Lipid nanoparticles containing labile PEG-lipids transfect primary human skin cells more efficiently in the presence of apoE

Camilla Hald Gregersen\textsuperscript{a,b}, Razan Mearraoui\textsuperscript{a,b}, Pia Pernille Søgaard\textsuperscript{c}, Gael Clergeaud Veiga\textsuperscript{b}, Karsten Petersson\textsuperscript{a}, Andrew Urquhart\textsuperscript{b}, and Jens B. Simonsen\textsuperscript{a,*}

\textsuperscript{a} Explorative Formulation & Technologies, CMC Design and Development, LEO Pharma A/S, 2750 Ballerup, Denmark
\textsuperscript{b} Department of Health Technology, Technical University of Denmark, 2800 Kongens Lyngby, Denmark
\textsuperscript{c} In Vitro Biology, Molecular Biomedicine, Research and early development, LEO Pharma A/S, 2750 Ballerup, Denmark

* Corresponding author: jbsimonsen@gmail.com
ABSTRACT

Nucleic acid-based therapeutics encapsulated into lipid nanoparticles (LNPs) can potentially target the root cause of genetic skin diseases. Although nanoparticles are considered impermeable to skin, research and clinical studies have shown that nanoparticles can penetrate into skin with reduced skin barrier function when administered topically.

Studies have shown that epidermal keratinocytes express the low-density lipoprotein receptor (LDLR) that mediates endocytosis of apolipoprotein E (apoE)-associated nanoparticles and that dermal fibroblasts express mannose receptors. Here we prepared LNPs designed to exploit these different endocytic pathways for intracellular mRNA delivery to the two most abundant skin cell types, containing: (i) labile PEG-lipids (DMG-PEG2000) prone to dissociate and facilitate apoE-binding to LNPs, enabling apoE-LDLR mediated uptake in keratinocytes, (ii) non-labile PEG-lipids (DSPE-PEG2000) to impose stealth-like properties to LNPs to enable targeting of distant cells, and (iii) mannose-conjugated PEG-lipids (DSPE-PEG2000-Mannose) to target fibroblasts or potentially immune cells containing mannose receptors. All types of LNPs were prepared by vortex mixing and formed monodisperse (PDI ~0.1) LNP samples with sizes of 130 nm (±25%) and high mRNA encapsulation efficiencies (≥90%). The LNP-mediated transfection potency in keratinocytes and fibroblasts was highest for LNPs containing labile PEG-lipids, with the addition of apoE greatly enhancing transfection via LDLR. Coating LNPs with mannose did not improve transfection, and stealth-like LNPs show limited to no transfection.

Taken together, our studies suggest using labile PEG-lipids and co-administration of apoE when exploring LNPs for skin delivery.

KEYWORDS

Lipid nanoparticles; PEGylation; Apolipoprotein E; Low-Density Lipoprotein Receptor; Mannose; Epidermal keratinocytes; Dermal fibroblasts
1. INTRODUCTION

Lipid nanoparticles (LNPs) are nanometer sized particles, typically containing phospholipids, cholesterol, polyethylene glycol (PEG)-lipids, ionizable lipids and encapsulated nucleic acid cargo (Hald Albertsen et al., 2022). The approval of LNPs for siRNA-delivery to the liver to treat hereditary transthyretin-mediated amyloidosis (Onpattro® in 2018) (Adams et al., 2018) and their use in the mRNA-based COVID-19 vaccines Spikevax® (Baden et al., 2021) and Comirnaty® (Polack et al., 2020) from Moderna and Pfizer-BioNTech, respectively, have sparked a huge interest in using nucleic acid modalities to treat various types of diseases. The success of LNPs in delivering nucleic acids to target cells is ascribed to their ability to encapsulate large nucleic acid entities, protect these from degradation, and facilitate their cytosolic delivery. However, LNPs have not yet fully solved the fundamental problem of nanomedicines – how to efficiently target extrahepatic organs when administered intravenously. To circumvent this problem, many clinical trials which e.g. test cancer therapies/vaccines based on LNP-mRNA use intratumoral administration of LNPs (Hald Albertsen et al., 2022). Along these lines, the use of topically administered LNPs to treat local skin diseases may be an attractive approach to explore. However, no work to the best of our knowledge has been reported on LNP studies for topical use, likely due to the hard-to-penetrate stratum corneum (SC) barrier that comprises the outermost layer of the skin. Instead, intradermal injections as the local route of administration to the skin have been explored. A comprehensive study by Blakney et al. (2021) evaluated the transfection capability of different lipoplexes (mRNA associated at the surface of liposomes) across cell types in skin explants. They used a large flow cytometry panel to study single cell suspensions from skin explants in which lipoplexes were injected intradermally. They show that non-active targeting lipoplexes could transfect fibroblast cells in addition to other types of cells, including different kinds of immune cells. However, this study did not study keratinocytes, another important type of skin cells, and did not use LNPs containing the nucleic acid payload in the core. Dong et al. (2023) also used intradermal injection to administer vascular endothelial growth factor A (VEGFA)-mRNA encapsulated into LNPs with a lipid composition similar to that of Onpattro®, to successfully promote wound healing in a skin wound mouse model. These studies showcase that intradermal injections can be a way of overcoming the SC for LNP administration. That said, many skin diseases are associated with impaired skin barriers and thus provide a possibility for nanosized delivery systems to transfect skin cells, including the viable keratinocytes that are present in the lower parts of the epidermis below the SC and fibroblasts situated below the epidermis in the dermis, among different types of immune cells. Several research studies have shown that nanoparticles, including liposomes and polymers, can be used as drug delivery vehicles into impaired skin (Alam et al., 2023; Boakye et al., 2015; Knudsen et al., 2011) or target hair follicles in intact skin (Boakye et al., 2015; Guan et al., 2023; Han et al., 2004; Lademann et al., 2007). Recently, a viral nanoparticle approach was proven successful in a clinical phase 3 trial for use as a topical gel to treat dystrophic epidermolysis bullosa (DEB) (Guide et al., 2022). The virus transducts keratinocytes with DNA encoding the full COL7A1 gene that is mutated in DEB patients and thereby enables the production of the type VII collagen protein that is essential for skin integrity. This example showcases that nanoparticle-based nucleic acid delivery indeed is possible in the context of a blistering skin disease. That said, LNPs have some advantages over viral vectors, including that they could be safer due to the absence of immunogenic viral proteins, can deliver larger nucleic acid payloads, and are easier to synthesize and manufacture at large scale.

Here we aim to evaluate the transfection potency of LNPs in the most abundant cells of the skin, the keratinocytes and the dermal fibroblasts. These cells are the target for gene delivery in genetic skin diseases that lead to impairments in skin integrity like DEB. Specifically, we prepare, characterize, and evaluate the transfection efficiency of three different classes of LNP formulations as shown in Figure 1A:

1. Onpattro-like LNPs. These have a lipid composition identical to the Onpattro® formulation, including an 1,2-dimyristoyl-sn-glycero-3-methoxypolyethylene glycol (DMG)-PEG lipid (Figure 1A),

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but contain mRNA instead of siRNA. Previous studies on LNP-siRNA (Onpattro®) accumulation in the liver show that the low-density lipoprotein receptor (LDLR) on hepatocytes mediates the LNP uptake. Accordingly, in apoE-deficient or LDLR-deficient mice, LNP-siRNA do not display the same gene silencing potency (Akinc et al., 2010). Hence, it has been suggested that Onpattro® LNPs exploit the apoE-LDLR pathway that is used for the uptake of cholesterol from endogenous remnant chylomicrons to the hepatocytes (Cooper, 1997) by dissociation of the labile saturated 14-carbon chained DMG-PEG into the biological environment followed by LNP association of apoE. Interestingly, shotgun proteomics of the different types of human skin cells (Dyring-Andersen et al., 2020) show a relative higher level of LDLR on keratinocytes compared to other skin cells including fibroblasts (Figure 1B). Hence, we wanted to test if the Onpattro-like LNPs can exploit the apoE-LDLR pathway in keratinocytes as depicted in Figure 1C.

(2) Stealth-like LNPs. These contain 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE)-PEG (Figure 1A), the PEG-lipid used in the long circulating Doxil® liposomes (Wei et al., 2016), also known as stealth liposomes. This saturated 18-carbon chained PEG-lipid is less prone to dissociate from LNPs compared to the DMG-PEG (Mui et al., 2013), used in Onpattro® (Adams et al., 2018) and the Onpattro-like formulation, and thus maintain the PEG-coating known to reduce opsonization and interactions with cells. Hence, this formulation potentially serves as a control to highlight the importance of using labile PEG-lipids like DMG-PEG to drive immediate cellular transfection in skin cells. Interestingly, the potential lack of immediate interactions with skin cells may enable stealth-like LNPs to reach distant tissues as illustrated in Figure 1C.

(3) Mannose-coated LNPs. These are based on mannose anchored to the tail of the PEG moiety on DSPE-PEG (Figure 1A). This PEG-lipid, DSPE-PEG-Mannose, is expected to stick well to the LNP surface like the bare DSPE-PEG in the stealth-like LNPs. The proteomic study discussed above (Dyring-Andersen et al., 2020) identified a higher level of the mannose receptor MRC2 (also known as endo180) on fibroblasts relative to keratinocytes. Other studies (East and Isacke, 2002; Hespanhol et al., 2005; Norregaard et al., 2020; Sheikh et al., 2000) support the existence of mannose receptors on dermal fibroblasts. The ability of MRC2 to mediate efficient endocytic uptake of mannose remains unclear, as the receptor is discussed in contradictory ways in different studies (East and Isacke, 2002; East et al., 2002; Sheikh et al., 2000). Indeed, a more established role of MRC2 is its ability to mediate endocytic uptake of collagen by fibroblasts and other cell types (Madsen et al., 2007). Uptake of collagen is an important process during wound healing and MRC2 has been reported to be upregulated in both fibroblasts and keratinocytes during this process (Honardoust et al., 2006). Another type of mannose receptor is MRC1, a highly effective endocytic receptor which is predominantly expressed on immune cells like macrophages and dendritic cells (also identified in Figure 1B) where it mediates internalization of mannosylated pathogens. Mannose-coated LNPs may thus transfect fibroblasts, but may alternatively also be efficient in transfecting immune cells like macrophages. Indeed, mannose has previously been used as targeting-ligand for intracellular uptake of mannose-coated polymeric nanoparticles via mannose receptors to genetically reprogram macrophages through delivery of exogenous mRNA (Zhang et al., 2019).

Taken together, we envision that the stealth-like LNPs can escape the keratinocytes and fibroblasts and potentially transfect distant cells upon local topical application of the LNPs. Mannose-coated LNPs may in contrast be able to actively target fibroblasts and may additionally be able to transfect skin-associated macrophages, which are known to contribute to pathogenesis in many diseases of the skin (Yanez et al., 2017). On the other hand, we expect that the Onpattro-like LNPs have the potential to exploit the apoE-LDLR pathway for uptake in keratinocytes, when the labile DMG-PEG lipids dissociate from the LNPs into the biological environment allowing the LNP to pick up endogenous and/or exogenous apoE (Akinc et al., 2010). To test the latter uptake mechanism, we investigate whether the presence of exogenous apoE in the aqueous medium and functionality of the low-density lipoprotein receptor (LDLR) (studied by inhibitory antibodies against LDLR) enhance the uptake of Onpattro-like LNPs in primary keratinocytes and fibroblasts. The presence of
endogenous apoE in skin fluids, co-administrating apoE with the LNPs or introducing apoE as a targeting moiety immobilized onto LNPs could potentially present different strategies to drive efficient LNP uptake in keratinocytes in vivo.

Figure 1. A) The molecular structure of the three different PEG-lipids (left) used to prepare the three different classes of LNPs (Onpattro-like, stealth-like and mannose-coated LNPs) that are being studied. All PEG-lipids used in this study contain PEG2000. Their proposed structures are shown (right). B) Relative expression of LDLR and mannose receptors (MRC1 and MRC2) across skin layers and relevant skin cells derived from shotgun mass spectrometry proteomics (Dyring-Andersen et al., 2020). The data shown has been recalculated from log2 values. C) Proposed cellular targets of Onpattro-like LNPs and mannose-coated LNPs in the skin, with stealth-
like LNPs expected to be shielded towards uptake by the keratinocytes and fibroblasts. Illustrations (A) and (C) are created with BioRender.com.
2. RESULTS AND DISCUSSION

2.1 Preparation of different PEGylated LNPs

The LNPs that we prepared were loaded with mRNA and contained a lipid composition identical or similar to Onpattro® (FDA, 2017), including (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA or MC3), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and PEG-lipids (Figure 1A) in a 50:38.5:10:1.5 molar ratio. All PEG-lipids contain a PEG-2000 moiety. We used commercially available mRNA encoding the enhanced green fluorescent protein (EGFP) that served as an mRNA model compound and allowed us to evaluate the transfection potency of the LNPs based on the fluorescence intensity from the translated EGFP. An N/P molar ratio of 6:1 was used for all LNP formulations. The only difference between the three classes of LNPs is the type of PEG-lipid used in the LNP formulation as shown in Figures 1A and B. While the Onpattro-like LNPs are based on DMG-PEG, and thus, contain a lipid composition identical to Onpattro®, stealth-like LNPs contain DSPE-PEG, and mannose-coated LNPs contain DSPE-PEG-Mannose. In addition to these three single PEG-lipid LNPs, three LNPs based on a 1:1 molar ratio between two different PEG-lipids were prepared. These include the ‘50% stealth-like LNPs’ containing 50% DSPE-PEG (the stealth PEG-lipid) and 50% DMG-PEG, ‘50% mannose-coated LNPs’ containing 50% DSPE-PEG-Mannose and 50% DMG-PEG, and ‘mannose-stealth-like LNPs’ containing 50% DSPE-PEG-Mannose and 50% DSPE-PEG, keeping the molar percentage of PEG-lipid constant (1.5 mol%). These two PEG-lipid component LNPs were included to learn whether the physicochemical and biological properties of LNPs depend on the surface density of the DSPE-PEG and DSPE-PEG-Mannose. Hence, we prepared in total six different LNP formulations based on three different PEG-lipids and three combinations thereof. An overview of the molar composition of these LNPs is presented in Figure 2A.

The LNPs were prepared in a simple fashion by vortex mixing (Figure 2B), as described in Materials & Methods. We performed optimization studies based on the Onpattro-like LNP formulation to prepare small and monodisperse LNPs with high encapsulation from vortex mixing (Figure S1). We learned that too high buffer concentration of the negatively charged citrate may compete with mRNA in binding to the positively charged ionizable lipids, explaining the observed increase in encapsulation efficiency (EE) using a citrate buffer (pH 4.4) of 25 mM compared to 100 mM (Figure S1B). Variations in lipid and mRNA concentrations (while keeping the N/P ratio and flow rate constant) have not been shown to alter LNPs characteristics when using the commercial microfluidic system NanoAssemblr™ to prepare LNPs (Carrasco et al., 2021). In contrast, the size, uniformity and
EE of LNPs prepared from vortex mixing may to a higher extent depend on the lipid and mRNA concentration, due to the more random mixing leading to heterogeneity in local concentrations and thus different kinetics for the self-assembly of the different components into LNPs. LNPs prepared with final lipid concentrations of 2.5 mM, 5 mM and 10 mM and constant N/P ratio revealed an increase in size when the lipid concentration increases, with rather stable PDI and EE (Figures S1C and S1D) across the concentrations. Based on these optimization studies, the preparation methods that formed LNPs with smallest size and high EE (citrate buffer of 25 mM and a final lipid concentration of 2.5 mM) were used for all the LNPs prepared by vortexing. We also prepared Onpattro-like LNPs using the NanoAssemblr™ Ignite to compare these to LNPs prepared by vortexing using identical bulk composition. The Ignite ensures proper mixing of the different LNP components leading to the preparation of uniform and reproducible LNPs. Hence, this type of equipment (incl. other microfluidic systems) is regarded the gold standard method for preparing LNPs. That said, we wanted to use the vortex mixing method (also referred to as solvent precipitation) to prepare the LNPs because a vortexer is a standard equipment in many laboratories and thus cheap (no expenses on a micro-fluidic equipment and single-use micro-fluidic cassettes) and easily accessible as compared to specialized microfluidic systems like the Ignite. Interestingly, the low lipid concentration (2.5 mM) used for the vortex mixing also allowed us to use a low amount of mRNA (20 µg) for each formulation, and thus, keep the overall costs per LNP formulation low. The latter feature is attractive in large LNP screening studies.

2.2 The physicochemical methods used to characterize the LNPs

The freshly prepared LNP samples were characterized by dynamic light scattering (DLS) to derive hydrodynamic diameter and polydispersity index (PDI), and by Ribogreen® RNA assay to calculate the total mRNA content and the EE. We learned that a slight increase in the EE is obtained in the dialyzed LNP samples (95 ± 2 % (n = 9) for Onpattro-like LNP formulations) as compared to the non-dialyzed (90 ± 5 % (n = 10) for Onpattro-like LNP formulations). We speculate that this is due to preferential adsorption of mRNA to the dialysis membrane and/or removal of free mRNA during the dialysis step.

2.2.1 Characterization of Onpattro-like LNPs prepared by vortex mixing and Ignite

Key to this study was to ensure that the vortex mixing preparation method produced uniform and reproducible LNPs. Hence, two scientists prepared in total 10 Onpattro-like LNP formulations to get a proper statistical basis for the evaluation of the batch-to-batch variations and the physicochemical quality of the LNPs prepared by vortex mixing. It is clear from the size (128 ± 6 nm), PDI (0.11 ± 0.01), and EE (90 ± 5 %) data presented in Figure 3 that uniform and reproducible Onpattro-like formulations were prepared independent of the formulation scientist that prepared the LNPs. That said, we observed that Onpattro-like LNPs prepared by the Ignite form smaller-sized LNPs (about 86 nm) in good agreement with previous studies on similar formulations (Carrasco et al., 2021; Zhang et al., 2020), with PDI values slightly lower than the Onpattro-like LNPs prepared with vortex mixing. The EE is also slightly higher compared to the vortexed LNP samples. To conclude, LNPs prepared by Ignite are generally smaller, slightly more monodisperse and with a slightly higher EE, and the formulation method is often more suitable for preparing larger scale batches of LNPs. However, we learned that vortex mixing can indeed be used to form uniform and reproducible LNPs at a scale suitable for in vitro testing. A study by Wang et al. (2023) supports the notion that vortex mixing can be used to prepare smaller scale batches of LNPs, reporting very similar size, PDI and EE for LNP-mRNA formulations with slightly different lipid compositions than in this study. Kamiya et al. (2022) questioned whether vortex mixing affected LNP-mRNA stability, reporting a collapse of the nanoparticle structure after 5 minutes of vortex mixing, while 30 seconds was suggested to be tolerable exposure time. In this study, just 15 seconds of vortex mixing was found sufficient for LNP-
mRNA formulation, suggesting no major effect on stability. Based on these findings, we decided to use the vortex method for the preparation of the other types of LNPs taking the low costs and simple and rapid LNP preparation associated with the vortex mixing method into account.

2.2.2 Characterization of the different PEGylated LNPs

Across the six LNP formulations prepared by vortexing (defined in Figure 2A), we observed a small variation in size corresponding to at highest ±25% with respect to the average size of the Onpattro-like LNPs (128 nm) (Table 1). Interestingly, we noticed that LNPs containing DSPE-PEG, including 50% stealth-like, stealth-like, and mannose-stealth-like LNPs gave rise to a smaller LNP size compared to Onpattro-like LNPs. The different packing parameters and hydrophobic match/mismatch with DSPC of DMG-PEG and DSPE-PEG could partly contribute to their size differences. We also observe that the introduction of a targeting-ligand, in our case mannose, to the DSPE-PEG lipid seems to alter the LNP size slightly (when comparing stealth-like (107 ± 13 nm) with mannose-coated LNPs (154 nm) or 50% stealth-like (95 nm) with 50% mannose-coated LNPs (129 ± 8 nm)). We do not expect that the packing parameters between DSPE-PEG and DSPE-PEG-Mannose are very different. We cannot rule out that the observed small size differences could be due to compositional heterogeneities in the DSPE-PEG and DSPE-PEG-Mannose products, including different PEG length variations and contaminations. Kim et al. (2021) prepared mannose-coated LNP-mRNA with a slightly different lipid composition. When exchanging C16-PEG ceramide with DSPE-PEG-Mannose, they also showed an increase in size when incorporating DSPE-PEG-Mannose. However, Zhou et al. (2022) showed that functionalization of LNPs with mannose (DSPE-PEG-MAN) did not alter the LNP structure when visualized by transmission electron microscopy (TEM). Importantly, all LNP formulations exhibit similar and acceptable PDI (~0.1) and EE (~90%) values.
Table 1. List of the different LNP formulations prepared by vortex mixing (except one formulation that was prepared by Ignite, specified in the table) and their physicochemical characteristics. The lipid composition of the different LNP formulations is illustrated in Figure 2A.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size [nm] (^a)</th>
<th>PDI (^a)</th>
<th>Encapsulation efficiency [%] (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onpattro-like LNP</td>
<td>128 ± 6</td>
<td>0.11 ± 0.01</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>Onpattro-like LNP (Ignite)</td>
<td>86 ± 8</td>
<td>0.07 ± 0.02</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>50% stealth-like LNP</td>
<td>95</td>
<td>0.11</td>
<td>90</td>
</tr>
<tr>
<td>Stealth-like LNP</td>
<td>107 ± 13</td>
<td>0.11 ± 0.00</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>50% mannose-coated LNP</td>
<td>129 ± 8</td>
<td>0.08 ± 0.01</td>
<td>91 ± 5</td>
</tr>
<tr>
<td>Mannose-stealth-like LNP</td>
<td>95 ± 3</td>
<td>0.11 ± 0.00</td>
<td>97 ± 0</td>
</tr>
<tr>
<td>Mannose-coated LNP</td>
<td>154</td>
<td>0.05</td>
<td>94</td>
</tr>
</tbody>
</table>

Data is presented as mean ± standard deviation of \(n\) formulation replicates. \(n\) for each of the formulations are (from top to bottom): 10, 2, 1, 3, 3, 2, 1.

\(^a\) Particle diameter and PDI derived from DLS.

\(^b\) The encapsulation efficiency values derived from RiboGreen RNA assay are obtained prior to the dialysis step.
2.3 Evaluating the transfection potency of the LNPs in keratinocytes and fibroblasts

2.3.1 Transfection of keratinocytes (HEKa and HEKn)

2.3.1.1 Transfection of HEKa cells with Onpattro-like LNPs

Keratinocytes represent the primary cell type of the epidermis and is the first cell type a formulation encounters when administered topically. Studying transfection efficiency and targeting strategies for either improving uptake in, or bypassing of, keratinocytes is therefore highly relevant for topical administration of LNPs. The keratinocyte phenotype is poorly reflected in cell lines and initial in vitro studies were therefore carried out in primary human keratinocytes from an adult donor (HEKa) in order to evaluate LNP-mRNA transfection efficiency and effects on cell viability.

![Figure 4](image)

Figure 4. Transfection of primary human keratinocytes from an adult donor (HEKa) with varying doses of Onpattro-like LNPs containing EGFP mRNA for 24 hours. A) Fluorescence images of HEKa cells expressing EGFP (green) stained with Hoechst dye (blue, nuclear marker), and DRAQ7 dye (red, marker of dying/dead cells). B) Transfection efficiency is shown as MFI (EGFP) and C) cell viability is shown as percentage of live cells with dead cells identified based on DRAQ7 fluorescence. Dunnett’s multiple comparisons test was performed, comparing the control (0 ng mRNA/well) to all doses of EGFP mRNA, showing no significant difference in cell viability between the control and any of the LNP doses for p = 0.05. D) Transfection efficiency (MFI) in HEKa cells transfected with different LNP formulations at varying doses. Data is presented as mean ± standard deviation (n = 3).

Different methods are used to quantify in vitro transfection, with flow cytometry and microscopy being the most frequently used methods (Blakney et al., 2021; Kim et al., 2021; Zhang et al., 2020).
The high content analysis system Operetta CLS™ allows for a combination of fluorescence microscopy and brightfield microscopy. This enables quantification of transfection efficiency and cell viability based on fluorescence whilst simultaneously gaining information on cell morphology and confluency in the brightfield channel. As opposed to flow cytometry, where cells are analyzed in suspension, microscopy allows for the cells to be analyzed while attached to the well surface they are cultured on. This is especially relevant for adherent cells like primary keratinocytes, since it allows for monitoring of morphological changes and continuation of the adherent culture conditions during imaging. Hence, we decided to use the Operetta system to assess our transfection studies. The translation process from qualitative signal from EGFP and stains (Figure 4A) to quantitative measures of transfection (Figure 4B) and viability (Figure 4C) is described in Materials & Methods. All transfection data is reported as mean EGFP fluorescence intensity (MFI) of EGFP positive cells while all viability data is reported as percentage of live cells in the population.

We observed that the Onpattro-like LNPs transfected HEKa cells in a dose dependent manner with no saturation reached at the tested concentrations (Figure 4B). Cell viability decreased slightly with increased dosing, indicating that some toxicity may be introduced (Figure 4C). Whether this is due to the LNPs or the expression of EGFP is not clear, since EGFP production itself is known to cause toxicity in cells over time (Liu et al., 1999). However, Dunnett’s multiple comparisons test shows no significant difference in cell viability between the control and either of the LNP doses for p = 0.05. Comparing LNP-mRNA to Lipofectamine-mRNA using similar doses of mRNA (100 ng/well) reveals that both LNP and Lipofectamine are able to transfect nearly 100% of HEKa cells (Figure S3A), yet a significantly higher EGFP expression is achieved by Lipofectamine as evident from MFI data (Figure S3B). Lipofectamine™ 3000 is a transfection reagent known for its high in vitro transfection efficiency. However, Lipofectamine has been linked to severe cytotoxicity, and is thus not useable for therapeutic applications (Wang et al., 2018). In contrast, LNPs have shown to be tolerable in humans when used for both siRNA and mRNA delivery (Adams et al., 2021; Baden et al., 2021; Polack et al., 2020).

Transfection efficiency for Onpattro-like LNPs prepared using either vortex mixing or Ignite was evaluated and compared in HEKa cells (Figures S4A and S4B). This study suggested that both lead to nearly 100% EGFP positive cells for the highest doses tested, with more pronounced differences observed for MFI data, where the Ignite LNPs led to higher transfection efficiency at high doses (~2 -fold at 500 ng mRNA/well). The observed difference in transfection is likely ascribed to size and structural differences in lipid composition introduced with the different formulation methods. Variations in LNP structure, both related to the external surface and the internal packaging of mRNA, could influence both cellular interactions and endosomal escape.

The following vortexed formulations from the three different LNP classes were tested for their transfection efficiency in HEKa cells: Onpattro-like LNPs, 50% Stealth-like LNP, Stealth-like LNPs and 50% mannose-coated LNP (Figure 4D). The data clearly reveals that by substituting the labile PEG-lipid, DMG-PEG, based on 14-carbon (C14) tail in the Onpattro-like LNPs with the less labile PEG-lipid, DSPE-PEG, based on C18 tail in the stealth-like formulation, the transfection efficiency of the LNPs is significantly reduced, by a factor of 6 for the 250 ng mRNA/well dose (Figure 4D). This observation is further supported by the 50% stealth-like LNPs (containing both C14/C18-PEG) resulting in a transfection efficiency corresponding to roughly an average of the transfection mediated by stealth-like and Onpattro-like LNPs independent on the LNP dose. Considering the high level of LDLR expression in keratinocytes (Figure 1B) and the presence of apoE even under serum-free culture conditions (normal conditions for culturing primary human keratinocytes) where apoE may derive from the bovine pituary extract (BPE) (the exact composition of BPE is not available, but has certain overlaps with serum) and/or be secreted by keratinocytes (Grehan et al., 2001), it is likely that the uptake of LNPs in the keratinocytes is similar to the proposed mechanism for the uptake of Onpattro® in hepatocytes (Akinc et al., 2019). This mechanism driven by the dissociation of the labile DMG-PEG lipid, association of apoE and LDLRmediate uptake as shown in Figure 1C for
Comparing 50% mannose-coated LNPs to 50% stealth-like LNPs (Figure 4D) reveal similar transfection levels at low doses. However, an increase in transfection efficiency for 50% mannose-coated LNPs is observed at higher LNP doses, although lower than the Onpattro-like LNPs. Hence, these data suggest that the mannose-ligand linked to the 50% mannose-coated LNPs does not improve the transfection when compared to the Onpattro-like LNPs in keratinocytes. We speculate whether the slight increase in transfection when comparing 50% mannose-coated LNPs with 50% stealth-like could be driven by size-dependent uptake, since the 50% mannose-coated LNPs are larger (129 ± 8 nm) than the 50% stealth-like LNPs (95 nm). The organization of the surface lipids on LNPs, and thus, the ability for DMG-PEG lipids to dissociate from the LNPs, may likely depend on the curvature/size of the LNPs. However, we cannot exclude that low background expression of the MRC2 receptor in keratinocytes (Figure 1B) could account for some of the transfection. As mentioned previously, other studies have reported MRC2 on keratinocytes (Honardoust et al., 2006).

2.3.1.2 Transfection of HEKn cells with Onpattro-like LNPs

It has been reported that HEK cells isolated from neonatal and adult donors differ in their degree of differentiation. HEKn cells have a less differentiated phenotype with expression markers that represent more basal-like keratinocytes and, while HEKa cells are characterized by markers of the more differentiated cells associated with the upper epidermal layers (Krejci et al., 2015; Mateu et al., 2016). This is reflected in a higher rate of migration and wound healing in HEKn cells, which is in agreement with wound healing being faster in neonatal stages of life than in adulthood (Mateu et al., 2016). In a therapeutic setting it is more attractive to deliver a functional gene to the proliferatively active cells of the basal layer, as these give rise to the cells that differentiate as they transition into the upper layers of the epidermis. Furthermore, in diseases where the aim is to restore skin integrity proteins that ensure firm adhesion of dermis and epidermis (like type VII collagen in DEB), these cells are the target cells (Ain et al., 2021). Hence, we decided to include transfection studies in the more basal-like HEKn cells and study the effect of apoE in more details, encouraged by our findings from the HEKa cell studies. The basal-like keratinocytes have been reported to have higher expression of LDLR compared to the highly differentiated cells in the upper epidermal layers (Mommaas-Kienhuis et al., 1987). LDLR, along with other lipoprotein receptors, is important for the uptake of lipids, including cholesterol, that is needed for their proliferation and differentiation towards more elongated keratinocytes, which eventually form the outermost skin layer stratum corneum, consisting of fully differentiated, dead keratinocytes (corneocytes) (Abd El-Latif et al., 2010).

Figure 5. The impact of apoE levels and LDLR activity on the transfection efficiency of Onpattro-like LNPs in primary human keratinocytes from a neonatal donor (HEKn) 24 hours post transfection. A) MFI and B) cell viability for cells transfected with varying doses of LNP. C) MFI for HEKn cells treated with 300 ng mRNA/well in
presence or absence of human apoE (0 μg/mL (-) or 10 (+) μg/mL apoE) and 1 hour pretreatment with increasing anti-LDLR antibody concentrations. Data is presented as mean ± standard deviation (n = 3).

As in the case of HEKa cells, we were able to transfect HEKn cells with the Onpattro-like LNPs (Figure 5A). We generally observed a low cell viability, which can be ascribed to the primary cells being more susceptible to death. However, no additional toxicity was related to transfection (Figure S5A) nor apoE at the highest dose of 10 μg/mL (Figure SSC). Microscopy images of HEKn cells (Figure S5B) reveal a round morphology with a high nuclear to cytoplasmic area ratio consistent with the basal keratinocyte characteristics of HEKn which neither transfection or the presence of apoE alters.

Next, we wanted to evaluate whether the apoE-LDLR mediated LNP uptake mechanism is relevant for these basal keratinocyte-like cells that in vivo likely are exposed to apoE/apoE-containing lipoproteins present in the dermal fluids. Proteomic data support the presence of apoE in the inner epidermis and dermis layers (Figure S6). Hence, we evaluated the effect of apoE on the transfection by adding pure apoE3 to the media prior to the LNP transfection in HEKn cells. ApoE3 is the most common isoform of apoE (apoE related to our studies will refer specifically to apoE3). The apoE concentration in human plasma is ~4.3 mg/dL (~43 μg/mL) (Rasmussen et al., 2019), and thus, slightly higher than the high apoE dose we tested. However, it should be noted that most of the apoE in plasma is associated with lipoproteins. The ability of keratinocytes to express apoE (Grehan et al., 2001), the lack of correlation between apoE plasma levels and apoE in skin fluids at the basal membrane, and limited information on the importance of how apoE is presented (free/containing low lipid content or associated with mature lipoproteins) to LNPs for efficient association of apoE with LNPs makes it difficult to define a biological relevant apoE concentration in our studies. Hence, the apoE as well as the anti-LDLR antibody concentrations were chosen based on similar studies in non-skin cells reported in the literature (Akinc et al., 2010; Chen et al., 2019; Kim et al., 2021; Suzuki and Ishihara, 2016). The following three apoE concentrations were used: 0.1, 1 and 10 μg/mL. To further study the involvement of LDLR in LNP uptake, LDLR was blocked using human anti-LDLR antibody at 1 and 10 μg/mL. The blocking effect was studied with and without the presence of 10 μg/mL apoE.

It is evident from Figure 5B that the transfection efficiency of LNP is highly related to the concentration of added apoE. HEKn cells receiving the highest dose of apoE (10 μg/mL) show a 17-fold increase in MFI compared to the LNP control (no added apoE), suggesting that the presence of apoE can significantly improve the ability of Onpattro-like LNPs to transfect HEKn cells. Next, we studied whether the apoE driven increase in transfection was facilitated by LDLR-mediated uptake of LNPs. This was tested by pretreating HEKn cells with anti-LDLR antibody prior to transfection with LNPs with and without the presence of apoE. Based on the significantly increased transfection observed for 10 μg/mL apoE (Figure 5B), this apoE dose was chosen for the following LDLR blocking study. This new study also showed an increase in transfection when adding 10 μg/mL apoE (Figure 5C), however, the increase was only 4-fold compared to the 17-fold shown in Figure 5B. It should be noted that the mRNA concentration, and thus the apoE/LNP ratio, is not the same in these two studies, but it serves to confirm the qualitative observation that apoE indeed has a positive impact on the transfection in HEKn cells. Figure 5C clearly shows a correlation between anti-LDLR antibody concentration and transfection efficiency, when apoE is present, suggesting the involvement of both apoE and LDLR in the uptake of LNPs. Interestingly, the HEKn cells pretreated with the highest dose of the anti-LDLR antibody (10 μg/mL) reached a similar transfection efficiency, regardless of whether apoE is present or not. The incomplete inhibition of EGFP expression may be attributed to the ability of the cells to internalize the LNPs by other cellular uptake mechanisms, which are not apoE/LDLR-dependent. Recent studies (Da Silva Sanchez et al., 2022; Dilliard et al., 2021; Miao et al., 2020) have shown that non-apoE/LDLR uptake mechanisms can facilitate LNP-mediated transfection. However, an involvement of apoE in LNP uptake can not be fully excluded, as a small amount of apoE is likely present in the cell media supplement (BPE) and keratinocytes are also known to be able to produce
apoE (Grehan et al., 2001). Hence, other endocytic apoE receptors such as the LRP8/apoE receptor 2 (apoER2) and very-low-density lipoprotein receptor (VLDLR) could also be involved (Figure S6). However, it seems unlikely that these receptors play a major role in the transfection since the anti-LDLR antibody data indicates that LDLR is the dominating receptor facilitating LNP transfection when apoE is added exogenously.

In conclusion, our keratinocyte transfection studies including the different LNP classes, the apoE/LDLR data, and two types of primary keratinocytes, collectively suggest that the labile DMG-PEG lipid mediates the transfection in keratinocytes to a high extent. Furthermore, the apoE/LDLR mechanism by which Onpattro® is expected to transfect hepatocytes (Akinc et al., 2019) may also drive the uptake and transfection of the Onpattro-like LNPs in HEKn cells, which mimic the therapeutically attractive basal keratinocytes, when apoE is added, as suggested in Figure 1C. Our data also suggest that other uptake mechanisms can facilitate Onpattro-like uptake when apoE is present at lower concentrations.

2.3.2 Transfection of fibroblasts (HDF)

After studying the most abundant cell type in the epidermis, we wanted to investigate the most abundant cell type that LNPs encounter in the dermis layer beneath the epidermis, namely the fibroblasts. These cells are known to engulf extracellular matrix components including denatured collagen via different endocytic receptors like MRC2 (Madsen et al., 2007). Hence, we envisioned that LNPs coated with mannose could enhance the transfection over the Onpattro-like LNPs, as the LDLR receptor level in fibroblasts are lower than in keratinocytes according to the proteomic data (Figure 1B).

Figure 6. Transfection of HDF cells with varying doses of Onpattro-like LNPs for 24 hours, including A) MFI and B) cell viability. C) MFI of cells treated with varying doses of Onpattro-like LNP, 50% mannose-coated LNP and mannose-stealth-like LNP. The four lower concentrations are highlighted in the box. D) MFI of cells treated with varying doses of Onpattro-like LNP, stealth-like LNP and mannose-coated LNP. Data in C) and D) originates from different studies and is therefore presented in separate figures. Data is presented as mean ± standard deviation (n = 3).
First, we studied whether the Onpattro-like LNPs could transfect HDF cells (Figure 6A). Interestingly, we observed an initial range of increasing transfection, followed by a decrease or stagnation in transfection. The turning point of 50 ng mRNA/well corresponds to the concentration where 100% of the cells are transfected (Figure S7). Potential explanations for this observation could be dilution of serum proteins responsible for the cellular uptake, yielding a net effect that there is more LNP surface area to the same amount of serum protein for high-dose LNP-mRNA. With higher doses of LNP-mRNA, there is also a higher probability of DMG-PEG reassociating with adjacent LNPs, hence reducing recruitment of apoE to the LNP. The same phenomenon of decreased transfection at high dosing was observed by Chen et al. (2019), who showed a negative correlation between gene silencing in HepG2 and increased LNP-siRNA concentrations. Cell viability is nearly 100% for HDF cells (Figure 6B), independent of dosing or transfection, which is higher than for both HEKa cells (Figure 4C) and HEKn cells (Figure S5A). A toxicity related explanation for the observed drop in transfection is therefore not expected.

Transfection efficiency for Onpattro-like LNPs prepared using either vortex mixing or Ignite was also evaluated and compared in HDF cells (Figures S4C and S4D). Like in HEKa cells, both lead to nearly 100% EGFP positive cells for the highest doses tested, with more pronounced differences observed for MFI data, where the Ignite LNPs generally led to higher transfection efficiency, ascribed to similar mechanisms as that discussed for HEKa cells.

Concluding that Onpattro-like LNPs can transfect HDF cells efficiently, especially for low doses of LNP-mRNA, lead us to testing whether we could further enhance transfection through direct targeting of MRC2, known to be expressed by fibroblasts (Figure 1B), by coating LNPs with mannose. Figures 6C and 6D show data on HDF cells transfected with mannose-coated LNPs of different composition. Focusing on the linear range of transfection in Figure 6C (see highlighted figure), revealed that Onpattro-like LNPs induce nearly double the EGFP expression compared to 50% mannose-coated LNPs, consisting of 50% DMG-PEG. The difference in transfection efficiency could therefore likely be ascribed to the DMG-PEG rather than the mannose ligand. To support this, we showed that mannose-stealth-like LNP, that differs from the 50% mannose-coated LNP by containing DSPE-PEG instead of the labile DMG-PEG, does not result in any transfection (Figure 6C). To study the effect of the mannose ligand, diminishing the effect of the DMG-PEG lipid, LNPs of similar PEG-compositions with (mannose-coated LNP) and without a mannose ligand (stealth-like LNP) were compared in Figure 6D. The same trend is however observed in Figure 6D, where neither stealth-like LNP nor mannose-coated LNP lead to transfection. It was investigated whether formulation dependent toxicity caused this lack of transfection, but since no significant difference in toxicity was observed between either of the LNP formulations in Figures 6C and 6D (Figure S8), this did not appear to be an influencing factor. In sum, these studies clearly show that the mannose ligand does not facilitate increased transfection and that the transfection in HDF cells is solely dependent on the proportion of the labile DMG-PEG lipid in the LNPs. The lack of MCR2-mediated transfection in HDF cells could be due to: (i) that the mannose-ligands on the PEG-lipid are not exposed to the MCR2 receptors on HDF cells but instead are buried in the PEG matrix. However, a study by Kim et al. (2021) showed significantly increased delivery of siRNA to liver sinusoidal endothelial cells (LSECs) for mannose-coated LNPs with a similar PEG-lipid molar ratio of 1.5 mol%, suggesting that the mannose ligand is accessible to the cells; (ii) MRC2 may be expressed at low levels in cultured HDF cells; (iii) Even though fibroblasts express the mannose receptor MRC2 (Figure 1B), it has been reported that only 10-20% of the total MRC2 in a cell is present on the cell surface, while the remainder is found intracellularly in the endocytic pathway (Sheikh et al., 2000) and thus not available to promote uptake of LNPs; and (iv) The mannose-coated LNPs bind to MRC2 without triggering endocytosis. This scenario, however, seems unlikely as Kim et al. (2021) showed an increase in siRNA in vivo delivery to liver sinusoidal endothelial cells (LSECs) containing mannose receptors for Mannose-coated LNPs compared to LNP formulations containing C16-PEG2000 ceramide.
2.3.2.1 The impact of apoE and LDLR on the transfection of HDF cells with Onpattro-like LNPs

Findings from Figures 6C and 6D suggest that transfection in HDF cells is solely dependent on the proportion of the labile DMG-PEG lipid in the LNPs. Hence, we decided to test whether the apoE-LDLR-mediated LNP uptake mechanism is also relevant for fibroblasts, despite the expression of LDLR being lower than in keratinocytes (Figure 1B). We used a similar study design for the HDF cells as for HEKn cells, with the only variation being the concentrations of LNP-mRNA and apoE (Figure 7). These were adjusted to ensure quantifiable transfection and accordingly, doses of LNP-mRNA within the linear transfection range were chosen for the following studies based on findings from Figure 6A.

Figure 7A indicates an increase in transfection when cells are treated with 0.1 μg/mL apoE, while for higher concentrations of apoE, the transfection decreases below that of untreated cells. The latter outcome could be explained by a similar effect observed for increased dosing of LNP-mRNA in Figures 6A and 6C. Observing the cell morphology in Figure 7B reveals characteristic flat and elongated fibroblasts that does not change upon exposure to LNP-mRNA with and without addition of 0.1 μg/mL apoE. Next, we wanted to study the importance of the LDLR on the transfection with and without apoE addition by introducing the anti-LDLR antibody. For this study, we decided to use a concentration of 50 ng mRNA/well corresponding to a concentration close to the maximum transfection observed in Figure 6A and 0.05 μg/mL apoE. In Figure 7C, a trend similar to HEKn cells (Figure 65C) is observed for HDF cells, namely that the transfection efficiency is not only significantly increased by the addition of apoE (when the dosing is not too high), but also hindered by anti-LDLR antibody blocking the LDLR. Further, we show that blocking the LDLR in the absence of exogenous apoE did not change the transfection level significantly. This indicates that other pathways than the apoE-LDLR mediated uptake contribute to transfection. The studies combining apoE addition and
anti-LDLR antibody show that the apoE-LDLR pathway is the major contributor to the increased transfection promoted by the exogenous addition of apoE, just like in the case of the HEKn cells.

2.4 Comparison of keratinocytes and fibroblasts

We started out proposing that the LNP design could potentially enable Onpattro-like LNPs to target keratinocytes and mannose-coated LNPs to target fibroblasts, based on findings from a comprehensive proteomics study in skin (Dyring-Andersen et al., 2020). However, our findings show that the mannose-coating of LNPs did not lead to enhanced transfection in fibroblasts, and in fact had the opposite effect. Li et al. (2018) developed active-targeting lipid nanoparticles containing a keratinocyte-targeting peptide sequence conjugated to DSPE-PEG, permanent and ionizable cationic lipids, DOPE and DSPE-PEG-amine loaded with locked nucleic acid (LNA) modified anti-miR. These keratinocyte-targeting LNPs showed a high preferential uptake in immortalized human keratinocytes (HaCaT cells) compared to the non-targeted LNP formulation and showed minimal transfection in immortalized human fibroblasts (BJ cells). This study suggests that other targeting ligands than mannose could potentially drive the desired preference for uptake in one skin cell over another, to facilitate treatment of diseases manifesting primarily in single cell types.

While the Onpattro-like LNPs were the best transfection vector in both keratinocytes and fibroblasts, a direct quantitative comparison between Onpattro-like LNP transfection across the two cell types/studies is difficult to perform due to a number of reasons: (i) the number of cells seeded is different due to the difference in cell size of keratinocytes and fibroblasts, which means that the cell number required for optimal density differs between the two cell types; (ii) the cells are cultured in different cell media which deviates with regards to many components including the content of apoE and sink conditions for DMG-PEG adsorption; (iii) the cells exhibit different levels of background fluorescence; (iv) settings for the Operetta-based measurements (e.g. exposure time) need to be optimized to the individual cell type. However, when using the percentage of EGFP positive cells as a function of dose (Figure 8), still considered a semi-quantitative approach, we observed that HDF cells reached almost 100% EGFP positive cells at 50 ng mRNA/well while more than 250 ng mRNA was needed to get close to 100% EGFP positive cells in case of HEKa cells. We speculate that the presence of 10% serum, containing both apoE, other proteins, and serum lipoproteins capable of hosting the DMG-PEG lipid, could mediate the higher transfection efficiency observed at lower doses of LNP-mRNA in HDF cells (Figure 6A) compared to HEKa cells (Figure 4B) and especially HEKn cells (Figure 5A). It should be noted that data is based on a single donor for each cell type, meaning that biological variance cannot be completely ruled out as a partial explanation for some of the observations. Our studies mainly focus on apoE-LDLR-mediated uptake, however non-apoE-LDLR mediated uptake of LNPs has been reported in in vivo studies (Da Silva Sanchez et al., 2022).

![Figure 8. Transfection of HDF, HEKa and HEKn cells with varying doses of Onpattro-like LNP for 24 hours. Transfection is reported as % EGFP positive cells. Data originates from three independent studies. Data is presented as mean ± standard deviation (n = 3).](https://ssrn.com/abstract=4604235)
Exploring the effect of adding apoE surprisingly revealed a factor ≈4 difference in transfection efficiency for both HEKn cells (Figure 5C) and HDF cells (Figure 7C). This data combined with the observation that blocking the LDLR in the absence of exogenous apoE did not decrease transfection, suggests that approximately 25% of the uptake is driven by an apoE-LDLR-independent uptake mechanism in both cell types under the tested conditions. By blocking the LDLR with 10 μg/mL anti-LDLR antibody, the transfection efficiency in HEKn cells is lowered to the same level for cells that either have or have not received apoE (Figure 5C). A similar effect is almost achieved for HDF cells (Figure 7C), indicating that the increased transfection mediated by exogenous apoE is for the most part facilitated by the LDLR in both cell types. We rule out lack of complete blocking of LDLR when using 10 μg/mL anti-LDLR antibody in both cell types, because the decrease in transfection going from 1 to 10 μg/mL anti-LDLR is considered low when comparing the difference in transfection with and without 1 μg/mL anti-LDLR.

A significant difference between keratinocytes and fibroblasts is observed in their cell viability, with keratinocytes displaying a viability of around 70% for HEKa (Figure 4C) and 50% for HEKn (Figure S5A) while fibroblasts approximated 100% viability (Figure 6B). This in vitro observation is consistent with primary keratinocytes generally having lower viability than primary dermal fibroblasts when kept in culture. This may be due to the challenge of replicating the environment keratinocytes are exposed to in vivo with limited exposure to e.g. nutrients and growth factors from the circulations. In contrast, fibroblasts to a large degree are able to produce the optimal environment for their own growth by secreting matrix components and growth factors. This ability of fibroblasts to create a growth niche is frequently exploited for growth of more difficult cell types, where fibroblasts can be used to condition medium to support the growth of other cell types e.g. as feeder layer for keratinocytes (Auxenfans et al., 2009).
3. CONCLUSIONS

First, we show that vortex mixing produces reproducible LNPs. Two scientists prepared in total 10 LNP formulations based on the lipid composition used in Onpattro®, but with an mRNA encoding EGFP as payload (in this study referred to as Onpattro-like LNPs). A low degree of variation across these 10 LNP batches based on the same composition were achieved with respect to size (128 ± 6 nm), PDI (0.11 ± 0.01) and EE (90 ± 5%). Hence, vortex mixing seems to present a cheap, fast, high throughput, easily accessible, and reproducible alternative to the use of commercial microfluidic systems to prepare LNPs. Onpattro-like LNPs prepared using NanoAssemblr™ Ignite were smaller (86 ± 8 nm), slightly more monodisperse (PDI 0.07 ± 0.02) and with a slightly higher EE (92 ± 3%). These smaller sized LNPs may be more favorable for topical applications. Onpattro-like LNPs prepared using either vortex mixing or Ignite transfected nearly 100% of HEKa and HDF cells at the highest doses tested, however Ignite LNPs generally led to higher MFI. The observed difference in transfection is likely ascribed to size and structural differences in lipid composition, influencing cellular interactions, endosomal escape and internal packaging of mRNA. That said, our results support the reported understanding that vortexed LNPs can be a useful approach for small-batch screening applications, like studying formulation-dependent differences in transfection efficiency, but is not suitable for LNP manufacturing.

We show that the Onpattro-like LNPs which are prepared by vortex mixing and include the labile PEG-lipid DMG-PEG can transfect primary keratinocytes and fibroblasts, the most abundant cell types in the epidermis and dermis, respectively, with no significant changes in viability. Furthermore, we show that addition of apoE enhances the transfection potency of the Onpattro-like LNPs in both skin cell populations and identify LDLR as the receptor responsible for mediating the apoE-induced increase in transfection efficiency by using LDLR blocking antibodies. Hence, although the LDLR expression level is much lower in fibroblasts than in keratinocytes, according to a recent proteomic study (Dyring-Andersen et al., 2020), our data suggests that the apoE-LDLR pathway also is relevant for LNP-mediated transfection in fibroblasts. We further show that mannose-coating of LNPs based on DSPE-PEG-Mannose did not improve transfection in fibroblasts, despite that studies have identified a relatively high level of mannose receptors on fibroblasts (Dyring-Andersen et al., 2020). We envision that the mannose-coated LNPs could instead be used to transfect skin associated macrophages. Stealth-like LNPs based on DSPE-PEG show, as expected from their stealth design, limited to no transfection in keratinocytes and fibroblast, respectively.

Our studies suggest the use of labile PEG-lipids and co-administration of apoE when exploring LNPs for nucleic acid delivery to skin cells. To optimize such studies, including increasing LNP transfection potential, we propose to evaluate more potent and degradable ionizable lipids for the delivery of mRNA-based payload to skin cells. That said, we cannot rule out that endogenous apoE concentrations in the skin fluids can be sufficient to mediate LNP transfection in the basal keratinocytes and fibroblasts. The largest hurdle for efficient transfection in vivo is the ability to deliver the LNPs to the target cells. Towards that end, research and clinical studies have shown that nanoparticles applied topically can be targeted to the hair follicles and penetrate impaired skin. Hence, our studies provide valuable insights that could guide future research in the development of topical products based on LNP loaded with therapeutic nucleic acids.

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4604235
4. MATERIALS AND METHODS

4.1 Materials

DSPC, Cholesterol and DSPE-PEG2000 were supplied from Sigma-Aldrich, DMG-PEG2000 was supplied from Avanti polar lipids, DSPE-PEG2000-Mannose was supplied from BioChemPeg and D-Lin-MC3-DMA was supplied from MedChemExpress. CleanCap® EGFP mRNA was supplied from Trilink BioTechnologies. NanoAssembr™ Ignite NxGen Cartridges were supplied from Precision Nanosystem. Slide-A-Lyzer™ Dialysis Cassettes with 10K molecular weight cut-off (MWCO) were supplied from ThermoFisher Scientific. Quant-iT™ Ribogreen® RNA assay kit was supplied from ThermoFisher Scientific while Triton X-100 was supplied from Sigma Aldrich. Human adult epidermal keratinocytes (HEKa) was supplied by ATCC, human neonatal epidermal keratinocytes (HEKn) was supplied from Cell Applications and human dermal fibroblasts (HDF) were obtained from LEO Pharma A/S. Dulbecco’s Modified Eagle Medium, EpiLife™ medium, human keratinocyte growth supplement (HKGS), FBS, Dulbecco’s Phosphate Buffered Saline (DPBS 1X), TrypLE™, Lipofectamine™ 3000, Opti-MEM™, DRAQ7™ and Hoechst 33342 were all supplied from ThermoFisher. Human apoE3 was supplied from Sigma Aldrich and human LDLR antibody was supplied from Bio-Techne.

4.2 Preparation of LNPs

4.2.1 Vortex mixing

LNPs containing EGFP mRNA were prepared by vortex mixing of lipids in organic phase and mRNA in aqueous phase. First, the organic and aqueous phases were mixed in an 1.5 mL eppendorf tube by adding the aqueous phase into the organic phase using a pipette. This step was immediately followed by vortexing for 15 seconds at 2500 rpm. All formulations had a final volume of 400 µL at an aqueous to organic phase ratio of 1:3 (v/v) ratio. Lipid- and mRNA concentrations were kept constant to ensure an N/P ratio of 6:1, with a final EGFP mRNA concentration of 50 μg/mL and a final lipid concentration of 2.5 mM. EGFP mRNA was dissolved in citrate buffer (25 mM, pH 4.0). Lipid components were dissolved in ethanol, preparing individual lipid stock solutions for each lipid, prior to mixing appropriate volumes of each stock solution to achieve the desired lipid molar ratios.

Onpattro®-like LNPs had a lipid composition of DSPC:MC3:Cholesterol:DMG-PEG at molar ratios 10:50:38.5:1.5. Stealth-like LNPs had a lipid composition of DSPC:MC3:Cholesterol:DSPE-PEG at molar ratios 10:50:38.5:1.5. 50% stealth-like LNPs had a lipid composition of DSPC:MC3:Cholesterol:DMG-PEG:DSPE-PEG at molar ratios 10:50:38.5:0.75:0.75. 50% mannose-coated LNPs had a lipid composition of DSPC:MC3:Cholesterol:DMG-PEG-Mannose at molar ratios 10:50:38.5:0.75:0.75. Mannose-stealth-like LNPs had a lipid composition of DSPC:MC3:Cholesterol:DSPE-PEG:DSPE-PEG-Mannose at molar ratios 10:50:38.5:0.75:0.75. Mannose-coated LPNs had a lipid composition of DSPC:MC3:Cholesterol:DSPE-PEG-Mannose at molar ratios 10:50:38.5:1.5. LNP formulation were left to equilibrate at room temperature for 1 hour and were subsequently characterized and dialyzed against 400 mL HEPES buffer (20 mM, pH 7.4) for 2 hours at room temperature using Slide-A-Lyzer™ Dialysis Cassettes with 10K MWCO.

4.2.2 Microfluidic mixing using Ignite

For comparison, Onpattro-like LNPs were prepared using the Precision NanoSystems NanoAssembr™ Ignite instrument. The N/P ratio was kept constant at 6:1 but the final EGFP mRNA concentration was 130 μg/mL and the final lipid concentration was 6.7 mM. A total volume of 1.8 mL was prepared, including 0.15 mL start waste and 0.05 mL end waste, at an aqueous:organic phase.
flow ratio of 1:3 (v/v) and a flow rate of 9 mL/min. The resulting formulations were dialyzed as previously stated.

4.3 Preparation of Lipofetamine

Lipofectamine-mRNA was prepared by rapid mixing of LipofectamineTM 3000 dissolved in Opti-MEMTM (1:33 v/v) and EGFP mRNA dissolved in Opti-MEMTM (1:25 v/v) followed by 10 seconds of vortexing.

4.4 Characterization of LNP

4.3.1 Size and PDI

The hydrodynamic diameter (size) and polydispersity (PDI) of the LNPs were determined using Malvern Zetasizer Nano®. Each LNP formulation was 7-fold diluted (corresponding to 7 μg mRNA/mL) in HEPES buffer (20 mM, pH 7.4). The measurements were performed at 25°C with laser emission at 633 nm and a detection angle of 173°. The solvent viscosity was set to 0.89 cP. All size and PDI measurements were repeated on three individual samples from the same LNP batch.

4.3.2 mRNA encapsulation efficiency

The mRNA encapsulation efficiency (EE) was determined by the RiboGreen® RNA assay using samples of non-dialyzed LNPs (EE values were also calculated for the Onpattro-like LNPs after dialysis). Each LNP sample was diluted into 1X Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5 in DEPC treated water). LNP samples were 50-fold diluted in 1X TE buffer (quantifying unencapsulated mRNA) and in 1X TE buffer with 4% Triton X-100 (quantifying total mRNA, both encapsulated and unencapsulated). LNP samples treated with Triton X-100 were incubated for at 60°C, with stirring at 300 rpm, for 30 minutes to disrupt the LNPs and release the encapsulated mRNA. LNP samples were prepared in triplicates and added to a 96-well plate. 100 μL of 200-fold diluted RiboGreen® RNA reagent was added to each sample leading to a 100-fold dilution of mRNA (500 ng/mL). The plate was incubated for 15 minutes prior to measuring fluorescence intensity on the Varioskan™ LUX multimode microplate reader with excitation wavelength 490 and emission wavelength 530 nm.

The EE was calculated as follows:

\[
EE(\%) = \left(\frac{\text{Total mRNA} - \text{unencapsulated mRNA}}{\text{Total mRNA}}\right) \times 100\%
\]

Same procedure was applied for dialyzed LNPs to measure total concentration of mRNA in LNP samples for in vitro studies. Quant-iT™ Ribogreen® RNA reagent was 200-fold diluted in 1X TE buffer. 1 μg/mL of 16S and 23S ribosomal RNA was diluted down to 0, 20, 100, 500 and 1000 ng/mL in 1X TE buffer and added to a 96-well plate. Quant-iT™ RiboGreen® RNA reagent was added to the 96-well microplate plate. A standard curve was made based on the fluorescence intensity measured by Varioskan™ LUX multimode microplate reader. The standard curve was used to calculate mRNA concentration in LNP samples.

4.4 In vitro studies

4.4.1 Cell culture conditions

HEK293 and HEK293T cells were thawed at passage 3 and 2 respectively and cultured in EpiLifeTM medium supplemented with HKGS and antibiotics. The thawing media was washed out by an 8 minute 170 RCF centrifugation step to remove DMSO from the culture medium. 2 · 10^6 cells were thawed into a
T175 flask. The cells were split every 3-4 days using TrypLE detachment. TrypLE Neutralizer was used for neutralization of trypsin activity. Cells were not used in experiments above passage 5.

HDF cells were thawed at passage 3 and cultured in Dulbecco’s Modified Eagle Medium/Nutrition Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. The cells were split once a week and kept below passage 10 for experiments.

For experiments HDF cells were seeded at 3.500 cells/well in Cell Carrier Ultra 96-well plates. HEKa and HEKn cells were seeded at a density of 10.000 cells/well. For studies involving apoE addition, cells were treated with human recombinant apoE3 at concentrations of 0.05, 0.1, 0.5, 1 or 10 µg/mL added directly to the cell media prior to cell seeding and LNP transfection. For studies involving LDLR inhibition, cells were pretreated with human anti-LDLR antibody at concentration of 1 or 10 µg/mL at 37°C for 1 hour prior to transfection, to allow the antibodies to bind and block LDLR.

4.4.2 Cell transfections

Cells treated with LNP-mRNA or Lipofectamine-mRNA all received 10 µL LNP-mRNA or Lipofectamine-mRNA dissolved in HEPES buffer (20 mM, pH 7.4) at varying concentrations as stated in the figure legends. The plate was incubated at 37°C for 24 hours prior to cell staining and analysis. Non-treated cells were included as negative control in all experiments.

4.4.3 Fluorescent and brightfield microscopy

Fluorescent and brightfield imaging of the EGFP-transfected cells was carried out in PerkinElmer Operetta CLS™ and analyzed using the Harmony 4.9 Software. To study cell viability, cells were stained with Hoechst (nuclear marker) and DRAQ7 (marker of dead/dying cells) prior to analysis. Cell media was removed from the 96-well plate and 50 µL DMEM/F12 (HDF) or EpiLife (HEKn) containing Hoechst (1:3000 v/v) and DRAQ7 (1:100 v/v) was added to each well. Cells were incubated for 10 min at 37°C and the staining media was replaced with 100 µL DMEM/F12 (HDF) or EpiLife (HEKn). Cells were incubated for 30 minutes at 37°C prior to capturing images to reduce stress from the media exchange. 9 images per well were captured for the following channels: brightfield, EGFP, DRAQ7 and Hoechst. The total number of cells was determined using the HOECHST channel for segmentation of nuclei. Live cells were defined based on the nuclear intensity of DRAQ7 being below an arbitrary threshold. Viability was thus defined as (live cells/total cells)*100. It should be kept in mind that the cell staining procedure involves exchange of culture medium, potentially resulting in the removal of unattached dead cells which could affect the analysis. Continuous evaluation of the cell count, however, excluded this phenomenon to be significant, as the cell count for all doses of LNPs were comparable to untreated cells (Figure S2). A similar gating strategy was used to define transfected (EGFP positive) cells as live cells with nuclear intensity of EGFP above an arbitrary threshold set based on intensities observed in non-transfected cells to ensure close to 0% EGFP positive cells in control wells. Transfection was additionally reported as mean nuclear EGFP intensity (MFI) in all EGFP positive cells, subtracting average background fluorescence from negative control cells.
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SUPPLEMENTARY MATERIAL FOR:

Lipid nanoparticles containing labile PEG-lipids transflect primary human skin cells more efficiently in the presence of apoE

Camilla Hald Gregersen⁠ᵃᵇ, Razan Mearraoui⁠ᵃᵇ, Pia Pernille Søgaard⁠ᶜ, Gael Clergeaud Veiga⁠ᵇ, Karsten Petersson⁠ᵃ, Andrew Urquhart⁠ᵇ, and Jens B. Simonsen⁠ᵃ,*

ᵃ Explorative Formulation & Technologies, CMC Design and Development, LEO Pharma A/S, 2750 Ballerup, Denmark
ᵇ Department of Health Technology, Technical University of Denmark, 2800 Kongens Lyngby, Denmark
ᶜ In Vitro Biology, Molecular Biomedicine, Research and early development, LEO Pharma A/S, 2750 Ballerup, Denmark

* Corresponding author: jbsimonsen@gmail.com
Figure S1. Optimization of LNP characteristics prepared using vortex mixing method. The effect of citrate buffer molar concentration on A) size (bar) and PDI (dot) and B) encapsulation efficiency. Lipid concentration is kept constant at 5 mM. The effect of lipid concentration on C) size (bar) and PDI (dot) and D) encapsulation efficiency. Citrate buffer molar concentration is kept constant at 25 mL. Data is presented as mean ± standard deviation of technical replicates (n = 3).

Figure S2. Viability reported as number of live cells within the analyzed area of a well, for HEKa cells treated with varying doses of Onpattro-like LNP for 24 hours. Data is presented as mean ± standard deviation (n = 3).
Figure S3. Transfection of HEKa or HDF cells with Onpattro-like LNP-mRNA and Lipofectamine-mRNA with similar doses of mRNA (100 ng/well) for 24 hours. Transfection is reported as A) % EGFP positive cells and B) MFI for HEKa cells and C) % EGFP positive cells and D) MFI for HDF cells. Data is presented as mean ± standard deviation (n = 3).

Figure S4. Transfection of HEKa or HDF cells with varying doses of Onpattro-like LNP for 24 hours, prepared using either vortex mixing or Ignite. Transfection is reported as A) % EGFP positive cells and B) MFI for HEKa cells and C) % EGFP positive cells and D) MFI for HDF cells. Data is presented as mean ± standard deviation (n = 3).
Figure S5. A) Cell viability for HEKn cells transfected with varying doses of Onpattro-like LNPs for 24 hours (transfection data presented in Figure 5A). B) Fluorescence microscopy and brightfield microscopy images of HEKn cells treated with 150 ng mRNA/well and either 0 (-) or 10 (+) μg/mL apoE. Cells are stained with Hoechst dye (blue) and DRAQ7 dye (red). E) MFI for HEKn cells treated with 300 ng mRNA/well in presence or absence of human apoE (0 μg/mL (-) or 10 (+) μg/mL apoE) and 1 hour pretreatment with increasing anti-LDLR antibody concentrations. C) Cell viability for HEKn cells treated with 150 ng mRNA/well in presence or absence of human apoE (0 μg/mL (-) or 10 (+) μg/mL apoE). Data is presented as mean ± standard deviation (n = 3).
Figure S6. Relative expression of apoE, LRP8/apoE receptor 2 (apoER2) and VLDLR across skin layers and relevant skin cells derived from shotgun mass spectrometry proteomics (Dyring-Andersen et al., 2020). The data shown has been recalculated from log$_2$ values.
Figure S7. Transfection of HDF cells with varying doses of Onpattro-like LNP for 24 hours. Transfection is reported as % EGFP positive cells. Data is presented as mean ± standard deviation (n = 3).

Figure S8. Cell viability evaluated in HDF cells 24 hours post transfection with varying doses of different LNP formulations, including A) Onpattro-like LNP, 50% mannose-coated LNP and mannose-coated LNP and B) Onpattro-like LNP, stealth-like LNP 100% and mannose-coated LNP. Data is presented as mean ± standard deviation (n = 3).
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