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Original Article

Design of lipid nanoparticles for *in vitro* and *in vivo* delivery of plasmid DNA

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Abstract

Lipid nanoparticles (LNPs) containing distearoylphosphatidlycholine (DSPC), and ionizable amino-lipids such as dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA) are potent siRNA delivery vehicles *in vivo*. Here we explore the utility of similar LNP systems as transfection reagents for plasmid DNA (pDNA). It is shown that replacement of DSPC by unsaturated PCs and DLin-MC3-DMA by the related lipid DLin-KC2-DMA resulted in highly potent transfection reagents for HeLa cells *in vitro*. Further, these formulations exhibited excellent transfection properties in a variety of mammalian cell lines and transfection efficiencies approaching 90% in primary cell cultures. These transfection levels were equal or greater than achieved by Lipofectamine, with much reduced toxicity. Finally, microinjection of LNP-eGFP into the limb bud of a chick embryo resulted in robust reporter-gene expression. It is concluded that LNP systems containing ionizable amino lipids can be highly effective, non-toxic pDNA delivery systems for gene expression both *in vitro* and *in vivo*.

Key words: Lipid nanoparticles; Plasmid; Gene delivery; Nanomedicine; Gene expression

Sophisticated delivery technologies are required to enable the therapeutic potential of macromolecules such as small interfering RNA (siRNA), mRNA or plasmid DNA (pDNA). These macromolecules are susceptible to breakdown in biological fluids, do not accumulate at target sites following systemic administration and cannot access intracellular sites of action even if they arrive at target cells.[1,2] Viral vectors are tedious to construct and manufacture, have limited carrying capacity[3] and are often immunogenic.[4–6] This has led to considerable efforts to develop non-viral carrier systems for delivery of nucleic acid based drugs. Lipid nanoparticle (LNP) systems containing optimized ionizable amino lipids have demonstrated considerable utility for delivery of siRNA for silencing genes in hepatocytes following intravenous (IV) administration.[7]

LNP delivery systems for pDNA must protect the pDNA from breakdown, facilitate uptake into target cells and encourage cytosolic release of encapsulated pDNA and then support pDNA entry into the nucleus.[1,8] The amino-lipids used in LNP formulations are optimized for endosomal uptake and cytosolic release but would not be expected to facilitate nuclear delivery.[9] As a result LNP-pDNA formulations would only be expected to be potent transfection reagents in dividing cells, where the nuclear membrane is temporarily compromised.[10] The inability of pDNA to cross the nuclear membrane has long been recognized as the major limitation of non-viral gene expression systems.[9,11–13] Here we focus on optimization of pDNA transfection in the presence of active cell division where reporter expression has been shown to be positively correlated with the number of cytosolic plasmids per cell.[10,14]

LNP-siRNA systems optimized to achieve maximum gene silencing potency in hepatocytes following IV administration in mice[15] contain DLin-MC3-DMA (MC3), DSPC, cholesterol and a polyethyleneglycol (PEG)-lipid at mole ratios of 50/10/38.5/1.5. The role of DSPC is poorly understood, whereas DLin-MC3-DMA

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exhibits an optimized pKa value that leads to dramatically enhanced potency.[7,15] Optimization of LNP lipid components requires maximizing the potential for inducing non-bilayer (H_{II}) phase to destablilize the endosomal membrane following uptake of the LNP into the cell.[16] Accordingly, we have examined the role of increased unsaturation in the PC component or substitution by unsaturated phosphatidylethanolamine (PE), which is expected to encourage non-bilayer structure.[17–19]

Here we show that LNP lipid mixtures that are optimized for siRNA cargos are not notably efficient for delivery and expression of pDNA. The potency of LNP-pDNAs *in vitro* can, however, be considerably improved by modifying the lipid composition and the amine-to-phosphate charge ratios. Substitution of unsaturated versions of PC for DSPC resulted in substantial improvements as did changes in the amino-lipid employed. It is shown that these systems have considerable *in vivo* activity in a chick embryo model. These results are discussed in terms of the requirements for different LNP components according to the cargo and ways in which further improvements in potency may be achieved.

Methods

Materials

The helper lipids 1,2-dioleoyl-*sn*-glycero-3-phosphorylethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (DSPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1,2-dilinoleoyl-*sn*-glycero-3-phosphorylcholine (DLinPC), and 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphorylcholine (DPoPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The amino-lipids heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA), and 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) were obtained from Biofine International (Vancouver, BC). Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO). (*R*)-2,3-bis(octadecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate (PEG-DMG; PEG-lipid), 1,2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA) and 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP) were synthesized as previously described.[20,21,22]

Preparation of plasmid-containing LNP

LNP-pDNA was prepared as previously described.[23] Amino-lipids, helper lipids, cholesterol and PEG-DMG were dissolved in ethanol at a molar ratio of 50/10/39/1. Purified pCI-FLuc (expressing Firefly Luciferase) or pCAX-eGFP (expressing eGFP,[24]) was dissolved in 25 mM sodium acetate pH 4 to 0.116 mg/ml. The solutions were mixed using a micromixer (Precision Nanosystems, Vancouver, BC, Canada). For large-scale formulations, mixing was performed through a T-junction mixer as previously described.[25] All formulations were produced at 0.029 mg DNA per μmol lipid (corresponding to N/P charge ratio of 6.0), unless otherwise stated.

Analysis of lipid particles containing DNA

Particle size, lipid concentration, pDNA entrapment, and total pDNA concentration were measured as previously described.[23,26] Zeta potential was measured as previously described.[27]

Cryo-transmission electron microscopy (Cryo-TEM) analysis of LNP-pDNA morphology

LNPs were imaged with a FEI Tecnai G20 TEM (FEI, Hillsboro, OR) using the method previously described.[28]

In vitro transfection studies of cultured cells

HeLa, HepG2 and Hep3B cells were cultured in DMEM with 10% FBS. PC12 and MCF7 cells were cultured in RPMI 1640 with 10% FBS. LNPs were diluted into medium at 0.75-6.0 μg/ml pDNA. The luciferase assay (Steady-Glo Luciferase kit, Promega, Madison, WI) was performed 24 h post-treatment. In the case of serum-free treatments, LNP-pDNA systems were diluted into medium alone. To study the role of ApoE, wild-type (C57BL/6) and ApoE$^{-/-}$ (B6.129P2-Apoe^{tm1Unc}/J) mouse sera were purchased from The Jackson Laboratory (Bar Harbor, ME). LNP-pDNA systems were diluted into medium containing 10% of each serum. Measured luminescence was normalized to protein content as measured by the Pierce BCA Protein Assay (Life Technologies, Carlsbad, CA).

In vitro LNP uptake measurement

Cellular uptake of LNP formulations was performed as previously described.[29] LNP-pDNAs labeled with DiI-C18 (0.2 mol% total lipid) were used. Plates were imaged using a Cellomics Arrayscan VTI HCS reader (Thermo Scientific, Pittsburg, PA).

Transfection of primary embryonic mesenchyme and analysis of reagent toxicity

White leghorn chicken embryos at stage 24[30] were removed and dissected in Hanks buffered saline solution (HBSS). Forelimbs were pooled and treated for 1 h in dispase to remove epithelial ectoderm. Limb mesenchyme was dissociated, and resuspended in DMEM with 5% FBS and 1% penicillin/streptomycin. Cells were plated onto 96-well plates at a density of 2×10^5 or 2×10^7 cells/ml and treated with 0-40 μg/ml LNP-pDNA. Lipofectamine was used as per manufacturer's protocol. Cultures were incubated overnight at 37 °C and resuspended in PBS buffer for expression analysis using a BD LSRII flow cytometer. Cell survival was assessed in low-density cultures after 24 h of treatment, by counting the adherent cells remaining after PBS washes. Viability was determined based on average counts of all adherent cells within a single field of view (100×) normalized to cell counts of untreated cultures.

In vivo transfection by injection of LNP-pDNA

Stage ~19-20 white leghorn chicken embryos were stained with neutral red dye, and a small tear was made in the extraembryonic membranes over the forelimb. LNP formulations mixed with ~0.1% Fast Green dye was injected at the distal forelimb at 10 μg/ml pDNA, using a Picospritzer II microinjector (Parker Hannifin Corp). Eggs were incubated overnight. Embryos were rinsed and fixed in 4% paraformaldehyde in PBS, and GFP fluorescence was recorded with a Leica MZFLIII stereofluorescence microscope.

GFP immunofluorescence

Paraffin sections were dewaxed by three 5-min washes in xylene, followed by rehydration in a series of ethanol washes (100% to 50%). Antigen retrieval was achieved by steaming sections with 10 mM sodium citrate (pH 6.0) for 15-20 min. Sections were blocked in 2% BSA/PBS and incubated overnight at 4 °C in anti-GFP primary antibody (Synaptic Systems). The slides were rinsed with PBS, and secondary antibody (Alexa Fluor 488 goat-anti-rabbit) was applied for 1 h at room temperature. Immunostained slides were mounted with Prolong Gold (Invitrogen) containing DAPI nuclear counterstain, and photographed using a Zeiss wide-field fluorescence microscope.

Statistical analysis

Statistical analyses were performed for all quantitative data using GraphPad. Where applicable, two-way ANOVAs were performed using the Bonferroni multiple comparison test and confidence level of 0.001. Similarly, where applicable one-way ANOVAs were performed using the Tukey multiple comparison test and confidence level of 0.001. Asterisks indicate $P < 0.0001$.

Results

Replacement of saturated PC by unsaturated PC and PE in LNP formulations of pDNA enhances transfection properties

Previous work has shown that pDNA can be efficiently encapsulated into LNP systems containing amino-lipids, cholesterol, DSPC and a PEG-lipid using a microfluidic mixing formulation technique.[23] Studies of LNP formulations of siRNA have established that lipid compositions consisting of DLin-MC3-DMA/DSPC/cholesterol/PEG-lipid (50/10/38.5/1.5; mol/mol) provide optimal hepatocyte gene silencing *in vivo* following IV administration.[15]

We first investigated whether DSPC was the most appropriate “helper” lipid in pDNA formulations. In the case of LNP-siRNA systems replacement of DSPC by other lipid species detracts from gene silencing potencies.[31] However a guiding principle for design of LNP lipid components concerns their ability to induce non-bilayer (H_{II}) lipid structures, which play a role in the membrane fusion events leading to intracellular delivery of macromolecules such as siRNA.[7,15,16] It is well established that unsaturated phospholipid species, such as DOPE, are more compatible with H_{II} structure.[32–34] We therefore tested the effect of substituting unsaturated versions of PC and DOPE (Figure 1, *A*) on the transfection efficiencies of LNP-pDNA systems.

As shown in Figure 1, *B*, replacement of DSPC by DOPC and SOPC in LNP-pDNA systems resulted in transfection potencies that were approximately 50-fold greater than could be achieved for DSPC-containing LNP. More unsaturated versions of PC (DLinPC) did not further improve transfection potencies and substitution of DSPC by DOPE resulted in only a modest (~3-fold) increase in transfection potency. The importance of the presence of PC or PE is indicated by the fact that little or no transfection was observed for formulations where such “helper” lipids were absent.

LNP-pDNA transfection potencies can be increased by substitution of DLin-KC2-DMA for DLin-MC3-DMA

It is well established that the species of amino-lipid can strongly affect the gene silencing potencies of LNP.[7,15] Here we investigated four amino-lipids that span the range of activities observed for siRNA. The relative potencies of these lipids follow the series DLin-MC3-DMA >> DLin-KC2-DMA >> DLinDMA >> DLinDAP (structures in Figure 2, *A*). In order to determine whether the same potency profile is observed for LNP-pDNA systems *in vitro*, formulations containing these four lipids were generated. LNPs containing DLin-KC2-DMA were significantly more effective transfection systems (Figure 2, *B*); when HeLa cells were incubated with LNP-pDNA at concentrations of 0.75 and 1.5 μg/ml pDNA, DLin-KC2-DMA-based LNP showed a 5-fold and 12-fold improvement in luciferase expression over LNP containing DLin-MC3-DMA, respectively. At 3.0 μg/ml pDNA, this improvement was approximately 7-fold (Figure 2, *B*). Differences in uptake of LNPs containing the various amino-lipids did not account for the difference in expression profile (Figure 2, *C*). In particular, uptake profiles correspond to DLinDMA > DLin-KC2-DMA > DLin-MC3-DMA > DLinDAP, while transfection potency is in the order of DLin-KC2-DMA >> DLin-MC3-DMA > DLinDMA > DLinDAP.

We next determined whether substituting an unsaturated PC for DSPC in formulations containing DLin-KC2-DMA provided further improvements in transfection potency. At 0.75 μg/ml pDNA, LNP containing DOPC and DLin-KC2-DMA exhibited a 3-fold enhancement in expression compared to LNPs containing DSPC and DLin-KC2-DMA, while LNP containing SOPC achieved a 4-fold improvement (Figure 2, *D*). The combined benefits of substituting an unsaturated PC for DSPC and DLin-KC2-DMA for DLin-MC3-DMA are substantial at lower pDNA concentrations (0.75 μg pDNA/ml); LNP containing SOPC and DLin-KC2-DMA exhibited transfection potencies that were more than 50-fold higher than LNPs containing DSPC and DLin-MC3-DMA.

Previous work has shown that the amount of amino-lipid in LNP affects the pDNA packing and sequestration from the external medium[35] and for siRNA, levels of amino-lipid below 40 mol% or greater than 60 mol% result in poor gene silencing properties.[7,15,36] Further the LNP potency is dependent on the amino-lipid to phosphate or nitrogen-to-phosphorus (N/P) ratio.[26,37] LNP-pDNA systems containing DLin-KC2-DMA and SOPC were formulated at N/P ratios between 3 and 15. As shown in Figure 2, *E*, maximum luciferase expression was observed at the N/P ratio of 6. Higher N/P values also exhibited reduced particle diameters (75 nm for $N/P = 6$ compared to 130 nm for $N/P = 3$).

Influence of helper lipids on LNP-pDNA accumulation into heLa cells

The transfection potency of LNP-pDNA systems depends on the amount of LNP that is accumulated into target cells and the efficiency of pDNA delivery to the cell cytoplasm. To determine the role of uptake, LNP internalization was tracked using the lipophilic

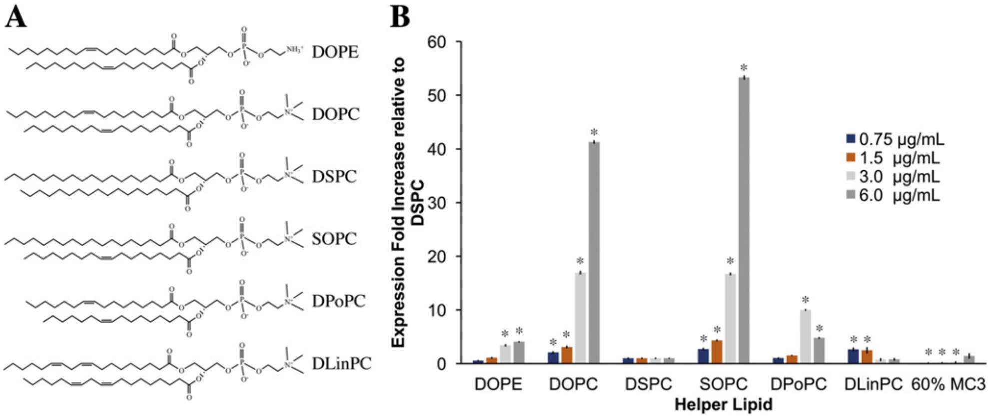


Figure 1. LNP-pDNA systems containing unsaturated PCs exhibit improved transfection properties *in vitro*. (**A**) Structures of "helper" lipids (HL) tested. (**B**) Luciferase expression following incubation of cultured HeLa cells with LNP-pCI-FLuc (0.029 mg DNA per µmol lipid) for 24 h at pDNA concentrations of 0.75-6.0 µg/ml. LNP lipid composition MC3/HL/cholesterol/PEG-lipid (50/10/38.5/1.5; mol/mol). Luciferase expression was normalized to expression achieved for DSPC-LNP at the same pDNA dose. Results represent average ± s.d. of three independent experiments. The * symbol indicates P values <0.0001 as determined using an ANOVA analysis/Bonferroni multiple comparisons test comparing expression for LNP containing HL to DSPC-LNP at the same pDNA dose.

fluorophore DiI-C18. It was found that LNP containing DOPE are internalized less than their PC-containing counterparts (Figure 2, *F*), whereas SOPC-LNPs maintain highest uptake over all doses, which could contribute to the higher transfection levels noted for LNP containing SOPC. Interestingly, in the absence of serum, DOPE- and DOPC-LNPs both exhibit low levels of uptake (Figure 2, *F*), and low levels of transfection (Supplementary Figure S2). When FBS is replaced by wild-type (WT) or ApoE$^{-/-}$ mouse serum in the culture medium, the transfection potency of LNP-pDNAs is effectively abolished for the ApoE$^{-/-}$ medium (Supplementary Figure S3). This result is consistent with previous *in vivo* studies where LNP-siRNA systems (that are highly effective for liver gene silencing in wild type mice *in vivo*) are ineffective in ApoE knockout mice.[38] The ApoE requirement has been ascribed to a need for ApoE to be adsorbed onto the LNP surface to achieve efficient uptake into cells *via* the LDL receptor family.

LNP formulations of pDNA prepared by microfluidic mixing exhibit solid core structures and monodisperse populations

To determine whether the different transfection properties of LNP-pDNA systems could be related to different structural features of LNP composed of different lipids, cryo-TEM studies were performed. As shown in Figure 3, LNP-pDNA systems exhibited electron dense core structure consistent with a hydrophobic interior consisting of plasmid associated with amino-lipid[23] independent of the whether the helper lipid was DSPC, SOPC, DOPC or DOPE. In addition, for all helper lipids tested, LNP-pDNAs achieved essentially complete entrapment of plasmid (greater than 90%), with particle sizes within the range of 65-75 nm and polydispersity indices less than 0.1 (Supplementary Table S1).

LNP-pDNA systems containing unsaturated PCs and DLin-KC2-DMA are effective transfection reagents for a variety of cell lines

The transfection properties of LNP-pDNA systems containing various helper lipids were tested in a range of mammalian cell lines (Figure 4) including cell originating from liver (HepG2 and Hep3B), adrenal gland (PC12), mammary gland (MCF7 and MDA-231), and ovaries (JHOC-9). LNP-pDNA systems containing DOPC and SOPC achieved the highest levels of transfection (Figure 4). In most cases LNPs containing SOPC were most potent, however for MDA-231 cells (Figure 4, *E*), LNPs containing DOPC were considerably more potent.

One observation common to all the transfection results is that when cells were incubated with pDNA at concentrations of 3 µg/ml pDNA or higher, transfection potency was diminished. A possible cause is the higher concentrations of LNP-associated PEG-lipid at high LNP concentrations. As noted elsewhere[37,39] the PEG-lipid employed can exchange rapidly to other membrane bound systems in times on the order of minutes, possibly disrupting uptake or endosomal release. In order to test whether this effect could be contributing to reductions in transfection potency at higher lipid doses, PEG-lipid micelles were added to 0.75, 1.5, and 3.0 µg/ml LNP-pDNAs to achieve a final PEG-lipid concentration of 5.75 µg/ml (corresponding to the PEG-lipid dose at 6 µg/ml pDNA). The addition of PEG-lipid micelles drastically reduced the transfection by LNP-pDNA systems (Figure S1, *A*). Interestingly, decreased uptake did not fully account for the decrease in expression. At 1.5 µg/ml pDNA, a 2.3-fold reduction in uptake corresponded to a 16-fold decrease in luciferase expression (Figure S1, *B*).

LNP-pDNA systems can be efficient, non-toxic reagents for transfection of primary cells both in vitro and in vivo

The transfection properties of LNP-pDNA formulations containing DLin-KC2-DMA and different helper lipids were first tested in isolated primary embryonic mesenchymal cells from white leghorn chick embryos. Following incubation with LNP-pCAX-eGFP, cells were analyzed by flow cytometry for GFP expression. Fluorescence microscopy of low-density cultures showed GFP expression in more than 85% of cells treated with all four helper lipid formulations (Figure 5, *A-D*). At the highest DNA dose, LNPs containing DOPE were also observed to effectively transfect high-density cultures (85-90% transfection, Figure 5, *E*). A higher concentration of cells expressing GFP was observed in

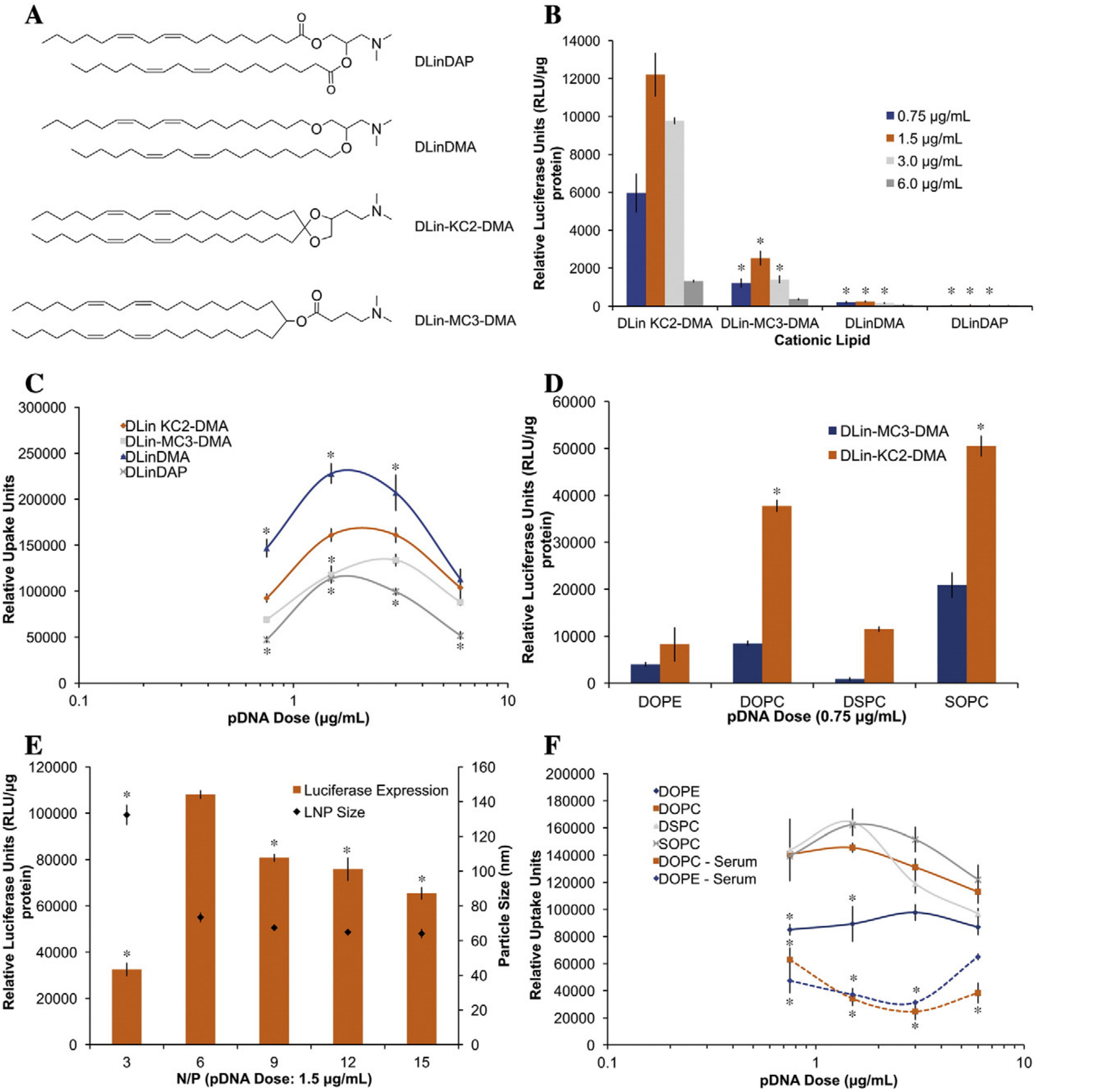


Figure 2. LNP-pDNA systems containing DLin-KC2-DMA and unsaturated PCs exhibit optimized transfection properties in HeLa cells. (**A**) Structures of amino-lipids tested. (**B**) Luciferase expression in HeLa cells following incubation with LNP-pCI-FLuc containing the amino-lipids shown in panel **A** (substituted for MC3). (**C**) Uptake of LNP-pCI-FLuc containing various cationic lipids into HeLa cells as a function of dose. (**D**) Luciferase expression in HeLa cells following incubation with LNP-pCI-FLuc containing DLin-KC2-DMA or DLin-MC3-DMA and one of the helper lipids DOPE, DOPC, DSPC or SOPC (substituted for DSPC). (**E**) Dependence of the transfection properties and particle size of LNP-pCI-FLuc containing DLin-KC2-DMA and SOPC on the N/P ratio in HeLa cells. (F) Uptake of LNP-pCI-FLuc containing DLin-KC2-DMA-LNPs and various helper lipids in the presence (solid lines) or absence (dotted lines) of serum as a function of LNP dose. Results represent the average ± s.d. of 3 experiments. The * symbol indicates *P* values <0.0001 determined using an ANOVA analysis/Bonferroni multiple comparisons test. Panel **B/C**: Expression compared to LNP containing DLin-KC2-DMA at the same pDNA dose. Panel **D**: Expression compared to LNP containing DLin-MC3-DMA for each helper lipid. Panel **E**: Expression compared to expression achieved for N/*P* = 6. Panel **F**: Uptake compared to LNP containing SOPC.

the chondrogenic condensations (white arrow, Figure 5, *E*). Other LNP formulations did not achieve similar transfection levels in high-density cultures (Figure 5, *H*).

Quantification of GFP-positive cells by flow cytometry (Figure 5, *F-H*) indicated that all LNP-pDNAs are able to achieve higher transfection efficiency (~90%) compared to Lipofectamine (~50%). Further, the higher percentage of viable cells indicated that the LNP-pDNA formulations were significantly less toxic than Lipofectamine. Lipofectamine treatments resulted in 33% cell viability, whereas the LNP-pDNA formulations resulted in more than 85% cell viability (Figure 5, *I*), in addition to higher transfection efficiencies (Figure 5, *G*).

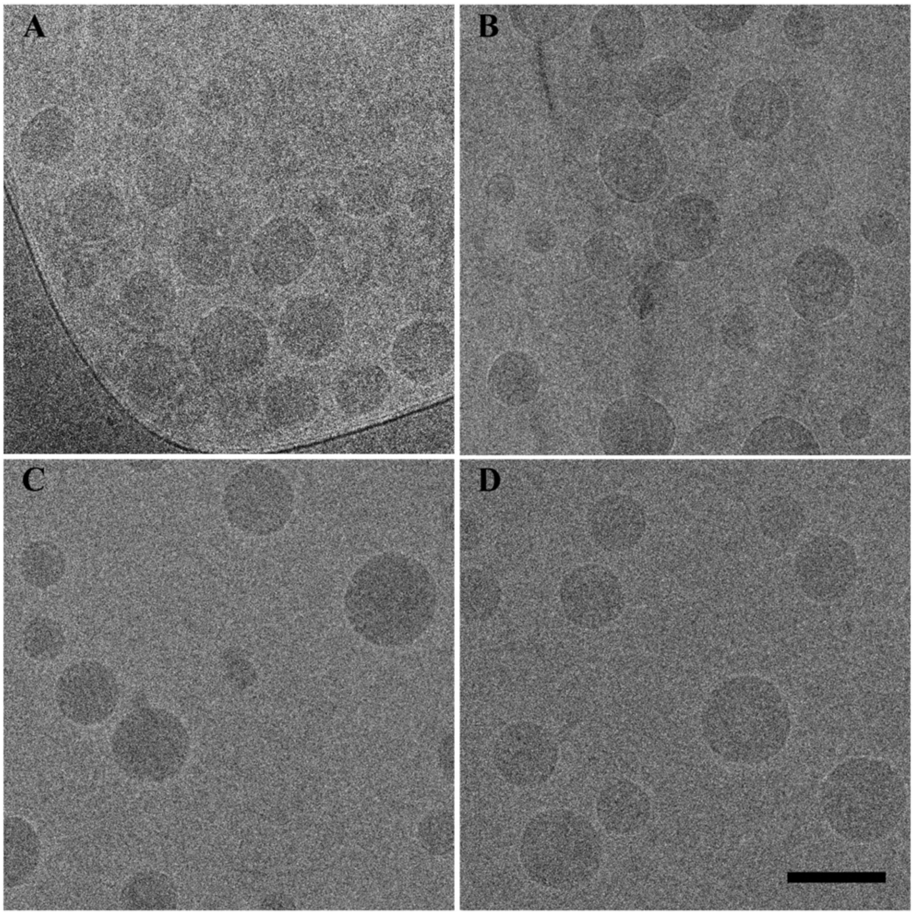


Figure 3. LNP-pDNA systems exhibit electron dense "solid core" structure as visualized by cryo-TEM. Cryo-TEM micrographs of LNP-pCI-FLuc containing DLin-KC2-DMA and various "helper" lipids: (**A**) DOPE, (**B**) DOPC, (**C**) DSPC, and (**D**) SOPC. Bar = 100 nm.

We next tested LNP-mediated pDNA transfection *in vivo* using the chick embryo model.[40] LNP-pDNA injections into the limb buds of chick embryos (stage 19-20) resulted in high levels of GFP expression (Figure 6, *E-H*). At stage 24, high levels of GFP expression were observed for all LNP-pDNA formulations but the highest levels of transfection were observed with DOPE-LNP (Figure 6, *A*, *E*). The relative expression levels followed the order DOPE > DOPC > DSPC > SOPC (Figure 6, *E-H*). Immunostaining of tissue sections of the limb bud further confirmed the presence of cytosolic GFP in mesenchymal cells (Figure 6, *I*). Interestingly, GFP-positive cells were observed in the developing cardiac tissue (Figure 6, *G*, white arrow), suggesting diffusion of LNP from the site of injection into the vasculature. Isolation and tissue sectioning of the cardiac region confirmed GFP expression (Figure 6, *J*). No developmental abnormalities were observed following LNP-pDNA administration.

Discussion

The results presented here show that the lipid composition of potent LNP formulations of pDNA for transfecting dividing cells *in vitro* differs significantly from the composition of LNPs optimized for *in vivo* delivery of siRNA. Furthermore, the lipid composition of optimized LNP-pDNA systems can differ according to cell lines being transfected. Finally, these systems are also effective and nontoxic transfection reagents for dividing primary cells both *in vitro* and *in vivo*. There are three points that warrant further discussion. The first concerns the differences between LNP formulations of siRNA and pDNA, and why the amino-lipid requirements for optimum transfection with LNP-pDNA *in vitro* should differ from those for LNP-siRNA formulations *in vivo*. The second point concerns the role of helper lipids and PEG-lipids. Finally, we remark on the utility of LNP-pDNA containing ionizable amino lipids for transfection of primary cells *in vitro* and *in vivo*.

An obvious difference between LNP-siRNA systems and LNP formulations of pDNA concerns the size of the pDNA. The siRNA 21-mer duplexes contain 42 negative charges, at the optimum amino-lipid to siRNA phosphate (N/P) charge ratio of three this corresponds to approximately 300 siRNA oligonucleotides per 70 nm diameter LNP. In the case of the Luc-pDNA (7.2 kb; 1.44×10^4 negative charges), an N/P charge ratio of six corresponds to 8.64×10^4 amino-lipids per pDNA. Assuming a lipid density of 0.9 g/ml this corresponds to a total lipid volume of 1.88×10^5 nm^3 for the LNPs employed here which have a diameter of ~75 nm (Figure 2, *E*). The volume of a single pDNA of 7.2 kb is 5.61×10^3 nm^3. Thus the total volume, excluding

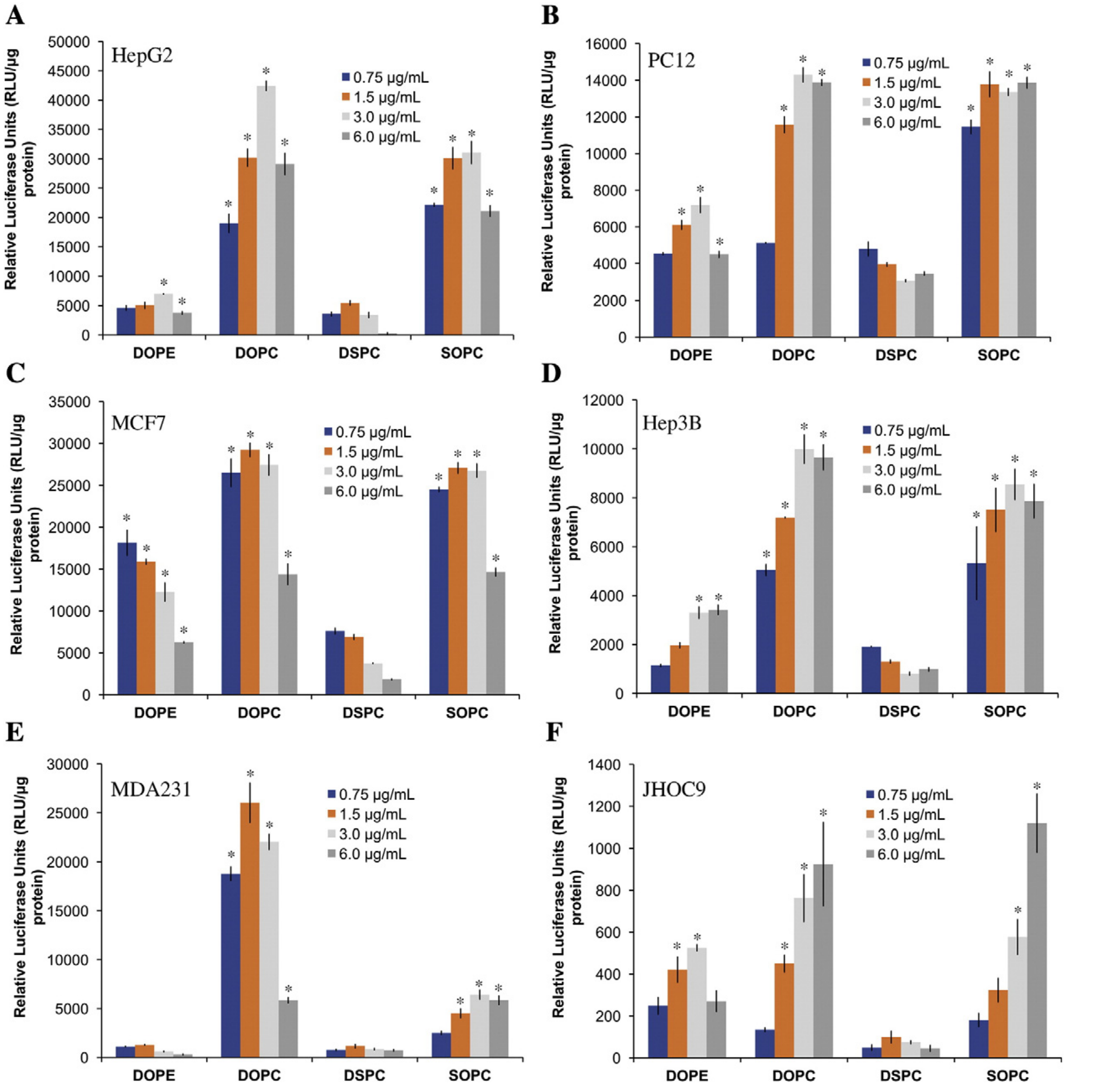
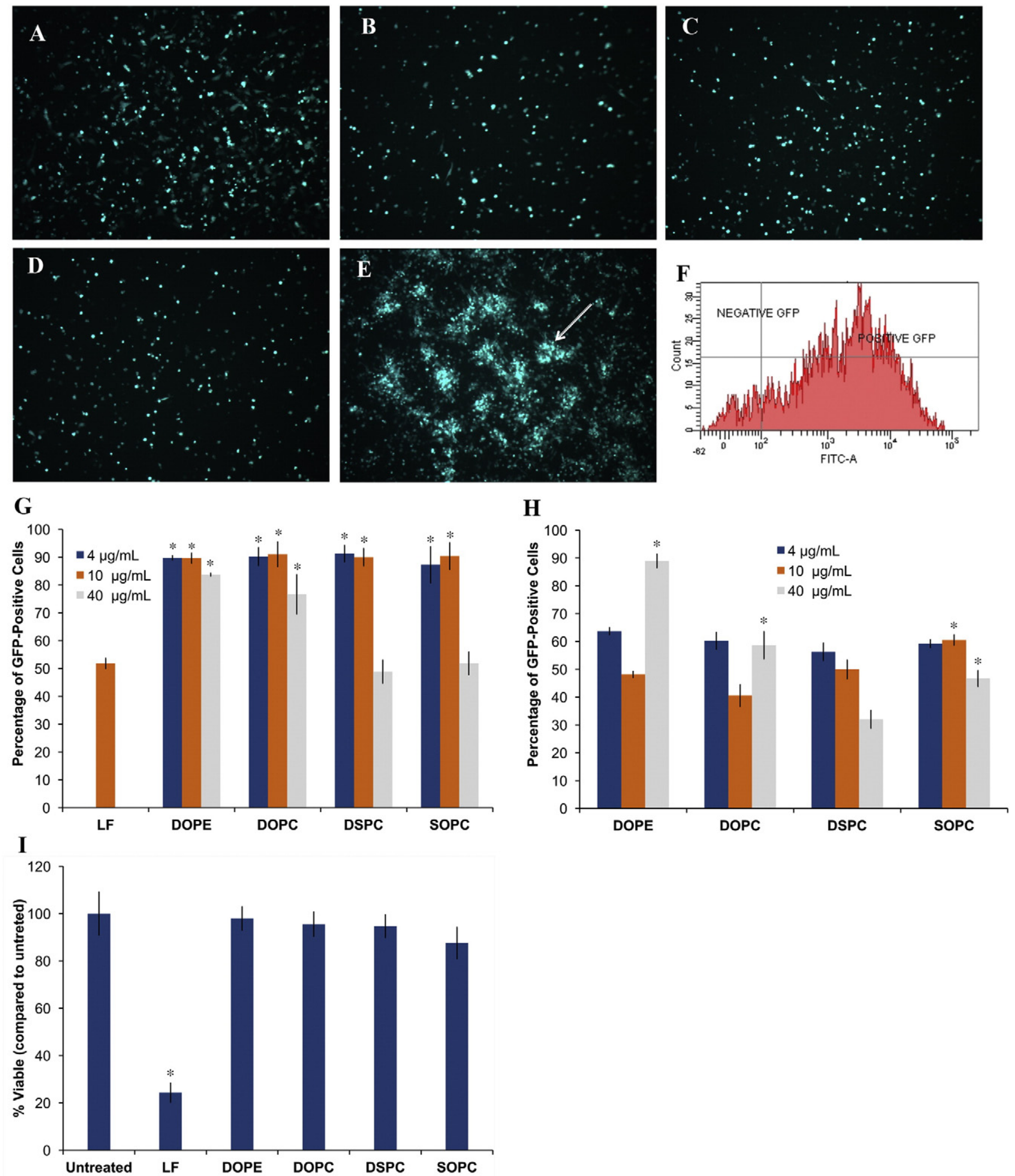


Figure 4. LNP-pDNA systems containing DLin-KC2-DMA and unsaturated PCs are efficient transfection reagents for a variety of mammalian cell lines. Luciferase expression following incubation of LNP-pCI-FLuc containing DLin-KC2-DMA and a variety of helper lipids in (**A**) HepG2, (**B**) PC12, (**C**) MCF7, (**D**) Hep3b, (**E**) MDA231 and (**F**) JHOC9 cell lines in culture using similar protocols as indicated for Figure 1. LNP-pDNA concentrations corresponded to 0.75-6.0 µg/ml pDNA; the relative luminescence was normalized to cellular protein. Results represent the mean ± s.d. of three experiments. The * symbol indicates P values <0.0001 as determined using an ANOVA analysis/Bonferroni multiple comparisons test comparing expression with various formulations to that achieved for LNP containing DSPC at the same pDNA dose.

water, of an LNP containing a single pDNA is 1.94×10^5 nm^3, which in turn corresponds to a diameter of 72 nm. It can be concluded that each LNP-pDNA system employed here contains one plasmid per particle.

At an N/P of three, the LNP-pDNA systems were larger (diameter 135 nm) than for an N/P ratio of six (diameter 75 nm). The smaller size at higher amino-lipid contents likely reflects a more condensed structure of the encapsulated pDNA. This is supported by the close correspondence between the theoretical and actual sizes of LNP-pDNA systems at the N/P ratio of six. The enhanced transfection observed at N/P values of six could arise due to improved uptake of the smaller systems, greater transfection potency of the condensed pDNA or the need for more amino-lipid to result in more complete endosomal disruption to release the large pDNA construct.

It is perhaps not surprising that optimized lipid compositions for LNP-pDNA systems for transfecting dividing cells *in vitro* differ from the optimized lipid compositions developed for LNP-siRNA systems for hepatic gene silencing *in vivo*. We show here that incorporation of DLin-KC2-DMA, rather than



DLin-MC3-DMA, results in improved transfection potencies. This improvement did not arise primarily due to differences in uptake as LNPs containing DLin-KC2-DMA exhibited only modestly improved uptake (~1.5-fold). DLin-KC2-DMA exhibits a higher pKa than DLin-MC3-DMA (6.7 *vs*. 6.4[15]) and this could account for the improved properties *in vitro*. DLin-MC3-DMA exhibits optimized properties *in vivo* where the pKa must be low enough so the LNP is not highly ionized at physiological pH (which leads to toxicity and rapid clearance by immune cells) but is high enough so that it is sufficiently ionized at endosomal pH that it can interact with endogenous anionic lipids to disrupt the endosomal membrane. For *in vitro* or direct

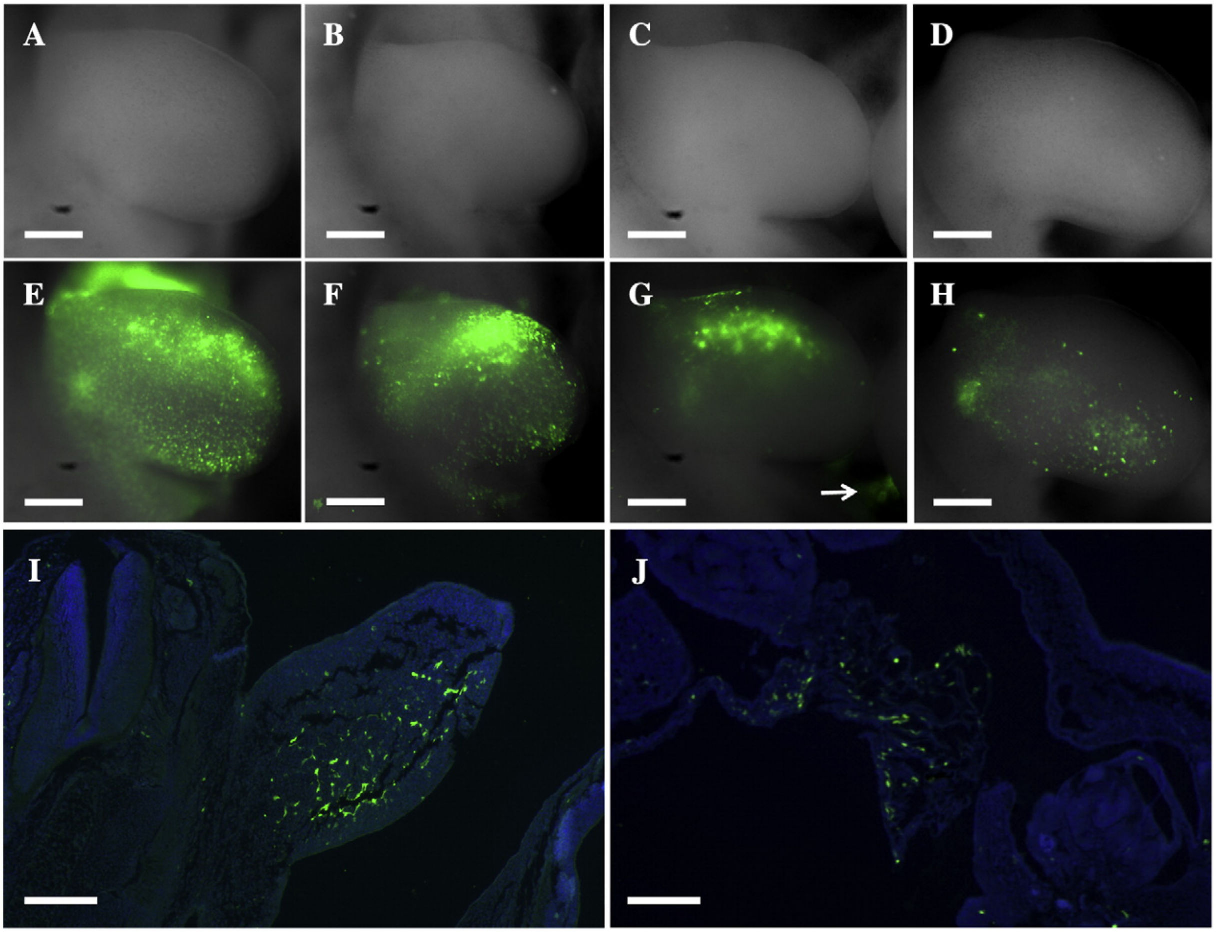


Figure 6. LNP-pDNA systems containing DLin-KC2-DMA and a variety of helper lipids are efficient transfection reagents *in vivo*. LNP-pCAX-eGFP systems were injected (~10-50 nL of 10 μg/mL LNP-pDNA) into the forelimb buds of stage 19-20 chicken embryos. Panels **A-D** show micrographs of limb buds at stage 24 (20-24 h after injection). Fluorescence microscopy of these limb buds revealed large numbers of cells expressing eGFP for LNP-pDNA systems containing all four helper lipids: (**A, E**) DOPE, (**B, F**) DOPC, (**C, G**) DSPC and (**D, H**) SOPC with LNP containing DOPE exhibiting the highest transfection rates (**A, E**). In some cases LNP-pDNA injections penetrated local vasculature resulting in significant transfection in heart tissues (arrow in **G**), suggesting that LNPs in the bloodstream may pool in the embryonic heart. (**I**) Micrograph of the injected forelimbs transfected with DOPE-LNP showing extensive cytosolic eGFP expression within injected regions using immunofluorescence staining. (**J**) Sections through the heart tissues again displayed EGFP antibody staining. Scale bars = 250 μm.

injection *in vivo* applications, the constraint of lower charge at physiological pH is relaxed, allowing use of lipids with higher pKa values. It may be noted that at a putative endosomal pH of 6, approximately 84% of DLin-KC2-DMA molecules would be positively charged compared to 72% of DLin-MC3-DMA molecules. As indicated elsewhere[7,16,41] positively charged lipids interact with endogenous anionic lipids to induce non-bilayer, membrane lytic structures that would enhance release of the pDNA into the cell cytoplasm, thus the presence of more positive charge would correlate with increased transfection.

While LNP-siRNA systems require DSPC for optimized activity *in vivo*, the requirements for optimized transfection by LNP formulations of pDNA *in vitro* are different. The requirement for a "helper" lipid (such as PC or PE) is maintained, however, incorporation of unsaturated PCs (SOPC and DOPC) results in significantly more potent transfection systems. This

Figure 5. LNP-pDNA systems containing DLin-KC2-DMA and a variety of helper lipids are efficient, non-toxic transfection reagents for primary avian embryonic cells *in vitro*. LNP-pCAX-eGFP systems were incubated (24 h, 4 μg/ml pDNA) with cultured mesenchyme cells (2×10^5 cells/ml). Fluorescence micrographs revealed significant GFP expression for LNPs containing various helper lipids: (**A**) DOPE, (**B**) DOPC, (**C**) DSPC, and (**D**) SOPC. (**E**) Fluorescence micrographs of high-density cultures (2×10^7 cells/ml) demonstrated efficient transfection by LNP-DNA systems containing DOPE. These LNP were able to penetrate the prechondrogenic structures formed in these cultures (arrow). (F) Flow cytometry analysis for DOPE-containing LNPs; significant numbers of fluorescent cells were detected for all formulations. GFP-positive cell counts (as a % of the total cell count) following incubation with 4, 10 or 40 μg/ml of LNP-pDNA are shown for (**G**) low-density and (**H**) high-density cultures. The transfection reagent Lipofectamine (LF) was employed as a control (see Methods). Results represent the mean ± s.d. percentage of GFP-positive cells (4 independent experiments). (**I**) Cell counts of low density cultures, normalized to untreated controls, after 24 h of treatment, prior to trypsinization for flow cytometry analysis. Results represent the mean ± s.d. of counts of three areas within the treatment wells. The * symbol indicates P values <0.0001 as determined using an ANOVA analysis/Bonferroni multiple comparisons test: Panel **G**, all values compared to LF; Panel **F**, values compared to LNP containing DSPC at the same dose; Panel **I**, values compared to untreated.

could be related to an enhanced ability to induce non-bilayer structures for more unsaturated lipids.[34] The effect is substantial, as SOPC in place of DSPC improves transfection potencies of LNP-pDNA systems for HeLa cells 5-fold, and in other cell lines tested (Figure 4) to levels that are (in some cases) far superior to levels achieved with commercial transfection reagents, without attendant toxicity. The requirement for PC as compared to DOPE appears related to improved uptake of LNP containing unsaturated PC as LNPs containing DOPE were internalized significantly less than LNPs containing PC. The differences in optimized lipid compositions between different cell lines likely reflect differing abilities to bind serum proteins (such as ApoE[38,42]) that trigger uptake into these cells.

The final lipid constituent to be discussed concerns the PEG-lipid. As noted in Figure 4, levels of expression in tissue culture do not increase with concentrations of pDNA above 1.5-3.0 μg/ml regardless of helper lipid. This lack of correspondence appears to be due to the high levels of PEG-lipid that are present when high concentrations of LNP-pDNA systems are employed. It is well known that a stable PEG coating renders LNP systems ineffective[43] due to reduced interactions with target cells and limited uptake. The PEG lipid employed here has short (C14) acyl chains that result in a PEG-lipid that can dissociate from the LNP after injection *in vivo*, resulting in potent LNP-siRNA systems.[39,44] *In vivo* there are many “sinks” that the PEG-lipid can associate with including lipoproteins, cells in the circulation and endothelial cells. However, the major sink *in vitro* would be serum lipoproteins and albumin, and potentially the plasma membranes of cells. It appears that at high concentrations of LNP-pDNA the equilibrium concentrations of PEG-lipid in the LNP are sufficient to inhibit uptake (Figure S1, *A*).

The final topic of discussion concerns the utility of these LNP-pDNA systems for transfecting primary cells both *in vitro* and *in vivo*. LNP-pDNA systems generated here achieved higher transfection efficiencies in primary cells than Lipofectamine. In contrast to cultured cells, the transfection properties for primary cells did not depend on the species of helper lipid in the LNP. LNP formulations with all helper lipids achieved near complete transfection (Figure 5, *G*) without appreciable toxicity in the low-density cultures (Figure 5, *I*). In high-density cultures, all LNPs achieved an appreciable level of transfection (near 60%) at 4 μg pDNA/ml with LNP-pDNA systems containing DOPE achieving 90% transfection at 40 μg pDNA/ml.

The observations that LNP-pDNA systems containing DLin-KC2-DMA and DOPE achieve mosaic transfection upon subcutaneous injection in the chick embryo limb bud suggest that they are highly effective transfection agents *in vivo* and could enable the study of gene expression in a developmentally significant manner. For example, gene editing plasmids coding for CRISPR/Cas9 require uptake of multiple plasmids in a single cell.[45] It may be concluded that LNPs containing ionizable amino-lipids are promising systems for applying genome editing tools to the chicken embryo.

In summary, the results presented in this study show that LNP formulations of pDNA containing ionizable cationic lipids can be highly effective transfection reagents *in vitro* that are significantly less toxic than commercial reagents such as Lipofectamine. LNP systems containing DLin-KC2-DMA and unsaturated helper lipids are also potent systems for transfecting primary cells *in vitro* and *in vivo*. It is anticipated that these systems will be of considerable utility for transfection of developing tissues *in vitro* with attendant potential for gene editing applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2016.12.014.

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