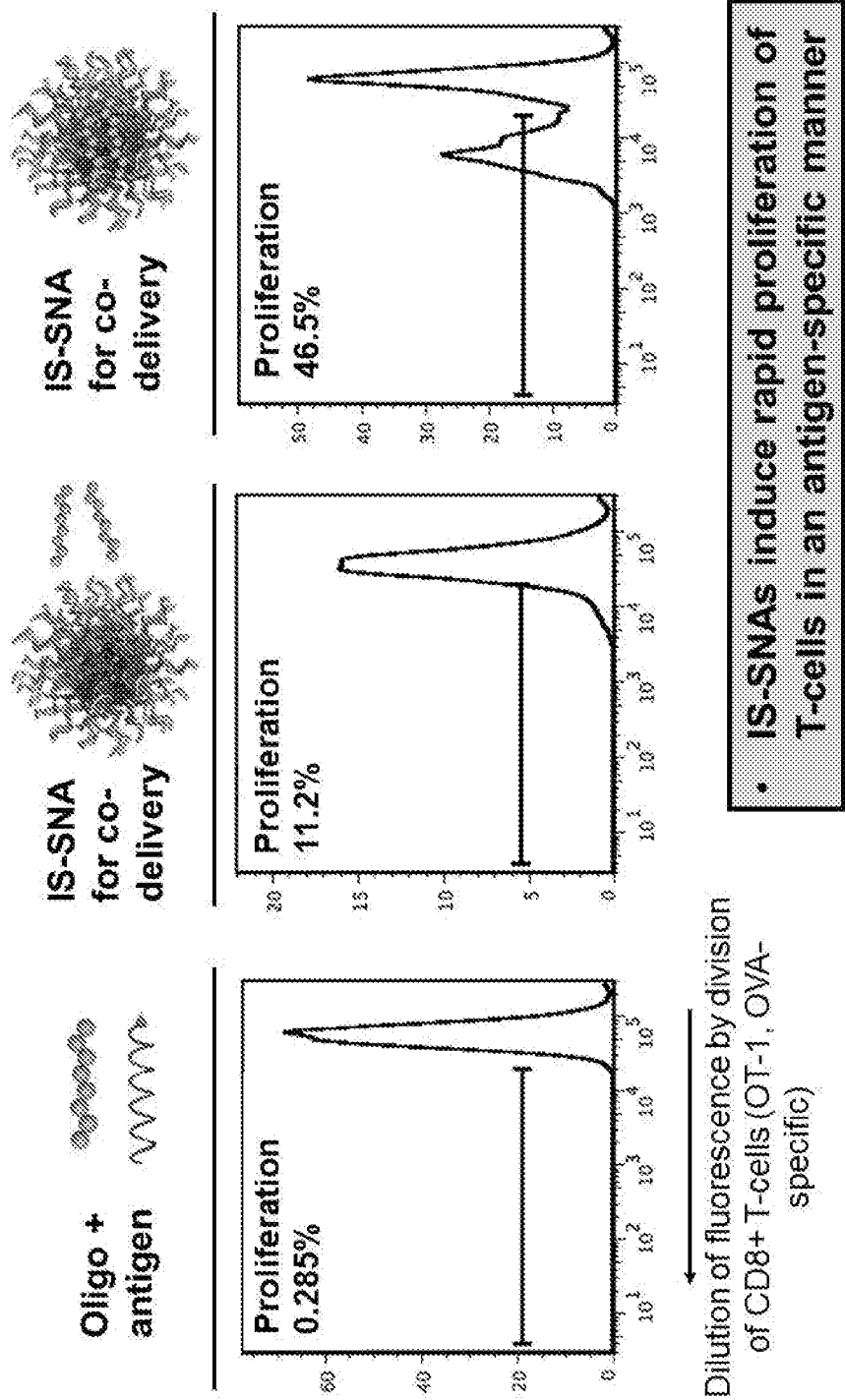
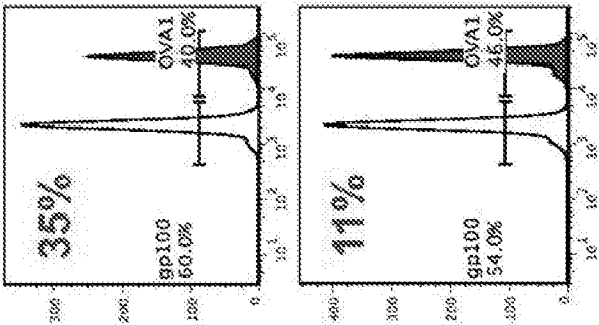
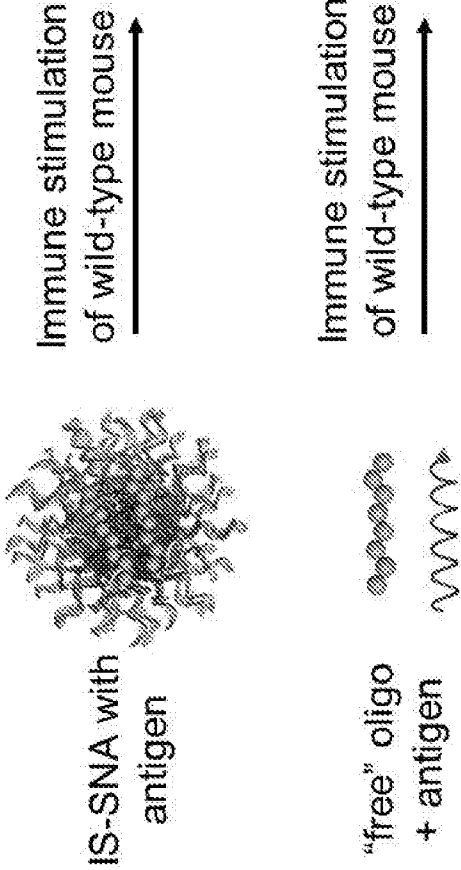


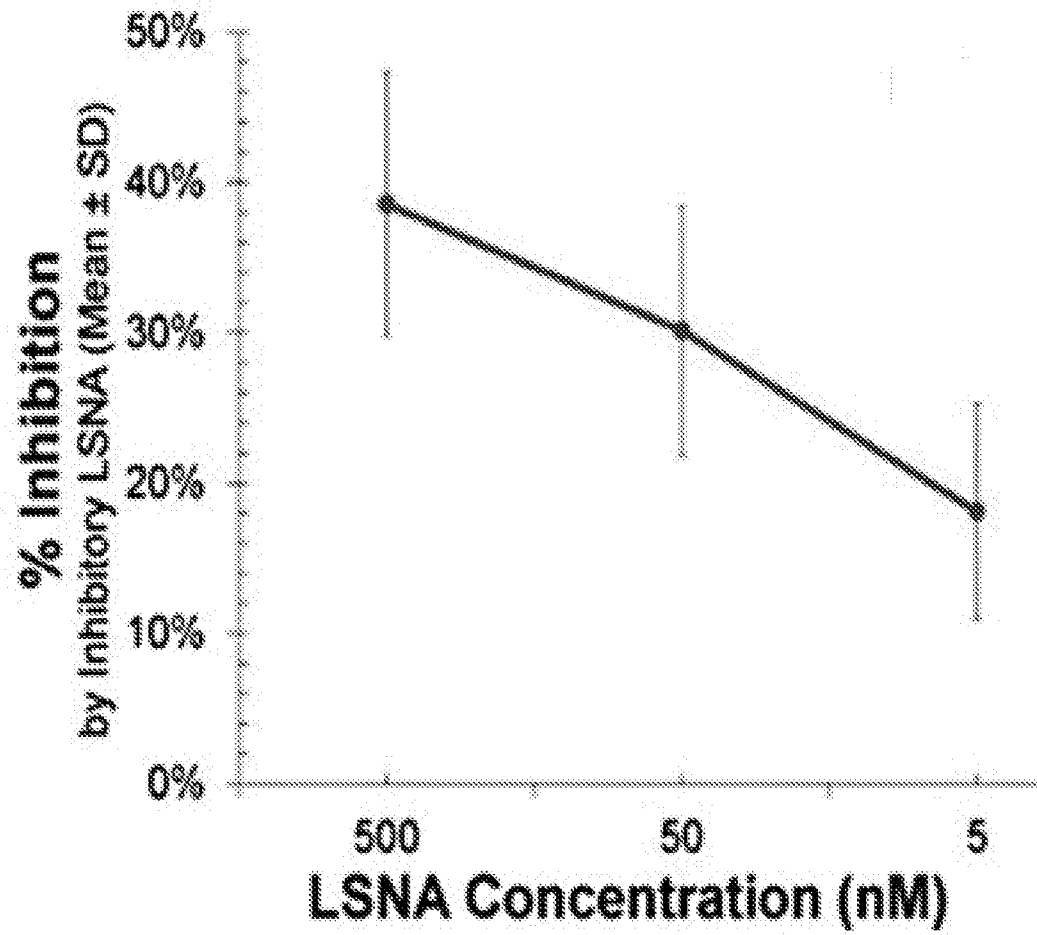
Figure 24

Antigen-targeted killing *in vivo*
(target cells injected into tail
vein of immunized mice)



• SNA architecture increases vaccine activity by 3x compared to peptide + adjuvant mixture

Figure 25

**Figure 26**

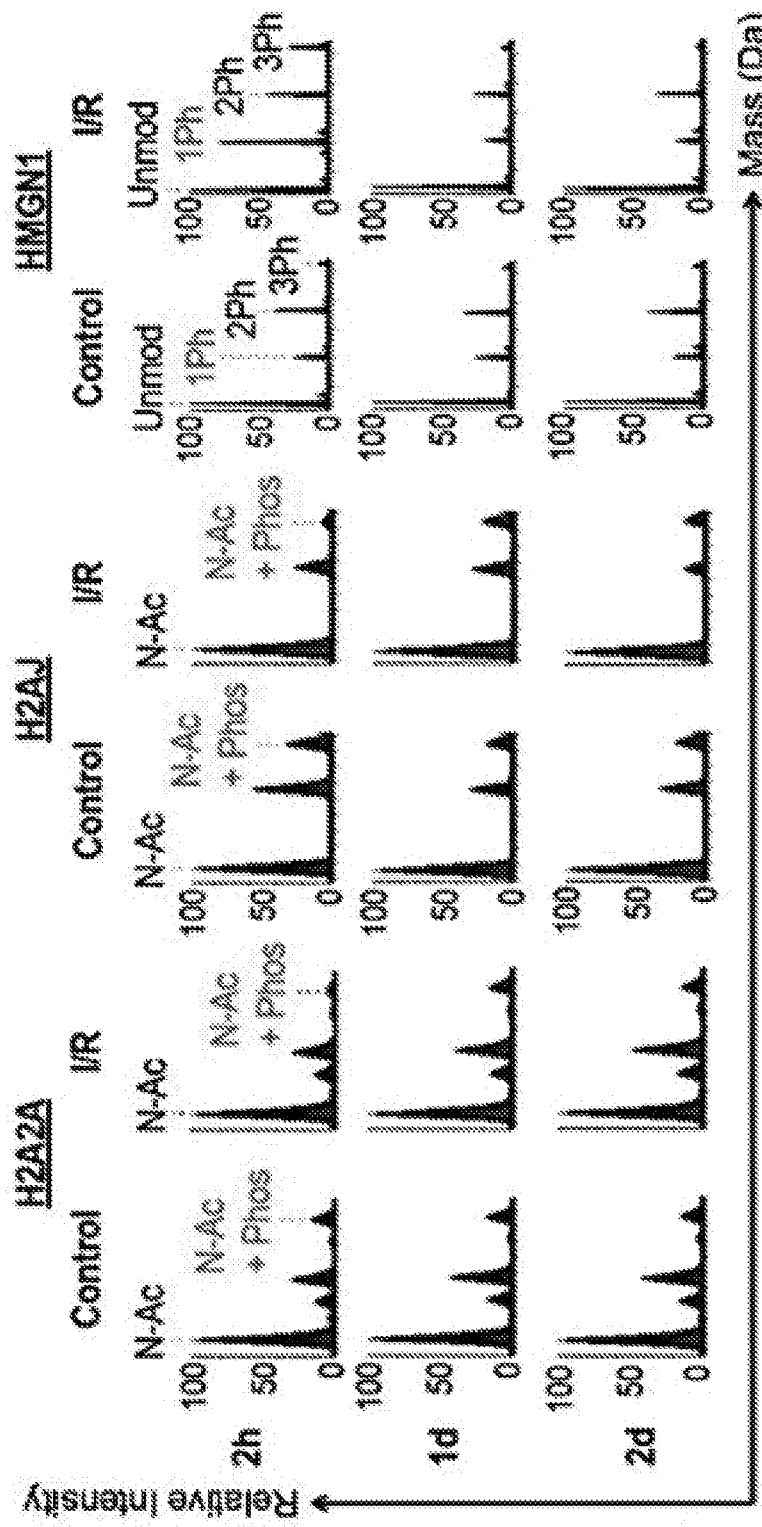


Figure 27

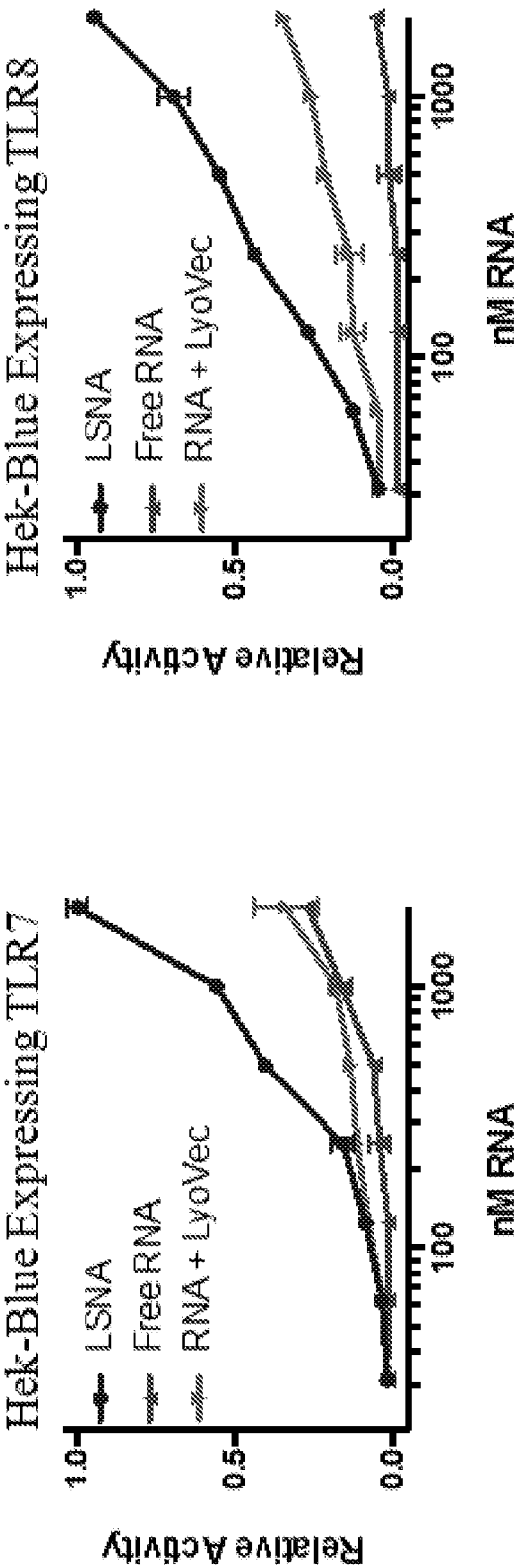


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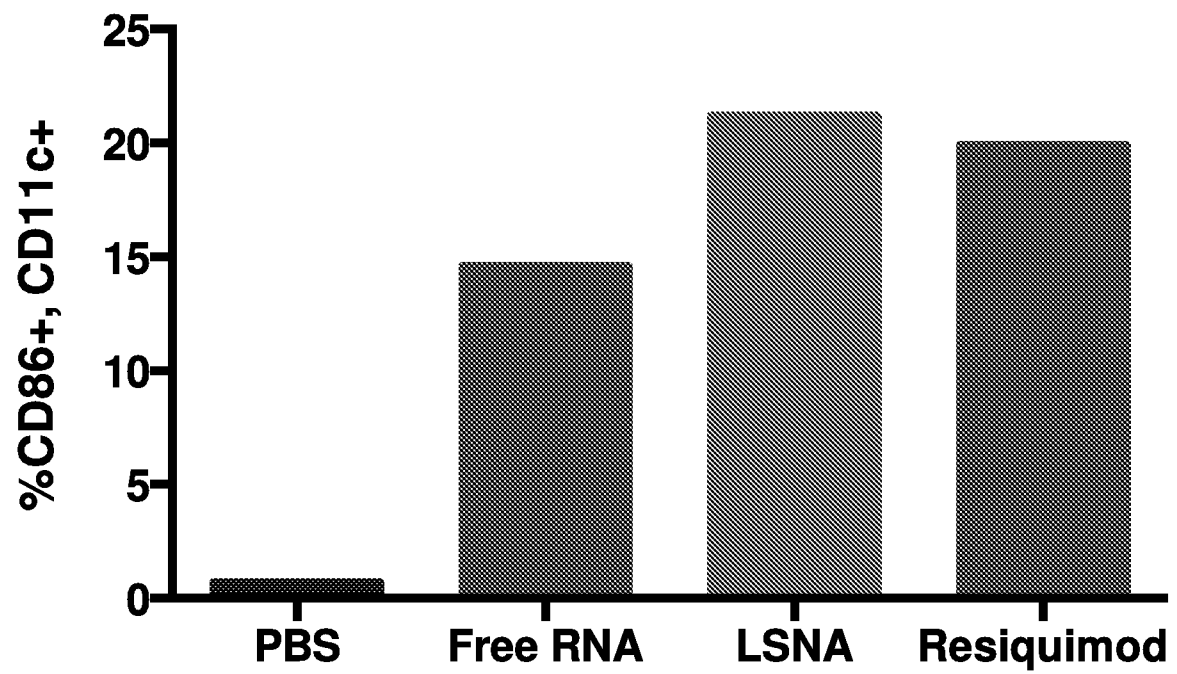
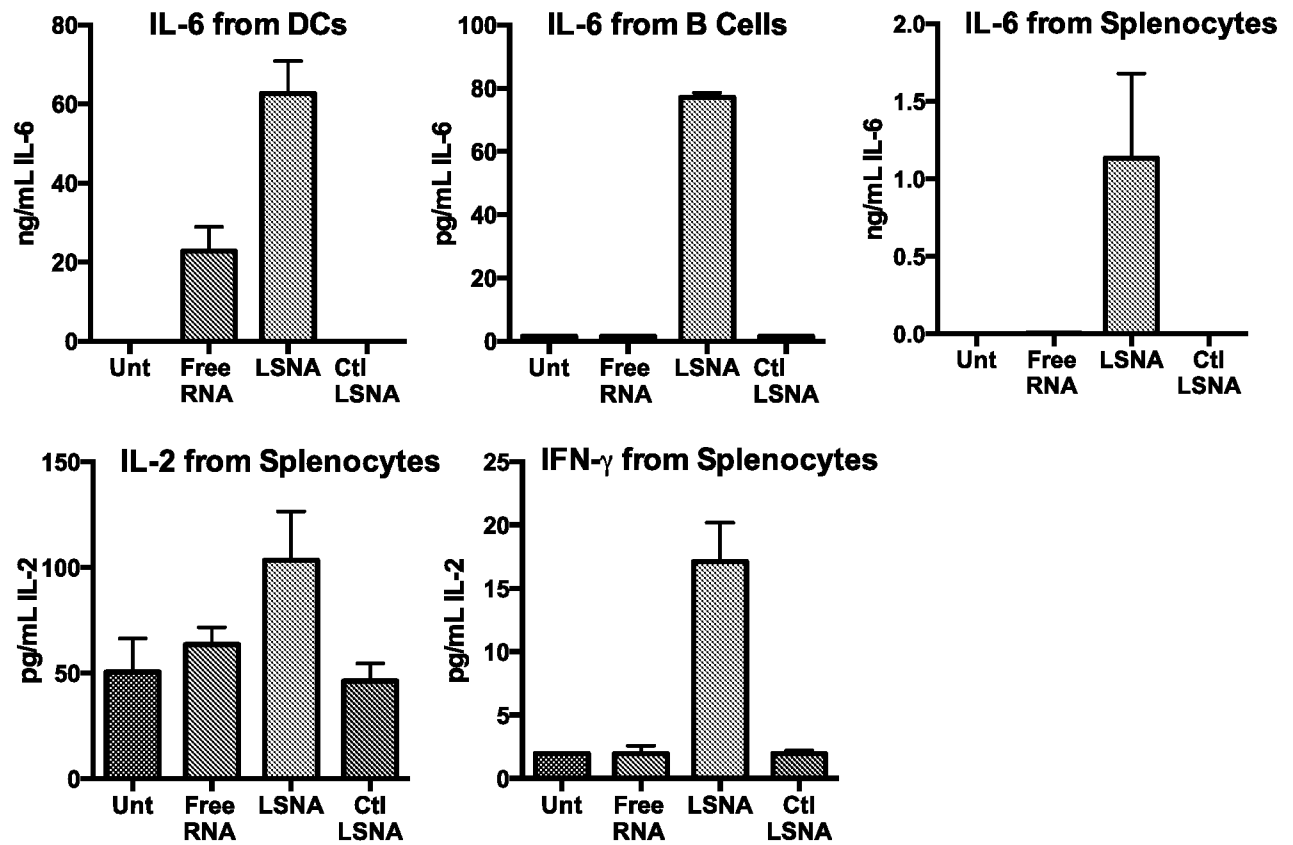


Figure 29

**Figure 30**

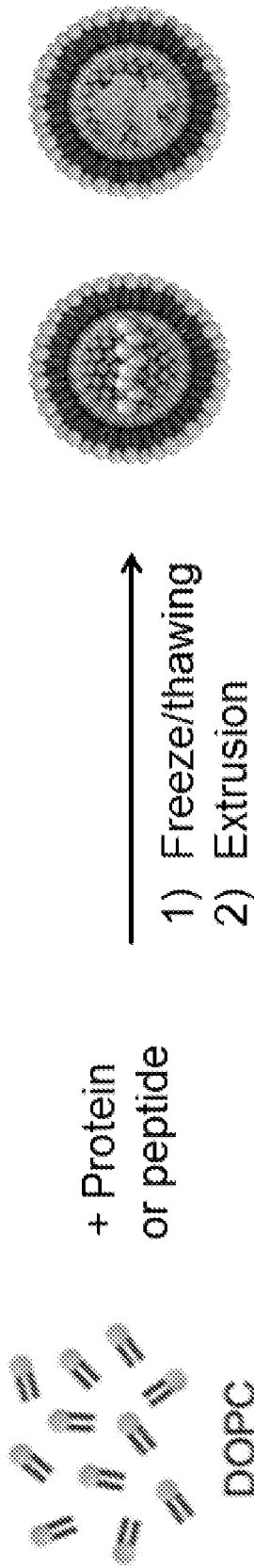
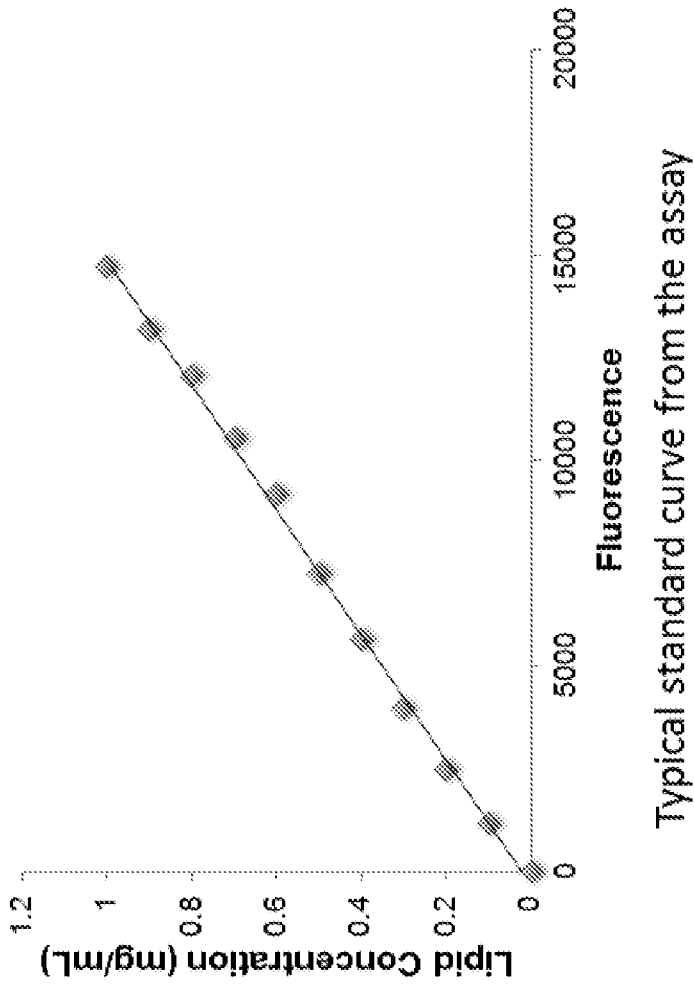
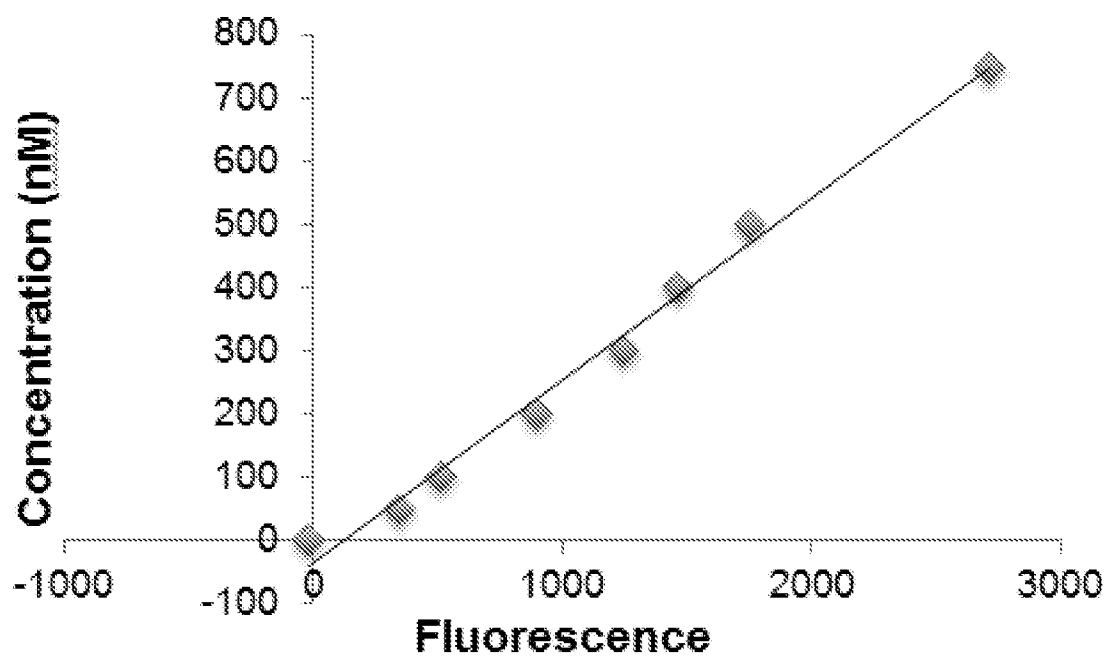


Figure 31



Greater than 94%
of the DOPC lipid is
incorporated into
liposomes.

Figure 32



Typical standard curve for fluorescent proteins

Figure 33

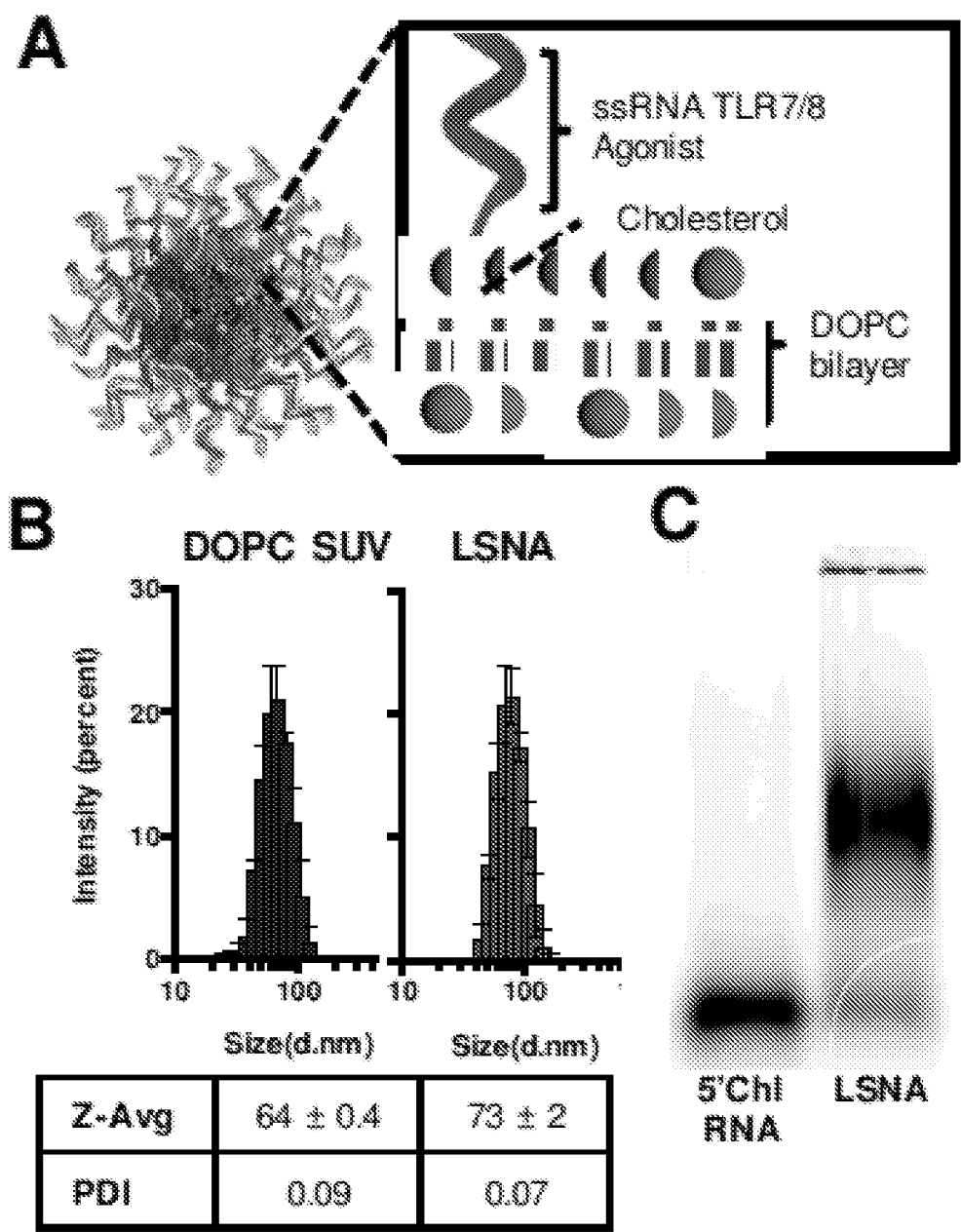


Figure 34

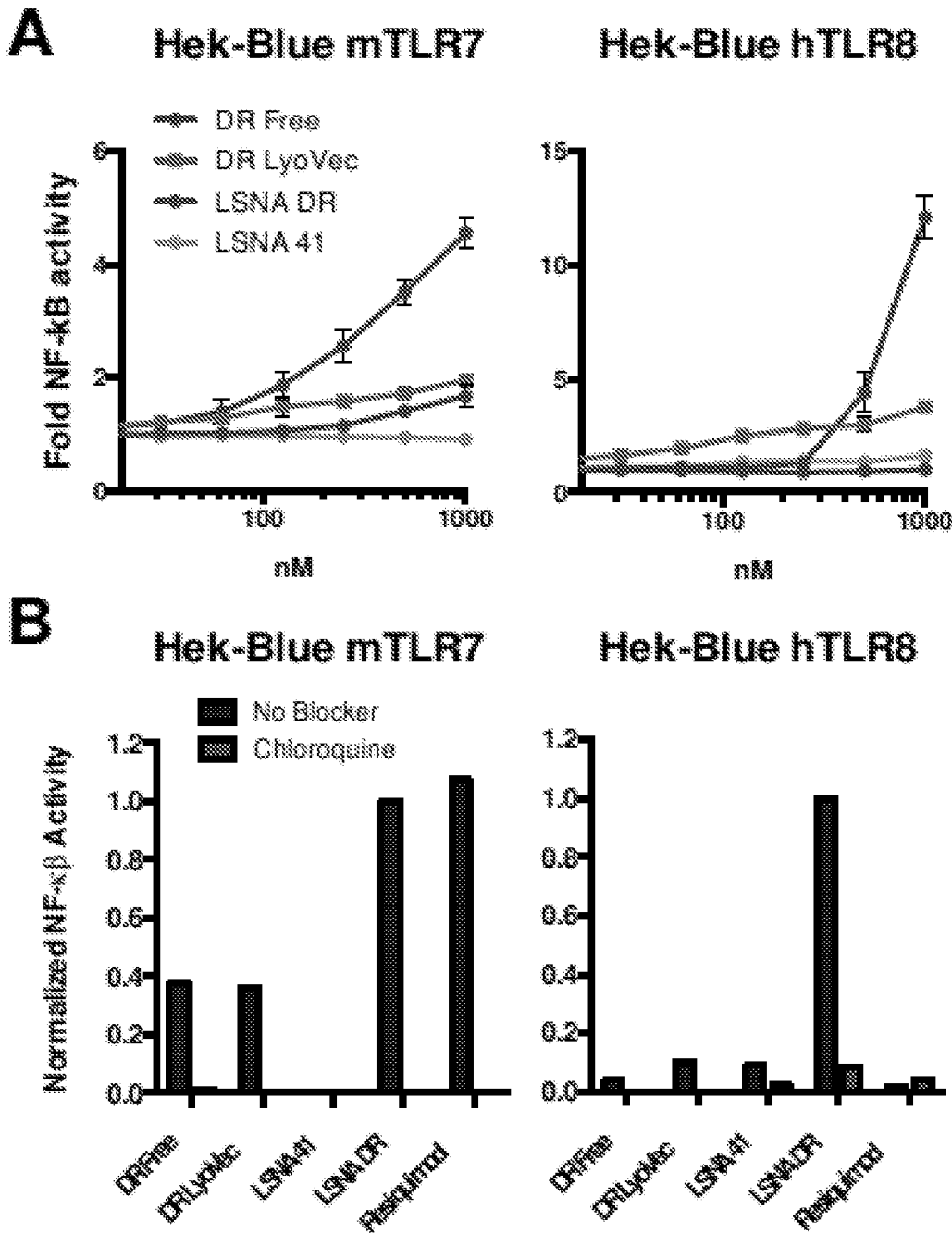


Figure 35

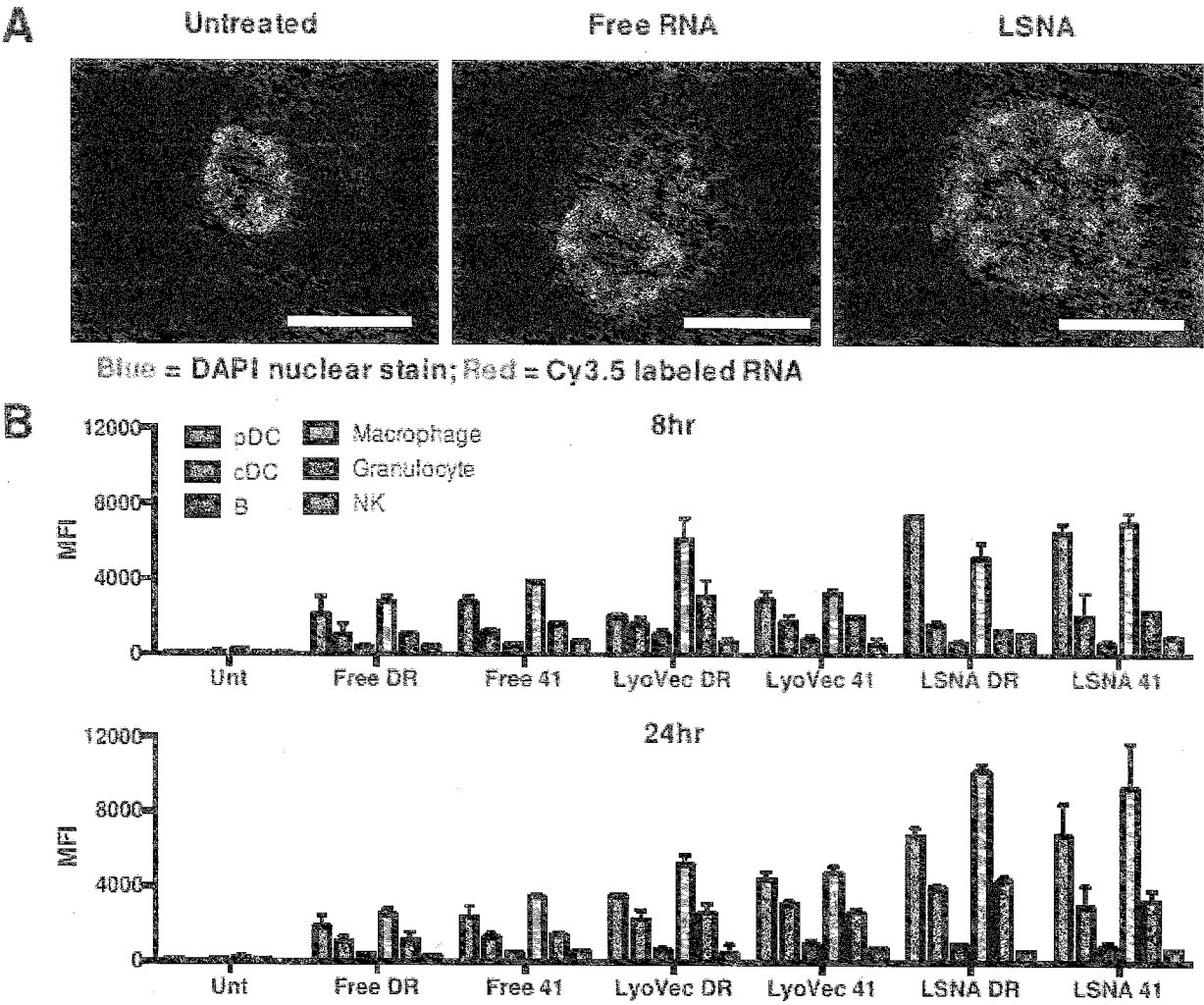


Figure 36

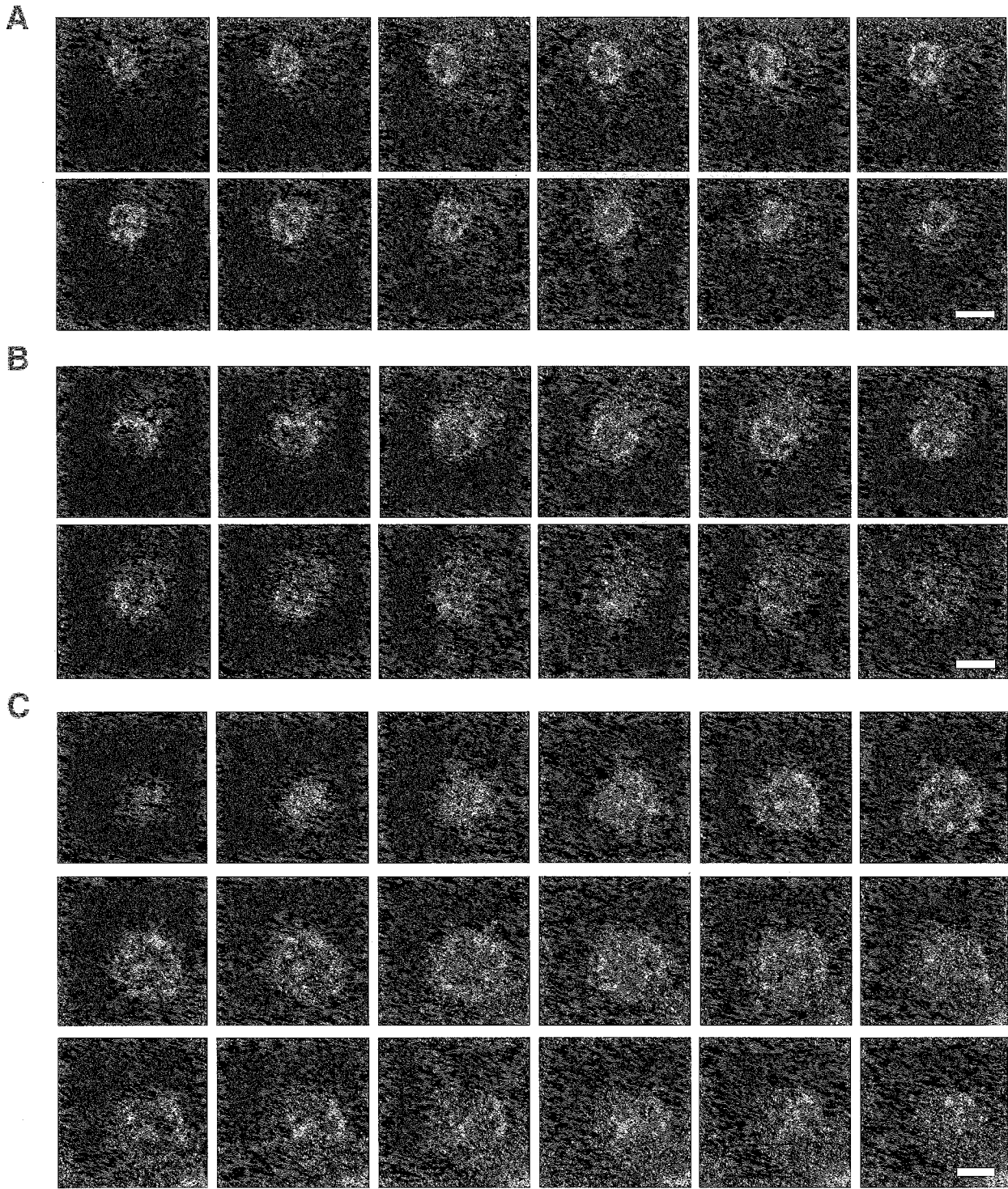
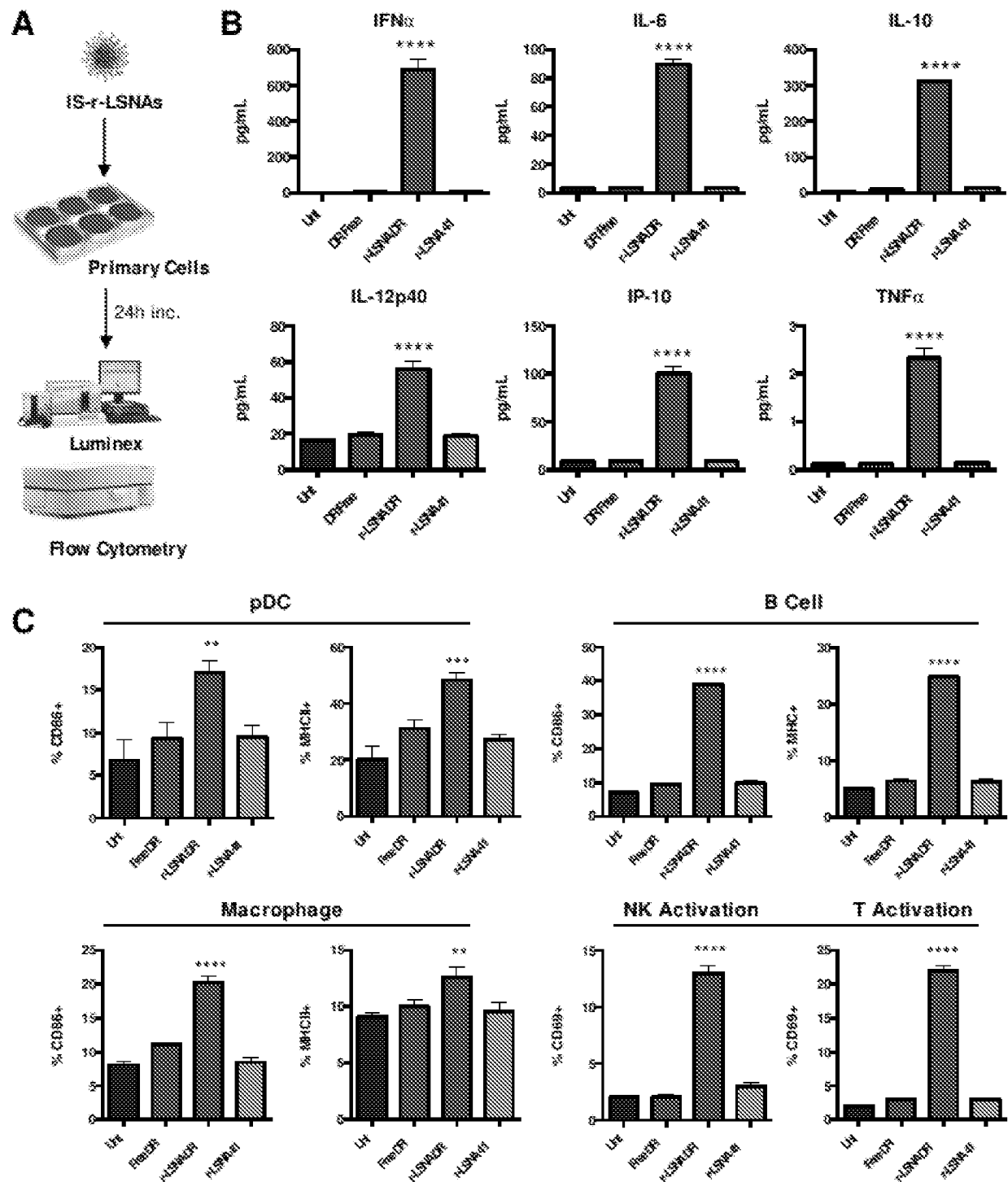


Figure 37

**Figure 38**

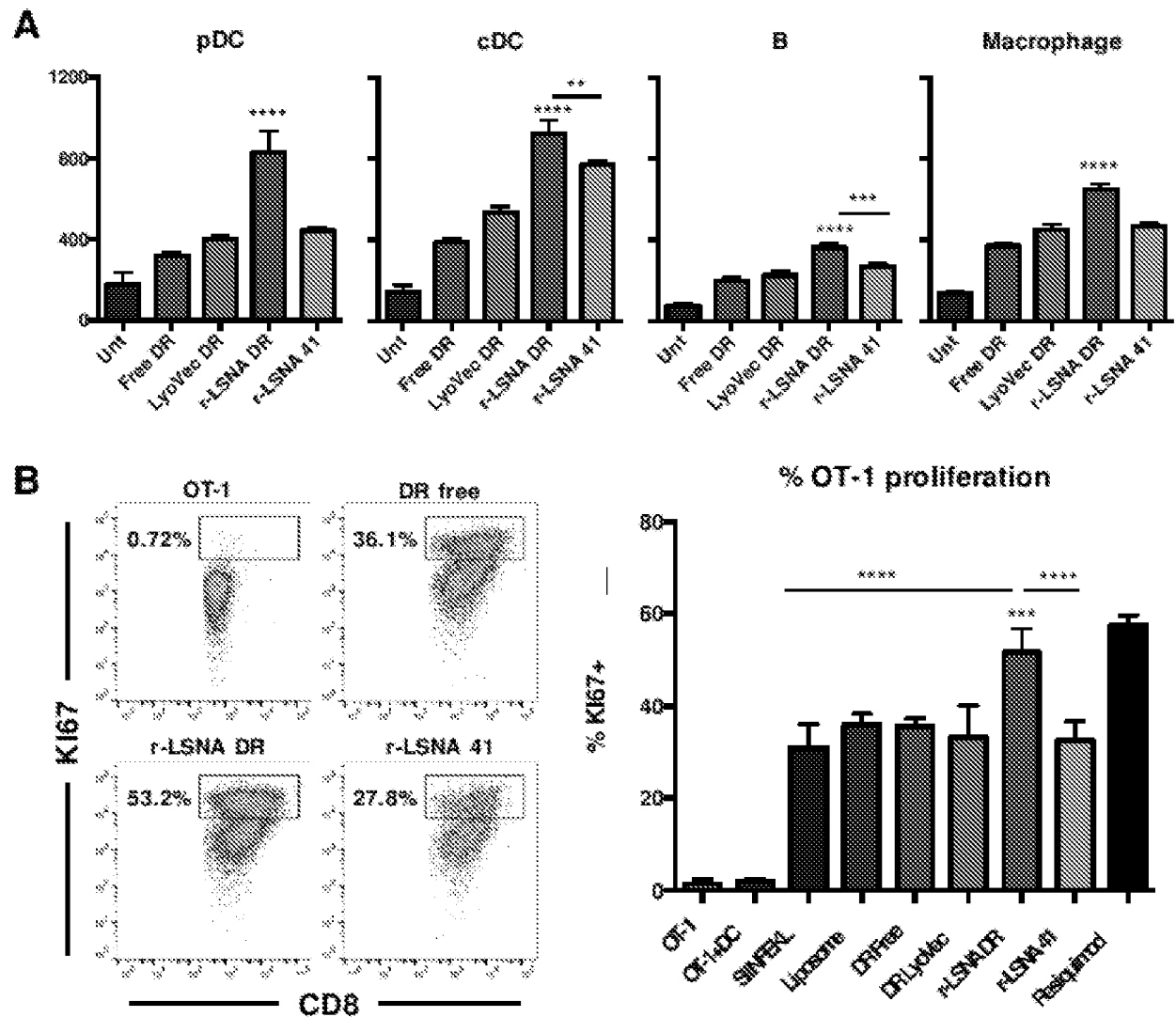
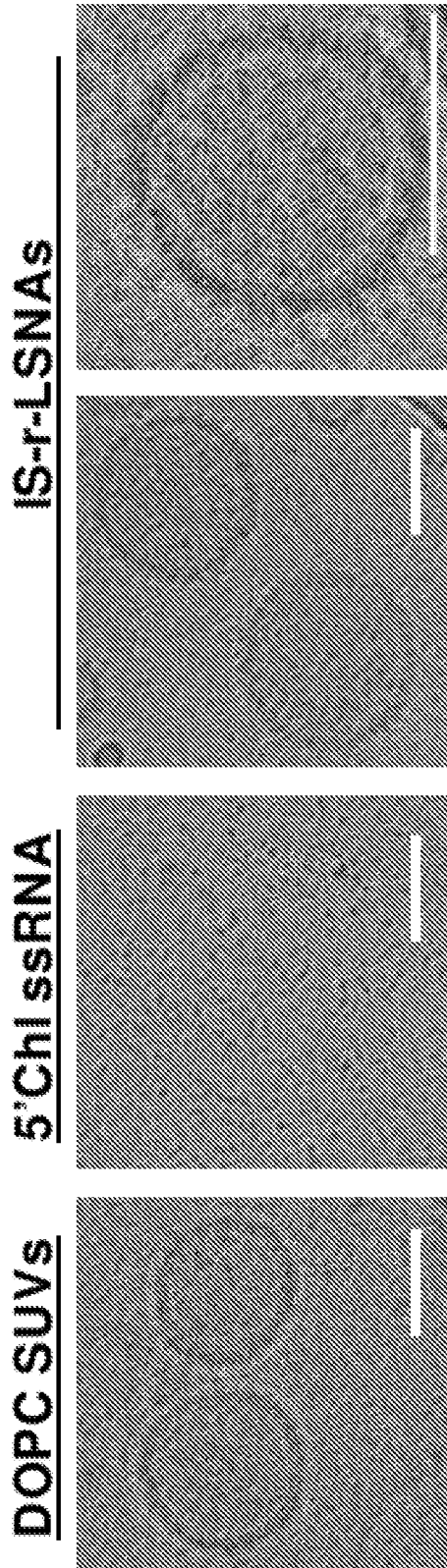


Figure 39

**Figure 40**

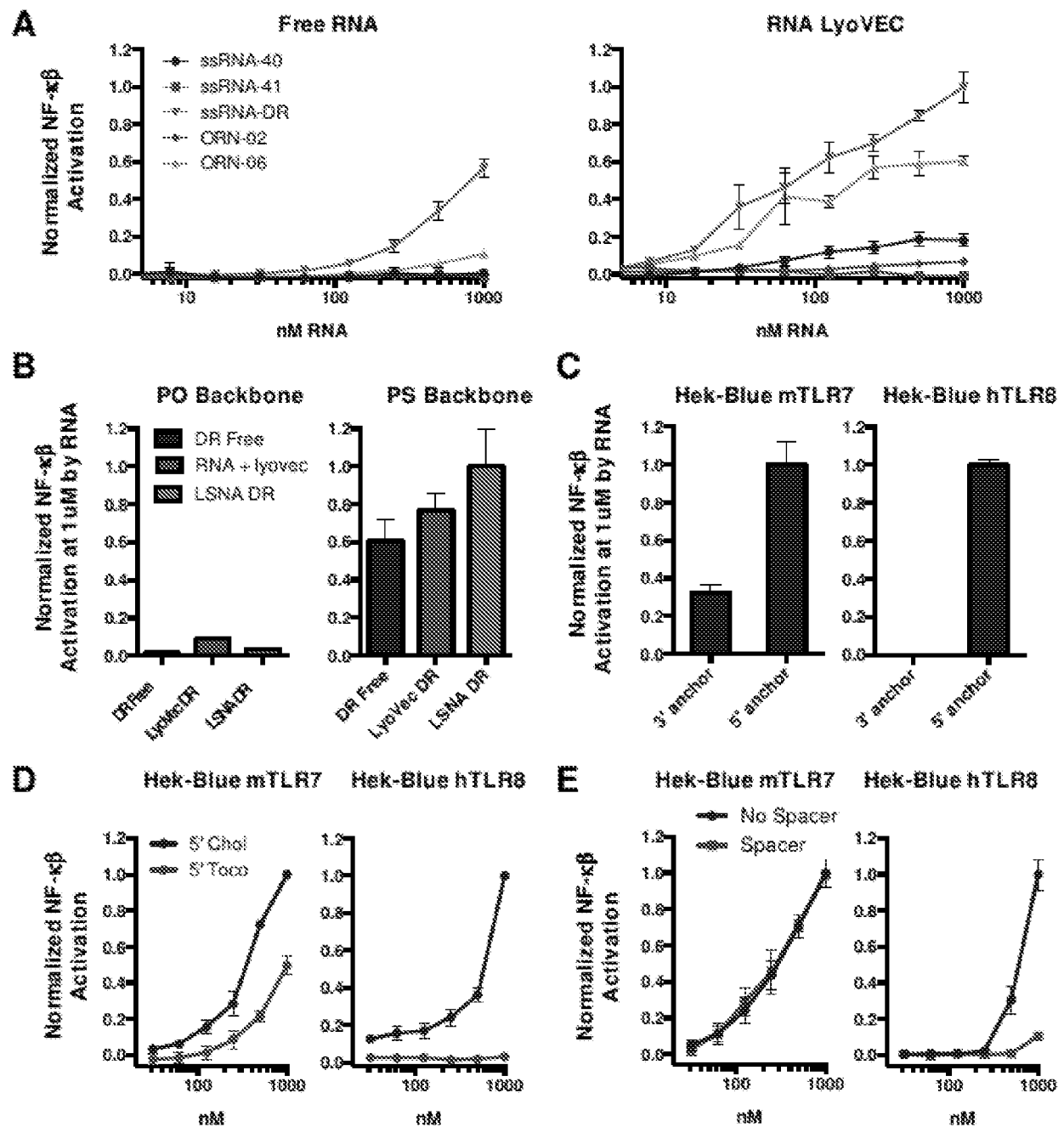
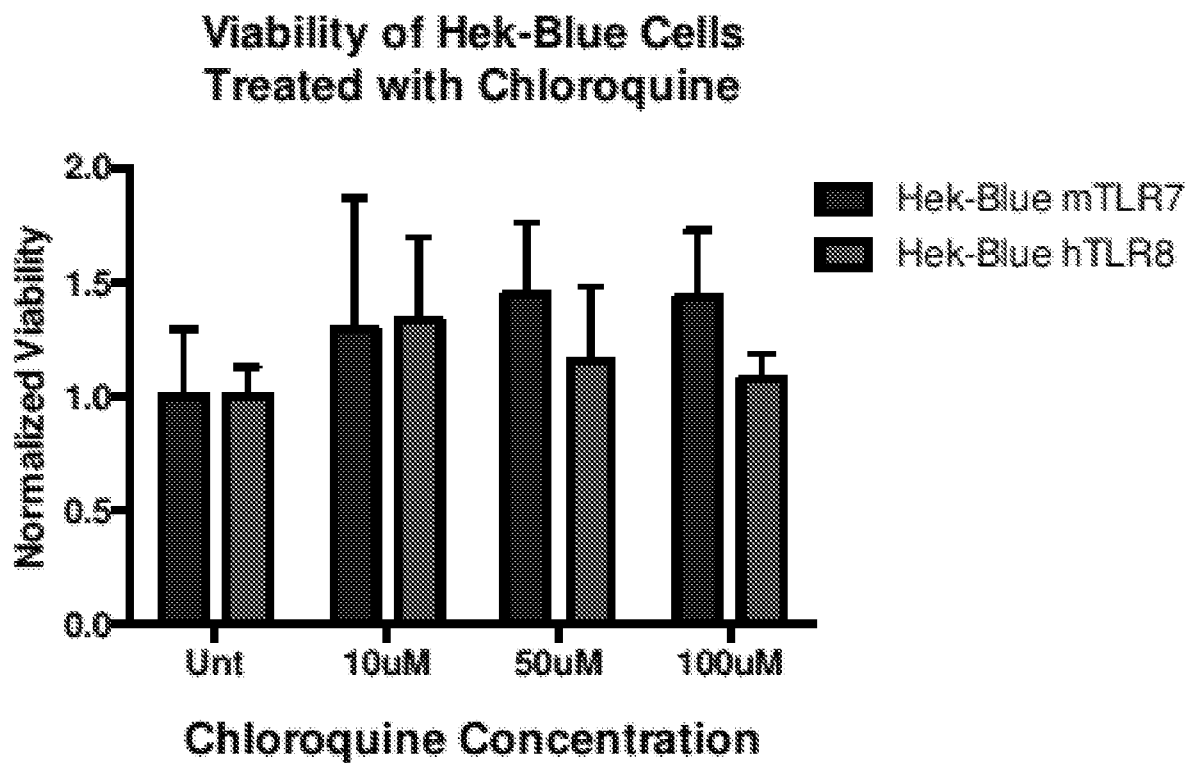


Figure 41



Figure 42

**Figure 43**

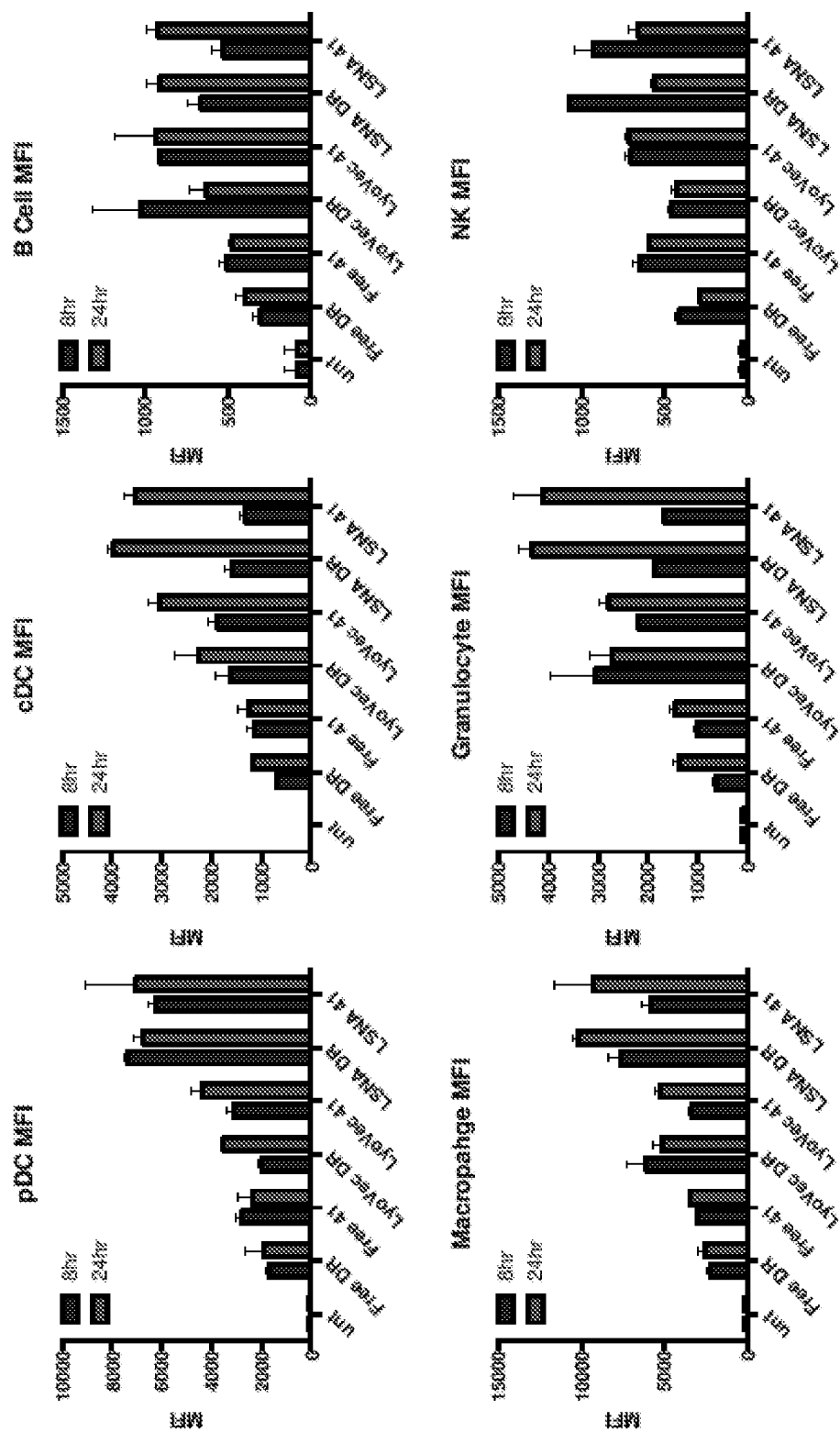


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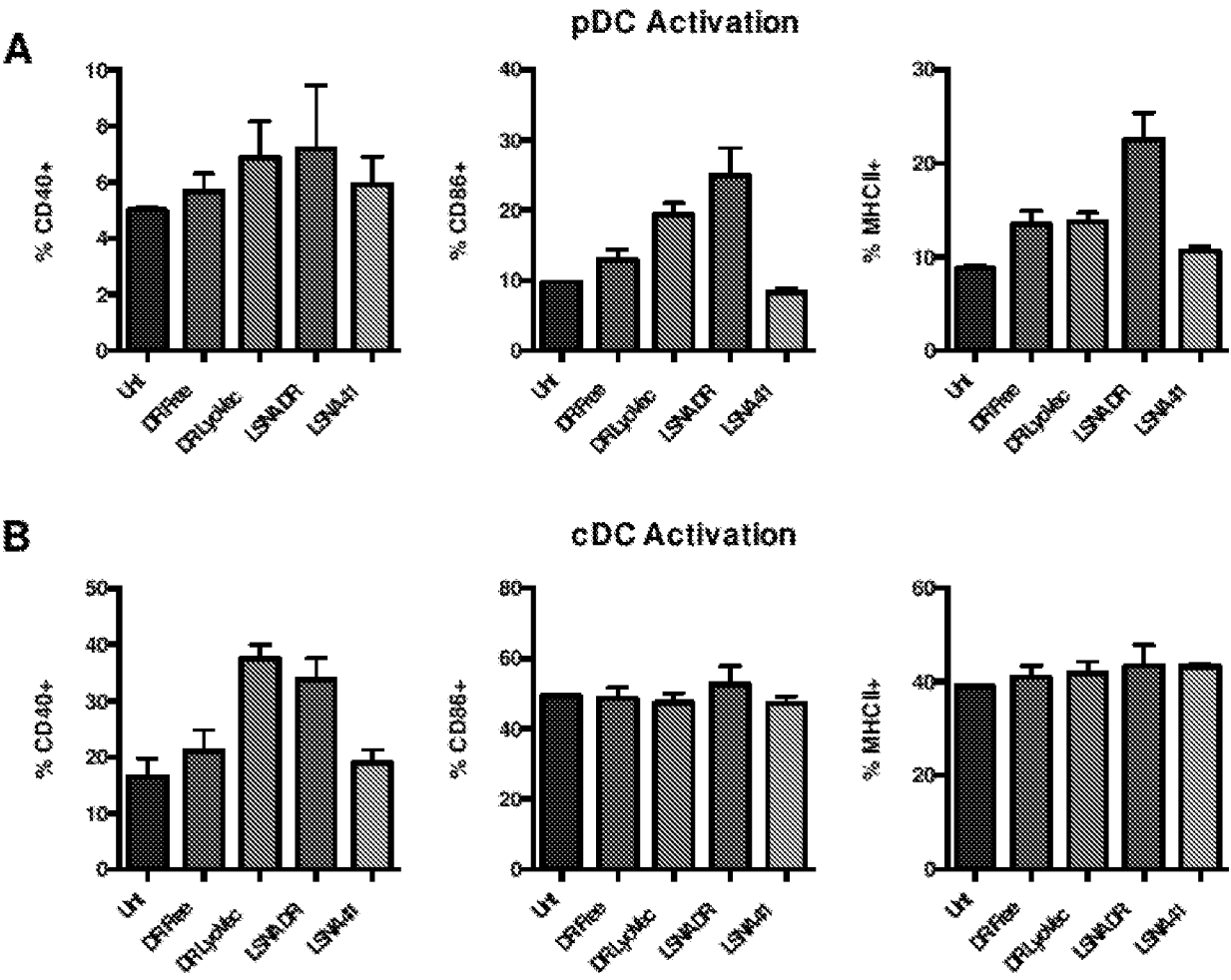


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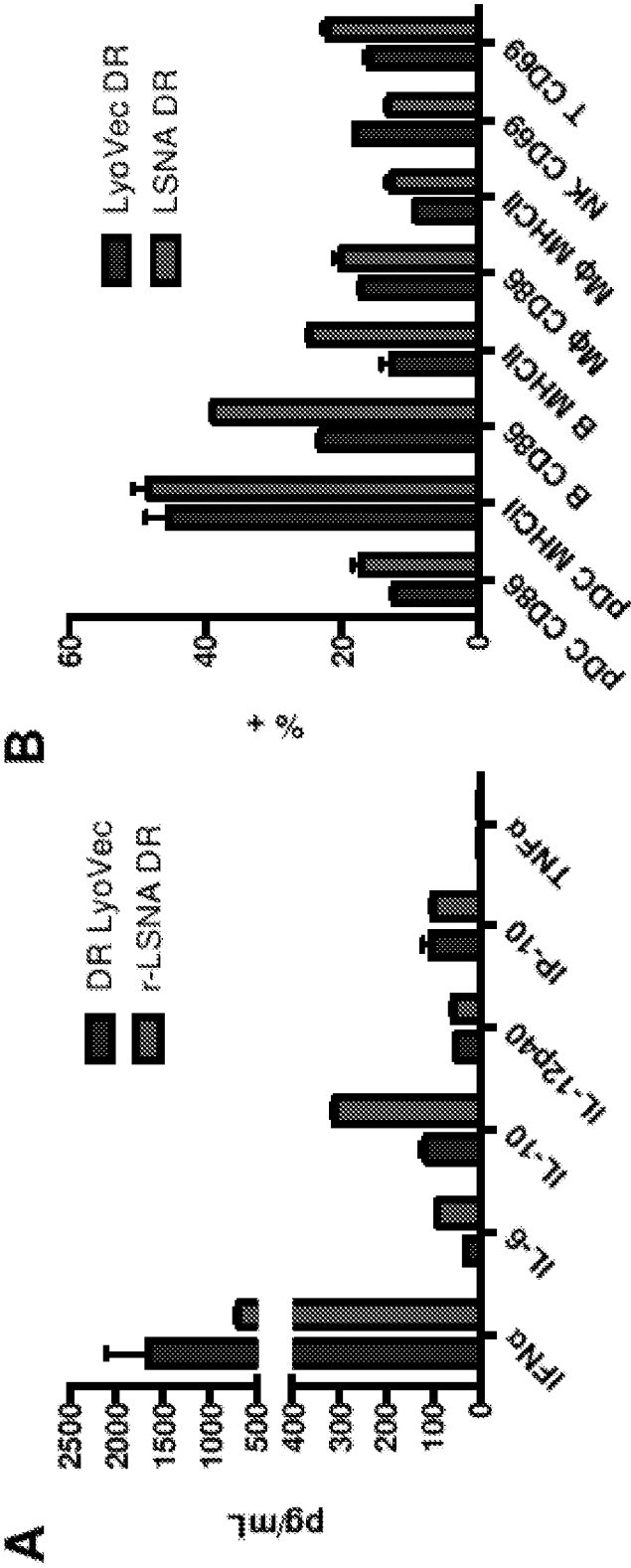


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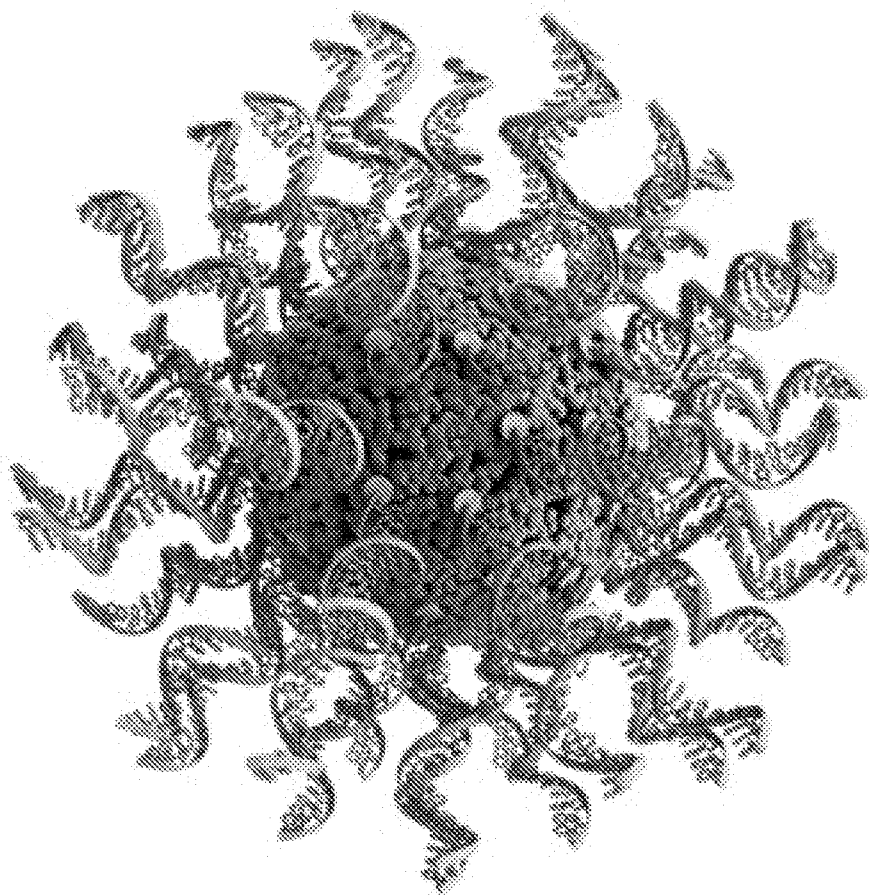
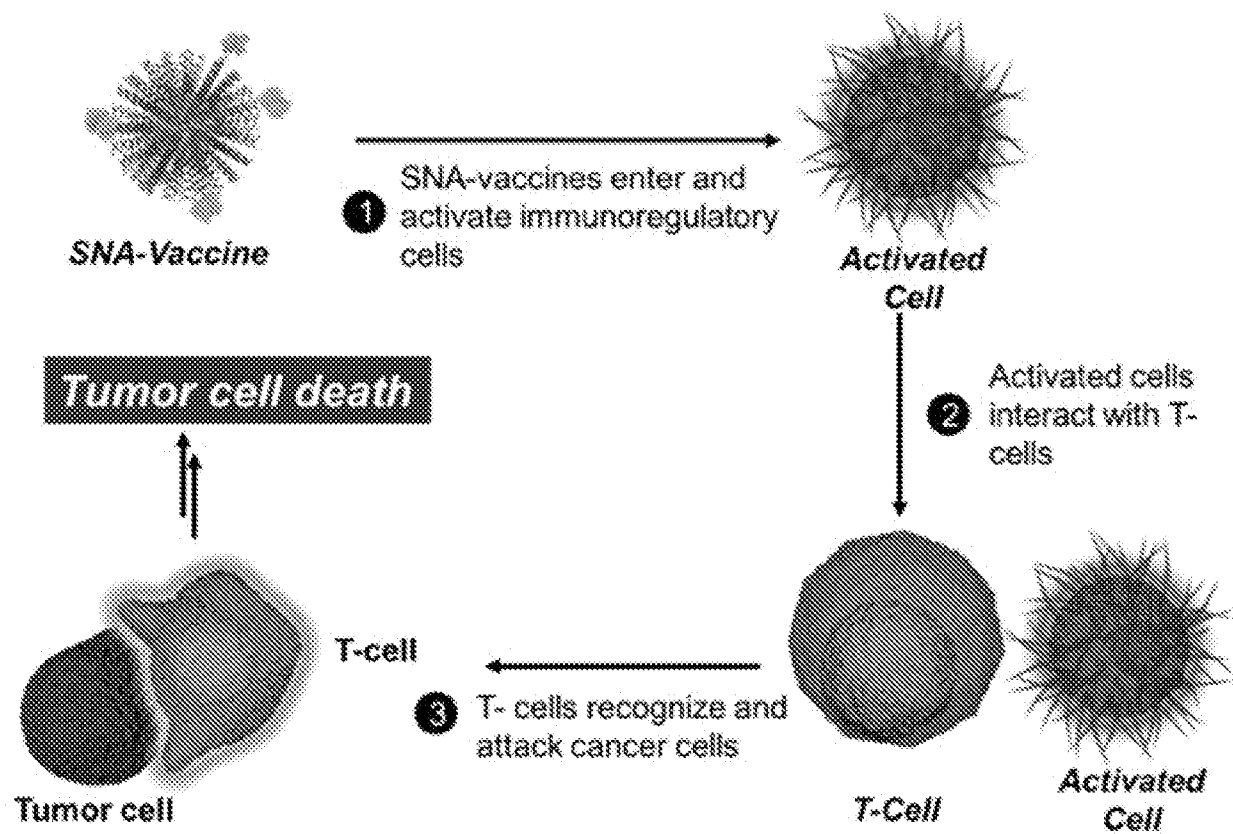


Figure 47

**Figure 48**

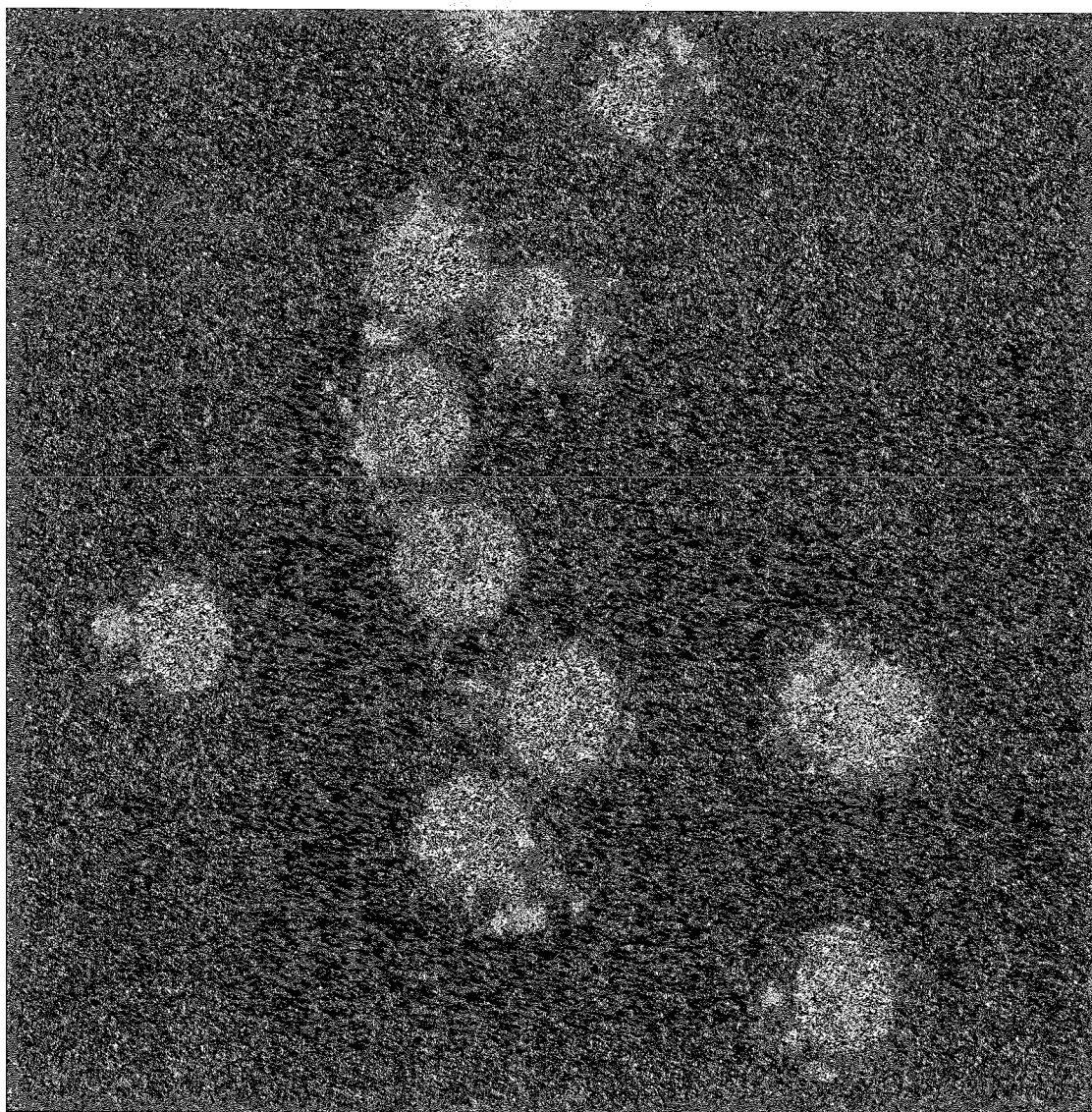
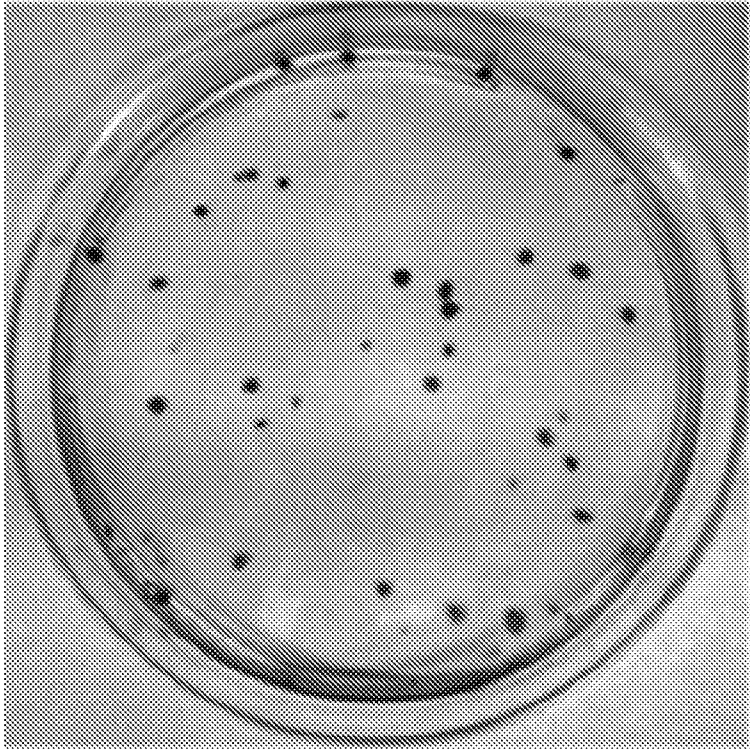
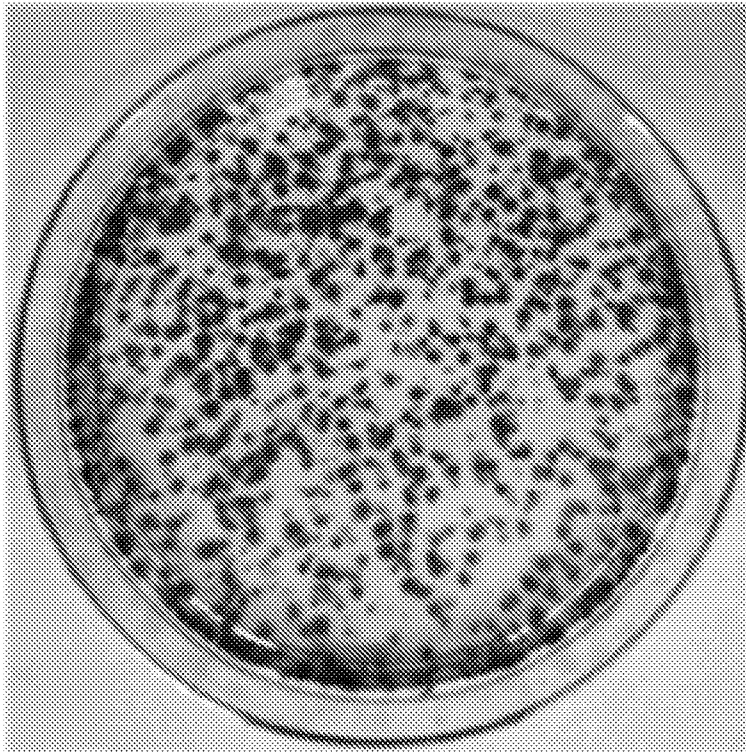


Figure 49



SNA Vaccine



Untreated

Figure 50

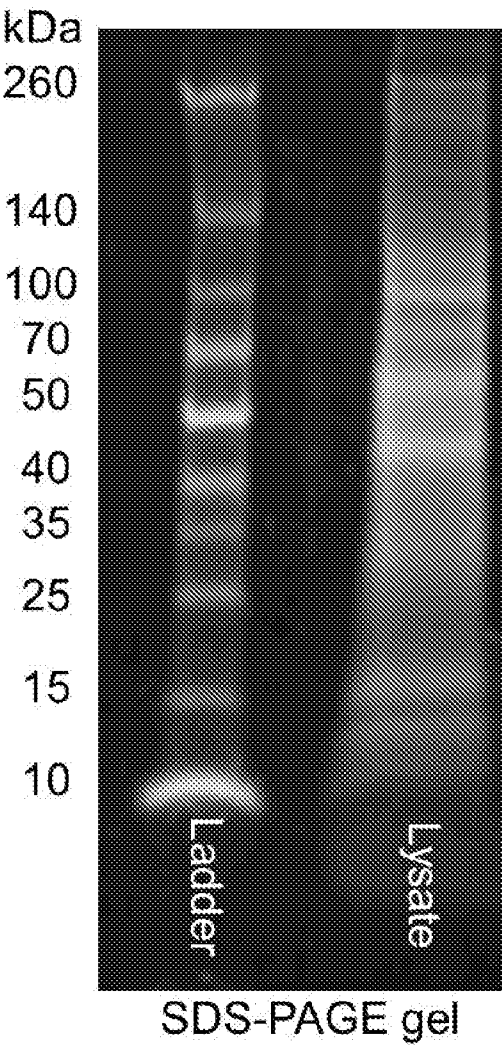
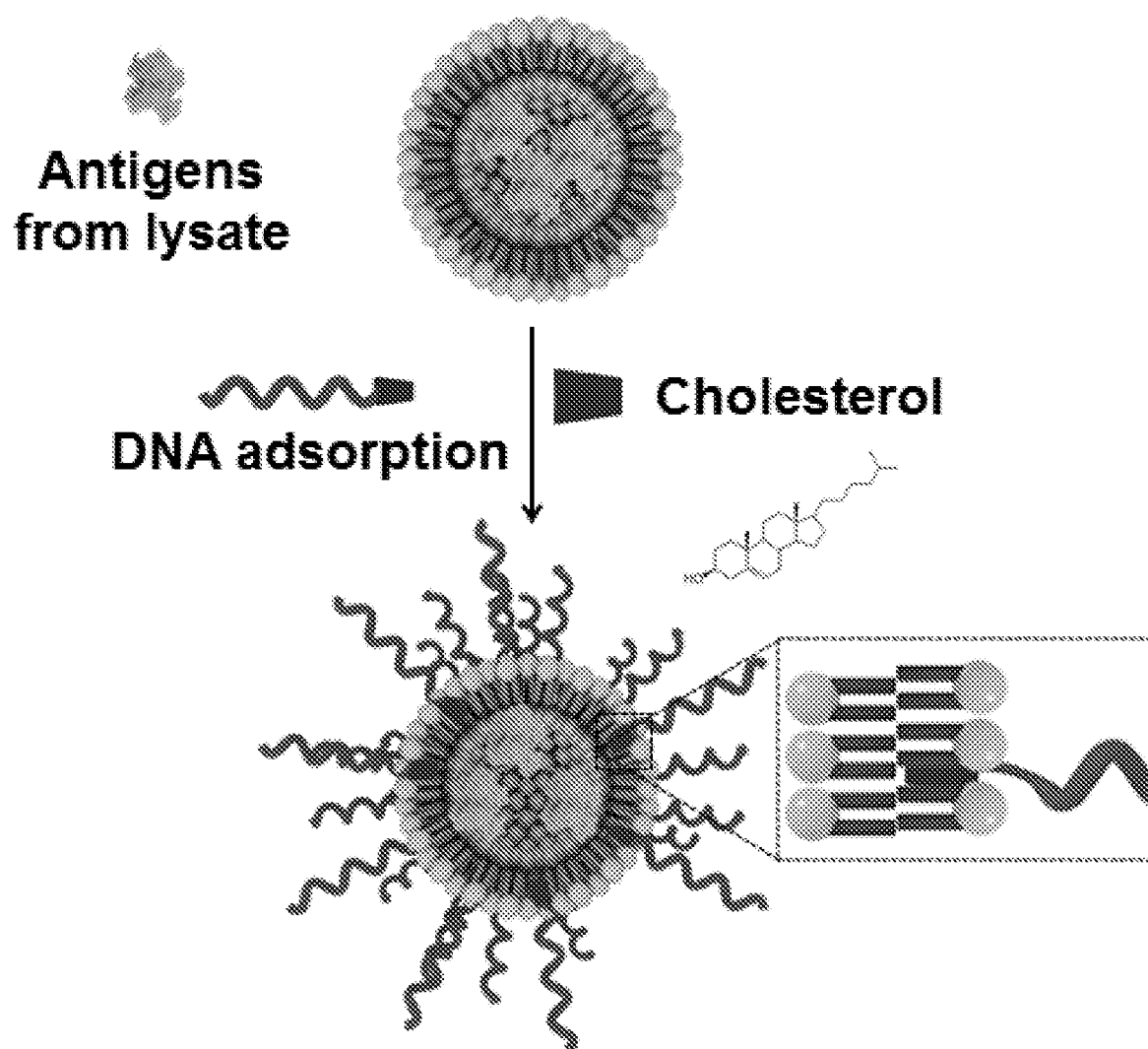


Figure 51

**Figure 52**

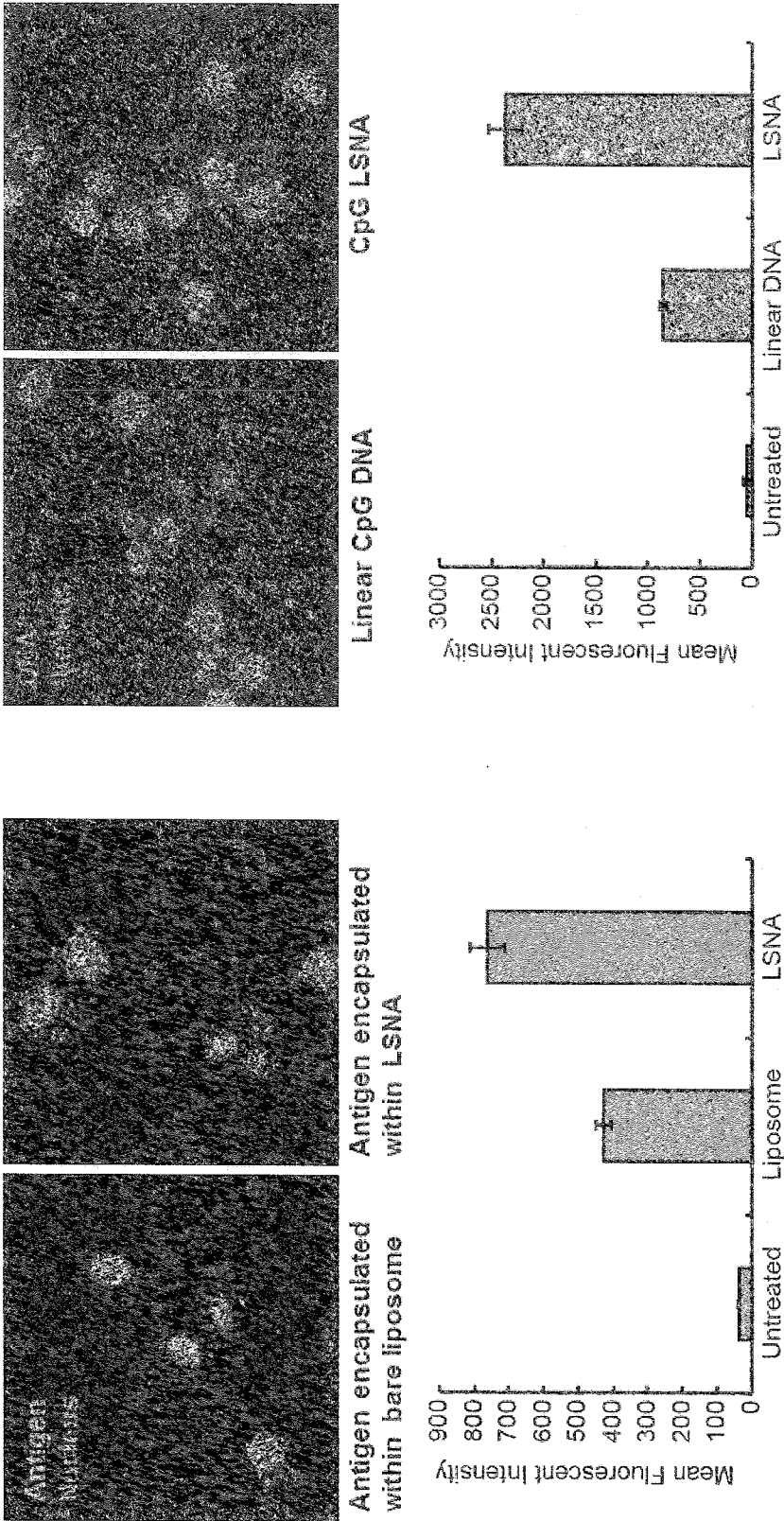


Figure 53

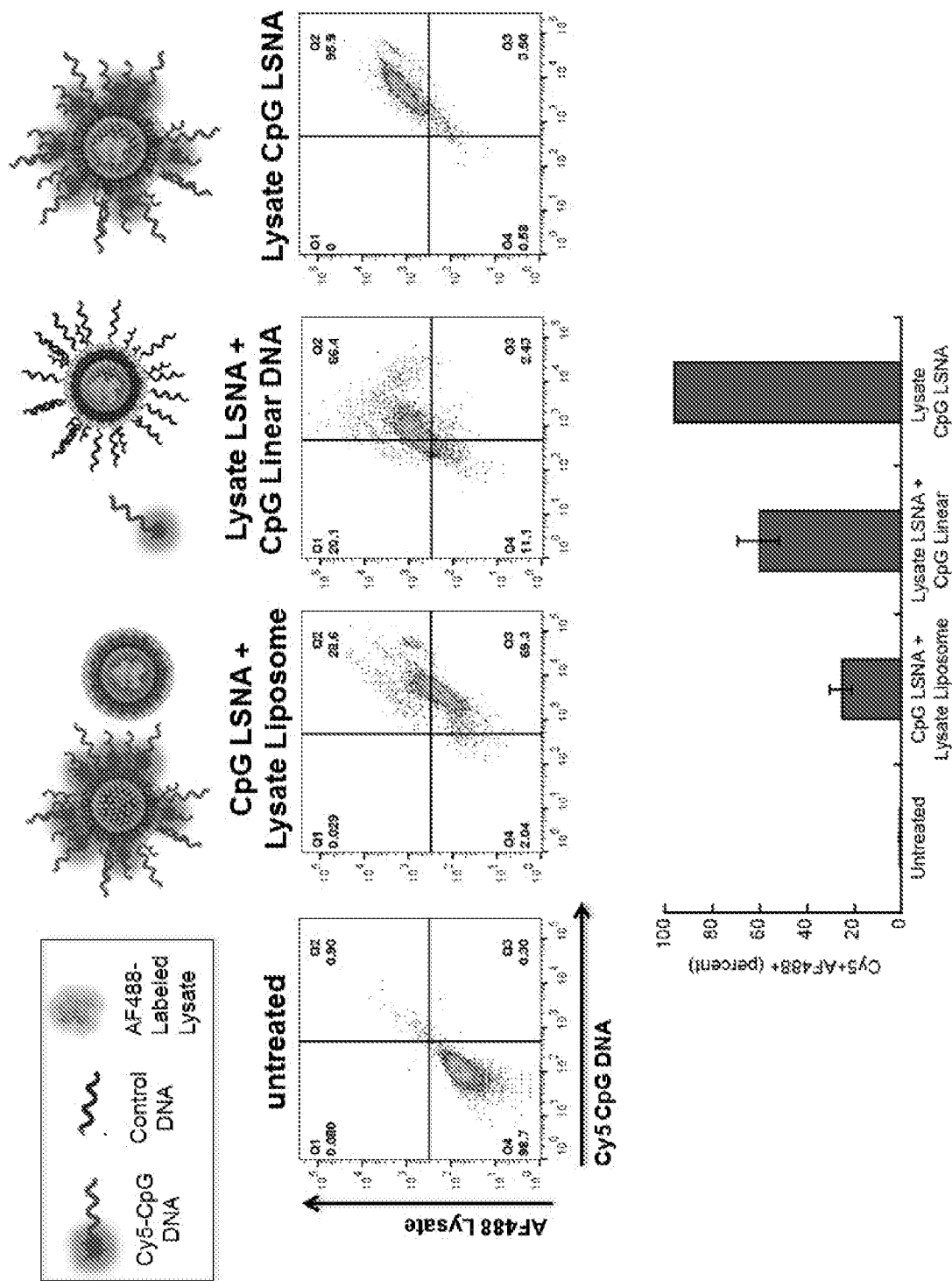


Figure 54

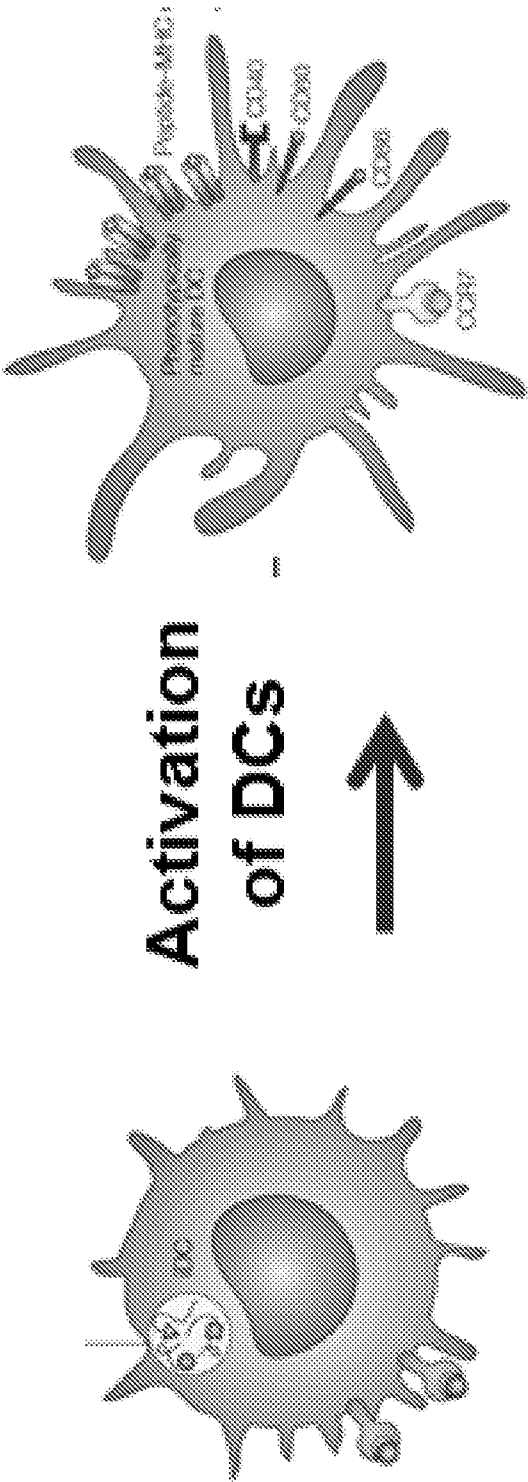
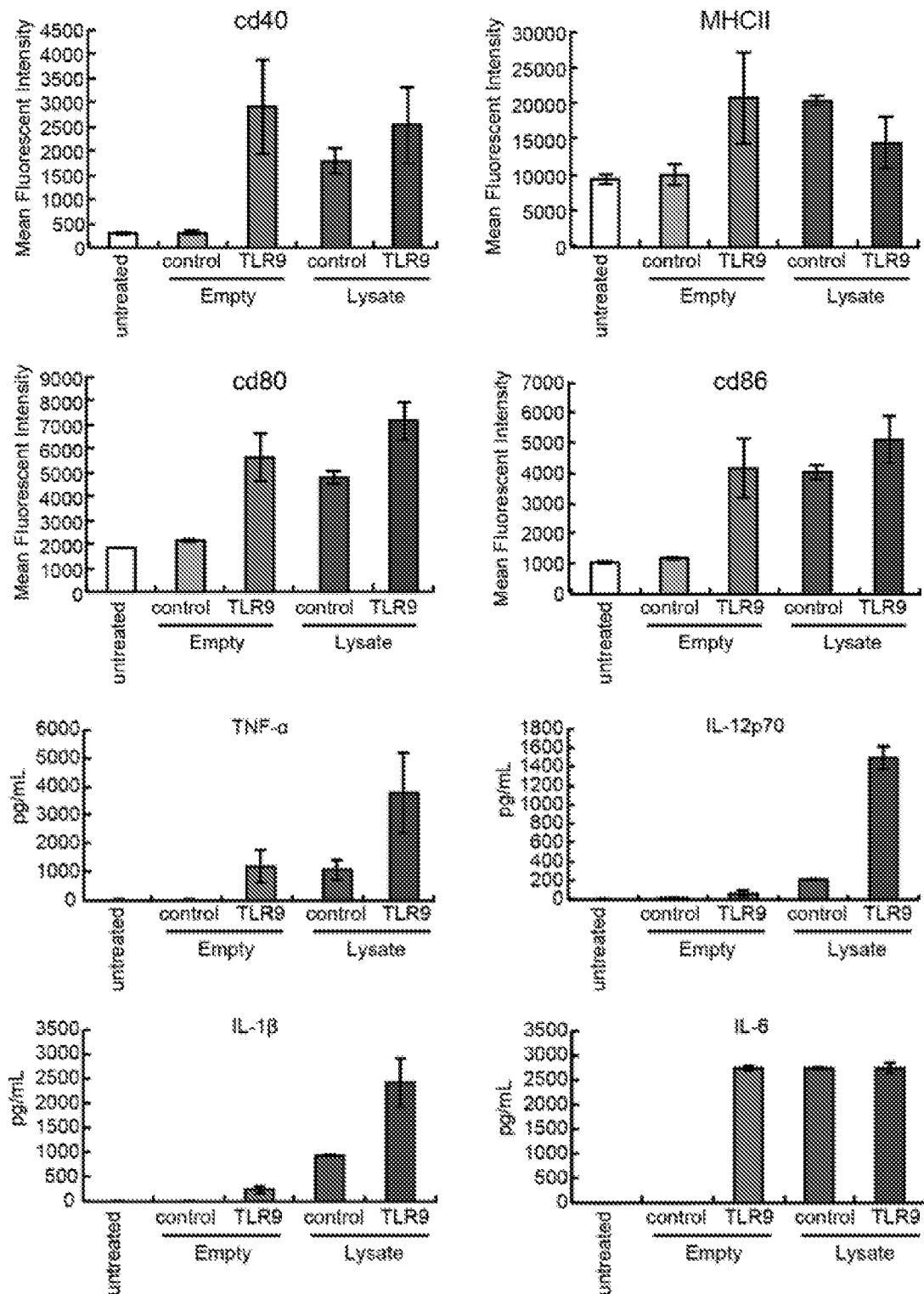
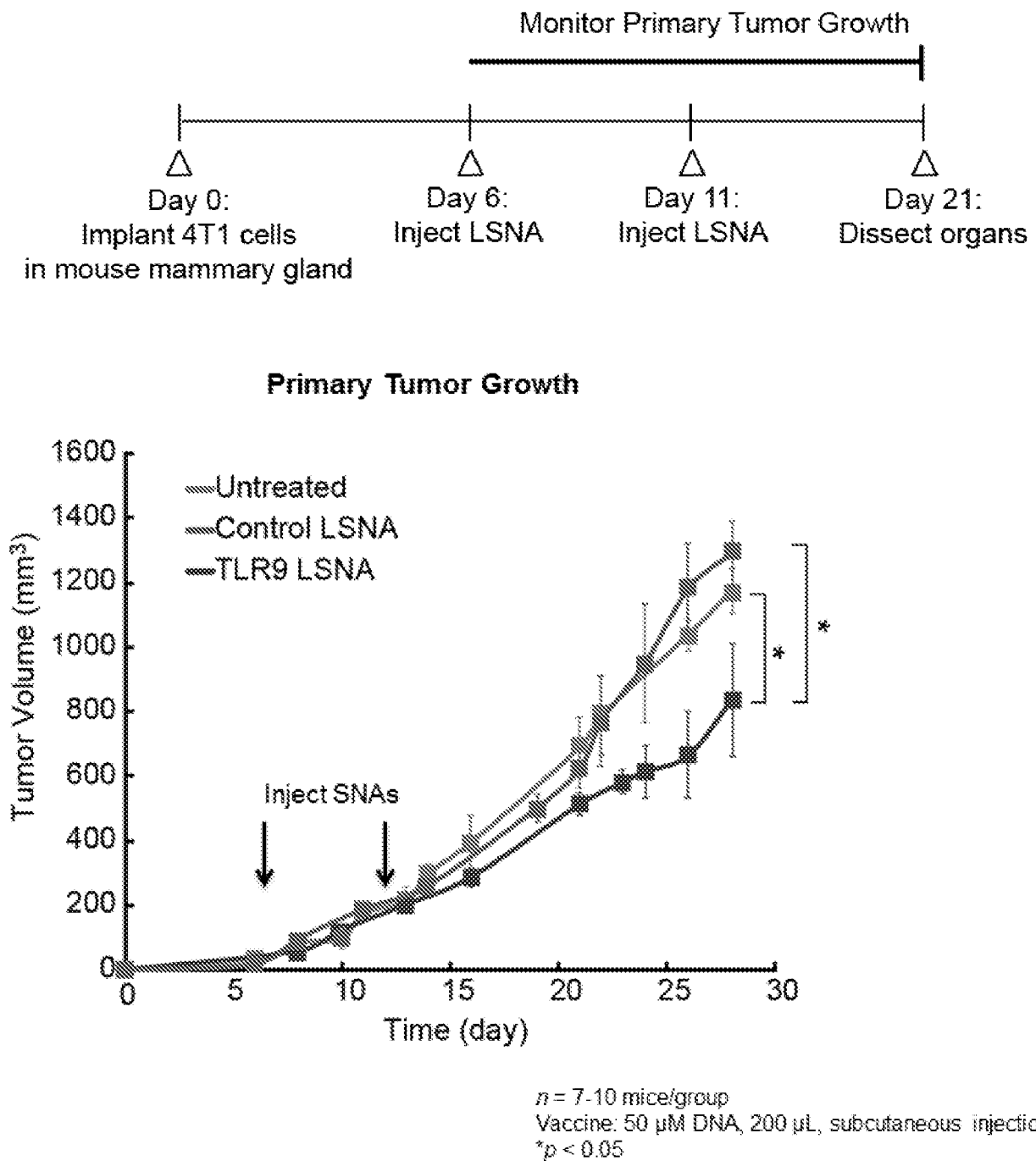


Figure 55

**Figure 56**

**Figure 57**

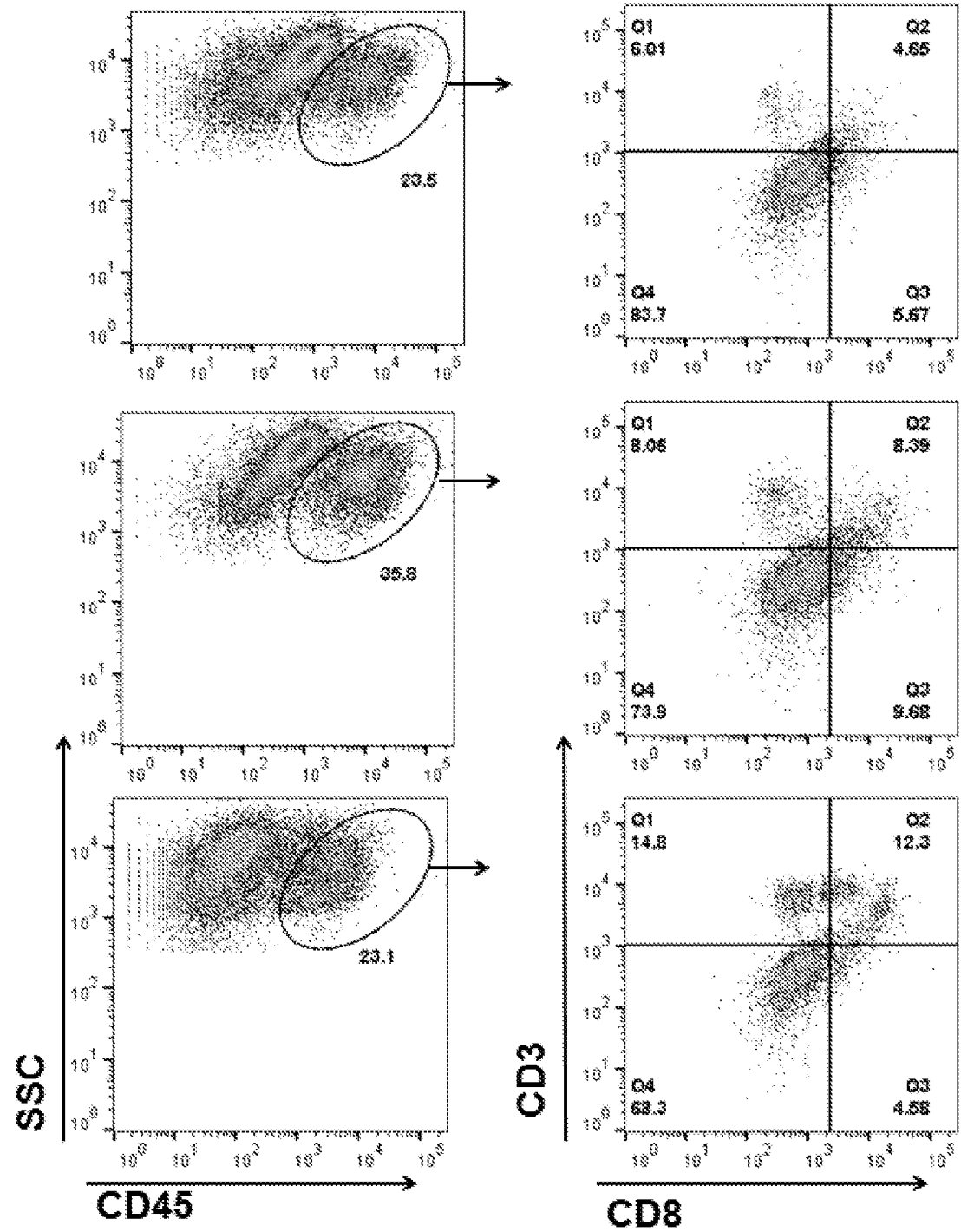
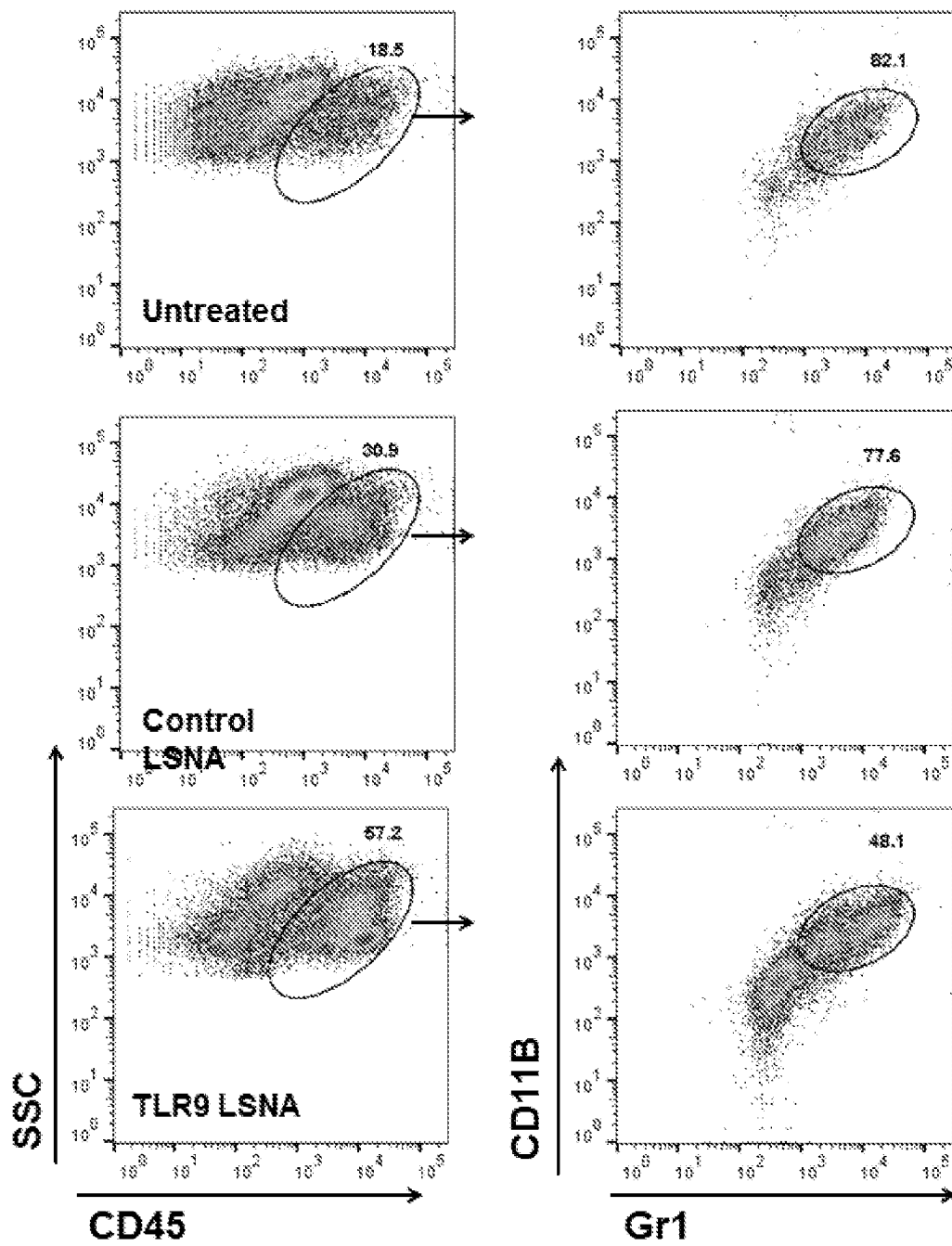


Figure 58

**Figure 59**

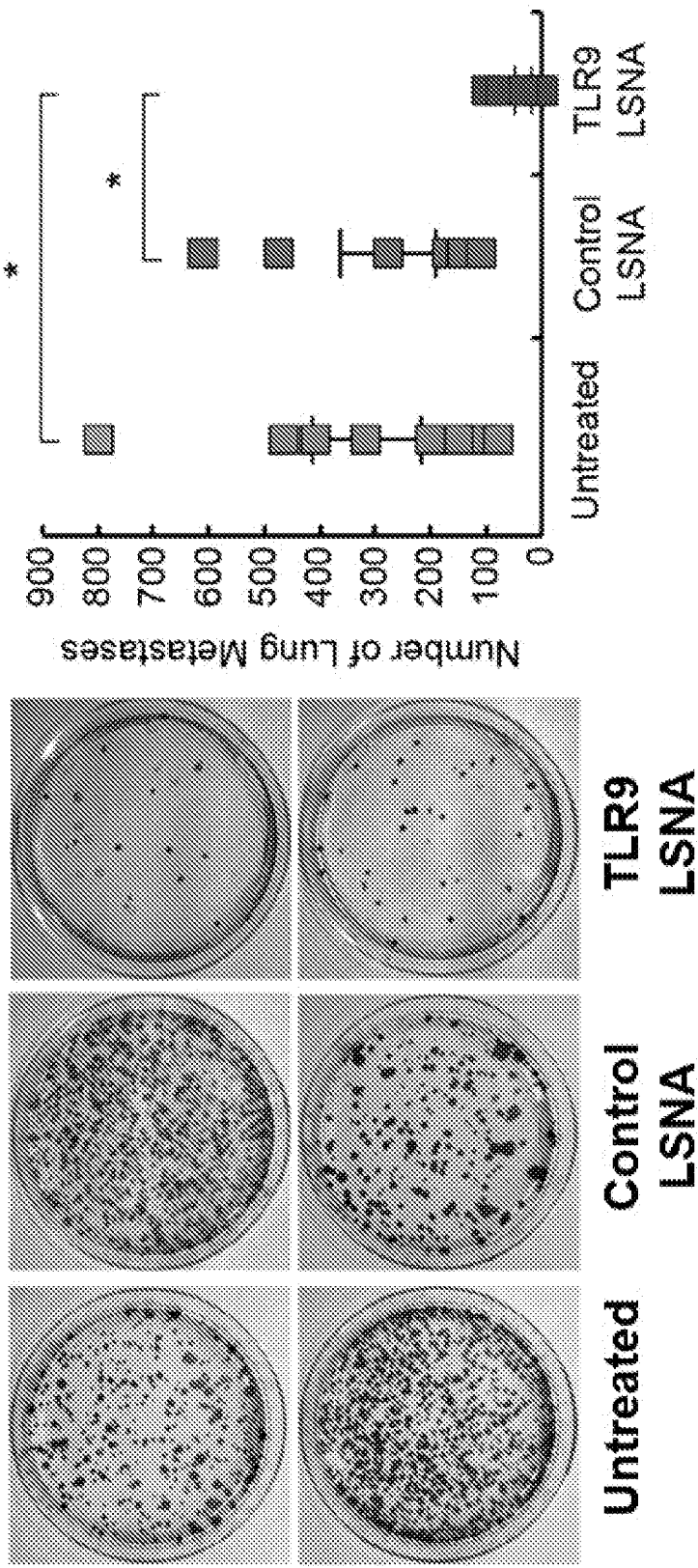
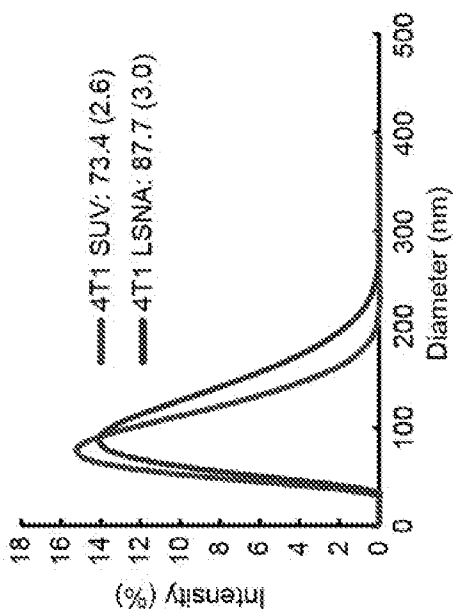
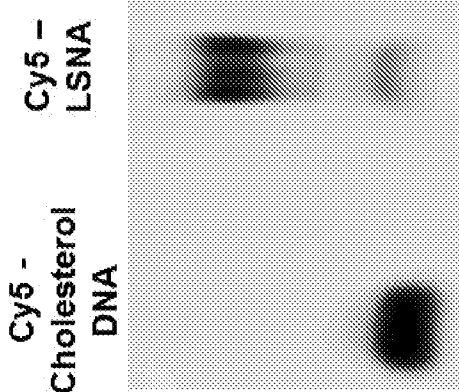
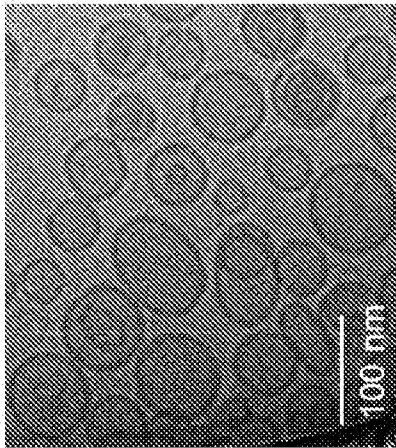


Figure 60



CryoEM of Protein Loaded Liposomes

Gel Electrophoresis and Dynamic Light Scattering Showing an Increased Diameter after DNA Functionalization

Figure 61

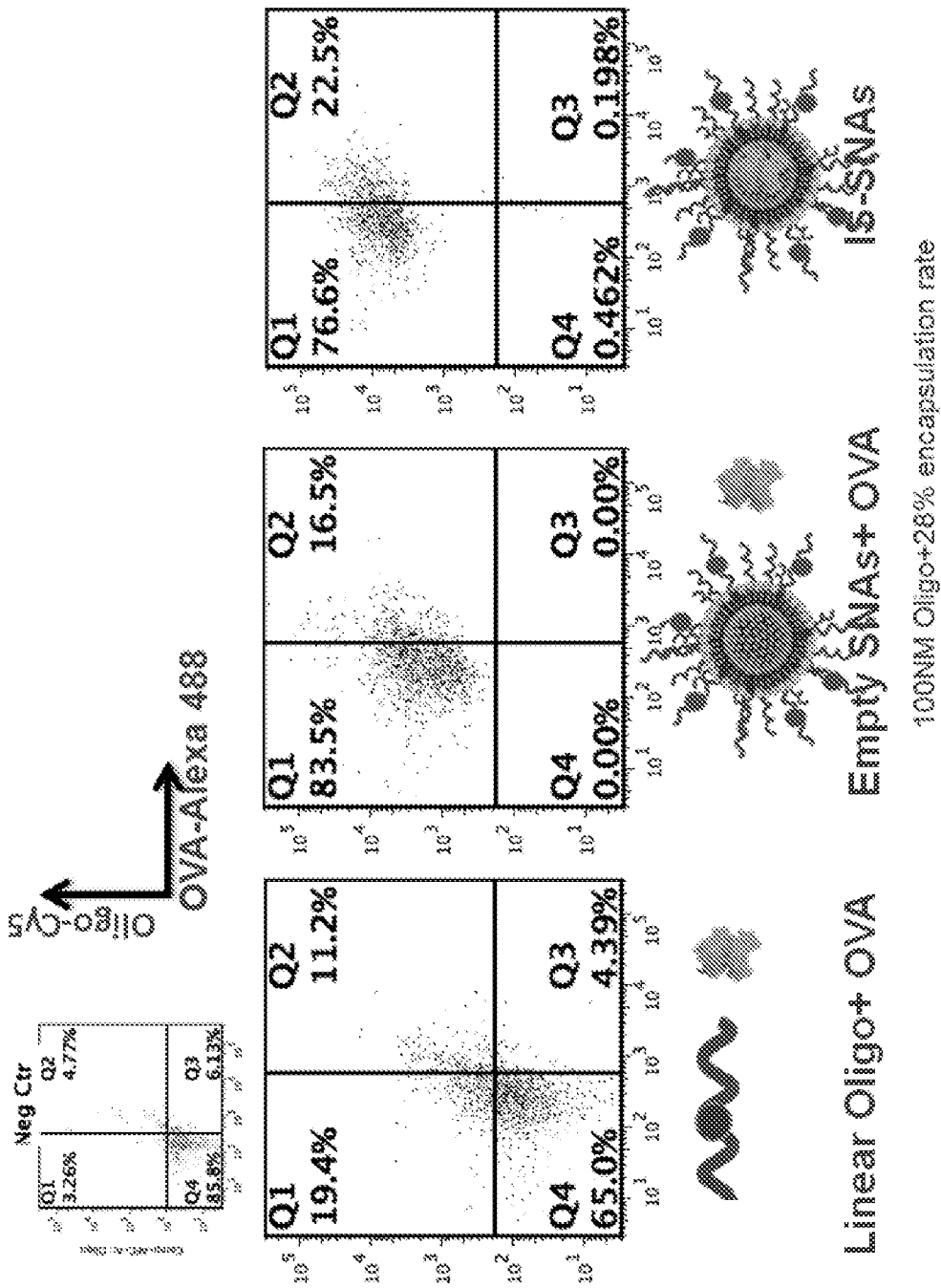


Figure 62