In Vivo Investigation of pDNA Delivery Using Various Lipid Nanoparticle Formulations in A549 and Huh7 Cell Lines

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KEY FINDING

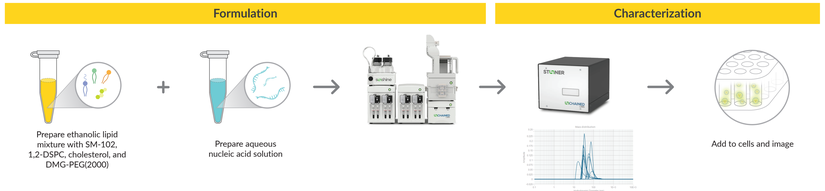
Encapsulation and transfection of eGFP-pDNA was successful using several ionizable lipid nanoparticles in Huh7 and A549 cell types.

INTRODUCTION

Lipid nanoparticles (LNPs) have shown remarkable success in delivering mRNA vaccines, notably against COVID-19, surpassing prior non-viral technologies in protein expression. This achievement has prompted interest in LNP-based therapeutics due to their ability to target specific organs and cell types.[1] However, mRNA's short half-life, lack of promoter regions for cell-specific and temporal control, and stability issues at room temperature or 4°C limit its utility. Plasmid DNA (pDNA) offers a promising alternative, providing prolonged transgene protein expression, incorporating cell type-specific or inducible promoters, enhanced storage stability, and fewer limitations on cargo size.[2] These benefits could enable treatments for diseases less accessible to mRNA-LNPs, such as chronic autoimmune disorders and neurodegenerative diseases. Despite these advantages, pDNA-loaded LNPs have been less explored due to concerns about toxicity observed in animal models, such as inflammation and mortality at therapeutic doses.[2] This study evaluates various LNP formulations for their ability to encapsulate GFP-encoding pDNA and transfect human hepatocyte (Huh7) and lung epithelial (A549) cell lines. By comparing encapsulation efficiency and transfection outcomes, we identified effective strategies for pDNA delivery using LNPs. These findings provide valuable insights into optimizing pDNA-LNP systems and potentially expanding their utility in gene therapy.

METHODS

LNP formulation and characterization



Ionizable Cationic Lipid	DLin-MC3-DMA	ALC-0315	SM-102	LP-01	4A3-SC8*	DOTAP*	SM-102/βS
Phospholipid	1,2-DSPC	1,2-DSPC	1,2-DSPC	1,2-DSPC	1,2-DOPE		1,2-DSPC
Sterol Lipid	Cholesterol	Cholesterol	Cholesterol	Cholesterol	Cholesterol		β-Sitosterol
PEGylated Lipid	DMG-PEG(2000)	ALC-0159	DMG-PEG(2000)	DMG-PEG(2000)	DMG-PEG(2000)		DMG-PEG(2000)
Lipid Molar Ratio ◊	50:10:38.5:1.5	46.3:9.4:42.7:1.6	50:10:38.5:1.5	45:9:44:2	11.9:50:11.0:23.8:2.4		50:10:38.5:1.5

◊ Ionizable cationic lipid:neutral phospholipid:cholesterol:PEGylated lipid;
**Selective organ targeting (SORT) LNP*

Figure 1 – Experimental Workflow.
LNPs were formed using a Sunshine™ microfluidics mixer (Unchained Labs, Pleasanton, CA, USA). The total flow rate was 10 ml/min with a flow rate ratio of 3:1. The organic phase consisted of a lipid mix in ethanol following various molar ratios. The aqueous phase contained either 200 µg/ml eGFP-pDNA or -mRNA in 50 mM sodium acetate, pH 4.5. LNPs were dialyzed against TBS, pH 7.4, overnight and stored at 4°C until use. A subset was flash-frozen in 10% sucrose and stored at -80°C for freeze-thaw (FT) analysis. LNP size and polydispersity (PDI) were assessed biophysically through dynamic light scattering (DLS). Payload concentration was determined following the Quant-iT™ dsDNA and mRNA Assay Kits (Invitrogen™). Huh7 and A549 cells were seeded for LNP treatment at 5,000 cells/well in 96-well plates. LNPs were added to the cells to achieve final nucleic acid concentrations of 62.5, 125, 250, or 500 ng/ml in the culture medium. 48 hours post-transfection, Hoechst 33342-stained cells were imaged using a Cytation 5 microscope. Transfection efficiency and GFP intensity was determined by counting GFP-positive cells and nuclei with Gen5 software.

RESULTS

Efficient encapsulation and optimal size distribution of pDNA-LNPs: Fresh and FT

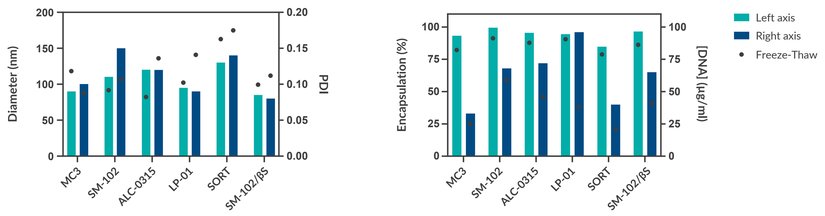
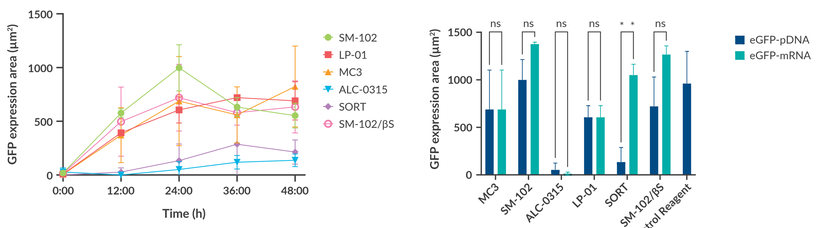


Figure 2 – Characterization of eGFP-pDNA LNP formulations prepared fresh and following treatment. (**A**) Comparison of PDI values and Z-average diameter from fresh and FT-treated LNPs. PDI and Z-average diameter were determined by DLS before and after FT cycles. (**B**) Encapsulation (%) and eGFP-pDNA concentration of LNPs before and after FT cycles. Encapsulation performance and pDNA concentration was assessed using the PicoGreen dsDNA standard (Invitrogen™). SM-102-containing β-sitosterol abbreviated SM-102/βS.

Successful transfection of human hepatocyte and lung epithelial cells

Huh7



A549

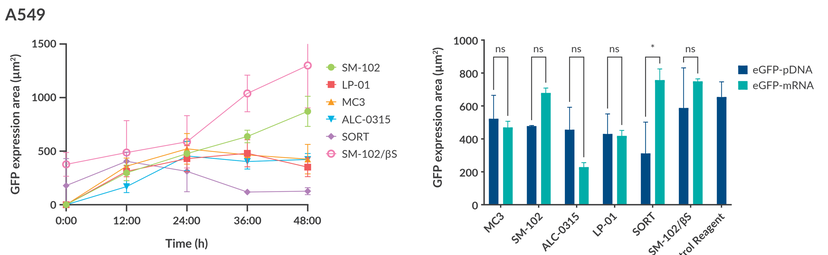


Figure 3 – GFP expression in hepatocytes (Huh7) and lung epithelial cells (A549) after treatment with 500 ng/ml of various LNP formulations containing either eGFP-pDNA or eGFP-mRNA.
Left: GFP expression area was measured over a 48-hour period, with images captured from each well of a 96-well plate every 12 hours. The total GFP-positive area per image was quantified using Gen5 image analysis software. **Right:** Comparison of GFP expression area at the 24-hour mark for eGFP-pDNA and eGFP-mRNA cargoes. Lipofectamine™ (Invitrogen™) served as a control reagent. Statistical significance: *p < 0.0131; **p < 0.0074; ns = not significant.

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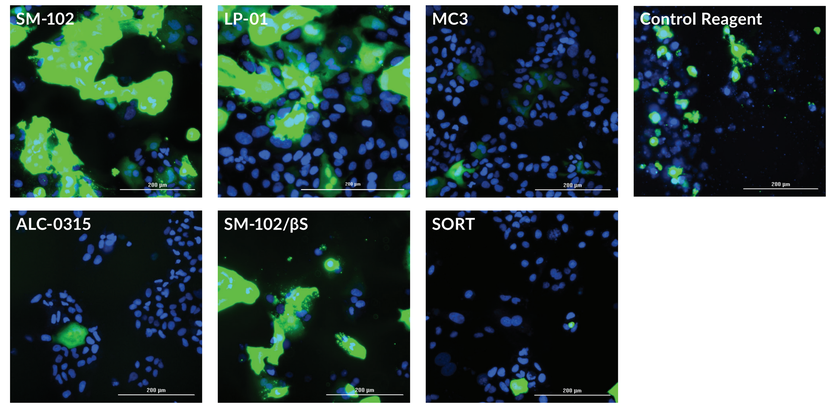

Figure 4 – eGFP-pDNA uptake by Huh7 hepatocytes in various LNP formulations and control.

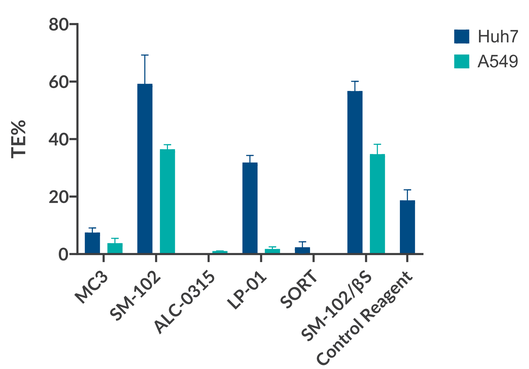

Figure 5 – Transfection efficiency (TE%) of GFP expression in Huh7 and A549 treated with 500 ng/ml of eGFP-pDNA-containing LNPs post freeze-thaw. TE% was calculated by dividing the number of GFP-positive cells by the total cell count across multiple wells, with results expressed as a percentage 48 hours post-transfection. Lipofectamine™ (Invitrogen™) was used as a control reagent.

CONCLUSIONS

- Plasmid DNA encoding GFP was successfully encapsulated in all tested formulations, achieving an average encapsulation efficiency of over 90% and a PDI below 0.10.
- GFP-pDNA LNPs maintained stability after a freeze-thaw cycle, with averaged biophysical characteristics remaining consistent (PDI <0.20 and encapsulation efficiency >85%).
- Transfection with pDNA-loaded LNPs showed comparable efficiency to mRNA controls (not shown) in both cell lines after freeze-thaw, outperforming the control reagent in SM-102, SM-102/βS, and LP-01 formulations.
- Replacing cholesterol with the analogue β-sitosterol in SM-102 resulted in the highest transfection efficiency of pDNA-containing LNPs, reaching 68.4% in Huh7 cells.

References
1. Hou, X., Zaks, T., Langer, R., *et al.* Lipid nanoparticles for mRNA delivery. *Nat. Rev. Mater.* **6(12)**, 1078-1094 (2021).
2. Patel, M.N., Tiwari, S., Wang, Y., *et al.* Enabling non-viral DNA delivery using lipid nanoparticles co-loaded with endogenous anti-inflammatory lipids. *bioRxiv* (2024).