Evaluation of the effects of storage conditions on spray-dried siRNA-LNPs before and after subsequent drying

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Key words: lipid nanoparticles, LNP, Onpattro®, RNA therapeutics, siRNA delivery, spray drying, pulmonary delivery, subsequent drying, storage, shelf life

Abstract

In an ideal world, pharmaceutical drugs would have infinite shelf life, no susceptibility to degradation, chemical reactions or loss of efficacy. In reality, these processes occur, however, making it desirable to extend a drugs' shelf life. Nucleic acid-based drugs are most commonly stored as aqueous suspension where they are vulnerable to microbial growth and degradation processes. Drying procedures, such as lyophilization and spray drying, help to reduce the products' residual moisture while increasing the products' shelf life and stability. The present study was designed to evaluate 90 days of storage of spray-dried siRNA-lipid nanoparticles (LNPs) at 4 °C and 25 °C. An updated Onpattro® composition modified with a positively charged helper lipid was used as the LNP carrier system. In an attempt to further reduce the residual moisture of our previously reported formulations, all LNP samples were subjected to a secondary drying step in the spray drying tower for 20 min. The measurement of physicochemical properties of spray-dried and subsequently dried LNPs resulted in sizes of 180 nm, PDI values of 0.1-0.15 and zeta potentials of +3 mV. Spray drying resulted in residual moisture levels of 3.6 - 4% and was reduced by subsequent drying to 2.8 - 3.1%. Aerodynamic properties after storage showed discrepancies depending on the storage conditions. MMADs remained at 2.8 µm when stored at 4 °C, whereas an increase to 5 µm at 25 °C was observed. Subsequent drying led to sizes of 3.6-3.8 µm, independent of the storage conditions. Spray-dried LNPs maintained bioactivity resulting in > 95% protein downregulation and confirming the lack of cytotoxic effects in a lung adenocarcinoma cell line. Furthermore, the spray-dried and subsequently dried LNPs stored for 3 months at 4 °C and 25 °C achieved up to 50% gene silencing of the house-keeping gene GAPDH after deposition on the mucus layer of Calu-3 cells. This study confirms the stability of spray-dried and subsequently dried LNPs over at least 90 days at 4 °C and 25 °C emphasizing the potential of dry powder inhalation of RNA-loaded LNPs as a therapy option for pulmonary diseases.

1. Introduction

In the last decade, nucleic acid-based drugs have demonstrated their potential of treating diseases by targeting their genetic construction plan. Nucleic acid therapeutics can accomplish long-term or curative effects through gene addition, inhibition, editing or replacement. [1] Commonly used nucleic acid carriers consist of polymers, peptides, lipids, inorganic materials or hybrid combinations. [2] Among them, lipid nanoparticles have become one of the most promising drug delivery systems for nucleic acids, driven for the most part by their use in the approved mRNA-based COVID-19 vaccines. [3-5] The storage of drugs and vaccines in general and mRNA-based vaccines in particular is an ongoing logistic problem. The latest updates from the European Medicines Agency (EMA) estimate the shelf life for mRNA vaccines from BioNTech/Pfizer and Moderna to a maximum of 2.5 months and 1 months, respectively, when stored at 4 °C. At storage temperatures of −80 °C and −20 °C, the shelf life increases to a 9-month and 6-month period, respectively. However, once opened, the vaccines needed to be administered within 24 h. [6-9] This discrepancy in storage life gives a hint of the difficulty in creating the ideal product conditions for long-term storage on a global supply level. Onpattro®, which is the first FDA-approved siRNA drug based on an LNP delivery system, shows a three-year shelf life when kept between 2 °C and 8 °C, provided that the drug vials are kept closed. [10] Further studies demonstrated that siRNA-LNPs remained stable at 2-4 °C for 6 to 18 months, maintaining their initial particle size and siRNA encapsulation efficiency. [11, 12] However, long-term storage on an aqueous basis can lead to microbial growth and chemical degradation. Hence, drying procedures are favorable for transferring the drug substance in a water-reduced storage environment. Lyophilization is one way of drying, performing a gentle procedure. In a freeze-dried form, mRNA-LNP vaccines could be conveniently shipped worldwide without the need for cooling or freezing. Ball et al. studied the impact of freeze drying on siRNA-LNPs, maintaining the bioactivity of the siRNA after 11-months storage at -80°C. [11]

In another study, Shirane et al. lyophilized ethanol-containing siRNA-LNPs, which maintained their gene knockdown efficiency *in vivo*. [13] The approach of lyophilization helped to prolong the shelf life. However, lyophilization of LNPs is not straightforward due to the complex composition of various lipids at certain ratios and the freezing process, which can alter the LNP composition. In addition, it is a very cost-intensive and time-consuming process. Therefore, other drying approaches, such as spray drying, can help overcoming these limitations. [14] Spray drying is a widely used method for improving the shelf-life of drugs. It is based on the principle of converting a liquid material into a dry powder, offering multiple advantages for industrial use, such as scale-up and encapsulation of bioactive ingredients into sugar matrices, extended storage stability due to decreased molecular mobility, shorter production time, and reduced production, transportation and storage costs. [15] The drying effect mainly relies on the excipient used. Crystalline sugars, such as mannitol, tend to reach residual moisture levels <1%, whereas spray-dried amorphous sugars, such as lactose, commonly result in moisture levels of 4 - 5%. [16, 17] Despite the higher moisture level in amorphous matrices, they are known to maintain a higher product stability and are preferred over crystalline sugars. Independent of the sugar matrix, the lower the moisture level in the product, the less susceptible the product to alterations or decline. [18] Thus, spray drying parameters need to be adapted to the material being spray-dried. Due to their higher melting point, polymer-based nanoparticles can be spray-dried at higher temperatures than lipid-based particle systems. Spray drying parameters need to achieve a balance between the biggest drying impact and the product's integrity. One approach to reducing the residual moisture of an amorphous sugar matrix would be the implementation of a subsequent drying step. A constant heat supply in the spray drying tower and the turbulent conditions in the collecting vessel would dry the product in a gentle way.
Various nanoparticle systems have been spray-dried focusing on the spray drying process itself to keeping the cargo intact and maintain nucleic acid bioactivity. [16, 19-21] Unfortunately, none of these studies evaluated the impact of a subsequent drying step or the long-term storage on the product. In a previous study, we have reported a successful spray drying procedure of LNP-siRNA formulations in 5% lactose solution (m/V). The LNP formulation was based on an updated Onpattro® composition and maintained its integrity and gene silencing efficiency on protein and mRNA levels both *in vitro* and *ex vivo.* [21] However, long-term assessments of spray-dried LNPs were not performed and need for further product development.

The aim of this study was to evaluate storage stability of spray-dried and subsequently dried LNPs over 3-month storage at 4 °C and 25 °C. The LNPs consisted of an updated Onpattro® formulation, using the positively charged helper lipid DOTAP ((+)LNP). Subsequent drying was performed for 20 min within the collection vessel, immediately following the spray drying process. Powders were analyzed and compared regarding physicochemical properties, siRNA losses, aerodynamic performance and siRNA integrity. The target range was set based on previous studies to obtain sizes after redispersion of around 150 nm, RNA losses below 15% and residual moisture levels below 5%, which was to be reduced to a maximum of 4% after subsequent drying. [21] Furthermore, aerodynamic sizes should range between 3-7 µm, and RNA bioactivity needed to be maintained. The LNPs' performance and siRNA bioactivity were tested *in vitro* in H1299 adenocarcinoma cells expressing enhanced green fluorescent protein (H1299-GFP). Moreover, in a more sophisticated *in vitro* setup, spray-dried and subsequently dried powders were added to mucus-secreting Calu-3 cells grown at the air-liquid interface and tested for gene silencing efficacy of the house-keeping gene GAPDH. The findings of this study were compared to previously reported data and between the different storage conditions. Unique insights on the performance of storage stability of spray-dried LNPs were obtained resulting in improved microparticle properties, particularly for pulmonary delivery.

2. Materials & Methods

2.1 Materials

Dicer substrate double-stranded siRNA targeting green fluorescent protein (DsiRNA EGFP, 25/27) (siGFP), dicer substrate double-stranded siRNA targeting the house-keeping gene GAPDH (DsiRNA GAPDH) (siGAPDH) and scrambled, non-specific control (siNC) were purchased from IDT (Integrated DNA Technologies, Inc., Leuven, Belgium). [21-24] Tris-EDTA buffer solution 100x (T9285), RPMI-1640 medium (R8758), EMEM medium, fetal bovine serum (FBS) (F9665), penicillin-streptomycin (P/S) (P4333), G418 disulfate salt solution (G8168), Dulbecco's phosphate buffered saline (D-PBS) (D8537), methylthiazolyldiphenyl-tetrazoliumbromid (MTT), Triton X-100 were obtained from Sigma-Aldrich, a subsidiary of Merck KGaA (Darmstadt, Germany). 1,2-Dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000 (PEG-DMG), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (DSPG) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) were bought from Avanti Polar Lipids, Alabaster, USA. The ionizable cationic lipid used is a sulfur-containing analog of DLin-MC3-DMA (pKa 6.3-6.6). [21, 25] InhaLac®230, lactose monohydrate for dry powder inhalers, was purchased from Meggle Group (Wasserburg, Germany). Quant-it™ RiboGreen DNA reagent, black 96-well plates (10307451), power SYBR™ green PCR master mix and Aquastar® water standard oven 1% were bought from Thermo Fisher Scientific (Schwerte, Germany). Pumpsil® tubings were obtained from Watson-Marlow GmbH (Rommerskirchen, Germany) and had an inner diameter and a thickness of 1.6 mm. White 96-well PCR plate and 0.2 mL PCR tubes were purchased from Biozym Scientific GmbH (Hessisch Oldendorf, Germany). PneumaCult ALI differentiation medium, hydrocortisone and heparin were bought from Stemcell Technologies (Vancouver, Canada). Transwell® polyester membrane cell culture inserts were purchased from Corning (New York, USA).

2.2. Preparation of lipid nanoparticles (LNPs) entrapping siRNA

LNP-siRNA formulations had a lipid composition based on the clinically approved Onpattro formulation and were prepared as previously described [3, 26, 27]. Briefly, lipid components (ionizable cationic lipid, helper lipid, cholesterol, and PEG-DMG) at molar ratios of 50:10:38.5:1.5 mol% were dissolved in ethanol to a concentration of 10 mM total lipid. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) was used to enable formation of LNP-siRNA systems with positive [(+)LNP] zeta potential. Purified siRNA (siNC, siGFP and siGAPDH) was dissolved in 25 mM sodium acetate pH 4 buffer to achieve an N/P ratio of 3, which is the charge ratio between the ionizable cationic head group on the lipid to the anionic phosphates in the RNA backbone. The two solutions were mixed through a T-junction mixer [28] at a total flow rate of 20 mL/min, and a flow rate ratio of 3:1 v/v (aqueous:organic phase). The resulting LNP suspension was subsequently dialyzed overnight against PBS pH 7.4, sterile filtered (0.2 µm), and concentrated to 1.0 mg/mL siRNA measured via Ribogreen assay [29].

2.3. Spray drying and subsequent drying of LNPs

For production of spray-dried and subsequently dried LNPs, the same method was chosen as described earlier. [21] Briefly, a B-290 spray drying tower (Büchi Labortechnik, Flawil, Schweiz) was used to spray dry siRNA-LNP formulations in a specified solvent (highly purified water (HPW) with lactose (InhaLac), 5% m/V, sterile filtered), at a pump rate of 1.4 mL/min and an inlet-temperature (T-In) of 100 °C resulting in a measured outlet-temperature (T-Out) of 62 °C ± 2 °C. Each individual sample of

LNP-siRNA formulations had a concentration of 30 µg siRNA in 5 mL 5% lactose solution (w/V). This resulted in an siRNA to sugar concentration of 0.12 µg siRNA/mg lactose. Subsequent drying of spray-dried powder was achieved by switching off the liquid feed and keeping the heat supply on for 20 min. Spray-dried and subsequently dried powders were filled into 20R vials, sealed and wrapped with parafilm. Samples were stored at 4 °C and 25 °C for 3 months (**Table 1**).

Table 1 Spray drying and subsequent drying schedule of various LNP formulations. Spray drying was performed using 5% lactose solution (m/V) at 62 °C outlet temperature. Storage temperatures consisted of 4 °C and 25°C and lasted for 3 months.

Name	Drying Time (min)	Storage temperature (°C)	Storage Time (months)
siGFP - (+)LNP siNC - (+)LNP siGAPDH - (+)LNP	0	4	3
siGFP - (+)LNP siNC - (+)LNP siGAPDH - (+)LNP		25	
siGFP - (+)LNP siNC - (+)LNP siGAPDH - (+)LNP	20	4	
siGFP - (+)LNP siNC - (+)LNP siGAPDH - (+)LNP		25	

2.4. siRNA quantification after spray drying of LNPs

The Quant-IT™ Ribogreen assay was adapted as described in Walsh et al. [29] Briefly, LNPs were either freshly prepared or redispersed as described above. For each reading, 50 µL of samples was transferred into a black 96-well plate and filled to 100 µL with 2% Triton X-100 solution. An siRNA standard curve was pipetted at 10.0, 5.0, 2.5 and 1.0 µL of a stock solution (20 µg/mL), resulting in final concentrations of 1.0, 0.5, 0.25 and 0.1 µg/mL, respectively. The plate was incubated at 37 °C for 60 min in a shaking incubator. Upon addition of the Ribogreen reagent at a 1:100 dilution, the fluorescence intensities were measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a TECAN Spark plate reader (TECAN, Männedorf, Switzerland). The siRNA loss was quantified by normalizing the siRNA amount of spray-dried and subsequently dried samples to the siRNA amount of fresh LNP samples.

2.5. Hydrodynamic diameter and zeta (ζ) potential measurements of spray-dried LNPs

Hydrodynamic diameters and polydispersity indices (PDI) were measured in disposable cuvettes (Brand GmbH, Wertheim, Germany) using the Zetasizer Nano ZS instrument (Malvern Instruments Inc., Malvern, U.K.). To measure the size and PDI of spray-dried and subsequently dried formulations at different storage conditions after redispersion, approximately 0.833 mg of spray-dried LNP powder was dissolved in 100 µL HPW. This equates to 0.1 µg of siRNA (1 µg siRNA/mL). For comparison, freshly prepared LNPs (c = 1 mg/mL) were diluted in 5% lactose to reach a concentration of 1 µg siRNA/mL. Refractive index and viscosity values of the dispersant were accounted for in the Zetasizer software. All samples were detected at a backscatter angle of 173°. Results are presented as hydrodynamic diameter size (nm) ± SD of three independent measurements. Zeta potentials were measured by Laser Doppler Anemometry (LDA) using a Zeta Cell (Zetasizer Nano series, Malvern, UK) containing a 6.5X dilution of the same 100 µL sample of the LNP suspensions. For each LNP formulation, measurements were presented as an average charge (mV) ± SD of three independent measurements.

2.6. Residual water content – Karl Fischer titration

The residual water content of spray-dried and subsequently dried LNPs powders stored at different conditions was determined by weighing 10 mg sample into 2R vials. A 1% water standard was prepared with approximately 40-50 mg powder. Empty vials served as blank values. For coulometric measurements, an Aqua 40.00 Karl Fischer Autosampler-Titrator with corresponding software from Analytik Jena AG (Jena, Germany) was used. The oven was heated to 100°C, and the final drift was set to less than 10.0 µg/min. Blank measurements were run and automatically subtracted from the standards and samples. Residual moisture measurements were considered valid if the 1% water standard measurement resulted in a value between 0.9 and 1.1%. Results are presented as mean residual moisture (%) ± SD of three independent samples.

2.7. Scanning Electron Microscopy (SEM)

A small amount of spray-dried and subsequently dried LNPs stored at various conditions was placed on top of a stub covered with double-sided carbon tape. The stub was then coated with carbon under vacuum for 40 s. The microparticles were examined using a FEI Helios G3 UC (Thermo Fisher Scientific, Schwerte, Germany).

2.8. Aerodynamic properties of spray-dried LNPs

For the analysis of the aerodynamic properties of spray-dried and subsequently dried powders after storage at 4 °C and 25 °C, procedures specified in the monograph 2.9.18, apparatus E, of the European Pharmacopoeia was performed using a next generation impactor (NGI) from Copley Scientific (Nottingham, UK). The measurement procedure was adapted as previously described. [21] Briefly, spray-dried or subsequently dried LNP powder was transferred into 2-3 hydroxypropylmethylcellulose capsules. Each capsule was loaded into a Handihaler® (Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany), hole-punched and discharged twice. Subsequently, the procedure for the Ribogreen assay as described in 2.4. was performed. Every stage of the NGI was washed with 2% Triton-X buffer. The induction port (IP) was washed with 5 mL, the pre-separator (PS) was pre-filled with 15 mL, small cups were filled with 2 mL and bigger cups were filled with 4 mL 2% Triton-X buffer solution. All parts were cautiously shaken and placed on a horizontal shaker for 20 min. Samples for a standard curve of fresh siRNA were prepared and topped up to 100 µL with 2% Triton-X buffer. As a control, freshly prepared LNPs at siRNA concentration of 1.0 µg/mL, similar to the redispersed samples, were prepared in 2% Triton-X buffer. Three aliquots of 100 µL from each stage were used for further analysis. All samples were pipetted into a black 96-well plate and placed in a shaking incubator for 60 min at 37 °C. Upon addition of Ribogreen reagent at a 1:100 dilution, the fluorescence intensities were measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a TECAN Spark. The mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD), fine particle dose (FPD), fine particle fraction (FPF) and powder recovery (%) were calculated as described in the European Pharmacopoeia, considering fine particles at sizes below 5 µm MMAD.

2.9. *In vitro* characterization of spray-dried LNPs

2.9.1 Cell Culture

The human non-small cell lung carcinoma cell line H1299 (ATCC CRL-5803) stably expressing enhanced green fluorescence protein (eGFP) was cultured in RPMI 1640 medium supplemented with 10% FBS, 1% P/S and 0.4% G418. Cells, starting from passage 9, were passaged every 3 days with 0.05% v/v trypsin and subcultured in 75 cm^2 flasks. Calu-3 cells were obtained from LGC Standards GmbH (Wesel, Germany). Cells were maintained in EMEM medium supplemented with 10% FBS and 1% P/S. Cells were passaged once 80% confluence was reached and subcultured in 75 cm^2 flasks.

H1299-GFP and Calu-3 cells were kept in a humidified atmosphere at 37 °C with 5% CO_2. All cell lines were mycoplasma-free and tested for mycoplasma every 3 months.

2.9.2 *In vitro* GFP protein downregulation in H1299 cells

To evaluate the *in vitro* gene silencing efficiency of siRNA-LNPs after spray drying and subsequent drying, H1299-GFP cells were seeded in a 24-well plate at a density of 25,000 cells per well in 500 µL medium at 37 °C and 5% CO_2 and incubated for 24 h. Powder samples were weighed at 0.833 mg, resuspended in 100 µL HPW, resulting in concentrations of 1 µg/mL, and added to 400 µL of fresh culture medium for 24 h incubation at 37 °C and 5% CO_2. The medium was then discarded and replaced with 500 µL of fresh medium, and the plates were further incubated for another 24 h. At the end of the incubation time, cells were washed with PBS, trypsinized and collected. After centrifugation at 400 rcf for 5 min, the supernatant was discarded and the cell pellet was washed two times in PBS before being resuspended in PBS with 2 mM EDTA. Samples were analyzed by flow cytometry (Attune® NxT, Thermo Fischer Scientific, Waltham, Massachusetts, USA), and the median fluorescence intensity (MFI) of GFP protein expression was measured using a 488 nm excitation laser, and the emitted light passing through a 530/30 nm band pass emission filter set was detected. All cell samples were gated by morphology for a minimum of 10,000 viable cells. Results are displayed as mean MFI values (%) ± SD for 3 independent samples.

2.9.3 *In vitro* cytotoxicity of spray-dried LNPs in H1299 cells

Cell viability after transfection with spray-dried and subsequently dried LNPs was tested via MTT assay as described previously.[30, 31] Briefly, 5,000 H1299-GFP cells per well were seeded in 100 µL medium into a transparent 96-well plate (BioLite 96 well multidish, Thermo Fisher Scientific, Rochester, New York, USA). The samples were prepared by redispersing 0.833 mg (1 µg siRNA) of powders in 100 µL of HPW. After 24 h, 90 µL of pre-warmed medium was added to each well and supplemented with 10 µL LNP samples, respectively. The plate was incubated for 24 h at 37 °C and 5% CO_2. As a full viability control, cells were incubated in 100 µL solutions consisting of 10 µL sterile 5% lactose solution (m/V) and 90 µL medium. After 24 h, the media was aspirated and 200 µL of MTT containing medium (0.5 mg/mL in serum-free RPMI-1640 medium) was added to each well. Cells were incubated for another 3 h at 37 °C and 5% CO_2. Subsequently, the cell culture medium was completely removed, and insoluble purple formazan crystals were dissolved in 200 µL DMSO. The plate was set on a horizontal shaker for 20 min for all crystals to dissolve. The absorbance was measured at 570 nm, corrected with background values measured at 680 nm, using a microplate reader (TECAN Spark, TECAN, Männedorf, Switzerland). The data are shown as mean ± SD as percentage of viable cells in comparison to untreated cells representing 100% viability.

2.9.4 *In vitro* GAPDH knockdown in Calu-3 cells

Calu-3 cells were seeded at a density of 250,000 cells onto uncoated Transwell® polyester cell culture inserts (6.5 mm, 0.4 µm pore size) in 100 µL medium (apical side), while 600 µL medium were added to the basolateral chamber. After 72 h, the apical medium was removed to obtain air-liquid interface conditions, while the medium from the basolateral chamber was replaced with 200 µL of PneumaCult™ ALI medium (STEMcell technology, Vancouver, Canada) and replaced every two days. Experiments were performed once TEER values ≥300 Ω*cm^2 were reached and a stable polarized epithelial layer was formed, as measured with an EVOM epithelial volt/Ω meter (World Precision Instruments, Sarasota, USA). For transfection of spray-dried and subsequently dried LNPs (siGAPDH-(+)LNP and siNC-(+)LNP) stored at different conditions, 8.33 mg (10 µg/mL) powder was directly poured on top of the mucus layer. The basal compartment consisted of 200 µL of PneumaCult™ ALI medium. Calu-3

cells were incubated for 48 h at 37 °C and 5% CO_2. Cells were harvested by washing the apical compartment with PBS. Subsequently, the cells were scraped carefully without braking the membrane and transferred into Eppendorf tubes. The RNA extraction protocol was carried out using the PureLink RNA mini kit according to the manufacturer's protocol. Remaining mRNA levels were quantified by RT-qPCR. In brief, cDNA was synthesized from total RNA using high-capacity cDNA synthesis kit (Applied Biosystems, Waltham, Massachusetts, USA). The obtained cDNA was then diluted 1:10, and a qPCR was performed using the SYBR™ Green PCR Master Mix (Thermo Fischer Scientific, Waltham, Massachusetts, USA) with primers for human GAPDH and β-actin (Qiagen, Hilden, Germany) for normalization. Cycle thresholds were acquired by auto setting within the qPCRsoft software (Analytic Jena AG, Jena, Germany). Three individual batches of spray-dried and subsequently dried LNPs (siGAPDH and siNC) were examined. The GAPDH silencing results are reported as mean percentages (%) normalized to values obtained after transfection with siNC ± SD.

2.10. Statistics, data analysis and presentation

All experiments were run in independent triplicates. Experimental data passed the D'Agostino-Pearson normality test and were analyzed for statistical significance using the One Way ANOVA repeated measurements using GraphPad Prism 5 software with Tukey's post-hoc test where $p>0.05$ was considered not significant (ns), * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data presentation was performed using GraphPad Prism 5 data science packages.

3. Results and Discussion

3.1. Characterization of spray-dried and subsequently dried LNPs

3.1.1 RNA quantification and physicochemical properties of spray-dried and subsequently dried LNPs

One of the most important quality criteria for all pharmaceutical processes is the quantification of drug loss after processing. Spray drying of LNPs and subsequent drying of the powder create additional stress on the nanoparticle delivery system and the RNA itself. In order to assess if the spray drying and subsequent drying process results in siRNA losses, an RNA quantification assay was performed. For all samples, not more than 10% of siRNA loss was detected (Figure 1) when spray-dryed at 62 °C outlet temperature. This result was independent of applying a subsequent drying step at the same temperature for 20 min and of storing the samples at 4 °C or 25 °C for 3 months. Moreover, it is perfectly in line with values obtained after spray drying only without subsequent storage. [21]

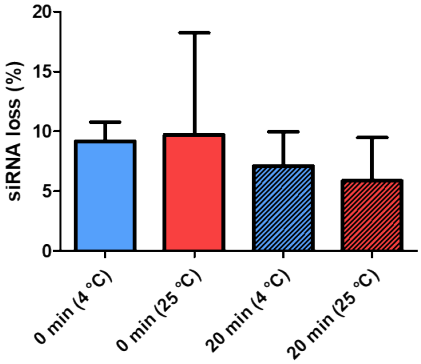

Figure 1 Quantification of siRNA losses after spray drying and subsequent drying for 20 min after 3 month storage at 4 °C and 25 °C. Spray drying of LNPs dispersed in 5% lactose solution (m/V) at an outlet temperature of 62°C. Each bar shown as mean ± SEM, n=3.

Subsequently dried LNP samples show a slight reduction in siRNA losses to 7-8%. However, the difference to only spray-dried LNPs is non-significant. The standard deviation of spray-dried powders stored for 3 months at 25 °C exceeded all other values, indicating that individual measurements varied more strongly. The spray drying procedure itself and the storage conditions could have been responsible for the preparation of a qualitatively lower performing batch. Overall, spray drying and subsequent drying of LNPs limited the loss of siRNA to a maximum of 10%, which is the process-mediated loss already observed directly after spray drying. [21] Hence, spray drying seems to be a suitable method for prolonging storage stability of RNA-loaded LNPs for a longer period at temperatures of 4 °C and 25 °C.

Another important aspect to look at are the physicochemical properties of spray-dried and subsequently dried LNPs being stored for 3 months. DLS measurements determine the nanoparticle sizes and quantify the size distribution. LNP microparticle powders were redispersed in HPW to prepare for measurements. All LNPs showed sizes of 180-200 nm after spray drying, subsequent drying and storage at 4 °C and 25 °C (Figure 2A), which are in line with values reported for freshly spray-dried LNPs. [21] The PDIs varied between 0.1 and 0.15 indicating a narrow size distribution after storage. Furthermore, a 5% PDI increase was observed for spray-dried powder without subsequent drying stored at 25 °C in comparison to samples stored at 4 °C. However, no significant differences were found between all LNP powders stored for 3 months. Compared with previously reported results, [21] the subsequent drying and storage at 4 °C and 25 °C for 90 days did not have an influence on the particle size or PDI. Furthermore, all samples showed zeta potentials between +2 - +3 mV (Figure 2B). As seen and explained before, the lactose matrix acts as a shield for charges and slowly dissolves fully. [21] Comparing drying times and storage conditions with each other, no variance in zeta potential was apparent. A disruption or change in LNP formulation would show obvious changes in zeta potential. Hence, the LNP structure seems to be maintained and the siRNA encapsulated during subsequent drying and over storage at 4 °C and 25 °C for 3 months.

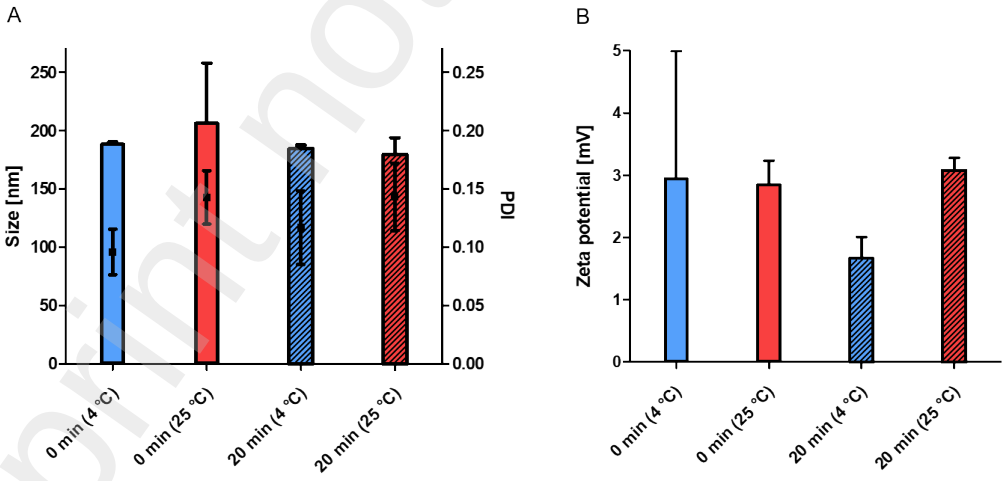

Figure 2 Physico-chemical properties of spray-dried (0 min) and subsequently dried (20 min, shaded) LNPs stored for 3 months at 4 °C (blue bars) and 25 °C (red bars). A) DLS measurements showing sizes and PDIs. B) Zeta potential measured via LDA. Spray-dried (0 min) and subsequently dried (20 min) samples were redispersed in HPW. Spray drying temperature was set to 62 °C outlet temperature. Mean ± standard deviation, n=3.

Storage conditions are one way of influencing a products' quality. Residual moisture can favor microparticle aggregation and microbial growth, a phenomenon to be prevented, especially during storage at room temperature. It is pivotal thus to determine the powder's residual moisture before

packaging and storing. [18] As shown in Figure 3, spray drying 5% lactose solution (m/V) resulted in a residual moisture level of 5%, which is in line with literature values. [17] Spray drying LNPs dispersed in 5% lactose solution (m/V) reduced the residual moisture to 3.6%. This level was kept constant while storing the samples at 4 °C. Applying a subsequent drying of 20 min reduced the residual moisture to 2.8%, showing a slight increase in moisture to 3.1% after storage at 25 °C. This increase was considered neglectable because no statistically significant difference was observed. As discussed before, the amorphous state of spray-dried lactose is favorable for LNP preservation. Moreover, a high glass transition temperature T_g value is necessary to keep the product stable over longer storage time. The T_g value is oppositely linked to the residual moisture level, meaning that the higher the residual moisture level, the lower the T_g value. [18, 21] Here, the moisture levels of spray-dried and subsequently dried LNPs in lactose were reduced in comparison to spray-dried 5% lactose solution. The decrease in residual moisture level favors the maintenance of the amorphous state of LNP powders, prolonging stability and avoiding nucleation and degradation processes over a longer storage time. In fact, storing the spray-dried LNP powders for 3 months at 4 °C (blue bars) and 25 °C (red bars) in 20R vials after sealing them did not result in any elevation of the residual moisture compared to the values measured directly after spray drying (white bars).

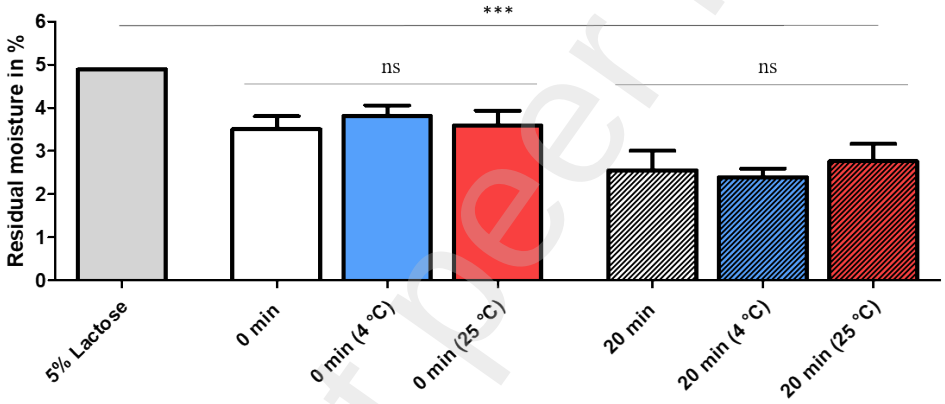

Figure 3 Residual moisture of spray-dried and subsequently dried LNPs. Subsequent drying lasted for 20 min. White bars show spray-dried samples directly analyzed, blue bars show samples stored at 4 °C and red bars show samples stored at 25 °C. All stored samples were kept sealed for 3 months. Mean ± standard deviation, n=3.

3.2.3 Geometric sizes and aerodynamic performance of spray-dried and subsequently dried LNPs

Spray-dried powders that are administered to the lungs need to meet size requirements in order to be delivered to the bronchioles or alveoli. A first indication can be drawn by measuring the geometric median diameter (GMD) via SEM. Information about the particles' surface and composition can be obtained. Prior measurements have shown spray-dried LNP sizes of 2-9 µm. [21] Subsequent drying of these powders and storing at 4 °C and 25 °C for 3 months led to geometric sizes of 3-8 µm (Figure 4). Therefore, the additional heat impact of subsequent drying did neither form microparticle aggregates nor merged powder particles. The sizes were unchanged, and the surface still appeared smooth, demonstrating ideal conditions of the subsequently dried powders for pulmonary administration.

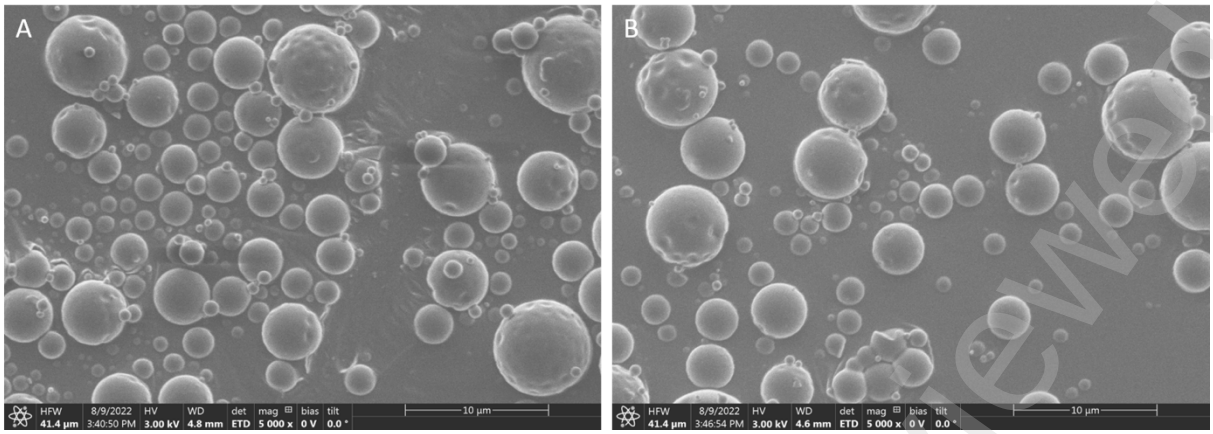

Figure 4 SEM pictures of spray-dried A) siGFP-(+)LNP after 20 min subsequent drying stored for 3 months at 4 °C, B) siGFP-(+)LNP after 20 min subsequent drying kept at 25 °C storage for 3 months. All samples were spray-dried in 5% lactose solution at 62°C outlet temperature.

Although the GMD provides a good impression of the actual sizes of spray-dried powders, the mass median aerodynamic diameters (MMAD) of porous materials commonly fall below the GMD. [32] For pulmonary application, ideal particle sizes of 3-7 µm are required. [33-35] A next generation impactor (NGI) was used to measure the aerodynamic performance of spray-dried and subsequently dried LNPs after storage at 4 °C and 25 °C for 90 days. In Table 2 *Microparticle characteristics of LNP formulations dispersed in 5% lactose solution spray-dried and subsequently dried at an outlet temperature of 62 °C assessed using a Next Generation Impactor (NGI). Subsequent drying lasted 20 min. Samples were stored for 3 months at 4 °C and 25 °C.* various comparisons between drying time and storage temperature are presented. Spray-dried LNPs that were stored at 4 °C resulted in an MMAD of 2.73 µm at a geometric standard deviation (GSD) of 1.85 µm. The fine particle fraction (FPF), which represents the mass percentage of spray-dried LNPs with an aerodynamic diameter below 5 µm, was detected at 30.1% at a recovery rate of 98%. Previous results reported a similar MMAD of 2.85 µm and a FPF value of 28.1%. [21] In comparison, spray-dried LNP powders that were subsequently dried and stored at 4 °C reported an increased MMAD of 3.78 µm. The FPF was reduced to 21.5% but the recovery rate remained at 99%. The subsequent drying at 62 °C outlet temperature could have caused the increase in MMAD by merging microparticles, leading to a reduced FPF value. Due to the reduced residual moisture after subsequent drying reduced, the opposite was expected as a result of protection against microparticles aggregation during storage. Nevertheless, sugar matrices tend to melt and merge if the T_g is exceeded, which was the case during secondary drying. Interestingly, the aerodynamic properties of spray-dried LNPs stored at 25 °C without secondary drying resulted in an MMAD of 4.98 µm, the highest MMAD value of all spray-dried samples. Even though the residual moisture level remained at 4% when samples were stored at 25 °C, a dominant increase in MMAD by more than 2 µm changed the characteristics of the spray-dried powder. It was concluded that 4% residual moisture is too high for storage at room temperature, resulting in microparticle aggregation over time. Accordingly, the FPF was reduced to 15.9% and the recovery rate sank to 71.3%. The reduction in recovery can be explained by microparticle aggregates retain the LNPs in the glued sugar matrix, preventing LNP release for quantitative siRNA detection. Furthermore, a bigger particle is less likely to be expelled by the Handihaler® application device, lowering the amount of siRNA detected. In addition, a big standard deviation for samples stored at 25 °C storage (0 min) implied batch to batch variability which became more obvious over storage time. However, a subsequent drying step clearly improved the powder characteristics after storage at 25 °C. The MMAD was stable at 3.60 µm (3.78 µm after storage at 4 °C), the FPF was increased to 22.4% (21.5% after storage at 4°C), and the recovery rate was enhanced to 83.9%. Therefore, it was concluded that applying a subsequent drying step for dry powder products containing LNPs that are intended to be stored at 25°C is advisable.

In fact, if the results are compared with regards to their drying conditions, the data reveal that aerodynamic properties of powders that underwent subsequent drying did not differ from each other. Solely, the recovery rate was 16% lower after 25 °C storage in comparison to 4 °C storage. In contrast, the comparison of spray-dried powders without subsequent drying resulted in an increased MMAD of almost 2-fold, a 2-fold reduction in FPF and a decrease of powder recovery by almost 27% for powders stored at 25 °C compared with powders stored at 4 °C. Nevertheless, the optimal MMAD range of 3 - 7 µm was met by all powders, facilitating ideal microparticle characteristics for dry powder pulmonary applications.

Table 2 Microparticle characteristics of LNP formulations dispersed in 5% lactose solution spray-dried and subsequently dried at an outlet temperature of 62 °C assessed using a Next Generation Impactor (NGI). Subsequent drying lasted 20 min. Samples were stored for 3 months at 4 °C and 25 °C. DD: dose delivered, FPD: fine particle dose, FPF: fine particle fraction, MMAD: mass median aerodynamic diameter, GSD: geometric standard deviation.

	0 min, 4°C	20 min, 4°C	0 min, 25 °C	20 min, 25 °C
DD	3.19 ± 0.34	3.80 ± 0.67	2.67 ± 0.38	1.96 ± 0.16
FPD (<=µm)	0.96 ± 0.08	0.80 ± 0.06	0.41 ± 0.21	0.43 ± 0.18
FPF (<=µm)	30.1 % ± 0.7 %	21.5 % ± 5.3 %	15.9 % ± 10.0 %	22.4 % ± 11.3 %
MMAD [µm]	2.73 ± 0.30	3.78 ± 0.49	4.98 ± 1.71	3.60 ± 0.70
GSD [µm]	1.89 ± 0.01	2.55 ± 0.66	2.03 ± 0.26	3.67 ± 2.55
Recovery	98.0 % ± 25.2 %	99.9 % ± 10.5 %	71.3 % ± 6.5 %	83.9 % ± 4.9 %

3.3. *In vitro* characterization of spray-dried LNPs

Present results established the loading and colloidal stability of siRNA-loaded LNPs after spray drying and storage and confirmed ideal aerodynamic properties of all spray-dried microparticles for pulmonary application. Hence, the bioactivity of spray-dried and subsequently dried LNPs after 3 months storage was assessed. Prior to bioactivity experiments, an MTT study revealed the lack of cytotoxic effects for all spray-dried and subsequently dried powders (Figure 5A). The reduced cell viability (approximately 80%) could result from lactose which can influence the osmotic pressure on cells resulting in decreased metabolic activity.

A gene silencing experiment targeting the enhanced green fluorescent protein stably expressed in H1299 (H1299-GFP) cells was performed using spray-dried and subsequently dried LNPs encapsulating scrambled, negative-control siRNA (siNC) or siRNA against GFP (siGFP) (Figure 5B**Figure 5** In vitro evaluation of spray-dried and subsequently dried LNPs. **A**) In vitro cytotoxicity evaluation via MTT assay in H1299 cells. **B**) Gene silencing effect of enhanced green fluorescent protein (eGFP) within a H1299-eGFP expressing cell line. siRNA concentration was 1 µg/mL. **C**) Gene silencing effect of house keeping gene GAPDH in mucus secreting Calu-3 cells. siRNA concentrations were 10 µg/mL. White samples were freshly spray-dried. Blue samples were stored for 3 months at 4 °C, and red samples were stored for 3 months at 25 °C. Mean ± standard deviation, n=3.). LNPs applied were freshly prepared samples (white bars), LNPs stored at 4 °C for 3 months (blue bars), or LNPs stored for 3 months at 25 °C (red bars). As established in a previous study, the siRNA concentration per transfection was 1 µg/mL

(55.7 nM siGFP/siNC). Throughout all samples and independent of the storage conditions, a highly significant GFP-knockdown of >95% was obtained. This matches previous results for gene silencing of spray-dried (+)LNPs. [21] Hence, the subsequent drying and storage for 90 days at 4 °C and 25 °C did not influence the bioactivity of the siRNA or LNPs, maintaining a very high gene silencing efficacy.

Following the investigation of successful protein knockdown, a mucus secreting cell line, Calu-3, was used to evaluate the gene knockdown efficiency on the mRNA level targeting the house-keeping gene GAPDH. Calu-3 cells were transfected by spreading spray-dried LNP powder, containing either siNC or siGAPDH, on the mucus layer that was grown on air liquid interface inserts. The knockdown efficiency was determined after 48 h incubation time. Figure 5C shows the %-values of GAPDH mRNA standardized by the β-actin mRNA levels and normalized against the values obtained after treatment with LNPs encapsulating negative control siRNA (siNC). Comparing the storage conditions for the same drying parameters, LNPs stored at 4 °C resulted in a GAPDH/β-actin expression of 55.9%, meaning a mRNA knockdown of 44.1% was achieved. However, storage at 25 °C without secondary drying resulted only in an mRNA knockdown of 29.2%. This reduction can be explained by the previous results of batch variety after storage at 25 °C. The bigger microparticle MMAD and lower FPF require longer dissolution and LNP release times and are less likely to diffuse through the mucus layer. Of course, a lower recovery rate will lead to a lower amount of available siRNA, adding to the discrepancy in mRNA knockdown levels of the powders stored at different temperatures. In contrast, subsequently dried LNP powders resulted in a mRNA knockdown of 50.7% and 44.3% for 4 °C and 25 °C storage, respectively.

As already discussed with regards to the aerodynamic performance of the spray-dried powders, LNP powders stored at 4 °C and subsequently dried powders stored at both temperatures do not differ in their performance. Only the spray-dried powders stored at 25 °C performed lower than others. Comparing samples stored at the same temperature, it can be appreciated that a subsequent drying step improved the GAPDH knockdown performance of LNPs stored at 25 °C, which performed comparably to their counterparts stored at 4 °C. Additionally, the mRNA silencing effect remained unchanged for samples stored at 4 °C. It can therefore be concluded that a residual moisture below 4% allows for siRNA-LNP storage at room temperature for at least 3 months.

This cell culture model was implemented to imitate real-life dry powder administration to the lungs, usually performed by dry powder inhalers. Here, powders must overcome natural barriers, such as lung mucus, before reaching the targeted cells. The lungs' mucus is constantly secreted, acting as a protective shield. Hence, any particle or drug that targets pulmonary cells needs to pass the mucus layer and a certain level of drug loss is expected. [21, 36, 37] Reaching GAPDH knockdown levels of 30-50% show the immense potential of LNPs for pulmonary delivery. Even a storage time of 90 days and storage conditions at room temperature did not harm the performance and maintained the LNP and siRNA stability and activity.

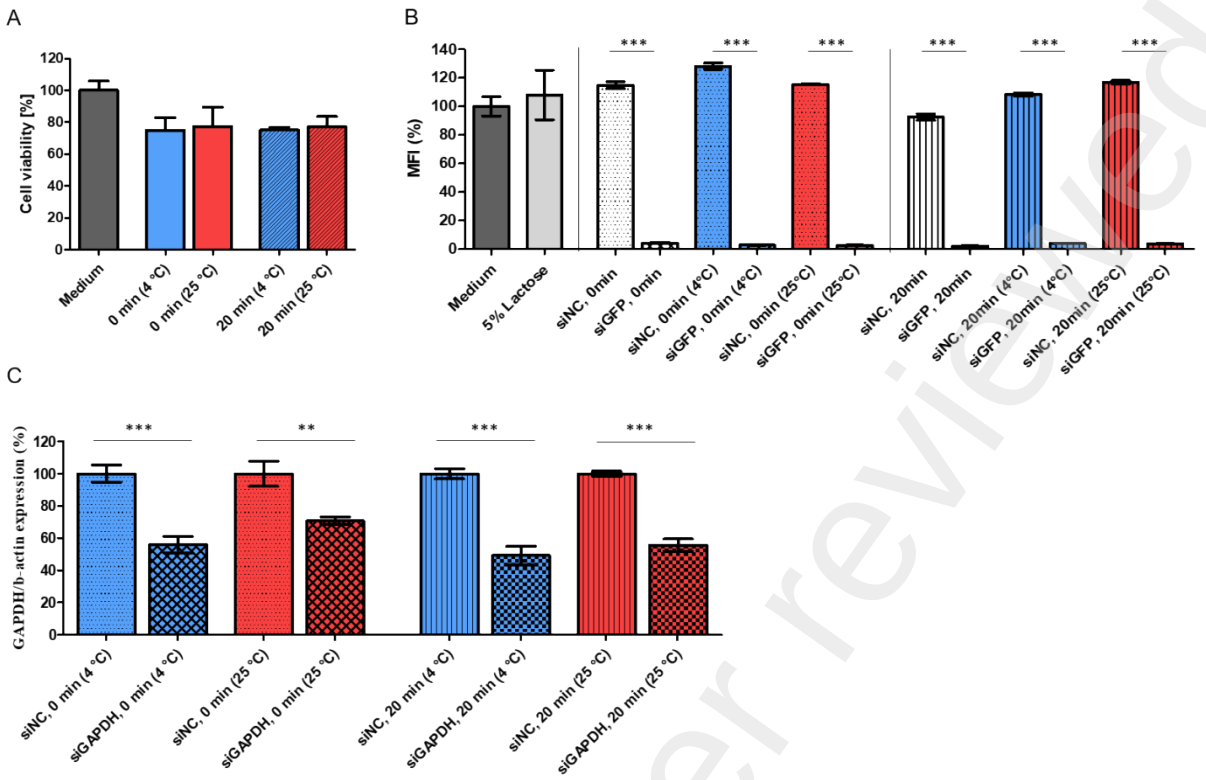

Figure 5 *In vitro* evaluation of spray-dried and subsequently dried LNPs. **A**) *In vitro* cytotoxicity evaluation via MTT assay in H1299 cells. **B**) Gene silencing effect of enhanced green fluorescent protein (eGFP) within a H1299-eGFP expressing cell line. siRNA concentration was 1 µg/mL. **C**) Gene silencing effect of house keeping gene GAPDH in mucus secreting Calu-3 cells. siRNA concentrations were 10 µg/mL. White samples were freshly spray-dried. Blue samples were stored for 3 months at 4 °C, and red samples were stored for 3 months at 25 °C. Mean ± standard deviation, n=3.

4. Conclusion

In the various phases of drug product development, identifying the conditions for long-term storage, it is pivotal to properly account for a scale-up of the manufacturing process. The aim of this study was to compare the stability of spray-dried and subsequently dried LNPs at storage conditions of 4 °C and 25 °C. Our previously established spray drying method for LNPs dispersed in 5% lactose solution (m/V) served as a starting point for the spray drying setup, parameters and the powder characterization. [21] Neither the spray drying, nor the subsequent drying or different storage temperatures resulted in siRNA losses >10%, underlining the preservation of siRNA inside the LNPs. Colloidal stability was reflected in measured nanoparticle sizes of about 180 nm for all spray drying and storage conditions. Furthermore, the size distribution remained narrow at PDI values from 0.1 - 0.15, and zeta potentials remained positive at +3 mV. The spray-dried microparticles showed residual moisture levels of about 4%, which was reduced to 3% applying the subsequent drying for 20 min. Storage at different temperatures did not lead to an increase in moisture levels. The spray-dried powder demonstrated optimal aerodynamic properties for pulmonary administration. Apart for the spray-dried powder, which did not undergo secondary drying and was stored at 25 °C, all samples' MMAD was between 2.8 and 3.6 µm, qualifying for administration to the lower respiratory tract. It was impactfully shown that a subsequent drying step can improve the MMAD and FPF when storing spray-dried LNPs at 25 °C. This improvement was not observed when stored at 4 °C. All samples resulted in efficient protein silencing of >95% in a lung adenocarcinoma cell line. As demonstrated before, LNPs can successfully pass the mucus layer present on mucosal tissues. Therefore, efficient GAPDH knockdown in mucus secreting Calu-3 cells was observed and resulted in a 50% gene silencing effect with all samples stored at 4 °C or subjected to secondary drying. In summary, our 90-days research on spray-dried and subsequently dried LNPs

confirms the conservation of LNP structure, cargo integrity and siRNA bioactivity both at storage temperatures of 4 °C or 25 °C after secondary drying, maintaining ideal properties for pulmonary delivery. Most importantly, it was shown that residual moisture below 4% allows for storage at room temperature for at least 3 months time.

Acknowledgements

The authors thank Prof Pieter Cullis and Dr Dominik Witzigmann for supporting this collaborative project and for providing the LNP formulations. The authors acknowledge Prof Marco Ciufolini and Dr. Fariba Saadati for providing the sulfur-containing lipid. This project was funded by the European Research Council (ERC-StG 637830 and ERC-2022-PoC1-101069308 to O.M.M)

Conflict of interest

O.M.M is an advisory board member for Coriolis Pharma GmbH, Corden Pharma GmbH, AMW GmbH and Carver BioSciences a consultant for AbbVie Deutschland GmbH, Boehringer-Ingelheim International GmbH and for PARI Pharma GmbH on unrelated projects. P.L. has consulted and received research grants from Lipoid, Sanofi-Aventis Deutschland and DSM Nutritional Products Ltd.

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