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CRS, General Meeting 2024
9-12 July 2024, Bologna, IT

Workshop on “The Upside and Downside of PEGylation and the Emerging Alternatives”

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Pharmanovation Consulting on behalf of Lipoid GmbH, Germany

DXPE MPEG-2000/5000 AS PHARMACEUTICAL EXCIPIENT: FROM RESEARCH TO PRODUCTS

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1. Introduction
2. Marketed Products
3. Research Projects
4. Suitability for LNPs?
5. Conclusions

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INTRODUCTION

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TOPIC OF SEMINAR

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LIPOID PE 14:0/14:0 – PEG 2000 (MPEG-2000-DMPE)	*N*-(carbonyl-methoxypolyethylene glycol-2000)-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt	CAS-No. 384835-59-0
LIPOID PE 18:0/18:0 – PEG 2000 (MPEG-2000-DSPE)	*N*-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt	CAS-No. 147867-65-0
LIPOID PE 16:0/16:0 – PEG 5000 (MPEG-5000-DPPE)	*N*-(carbonyl-methoxypolyethylene glycol-5000)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt	CAS-No. 384835-61-4

SYNTHESIS OF DXPE MPEG 2000/5000

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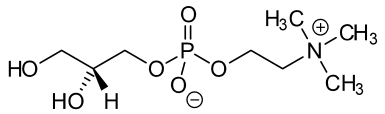
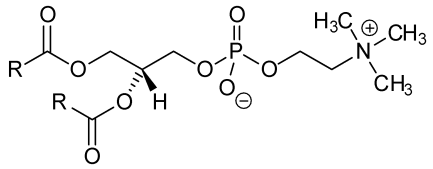
Soybean Phosphatidylcholine

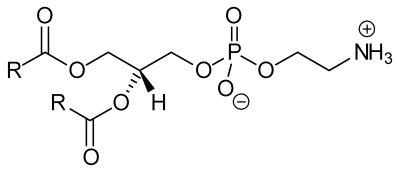
Alkaline hydrolysis



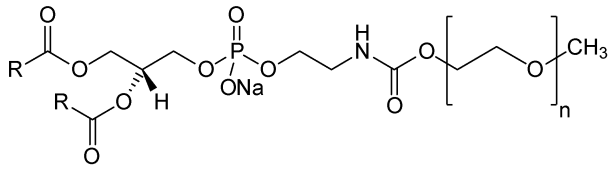
Acylation with fatty acids



Enzymatic head group exchange using Phospholipase D



Acylation, MPEG 2000/5000



DMPE-PEG 2000: R = $C_{13}H_{27}$; n = 44 (average)
DSPE-PEG 2000: R = $C_{17}H_{35}$; n = 44 (average)
DPPE-PEG 5000: R = $C_{15}H_{31}$; n = 112 (average)

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DSPE MPEG 2000: Solubility in Solvents

Ethanol (absolute):
Practically insoluble, or Insoluble (20 °C) (1 : 10000 and over)
Very soluble (35 °C) (Less than 1)

Ethanol (96 %):
Freely soluble (20 °C) (1 : 10)
Very soluble (35 °C) (Less than 1)

Acetone:
Practically insoluble, or Insoluble (20 °C) (1 : 10000 and over)
Very soluble (35 °C) (Less than 1)

Toluene:
Very soluble (40 °C) (Less than 1)

PHYSICO-CHEMICAL PROPERTIES DXPE MPEG 2000/5000

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Critical Micelle Concentration

Critical micelle concentrations (CMC) determined for DSPE-PEG with different PEG chain lengths (2000, 3000, and 5000) using a fluorescent probe were in the micromolar range (0.5–1.5 mM) **with higher CMC for longer PEG chain length**.(16:0 Lyso PC 4-8 µM, 18:0 Lyso PC 0.4 µM)

The size of micelles determined by quasi-elastic light scattering (QELS) showed **that micellar systems became heterogeneous when PC was added** at 25% for DSPE-PEG 2000, and 40% for DSPE-PEG 5000, respectively. Above these critical PC ratios a significant increase in aggregation number and formation of rod like particles were observed by small angle neutron scattering (SANS).

In **systems with different fractions 1,2-dipalmitoyl-sn-glycero-3-phosphocholine: DSPE-PEG(2000) PEGylation** leads to two features of the system: **(1) Spherical vesicles present a window of elevated chain-melting temperatures and (2) lipid packing shape-controlled liposome-to bicelle transition.** The first finding is significant for targets requiring multiple release sequences and the second enables tuning the release by composition and the PEG polymer length.

Viitala, Lauri, et al. "Shape and phase transitions in a PEGylated phospholipid system." *Langmuir* 35.11 (2019): 3999-4010.

Ashok, Beena, et al. "In vitro characterization of PEGylated phospholipid micelles for improved drug solubilization: effects of PEG chain length and PC incorporation." *Journal of pharmaceutical sciences* 93.10 (2004): 2476-2487.

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PRODUCTS WITH DXPE MPEG 2000/5000

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PRODUCT EXAMPLES WITH DXPE MPEG 2000/5000

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Product: **Doxil**
DS: Doxorubicin HCl
Dosage Form: Liposomes for iv administration
Indication: Cancer
Lipid Excipients: Cholesterol, HSPC, DSPE-MPEG 2000
Function of pegylated lipid: Stealth effect

Product: **Definity**
DS: Perflutren
Dosage Form: Microbubble suspension for iv administration
Indication: Diagnostic Ultrasound Enhancement
Lipid Excipients DPPA, DPPC, DPPE MPEG 5000
Function of pegylated lipid: Stabilisation

PRODUCT EXAMPLES WITH DXPE MPEG 2000/5000

Lipoid

Product: **IMCIVREE**
DS: Setmelanotide
Dosage Form: Solution for sc administration
Indication: Chronic weight management
Lipid Excipients DSPE MPEG 2000
Function of pegylated lipid: Complexation between phosphate group of PL and cationic peptide

PHARMACOPOEIAL STATUS of DXPE MPEG 2000/5000:

Monographs of DSPE MPEG 2000 expected in 2025 in USP/NF and CP

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RESEARCH WITH DXPE MPEG 2000/5000

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LIPODISKS WITH DXPE MPEG 2000/5000

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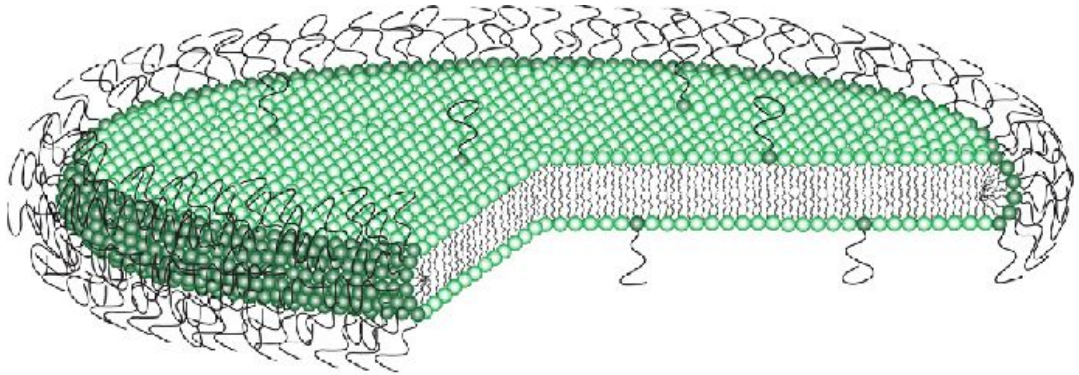
Features

- Lipodisks are **nano-sized lipid bilayers** stabilized into a discoidal shape by edge-active PEG-lipids
- Size-range from about **20 to 100 nm**
- **Larger loading capacity for lipophilic/hydrophobic drugs** per particle compared to liposomes
- Composition regarding type of lipids and PEG-lipids, can be adapted to optimize the loading of particular drugs
- Non-spherical particle shape may **favourably affect the blood circulation time and biodistribution**
- Display excellent **temperature, concentration and long-term stability**
- Animal studies indicate **plasma half-lives superior to those of, e.g., PEGylated liposomes**



Zetterberg, Malin Morin, et al. "Optimization of lipodisk properties by modification of the extent and density of the PEG corona." *Journal of colloid and interface science* 484 (2016): 86-96.

LIPODISKS WITH DXPE MPEG 2000/5000

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Example

Tumor-targeting **lipodisk nanoparticle** formulation with fixed ratio of two hydrophobic model drugs, **doxorubicin (DOX) and paclitaxel (PTX).**

pH-sensitive peptide (SAPSP) incorporated into lipodisks effectively enhanced the tumor-targeting and cell internalization.

The obtained co-loaded lipodisks were approximately 30 nm with a pH-sensitive property.

Efficacy tested in the ratiometric co-delivery of two drugs via lipodisks was confirmed in both the drug-resistant MCF-7/ADR cell line and its parental MCF-7 cell line *in vitro*, as well as in a tumor-bearing mouse model *in vivo* compared with a cocktail solution of free drugs.

Co-loaded lipodisks exerted improved cytotoxicity to tumor cells in culture, particularly to drug-resistant tumor cells at synergistic drug ratios.

In an *in vivo* xenograft mouse model, the anti-tumor ability of co-loaded lipodisks was evidenced by the **remarkable inhibitory effect on tumor growth** of either MCF-7 or MCF-7/ADR tumors.

It is concluded that for co-loaded lipodisks, cytotoxicity data *in vitro* could be used to predict their inhibitory activity *in vivo*, potentially **enhancing the clinical outcome of synergistic therapy.**

Feng, Chunlai, et al. "Ratiometric co-encapsulation and co-delivery of doxorubicin and paclitaxel by tumor-targeted lipodisks for combination therapy of breast cancer." *Inter*

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SUITABILITY OF DXPE MPEG 2000/5000 FOR LNPs?

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DXPE MPEG 2000/5000 ALTERNATIVE TO DMG-PEG IN LNPs ??

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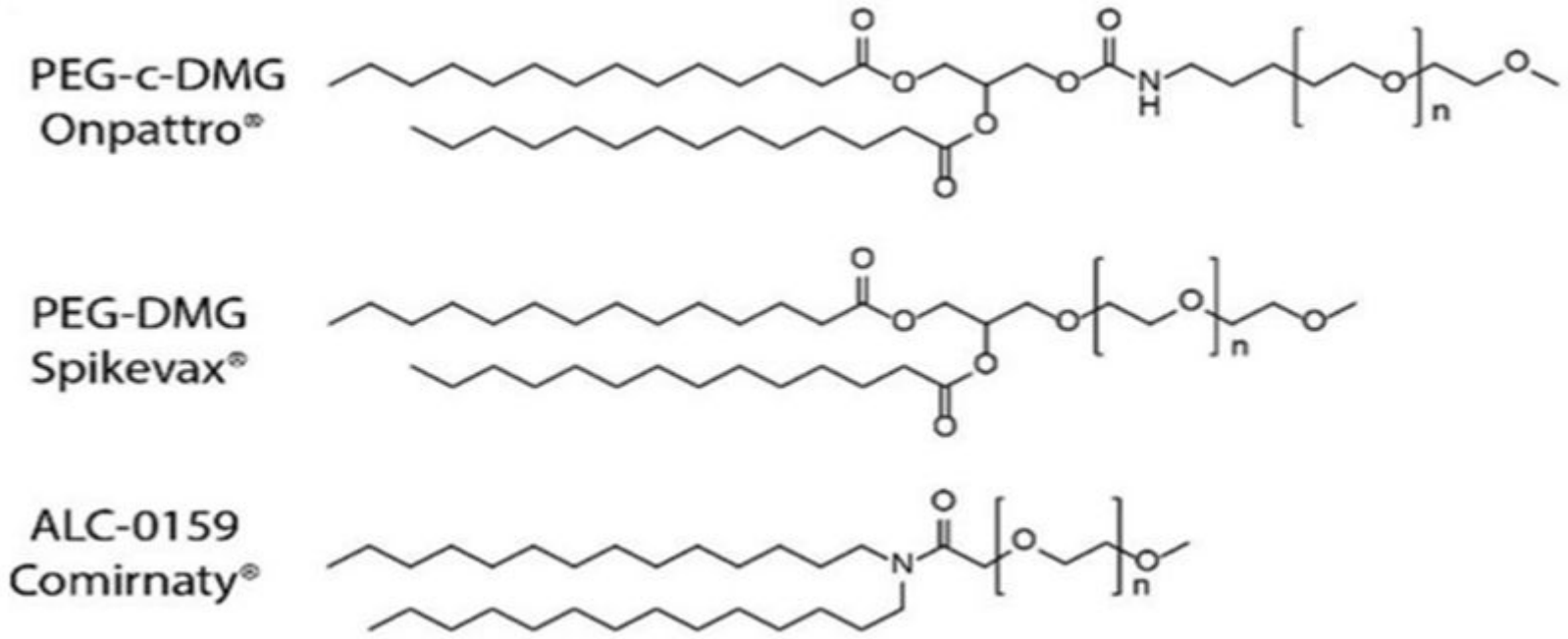


FUNCTION OF DMG-PEG IN LNPs

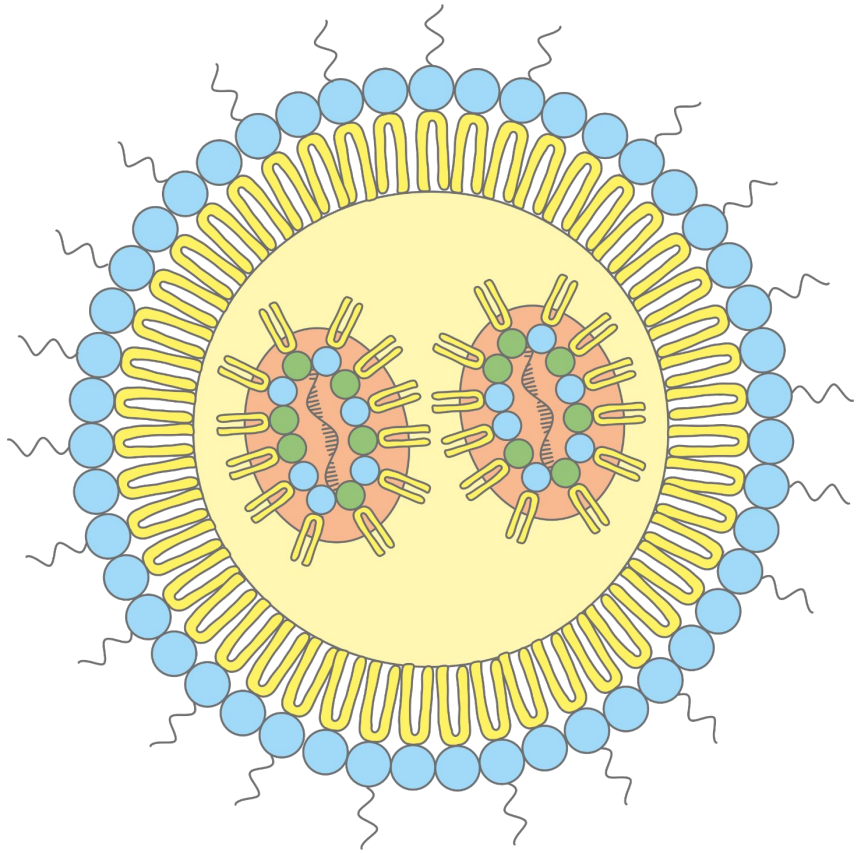
PEG-lipids constitute the smallest molar percentage of the lipid components in LNPs (typically ~1.5 mol%),

- Population size and dispersity
- LNP aggregation prevention
- Particle stability during both preparation and storage.

- Nucleic acid encapsulation efficiency/circulation half-life
- In vivo distribution
- Transfection efficiency
- Immune response

Influenced by the molar ratio of the PEG-lipid and structure and length of both the PEG chain and the lipid tail (alkyl/dialkyl chain(s)).

Downside: Immunogenicity



FUNCTION OF DMG-PEG IN LNPs: ONPATTRO /VACCINES

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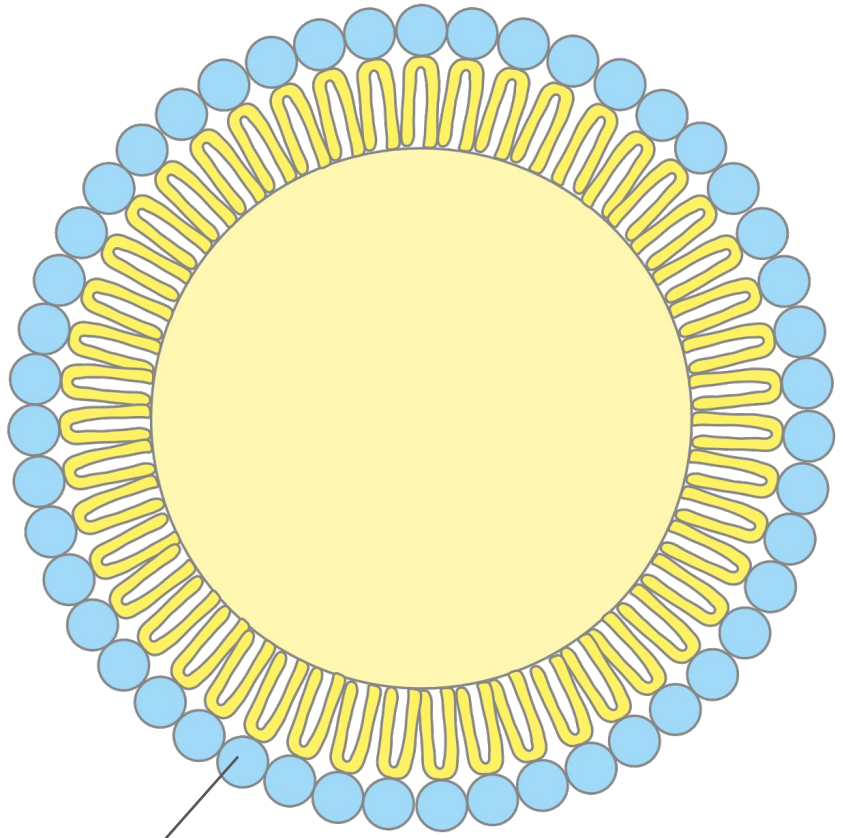
Diffusible short chain DMG-PEG 2000: leaves after injection LNPs to enable ApoE interaction and endocytic uptake:……

DMG-PEG main function is to prevent LNP aggregation

- Is DMG-PEG really needed in LNPs (for liver targeting and vaccines)?
- Are charged lipids to prevent aggregation of LNPs by means of charge repulsion possibly useful/better alternatives?
  - → PS, PG, PA, PI, CL, fatty acids are a standard method to stabilize liposomes and emulsions



ALTERNATIVES TO DMG-PEG IN LNPS: DXPE MPEG 2000/5000?

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High-throughput screening for PEG analogs

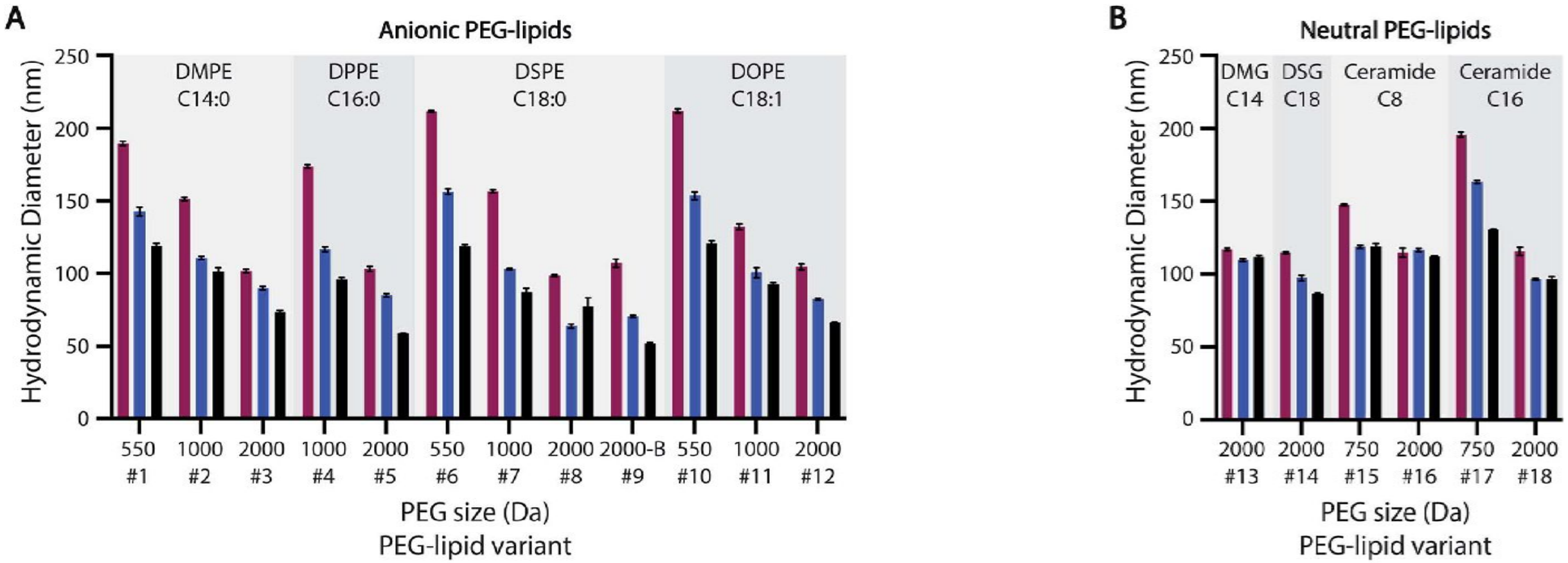
A low-volume, automated, high-throughput screening (HTS) workflow for the preparation, characterization, and in vitro assessment of LNPs loaded with a therapeutic antisense oligonucleotide (ASO) was developed. A **library of 54 ASO-LNP formulations with distinct PEG-lipid compositions was prepared** using a liquid handling robot and assessed for their physiochemical properties as well as gene silencing efficacy in murine cortical neurons.

LNPs with various PEGylated lipids were prepared using a high-throughput approach reported previously.32 Briefly, the ASO was dissolved at 93.9 mg mL1 in citrate buffer (25 mM, pH 4.0) and dispensed at 150 mL per well in a 96-well plate (Greiner Bio One 655101, NC, USA) using a TECAN Freedom EVO robotic liquid handler (Tecan Life Sciences, NC, USA). Using the automation setup, **different lipid mixtures composed of MC3, DSPC, cholesterol, and respective PEG-lipid analogues were prepared in ethanol at a molar ratio of 40 : 10 : (50 X) : X, where X ¼ 1, 3, or 5; and a total lipid concentration of 4 mM was used to maintain** N : P = 2 for the resulting ASO-LNPs.

Sarode, Apoorva, et al. "Predictive high-throughput screening of PEGylated lipids in oligonucleotide-loaded lipid nanoparticles for neuronal gene silencing." *Nanoscale advances* 4.9 (2022): 2107-2123.

ALTERNATIVES TO DMG-PEG IN LNPS: DXPE MPEG 2000/5000?

High-throughput screening for PEG analogues



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ALTERNATIVES TO DMG-PEG IN LNPS: DXPE MPEG 2000/5000?

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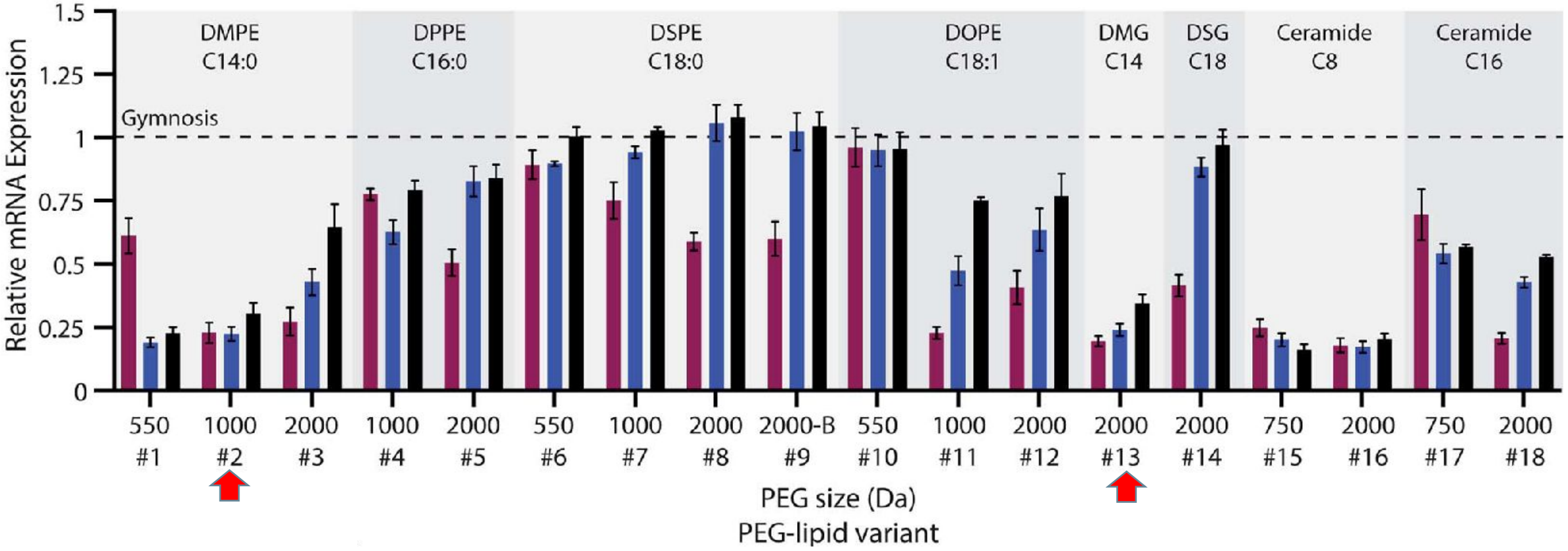
High throughput screening for PEG analogues



Sarode, Apoorva, et al. "Predictive high-throughput screening of PEGylated lipids in oligonucleotide-loaded lipid nanoparticles for neuronal gene silencing." *Nanoscale advances* 4.9 (2022): 2107-2123.

COMPARISON OF DMG-PEG WITH DMPE MPEG 2000/5000

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***In vitro* activity of different RNA-loaded lipid nanoparticle formulations in an air-liquid interface culture system mimicking the lungs: TEST FORMULATIONS**

Molar ratios	LNP 1	LNP 2	LNO 3 = Onpattro® like	LNP4
50	D-Lin-MC3-DMA	D-Lin-MC3-DMA	D-Lin-MC3-DMA	D-Lin-MC3-DMA
38.5	Cholesterol	Cholesterol	Cholesterol	Cholesterol
10	DOPE	DOPE	DSPC	DSPC
1.5	PEG-DMG	PEG-DMPE	PEG-DMG	PEG-DMPE

by Lipoid GmbH: Poster S. Rademacker et al, ***In vitro* activity of different RNA-loaded lipid nanoparticle formulations in an air-liquid interface culture system mimicking the lungs, 6th annual RNATx** Worcester, Massachusetts, USA

ALTERNATIVES TO DMG-PEG IN LNPS: DXPE MPEG 2000/5000?

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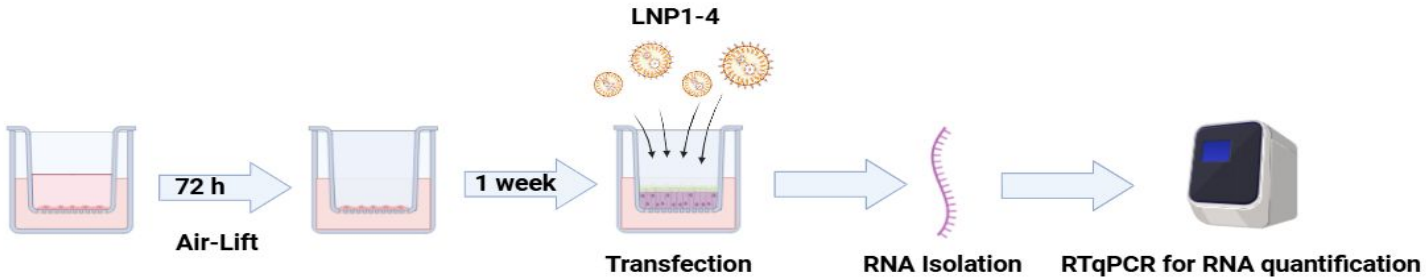
In vitro activity of different RNA-loaded lipid nanoparticle formulations in an air-liquid interface culture system mimicking the lungs: METHODS



To assess the suitability of the formulations for lung application, the LNPs were tested in an **air-liquid interface (ALI) system.** ALI cell culture systems mimic the lungs more realistically than conventional systems, as a **pseudostratified epithelium and mucociliary differentiation can be achieved.**

For the ALI cell culture, human lung cancer cells (Calu-3) were seeded on Trans wells. The airlift was performed and cells were transfected with the LNP formulations. **Uptake was followed with fluorescently labelled siRNA** (AF488) LNPs, labelled siRNA with Lipofectamine as a positive control and labelled but free siRNA as a negative control using flow cytometry.

For the **knockdown evaluation, the cells were transfected with siRNA GAPDH** encapsulated in LNPs, and noncoding RNA-loaded LNPs as a control. After RNA isolation and readout with RT-qPCR was performed, using beta-actin as the housekeeping gene for comparison.

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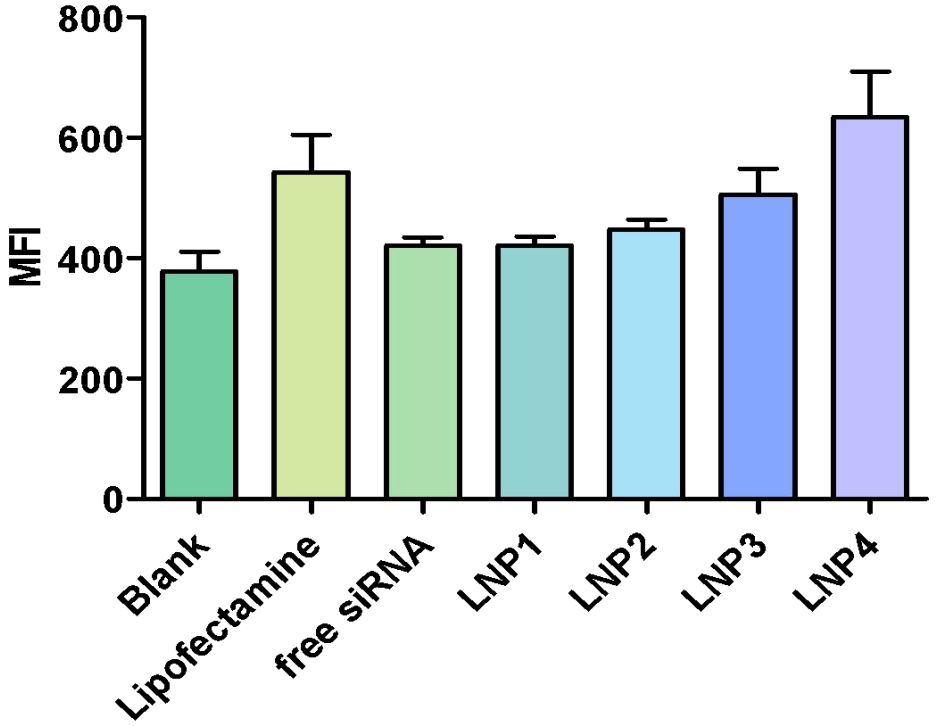
***In vitro* activity of different RNA-loaded lipid nanoparticle formulations in an air-liquid interface culture system mimicking the lungs: RESULTS**

In vitro cellular uptake of siRNA LNPs in Calu-3 cells in an ALI model



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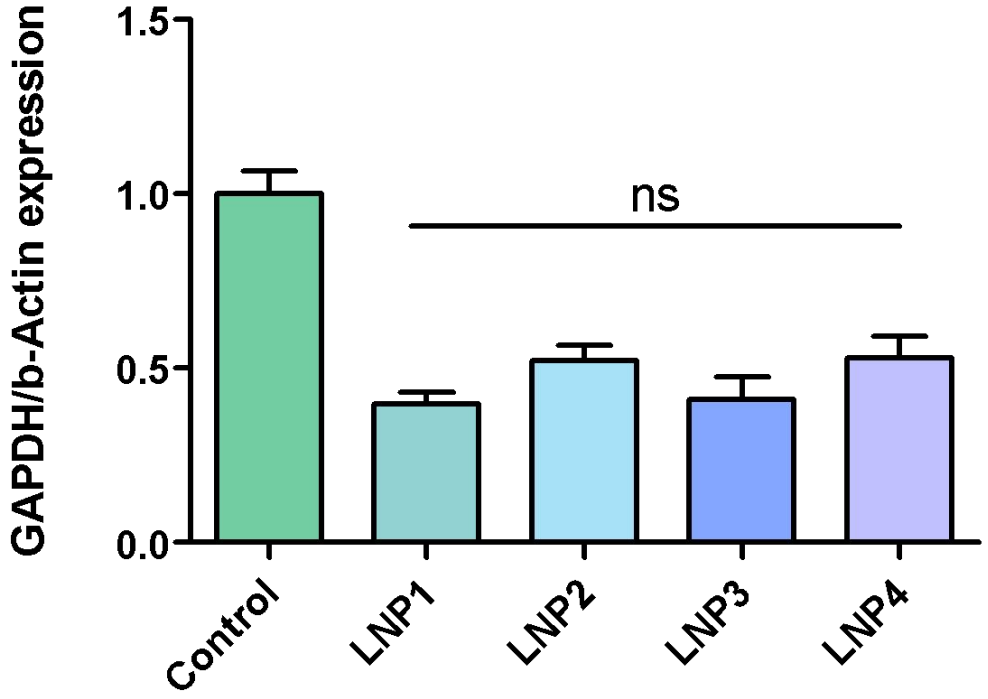
***In vitro* activity of different RNA-loaded lipid nanoparticle formulations in an air-liquid interface culture system mimicking the lungs: RESULTS**

In vitro gene knockdown of GAPDH in Calu-3 cells in an ALI model using LNPs with siRNA GAPDH



ALTERNATIVES TO DMG-PEG IN LNPS: DXPE MPEG 2000/5000?

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***In vitro* activity of different RNA-loaded lipid nanoparticle formulations in an air-liquid interface culture system mimicking the lungs: CONCLUSIONS**

- The various LNP formulations **do not exhibit notable differences concerning physicochemical properties**. However, **significant differences in gene knockdown efficiency and cellular uptake were observed among the siRNA formulations** but not for the mRNA formulations.
- The knockdown efficacy of the LNPs is **significantly influenced by the choice of the helper lipid.** Notably, the **superior performance of DOPE** in comparison to DSPC underlines the potential benefits of DOPE's cone-like structure for facilitating endosomal escape by promoting a hexagonal phase formation.
- Altering the **PEGylation of the LNPs** by using PEG-DMPE compared to PEG-DMG **neither influences the gene knockdown nor the gene expression efficiency**. **PEG-DMPE might however slightly improve the uptake** of the LNPs into the cells.
- The LNP formulations demonstrated efficient gene silencing and expression effects in submerged cultured lung cell lines.

SUMMARY

Take Home Messages

- DXPE MPEG 2000/5000 are versatile excipients
- They give STEALTH effects to particles and can be used as solubilizer, stabilizer, emulsifier and for surface coating of stents
- Lipodisks with DXPE MPEG 2000/5000 may be alternative drug carriers for lipophilic drugs compared to liposomes
- In LNPs short fatty acid chain DXPE MPEG 2000/5000 can be used as alternative to DMG-PEG
- In case LNP targeting outside the liver is desired DXPE MPEG 2000/5000 with longer fatty acid chains can be used
- Lipoid provides DXPE MPEG 2000/5000 at industrial scale fulfilling regulatory requirements
- Lipoid can offer strong regulatory support for DXPE MPEGs

FURTHER INFORMATION

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or consult our webpage: https://lipoid.com/en/