Novel Ether-Ionizable Lipids Complexed into Lipid Nanoparticles Enhance mRNA Delivery Efficiency

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Introduction

Lipid nanoparticles (LNPs) are effective drug delivery platforms for nucleic acids such as messenger RNA (mRNA). LNPs increase RNA stability, circulation time, cellular uptake, and tissue specificity. They are comprised of four lipid components including ionizable lipids, cholesterol, phospholipids, and lipid-conjugated poly(ethylene) glycol (PEG). The ionizable lipid is the active component of LNPs due to their pH-responsiveness; they are neutrally charged in physiological pH but become positively charged in acidic conditions. Upon cellular entry, LNPs become trapped in acidic endosomes, causing them to destabilize the endosomal membrane and release the RNA into the cytosol [1,2]. The chemical structure of these ionizable lipids plays an important role in drug delivery efficacy to various cells and tissues. For example, ether groups have been incorporated into ionizable lipids to increase delivery to monocytes [3,4]. However, a mechanistic of the role of ether groups for intracellular nucleic acid delivery remains to be elucidated. Here, we were interested in assessing how the number of oxygens in these ether-ionizable lipids impacts delivery. We developed a series of novel ionizable lipids, each containing different numbers of ether groups and various alkyl tail lengths, both of which are known to impacts nucleic acid delivery [5]. These ionizable lipids were synthesized into LNPs, characterized, and evaluated *in vitro*. Ultimately, this work will provide structure:function relationships between ionizable lipid design and delivery efficiency.

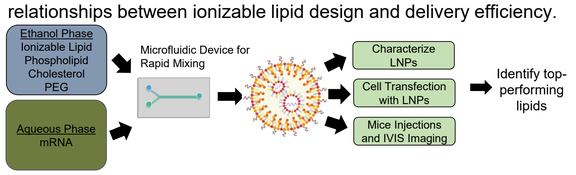

Figure 1. LNPs are synthesized by rapidly mixing lipid components in the ethanol phase and mRNA in the aqueous phase using a microfluidic device. We designed and synthesized the ionizable lipids used in this work to contain variable alkyl tail lengths and ether groups. LNPs were characterized and assessed for cellular delivery and *in vivo* biodistribution to understand structure:function relationships.

Ionizable Lipids are Comprised of a Polyamine Core and Alkyl Tails

Table 1. Ionizable lipid structures contain varying numbers of ether groups (1-3) as well as alkyl tail lengths (8 to 14 carbons).

Name	Tail Length	Number of Ether Groups
Lipid 1	8	1
Lipid 2	10	1
Lipid 3	12	1
Lipid 4	8	2
Lipid 5	10	2
Lipid 6	12	2
Lipid 7	14	2
Lipid 8	10	3
Lipid 9	12	3
Lipid 10	14	3

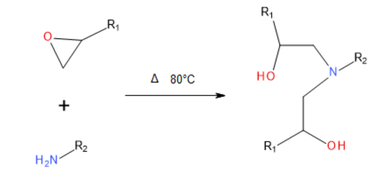

Figure 2. Epoxide-terminated tails are alkylated onto polyamine cores via epoxide-ring opening reactions. Ionizable lipids are purified by flash chromatography and characterized by nuclear magnetic resonance (NMR).

Ether-Ionizable Lipids Form LNPs with Anticipated Physicochemical Properties

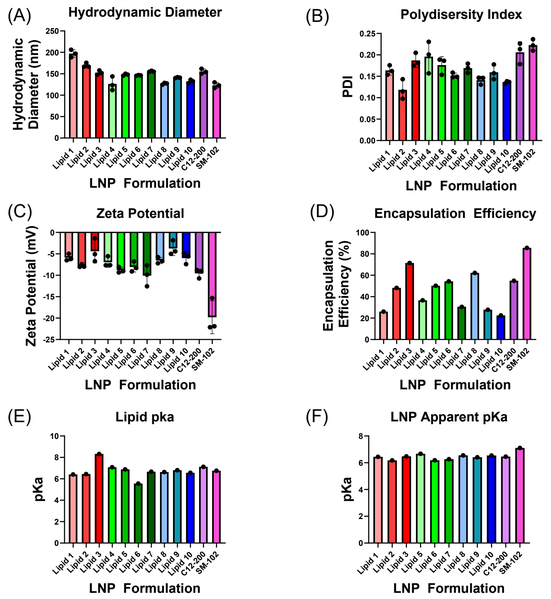

Figure 3. Characterization of LNPs including (A) hydrodynamic diameter, (B) polydispersity index, and (C) zeta potential by dynamic light scattering, and (D) encapsulation efficiency by Ribogreen assays. Apparent pKa of the (E) ether-ionizable lipids and (F) LNP formulations was quantified using fluorescent-based TNS assays.

Number of Ethers and Alkyl Tail Length in Ionizable Lipids Drives LNP Delivery Efficiency

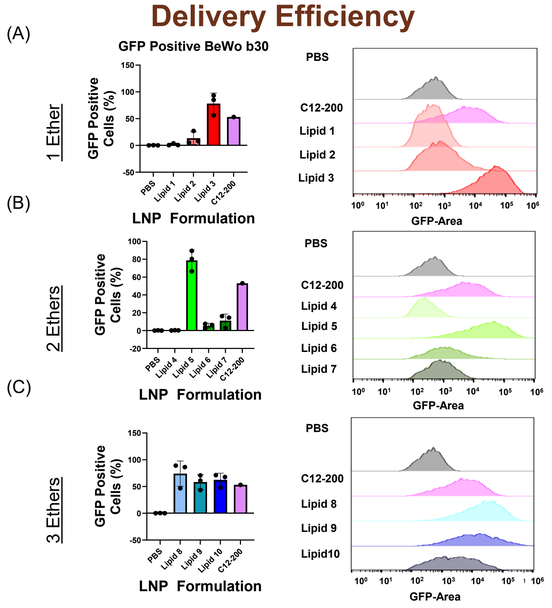

Figure 4. BeWo b30 placental cells were treated with LNPs formulated with the ether-ionizable lipids, commercially available lipid (C12-200), or PBS. The ionizable lipids contain (A) 1, (B) 2, or (C) 3 ether groups. The left panels quantifies the percentage of GFP-positive cells, and the right panel shows representative histograms.

Ionizable Lipid Structure Alters Tissue Biodistribution in Mice

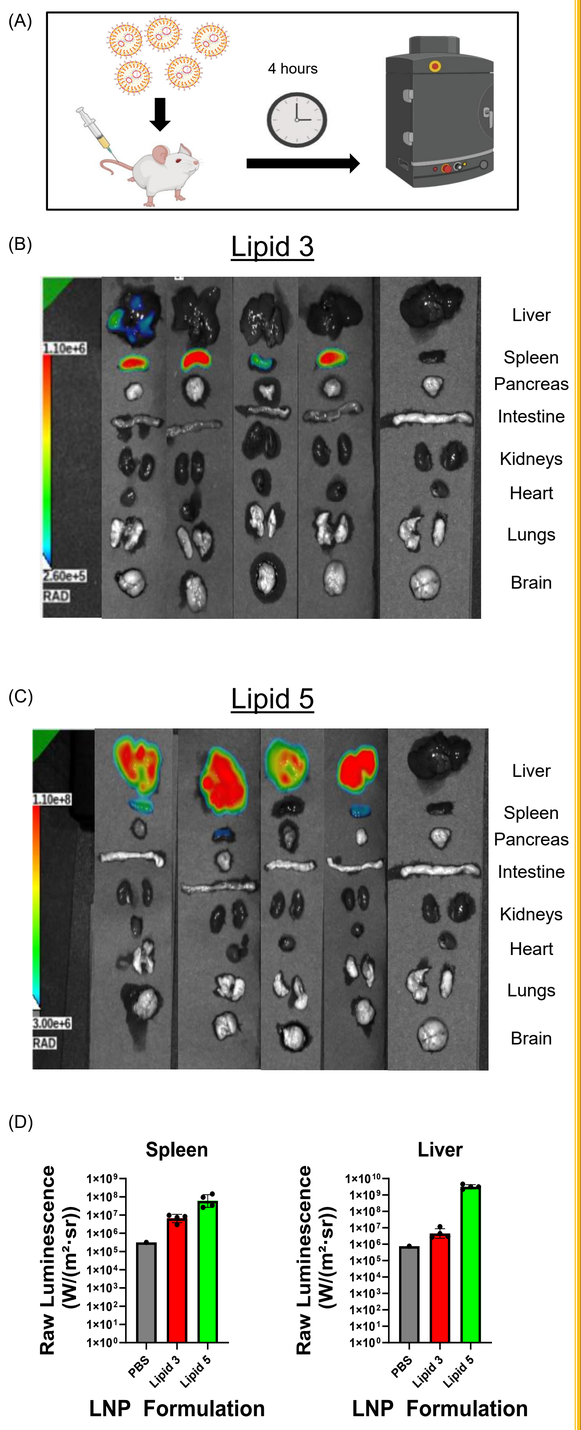

Figure 5. (A) C57BL/6 mice were injected with LNPs with encapsulated luciferase mRNA and biodistribution assessed by IVIS. Mice were intravenously injected at a dose of 1.0 mg/kg mRNA. LNPs comprised of (B) Lipid 3 or (C) Lipid 5 primarily accumulate in the spleen or liver, respectively. (D) Quantification of raw luminescence from IVIS data.

Mechanism of Uptake of LNPs Comprised of Top Ether-Ionizable Lipids

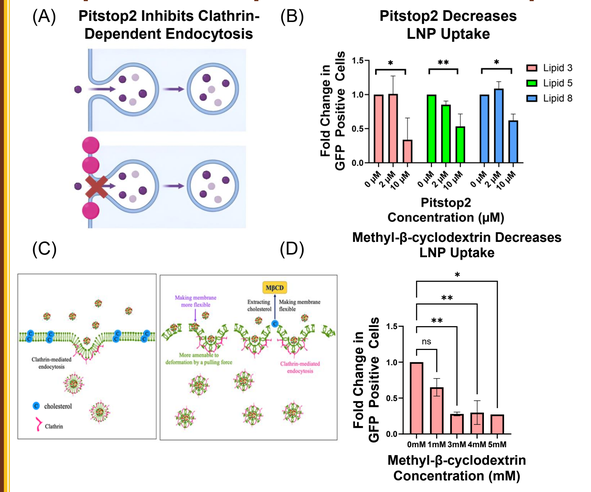

Figure 6. (A) Pitstop2 (pink circles) inhibits the clathrin-dependent endocytosis pathway. (B) Placental cells (BeWo b30 cell line) were pre-treated with various concentrations of Pitstop2 and then cells were treated with top-performing ionizable lipids complexed into LNPs with encapsulated GFP mRNA. (C) Methyl-β-cyclodextrin inhibits lipid-raft endocytosis pathway. Refer to [6]. (D) Cells were treated with various concentration of Methyl-β-cyclodextrin (MβCD) and treated with LNPs complexed with Lipid 3. Top-performing lipids include Lipid 3 (red), Lipid 5 (green), and Lipid 8 (blue). **p<0.0001 and *p<0.05 by one-way ANOVA.

Conclusion

We synthesized a series of novel ether-ionizable lipids and complexed these lipids into LNPs to evaluate structure:function relationships between lipid design and delivery efficiency. Further, we aimed to identify LNPs that enabled high mRNA delivery to placental cells, as future work will use these LNPs to treat diseases of pregnancy. LNPs prepared with Lipid 3, Lipid 5, and Lipid 8 were the top-performing ether-ionizable lipids based on GFP mRNA delivery to b30 trophoblast cells. These outperformed the benchmark lipid C12-200. Furthermore, IVIS imaging following intravenous injection in mice showcased that luciferase-loaded LNPs with Lipid 3 enable high delivery to the spleen with minimal delivery to the liver, whereas LNPs with Lipid 5 have high liver delivery. Last, our data demonstrates that LNPs are primarily taken up through clathrin-mediated and lipid-raft endocytosis. Future work will explore the therapeutic potential of these lipids to treat diseases.

References

[1] Samaridou et. al. Adv. Drug Deliv. Rev. 2020
[2] Han et. al. Nat. Commun. 2021
[3] Chu. et. al. Int. J. Pharm. 2025
[4] Mukalel. et. al. Adv. Funct. Mater. 2024
[5] Tilstra. et. al. J. Am. Chem. Soc. 2023
[6] Xu. et. al. Mol. Pharm. 2024

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