

Development of mRNA lipid nanoparticles for the delivery of gene editing tools to the lungs

S. Hak¹, S. Saxena², D. Guidone³, A. G. Maule⁴, J. Parot¹, A. Molska¹, F. Roda¹, F. de Carlos Hernandez¹, A. Colliva², S. Vodret², S. Zacchigna², A. Borrelli³, L.J.V. Galletta³, E. Gurrieri⁴, M. Stancampiano⁴, A. Cereseto⁴, S.E. Borgos¹

¹SINTEF, Department of Biotechnology and Nanomedicine, Trondheim, Norway

²International Centre for Genetic Engineering and Biotechnology, Cardiovascular Biology Laboratory, Trieste, Italy

³Telethon Institute of Genetics and Medicine, TIGEM, Pozzuoli, Italy

⁴University of Trento, Department CIBIO, Trento, Italy

Summary

Genome editing technologies hold great potential for correcting cystic fibrosis transmembrane conductance regulator (CFTR) mutations. However, functional delivery of such tools to cells in the lungs of cystic fibrosis (CF) patients, with overproduction of thick and sticky mucus, remains a formidable challenge and a main obstacle for the clinical translation of such therapies.

Recently, Siegwart *et al.* introduced the so-called SORT (selective organ targeting) principle and demonstrated that incorporation of cationic lipids endows lipid nanoparticles containing mRNA (mRNA-LNPs) with an increased ability to transfect cells in lungs upon their intravenous administration in mice¹. Based on that work, we here defined a variety of mRNA-LNPs with anticipated ability to transfect cells in lungs. Formulations with desirable physicochemical properties (diameter below 150 nm, monodisperse, stable, and high mRNA encapsulation efficiency) were tested for this ability in human lung epithelium grown in air-liquid-interface (ALI) cultures and mice. Two of the tested new mRNA-LNP formulations effectively transfected cells in these models.

To the best of our knowledge, this is only the second report demonstrating transfection of fully differentiated human lung epithelium in ALI cultures². Furthermore, we also demonstrated that after intratracheal instead of intravenous administration in mice, a significantly higher proportion of epithelial cells playing a role in CF pathology were transfected.

Key Message

We have identified mRNA-LNP formulations that transfect differentiated human primary lung epithelium and mouse lung epithelium after intratracheal administration *in vivo*. These mRNA-LNP formulations may allow for tackling the delivery challenge for mRNA to the lung.

Introduction

Genome editing technologies hold great potential for correcting cystic fibrosis transmembrane conductance regulator (CFTR) mutations. Several studies have demonstrated efficient recovery of mutated CFTR in experimental models of cystic fibrosis (CF)^{3,4}. And although various phase 1 clinical trials with mRNA to treat CF are underway⁵, reaching and transfecting cells in the lungs of CF patients with overproduction of thick and sticky mucus remains a formidable challenge. Hence, although a lot of progress is made on gene editing tools that can accurately repair a host of disease-causing mutations, the delivery challenge is currently a main obstacle for the clinical translation of such therapies in CF patients. In this study, we aim to develop novel mRNA-LNPs with improved ability to functionally deliver mRNA into the lungs to various cell types playing a role in CF pathology.

We designed and formulated a variety of mRNA-LNPs with anticipated ability to transfect cells in lungs. Several of these formulations are based on the so-called SORT principle introduced by Siegwart *et al.*, and contain cationic lipids that endow mRNA-LNPs with an increased ability to transfect cells in lungs upon their intravenous administration¹. Formulations with desirable physicochemical properties (diameter below 150 nm, monodisperse, stable, and mRNA encapsulation efficiency above 80%) were tested for their ability to transfect cells (using reporter systems) in fully differentiated human lung epithelium grown in air-liquid-interface (ALI) models and mice.

We have now identified 2 new mRNA-LNP formulations that effectively transfect cells in lungs in mice and ALI cultures. To the best of our knowledge, this is only the second report demonstrating transfection of fully differentiated human lung epithelium in ALI cultures^{2,6}. Furthermore, we also demonstrated that after intratracheal administration in mice, a significantly higher proportion of epithelial cells playing a role in CF pathological condition were transfected as compared to intravenous mRNA-LNP administration.

Methods and results

mRNA LNPs were prepared using a flow-mixing approach with a T-junction mixer (CapTite Interconnect Tee, LabSmith) connected to two automated syringe pumps (New Era pump systems). Lipids, dissolved in ethanol, were mixed with mRNA (CleanCap® Firefly Luciferase mRNA, TriLink Biotechnologies) in RNase-free 20 mM acetate buffer (pH 4.0) at a total flow rate of 12 ml/min with an organic-to-aqueous flow rate ratio of 1:3. Following formulation, mRNA-LNPs were dialyzed overnight against Tris-HCl buffer (10 mM, pH 7.4) using 10 KDa molecular weight cut-off dialysis cassettes (Slide-A-Lyzer™ G2, Thermo Scientific™). After dialysis, the mRNA-LNPs were supplemented with 10% w/v sucrose and characterised before storage at -20°C for 2 or 4 weeks, after which they were characterized again. The mRNA-LNPs were characterized for their size using dynamic light scattering (DLS), for their size and quality using multidetector field flow fractionation (MD-FFF) and for their mRNA encapsulation efficiency (EE%) using the Quant-it RiboGreen assay. We defined 7 LNP formulations (composed of mRNA, ionizable lipid, sterol, PEG-lipid, helper lipid) based on literature and our own preliminary data. **Table 1** shows the physicochemical characterization results for the formulations, and we found that only mRNA-LNP F and G were stable for up to 4 weeks, hence these formulations were tested in air-liquid-interface (ALI) cultures and in vivo in mice (formulated with TriLink CleanCap® Cre instead of Fluc mRNA) for their ability to transfect lung tissue. We also included a third formulation in the biological testing, mRNA-LNP H, which was the same as mRNA-LNP G, but in which we replaced the ionizable lipid used (this formulation had a typical DLS z-average and PDI of 100-120 nm and 0.20, and mRNA EE% >90%).

Table 1: Physicochemical characterization of 7 different mRNA-LNPs. The colour coding indicates good (green), borderline (orange), and poor (red) physicochemical characteristics.

Formulation	Day 1							Week 2/4						
	time-point	DLS		MD-FFF			Ribogreen	time-point	DLS		MD-FFF			Ribogreen
		Z-AVG (nm)	PdI (a.u.)	Rg (d.nm)	Rh (d.nm)	Recovery (%)	Encapsulation efficiency (%)		Z-AVG (nm)	PdI (a.u.)	Rg (d.nm)	Rh (d.nm)	Recovery (%)	Encapsulation efficiency (%)
mRNA-LNP A	1 day	94	0,16	87	102	47 %	98 %	2 weeks	107	0,26	359	482	25 %	97 %
mRNA-LNP B	1 day	101	0,17	82	100	94 %	88 %	2 weeks	252	>0,5	114	102	22 %	83 %
mRNA-LNP C	1 day	148	0,18	106	131	49 %	59 %	2 weeks	169	0,18	79	81	65 %	45 %
mRNA-LNP D	1 day	94	0,25	156	181	57 %	100 %	2 weeks	144	0,39	243	141	85 %	99 %
mRNA-LNP E	1 day	241	0,35	170	244	1 %	51 %							
mRNA-LNP F	1 day	103	0,14	60	72	104 %	90 %	4 weeks	114	0,15	62	70	95 %	85 %
mRNA-LNP G	1 day	78	0,16	81	86	97 %	97 %	4 weeks	100	0,23	64	68	93 %	89 %

To generate ALI cultures, we used human bronchial epithelial cells (from explanted lungs from non-CF individuals, which are more available than lungs from CF-patients) plated on porous membranes (cc3470 Transwell inserts) and kept these for two weeks under ALI culture condition to induce differentiation. We used two differentiation media, PneumaCult ALI and PneumaCult ALI-S. The former medium induces the formation of an epithelium of large airways: tall, columnar, and highly ciliated. The latter induces an epithelium with characteristics of small airways: more cuboidal and less ciliated. Short-circuit current recordings were carried out to ensure that the epithelia used for testing LNPs had the proper degree of differentiation. LNPs were added to the apical side of the ALI cultures at two doses (0.2 and 1 µg mRNA in 50 µl saline solution) for two hours. Before application of the LNPs, the epithelial surface was washed with a saline solution to remove mucus. After treatment, LNPs were removed and epithelia kept back under culture conditions. After 48 h, epithelia were lysed to determine luciferase

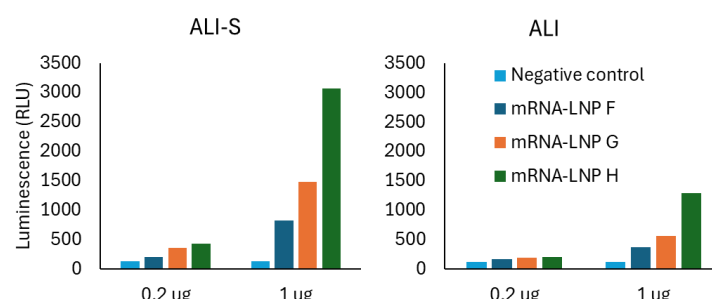


Figure 1: Luminescence measurements on lysed ALI cultures after transfection with 3 different LNP formulations. Especially evident in the ALI-S condition, mRNA-LNP G and H outperform mRNA-LNP F. Furthermore, mRNA-LNP H transfects the ALI cultures more efficient than mRNA-LNP G.

activity. We found a significant dose-dependent transfection of luciferase mRNA by LNPs as can be seen in **Figure 1**. Epithelia kept with PneumaCult ALI-S appeared more prone to mRNA transfection with our LNPs. mRNA-LNP H was the most effective as compared to mRNA-LNP F and G (mRNA-LNP F had the same composition as mRNA-LNP G, but lacks a cationic lipid), see **Figure 1**.

For in vivo testing, we formulated the LNPs with CRE mRNA and administered them intratracheally or intravenously (IV) to Rosa26-mTmG reporter mice. For intratracheal administration, 12-16 weeks old mice were anesthetized with ketamine/xylazine (100 mg/kg and 40 mg/kg respectively) and 30-60 μ l mRNA-LNP (5 μ g of CRE mRNA) dispersion was administered using a cannula and a Hamilton syringe, directly inserted into the trachea. For intravenous administration, the mRNA-LNPs were injected retro-orbitally in neonatal pups (P4) in a bolus of 40 μ l containing 5 μ g of CRE mRNA. Mice were kept in a controlled environment at 23 ± 2 °C, $50 \pm 10\%$ humidity, and 12h:12h light:dark cycle for 72 h. Food and water were provided ad libitum throughout the study.

Mice were sacrificed 72 hours after intratracheal administration or 1 week after intravenous administration and the lungs were collected and processed for histological analysis. After intratracheal administration, high levels of Cre-mediated recombination were observed, resulting in GFP expression throughout the lung parenchyma for mRNA-LNP G and H, while we were unable to detect transfection by mRNA-LNP F. For mRNA-LNP G, we observed a high percentage of transfected CFTR-expressing airway epithelial cells. Nearly 80% of GFP positive cells co-expressed uteroglobin, (SCGB1A1, a marker for secretory cells), 48% co-expressed SOX2 (a marker for epithelial cells in both airways and submucosal glands), and 75% co-expressed NKX2.1 (a marker for bronchial epithelial and alveolar type 1 cells). Approximately 20% of GFP-positive cells also expressed Prosurfactant C (a marker for alveolar type 2 cells), indicating that intratracheal administration transfected not only airway epithelial cells but also interstitial alveolar cells.

In contrast, intravenous administration of each of the mRNA-LNP formulations resulted in the transfection of predominantly endothelial and other interstitial cells, with minimal targeting of airway epithelial cells.

Discussion and Conclusion

The main objective in this study is to improve the functional delivery of mRNA to the lungs. So far, we have identified 2 new mRNA-LNP formulations that effectively transfect cells in lungs in mice and ALI cultures. To the best of our knowledge, this is only the second report demonstrating functional delivery of mRNA to very hard-to-transfect fully differentiated human lung epithelium in ALI cultures^{2,6}. In the ALI culture experiments, we decided to remove mucus because our data indicate that an important and limiting barrier is that differentiated epithelia themselves are hard to transfect as they have intrinsic mechanisms, other than mucus, that strongly reduce the entry of LNPs and the delivery of cargo. Therefore, we decided to remove the mucus layer to identify LNPs that are able to specifically overcome these mechanisms. We also observed that after intratracheal mRNA-LNP administration in mice, a significantly higher proportion of epithelial cells playing a role in CF lung pathology were transfected as compared to intravenous administration, after which predominantly endothelial cells were transfected. There are published data indicating that CFTR is expressed in endothelial cells and that its lack in CF may have deleterious effects^{5,6}. Therefore, induction of CFTR in endothelial cells may have a beneficial effect. Although there is no clear indication of a CF-defect in alveoli, CFTR is also normally expressed in alveolar cells and delivery to alveolar CF cells would restore a normal situation.

Taken together, although intravenous administration will be preferable to target CFTR mutations also in other organs than lungs, the first clinically approved mRNA-LNP technology for CF patients may well be based on inhalation, as seemingly a broader variety of cells potentially playing a role in CF are reached. Interestingly, inhalation is also the sole administration route used in ongoing clinical trials with mRNA-based therapeutics in CF⁵.

As a next step to assess whether our formulations can contribute to the clinical implementation of gene editing technology in CF, we are now pursuing formulations with mRNA encoding base editor technology (Cas nuclease SpG Cas9) selected for its proven efficiency in precisely and effectively repairing CFTR mutations⁴. Furthermore, we have synthesized a handful of novel proprietary cationic lipids which will also be tested in the same experimental set-ups as presented in this study, to further increase transfection efficiencies as well as biocompatibility of mRNA-LNPs.

References

- (1) Cheng, Q.; Wei, T.; Farbiak, L.; Johnson, L. T.; Dilliard, S. A.; Siegwart, D. J. Selective Organ Targeting (SORT) Nanoparticles for Tissue-Specific mRNA Delivery and CRISPR-Cas Gene Editing. *Nat Nanotechnol* **2020**, *15* (4), 313–320. <https://doi.org/10.1038/s41565-020-0669-6>.
- (2) Jiang, A. Y.; Witten, J.; Raji, I. O.; Eweje, F.; Maclsaac, C.; Meng, S.; Oladimeji, F. A.; Hu, Y.; Manan, R. S.; Langer, R.; Anderson, D. G. Combinatorial Development of Nebulized mRNA Delivery Formulations for the Lungs. *Nat. Nanotechnol.* **2024**, *19* (3), 364–375. <https://doi.org/10.1038/s41565-023-01548-3>.

- (3) Nicosia, L.; Harrison, P. T. CRISPR for Cystic Fibrosis: Advances and Insights from a Systematic Review. *Mol Ther* **2025**, S1525-0016(25)00472-1. <https://doi.org/10.1016/j.ymthe.2025.06.021>.
- (4) Amistadi, S.; Maule, G.; Ciciani, M.; Ensink, M. M.; De Keersmaecker, L.; Ramalho, A. S.; Guidone, D.; Buccrossi, M.; Galletta, L. J. V.; Carlon, M. S.; Cereseto, A. Functional Restoration of a CFTR Splicing Mutation through RNA Delivery of CRISPR Adenine Base Editor. *Mol Ther* **2023**, 31 (6), 1647–1660. <https://doi.org/10.1016/j.ymthe.2023.03.004>.
- (5) Le Ribeuz, H.; To, L.; Ghigna, M.-R.; Martin, C.; Nagaraj, C.; Dreano, E.; Rucker-Martin, C.; Girerd, B.; Bouligand, J.; Pechoux, C.; Lambert, M.; Boet, A.; Issard, J.; Mercier, O.; Hoetzenecker, K.; Manoury, B.; Becq, F.; Burgel, P.-R.; Cottart, C.-H.; Olschewski, A.; Sermet-Gaudelus, I.; Perros, F.; Humbert, M.; Montani, D.; Antigny, F. Involvement of CFTR in the Pathogenesis of Pulmonary Arterial Hypertension. *Eur Respir J* **2021**, 58 (5), 2000653. <https://doi.org/10.1183/13993003.00653-2020>.
- (6) Totani, L.; Plebani, R.; Piccoli, A.; Di Silvestre, S.; Lanuti, P.; Recchiuti, A.; Ciani, E.; Dell'Elba, G.; Sacchetti, S.; Patruno, S.; Guarnieri, S.; Mariggiò, M. A.; Mari, V. C.; Anile, M.; Venuta, F.; Del Porto, P.; Moretti, P.; Prioletta, M.; Mucilli, F.; Marchisio, M.; Pandolfi, A.; Evangelista, V.; Romano, M. Mechanisms of Endothelial Cell Dysfunction in Cystic Fibrosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **2017**, 1863 (12), 3243–3253. <https://doi.org/10.1016/j.bbadis.2017.08.011>.