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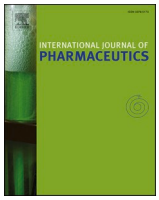

Formulation screening of lyophilized mRNA-lipid nanoparticles

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ABSTRACT

Lipid nanoparticles (LNPs) have demonstrated their therapeutic potential as safe and effective drug delivery systems for nucleic acids during the COVID-19 pandemic. However, one of the main challenges during technical CMC (Chemistry, Manufacturing, and Controls) development is their long-term stability at temperatures of 2–8 °C or higher, which may be improved by the removal of water by lyophilization. In this study, we identified lyo-/cryo-protectants for freeze-dried mRNA-LNP formulations beyond conventional excipients such as sucrose and trehalose as T_g-modifiers using polyA as a surrogate. Hydroxypropyl-beta-cyclodextrin, Kollidon® 12 PF (PVP), and dextran 40 kDa were tested in combinations to best stabilize the mRNA-LNPs during the lyophilization process as well as during storage for up to 6 months at 2–8 °C, 25 °C/60 % r.h., and 40 °C/75 % r.h.. We also tested the formulation principle including protectants in- and outside of the LNPs. Formulations were assessed for size, PDI, encapsulation efficiency, and properties related to the lyophilized dosage form. While 10 % (w/V) sucrose formulations successfully stabilized LNPs during the lyophilization process, they were not suitable for storage at temperatures beyond 2–8 °C. The most promising formulations for storage at higher temperatures were identified as 9 % (w/V) trehalose + 1 % (w/V) PVP with only a small increase in size over 6 months at 25 °C maintaining PDI and encapsulation efficiency. Results were verified with eGFP-mRNA-LNPs and tested in cell culture experiments. This study may serve as guidance for formulation scientists to further optimize freeze-dried mRNA-LNP formulations and eventually eliminate the cold chain for mRNA-LNP products.

1. Introduction

The messenger RNA (mRNA) vaccines Comirnaty® and Spikevax® showed the full potential of lipid nanoparticles (LNPs) as new delivery platform technology during the COVID-19 pandemic (Crommelin et al., 2021). In vitro production allowed the cost-conscious development of effective and safe vaccines in no time (Gote, 2023). They were produced in large amounts, e.g., three billion delivered Comirnaty® doses in 2021 (Warne, 2023); and the mRNA encoding for the spike protein was adjusted in only a few months after the Omicron variant emerged (Webb, 2022). However, one of the main challenges associated with technical CMC development of mRNA-LNPs is their stability (Schoenmaker, 2021). mRNA is sensitive, among others, to thermal and enzymatic degradation (Oude Blenke, 2022). Even a single alteration in the sequence (e.g. strand break, oxidation) in the long mRNA strand (1000 – 5000 nucleotides) can stop translation and lead to loss of efficacy. Therefore, mRNA COVID-19 vaccines have to be stored frozen at temperatures between −90 °C and −60 °C (Comirnaty® from BioNTech) and between −50 °C and −15 °C (Spikevax® from Moderna) respectively, which is challenging from a supply chain perspective including storage logistics and global distribution.

There are multiple approaches to improve the stability of mRNA-LNPs including the engineering of the mRNA on a molecular basis, optimization of the lipid composition of the delivery system, as well as optimization of the surrounding formulation of the LNP. Among others, the removal of water from the drug delivery system has been previously described as a promising formulation strategy to improve long-term stability and enable storage at temperatures of 2–8 °C or higher (Oude Blenke, 2022; Muramatsu, 2022). Since spray-drying may be problematic due to the use of high temperatures (Friis, 2023), lyophilization has been described as a promising approach for drying the formulations (Gote, 2023). In addition, it has been previously discussed that the

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secondary structure of the mRNA might play a decisive role for long-term stabilization (Oude Blenke, 2022). Moreover, the structural properties of LNPs also appear to influence the functionality and stability of mRNA-LNPs. In particular, the location of the mRNA – whether segregated into aqueous bleb structures or lipid-associated in the core – plays a crucial role (Simonsen, 2024).

Comirnaty® and Spikevax® are stored frozen and both vaccines contain sucrose as a cryoprotectant. While Spikevax® uses a Tris buffer, Comirnaty® used a phosphate buffer with potassium chloride and sodium chloride in the first generation vaccine, which was changed to Tris buffer for the second generation (EMA, 2023; EMA, 2024). Tris buffer has the advantage of acting as an "aldehyde sink" reducing the formation of mRNA-lipid adducts (Oude Blenke, 2022). Additionally, the switch to Tris buffer may eliminate the pH shift during the freezing step (Thorat and Suryanarayanan, 2019). As both vaccines contain sucrose and Tris buffer, they are promising formulations for lyophilization. In a recent study, Fan et al. proposed an acetate buffer at a pH of 5 yielding a superior biological read-out after lyophilization compared to other buffer systems such as PBS; and also reported promising results for a formulation containing Tris buffer (Fan, 2024).

Recent publications demonstrate that lyophilized mRNA-LNPs can be stored for several months at 2–8 °C and for a few weeks at 25 °C. Suzuki et al. used LNPs consisting of the newly synthetized, ionizable lipid L202, DSPC, cholesterol, and PEG-DMG. The encapsulated mRNA coded for the SARS-CoV-2 spike protein and the lyophilized LNPs were formulated in 20 mM Tris buffer with different sucrose concentrations. Samples with 16 % (w/V) sucrose showed consistent immunogenicity in mice after 1 month of storage at 5 °C and 25 °C (Suzuki, 2022). Muramatsu et al. manufactured LNPs of the ionizable lipid Lipid 10, DSPC, cholesterol, and PEG2000-c-DMA. They used mRNA encoding for firefly luciferase (fLuc) and influenza virus hemagglutinin. With a formulation of 5 mM Tris containing 10 % sucrose and 10 % maltose (w/V), they reached 24 weeks of stability at 4 °C and 12 weeks of stability at room temperature (Muramatsu, 2022). Meulewaeter et al. prepared LNPs using the ionizable lipid C12-200, DSPC, cholesterol, and DMG-PEG2000 and encapsulated enhanced green fluorescent protein (eGFP) or fLuc-mRNA. They tested Tris, phosphate, and PBS buffer in buffer capacities between 10 mM and 40 mM with 12.5 % (w/V) sucrose or trehalose. The samples were exposed to a continuous freeze-drying process. A formulation with 20 mM Tris and 12.5 % sucrose maintained the transfection properties after 12 weeks at 4 °C, 22 °C, and even at 37 °C. However, the formulation with 10 mM phosphate buffer and 12.5 % sucrose showed good results (Meulewaeter, 2023). Shirane et al. prepared LNPs with ssPalmO-Phe-P4C2, DOPC, cholesterol, and DMG-PEG2000 encapsulating luciferase or hEPO mRNA. The lipid solution was prepared in 90 % t-BuOH because the organic phase was not removed by dialysis but directly during the freeze-drying process. Sucrose was added to the LNPs before they were lyophilized. They tested different buffers (pH values and sodium chloride concentrations) and different sucrose concentrations and concluded that for their alcohol dilution-lyophilization method, acidic pH and low salt concentration were beneficial. Lyophilized LNPs protected with 16 % (w/V) sucrose preserved the gene expression efficiency for at least 4 months at 4 °C (Shirane, 2023). Highest stability was achieved by Ai et al., who lyophilized SARS-CoV-2 vaccines that showed no change in physiochemical properties and bioactivities after 6 months at 25 °C (Ai, 2023). However, they did not disclose the ionizable lipid, buffer system, or protectant used.

In sum, most of these studies use conventional sucrose or trehalose as a cryo- and lyoprotectant to stabilize the LNPs against stress during both the freezing drying process. While trehalose exhibits higher glass transition temperatures (T_g'/T_g), which are beneficial for more aggressive lyophilization cycle conditions and enable higher storage temperatures, trehalose crystallization during frozen storage needs to be investigated (Singh, 2011). In contrast, sucrose has a slightly lower T_g'/T_g and may crystallize and hydrolyze at elevated storage temperatures. Further tested sugars reported in literature are mannitol, lactose, and glucose, which were not successful in sufficiently stabilizing mRNA-LNPs during lyophilization or storage (Zhao, 2020; Li, 2023).

T_g'/T_g-modifiers have been described in the literature as promising excipients to increase the glass transition temperature of the freeze-concentrated solution (T_g'), as well as of the lyophilized cake (T_g). As a result, lyophilization cycles may be significantly shortened due to more aggressive primary drying, saving time and energy, and lyophilized products can be potentially stored at higher temperatures without a collapse or impairment of the lyophilized cake. Häuser et al. described the use of sucrose (Suc), hydroxypropyl-beta-cyclodextrin (CD), dextran 40 kDa (Dex), and PVP for lyophilization of monoclonal antibodies. Formulations with CD/Suc and HP-beta-CD/PVP/Suc performed better for the amorphous protein matrix than pure sucrose for 9 months of storage at 40 °C, potentially enabling storage at room temperature (Haeuser et al., 2020). In another publication, the same authors showed that lyo cycle time may be reduced by 50 % using a CD/Suc formulation (Haeuser et al., 2019). In a very recent study, Fan et al. evaluated the addition of various cryo/lyo-protectants including sucrose, maltose, PEG-1500 and PVP-K-12 as well as lysine for their ability to stabilize eGFP encoding mRNA during lyophilization. They concluded that PVP in combination with an acetate or Tris buffer might be promising excipients based on various analytical read-outs including cell culture experiments. However, they did not provide stability data on their formulations offering mechanistic insights about the influence of physiochemical and structural properties of lyophilized mRNA-LNPs on functionality (Fan, 2024). The formulation principle of using T_g'/T_g-modifiers to stabilize lyophilized mRNA-LNPs on long term storage and above 2–8 °C has not been tested. For the sake of clarity, T_g'/T_g-modifiers are only called T_g-modifiers later in this article.

The objective of this study was to identify T_g-modifiers that best stabilize mRNA-LNPs during the lyophilization process as well as during long-term storage, to guide future formulation development of lyophilized mRNA-LNPs. For this purpose, we included hydroxypropyl-beta-cyclodextrin, Kollidon® 12 PF (PVP), and dextran 40 kDa as T_g-modifying excipients into the study. While the optimization of pH and buffer system was not the focus of this study, we further studied the combination of conventional cryo-/lyo-protectants such as sucrose and trehalose with the T_g-modifiers, as well as the presence of sugars and T_g-modifiers inside the LNPs. This was achieved by adding protectants into the aqueous phase before the mixing step.

PolyA-LNPs with a lipid composition equivalent to the Moderna vaccine Spikevax® were used as a surrogate for mRNA-LNPs consisting of SM-102, cholesterol, DSPC, and DMG-PEG2000. Results were confirmed with eGFP-mRNA-LNPs. We determined several product attributes for the lyophilized product including T_g, cake appearance, residual moisture, and reconstitution time, as well as formulation attributes of the reconstituted solution including pH, osmolality, and T_g'. Critical quality attributes (CQAs) of the LNPs such as size/PDI and encapsulation efficiency were assessed before and after lyophilization and after storage up to 6 months at 2–8 °C, 25 °C/60 % r.h., and 40 °C/75 % r.h.. eGFP-mRNA protein expression was tested in cell culture experiments using HeLa cells.

2. Material and methods

2.1. Preparation of mRNA-LNPs

Nucleic acid solutions were prepared from either polyadenylic acid (polyA) (ABP Biosciences, Virginia, USA) or CleanCap EGFP-encoding mRNA (TriLink, San Diego, USA) in 50 mM citrate buffer pH 4.0 (citric acid, Thermo Fisher Scientific; sodium citrate tribasic, Sigma-Aldrich, St. Louis, USA). Stock solutions of the ionizable lipid SM-102 (BroadPharm, San Diego, USA), cholesterol (Sigma-Aldrich), the helper lipid DSPC (Lipoid, Ludwigshafen, Germany), and DMG-PEG2000 (Avanti Polar Lipids, Birmingham, USA) were prepared in ethanol

(Carl Roth, Karlsruhe, Germany) and mixed at a molar ratio of 50 : 38.5 : 10 : 1.5.

LNPs were prepared by rapid mixing using peristaltic pumps (P-1, GE Healthcare, Uppsala, Sweden) connected via a T-shaped PEEK union (Thermo Fisher Scientific, Rockford, USA) and PEEK capillary tubing (0.02 in ID, Thermo Fisher Scientific). The mixing was performed at a total flow rate (TFR) of 15 mL/min, a flow rate ratio (FRR) of 2:1 (aqueous:organic), and a N/P ratio of approximately 6.

LNPs were dialyzed overnight at 2–8 °C into a 20 mM Tris buffer pH 7.4 (Thermo Fisher Scientific) using Slide-A-Lyzer™ G3 dialysis cassettes (20 K MWCO, Thermo Fisher Scientific). The LNPs were formulated to a total solid content of 10 % (w/V) using the following lyo-/cryo-protectants as summarized in Table 1: sucrose (Suc) (Caelo, Hilden, Germany), trehalose (Trh) (Pfanstiehl, Waukegan, USA), HP-β-CD (CD) (Kleptose HPB, Roquette, Lestrem, France), Kollidon 12PF (PVP) (BASF, Ludwigshafen, Germany), and Dextran 40 (Dex) (Carl Roth). Selected LNPs were formulated with the protectant also present in the core of the LNPs, which was achieved by adding the respective protectant to the nucleic acid-citrate buffer solution before rapid mixing using a stock solution in 50 mM citrate buffer pH 4.0. The principle follows the findings by Arte et al. classifying lyoprotectants such as trehalose as non-membrane penetrating (Arte, 2024).

The formulations were filtered through 0.20 μm PVDF filters (Chromafil, Faust, Klettgau, Germany). The final nucleic acid concentration was verified between 10–15 μg/mL.

300 μL of LNP solution were filled into clear 2 mL Fiolax® vials (Schott, Müllheim, Germany) and partially closed with lyo stoppers (Datwyler Pharma Packaging, Alken, Belgium) before lyophilization.

2.2. Lyophilization and storage

Lyophilization was performed on a pilot freeze-dryer Epsilon 2-6D (Christ, Osterode, Germany). Samples were frozen at 1 K/min to −40 °C. The shelves were kept at −40 °C for 3 h. Primary drying was performed at 0.13 mbar and −20 °C. The end of primary drying was determined after 10 h by comparative pressure measurement (Pirani vs. capacitive diaphragm vacuum gauge). For secondary drying, the temperature was increased to 25 °C at a rate of 0.2 K/min and kept for 5 h. The vials were stoppered at 750 mbar, removed from the freeze-dryer, and capped (Crimp Caps N13 with center tear off, Macherey-Nagel, Düren, Germany) before storage at 2–8 °C, 25 °C/60 % r.h., or 40 °C/75 % r.h.. One vial of each formulation was analyzed after 1, 3, and 6 months as a technical triplicate. It should be noted that for the 40 °C storage conditions, a temperature deviation of 7 weeks at 17–23 °C between t_3 and t_6 has been detected.

2.3. Physico-chemical properties

pH and osmolality were determined using an InLab Micro electrode (Mettler Toledo) and a semi-micro osmometer K 7400 (Knauer, Berlin, Germany).

2.4. Dynamic light scattering

Dynamic light scattering (DLS) was performed using a Zetasizer Nano ZS (Malvern Panalytical, Kassel, Germany) to determine the size (z-average) and polydispersity index (PDI) of the LNPs. Samples were diluted 1:8 in 10 mM Tris containing 140 mM NaCl and equilibrated for 60 s to 25 °C. Detection occurred at 173° backscattering with automatic attenuation and triplicates were performed consisting of 10 runs of 10 s.

2.5. RiboGreen assay

The encapsulated and total nucleic acid concentrations were

Table 1
Formulation compositions and formulation attributes. Formulation composition: LNPs were formulated at a nucleic acid concentration between 10 – 15 μg/mL in a 20 mM Tris buffer pH 7.4 and a total solid content of 10 % (w/V). The lyo-/cryo-protectants are present outside the LNPs and for some LNPs present inside the core as well. (Suc = sucrose, Trh = trehalose, CD = HP-β-CD, PVP = Kollidon 12PF, Dex = Dextran 40). Formulation attributes: T_g' was measured using the liquid sample (t_0). Osmolality (t_0) and pH (average of t_0 and t_6) were measured after reconstitution with RNase-free water. Reconstitution time (average of all samples without collapse), T_g (t_0), and initial residual moisture (t_0) were analyzed using the lyophilized samples.

	Formulation composition				Formulation attributes					
No.	Nucleic acid	Protectant 1 (% w/V), outside	Protectant 2 (% w/V), outside	Protectant (% w/V), inside	T_g' (°C)	Osmolality (mOsm/kg)	Reconstitution time (s)	pH	T_g (°C)	Initial residual moisture (%)
1	polyA	10 % Trh	–	*liquid*	−29.5	289	–	7.3 ± 0.1	–	–
2	polyA	10 % Suc	–	–	−33.0	345	15 ± 4[1]	7.3 ± 0.1	42.2	2.5 ± 0.2
3	polyA	10 % Trh	–	–	−29.5	289	25 ± 6	7.3 ± 0.1	97.3	1.0 ± 0.2
4	polyA	7 % Trh	3 % CD	–	−24.7	269	49 ± 18	7.3 ± 0.1	104.0	0.4 ± 0.1
5	polyA	9 % Trh	1 % CD	–	−27.9	309	26 ± 4	7.3 ± 0.1	101.2	0.6 ± 0.0
6	polyA	5 % Trh	5 % PVP	–	−25.5	196	20 ± 6	7.3 ± 0.1	110.0	0.4 ± 0.0
7	polyA	7 % Trh	3 % PVP	–	−26.8	239	19 ± 5	7.1 ± 0.1	104.5	0.3 ± 0.1
8	polyA	9 % Trh	1 % PVP	–	−27.3	283	19 ± 5	7.2 ± 0.1	98.1	0.6 ± 0.2
9	polyA	9 % Trh	1 % Dex	–	−28.9	314	30 ± 5	7.3 ± 0.1	99.4	0.4 ± 0.1
10	polyA	10 % Trh	–	10 % Trh	−29.1	328	27 ± 7	7.3 ± 0.1	96.8	0.7 ± 0.2
11	polyA	7 % Trh	3 % PVP	7 % Trh + 3 % PVP	−26.5	266	23 ± 5	7.3 ± 0.1	105.1	0.4 ± 0.1
12	polyA	9 % Trh	1 % PVP	9 % Trh + 1 % PVP	−27.7	317	22 ± 5	7.2 ± 0.1	102.0	0.5 ± 0.1
13	eGFP-mRNA	9 % Trh	1 % PVP	9 % Trh + 1 % PVP	−27.5	315	20 ± 4	7.3 ± 0.1	Not measured	Not measured

[1] Cake collapsed after 6 months at 25 °C and after 1 month at 40 °C. Reconstitution time of collapsed sucrose cakes: 69 ± 21 s.

determined in technical triplicates on three different black 96-well plates (Greiner bio one, Frickenhausen, Germany) by Quant-iT RiboGreen Assay (ThermoFisher) as already published in Ruppl et al. (Ruppl, 2024). In brief, nucleic acid loaded LNPs were diluted with TE buffer or 2 % (V/V) Ecosurf buffer. 100 µL of a 400-fold diluted RiboGreen reagent was added to each well. After a 5-minute incubation protected from light, the plate was analyzed on a fluorescence plate reader (FLx800, BioTek, Vermont, USA) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The encapsulation efficiency (EE) was calculated as the percentage of the encapsulated nucleic acid relative to the total nucleic acid concentration.

2.6. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to determine T_g' according to the publication of Trenkenschuh et al. (Trenkenschuh et al., 2021). 20 µL of the liquid sample were filled into aluminum crucibles (Netzsch, Selb, Germany), which were hermetically sealed before measurement on a DSC 200 F3 (Netzsch) instrument. The samples were cooled at 10 K/min from 20 °C to −60 °C, held at −60 °C for 1 min, and reheated at 10 K/min to 20 °C. T_g' was reported as the inflection point of the glass transition during heating. The measurement was performed on two crucibles for each sample.

T_g of the lyophilized samples was analyzed on a DSC2500 Discovery (TA instuments, New Castle, USA). Approximately 5 mg of powder were weighed into T_{zero} aluminum pans (TA instruments) in a nitrogen-controlled glove box and hermetically sealed by using the T_{zero} press and dies kit (TA instruments). Two crucibles were prepared out of one vial and the average value was reported. Samples were heated from 0 °C to 160 °C at 10 K/min and cooled down again at the same rate. The second heating cycle to 160 °C at 10 K/min was used to detect the inflection point. For sucrose samples, the first cycle was used for data collection.

2.7. Cell culture

HeLa cells were cultured and seeded as previously described by Ruppl et al. (Ruppl, 2024). Briefly, 60,000 HeLa cells per well were seeded in 24-well plates in 1 mL cell culture medium. After 24 h, a fixed volume of LNPs was added to the cells corresponding to a final concentration of 0.5 µg/mL encapsulated mRNA after initial preparation. The volume was not adjusted to changes in EE. Untreated cells were used as a negative control and Lipofectamine 2000 (Thermo Fisher Scientific) as a positive control. Following 24 h of incubation, cell analysis was conducted using flow cytometry.

2.8. Flow cytometry

mRNA-induced eGFP expression in HeLa cells was examined as a technical triplicate using flow cytometry on a BD LSRFortessa instrument (Heidelberg, Germany). A total of 10,000 HeLa cells within the "living gate" (SSC-A/FSC-A) were captured and then subjected to doublet discrimination (FSC-H/FSC-A). GFP-positive cells were gated within the single-cell population, using a threshold of 0.5 % of the GFP signal from the background of the negative control. Results were presented as the percentage of GFP-positive cells and the median fluorescence intensity (MFI) of the single-cell population.

2.9. Product quality attributes of the lyophilizate

2.9.1. Cake appearance

Cake appearance was evaluated by visual inspection. Representative pictures were taken against a dark background.

2.9.2. Reconstitution time

Samples were reconstituted by the addition of 275 µL RNase-free water to the lyophilized sample. The vial was swirled and the reconstitution time was immediately measured using a stopwatch.

2.9.3. Karl-Fischer titration

Residual moisture of the lyo cakes was determined in triplicate by volumetric Karl Fischer titration using a Metrohm 915 KF Ti-Touch (Metrohm, Herisau, Switzerland). The samples were dissolved in anhydrous Methanol (Merck, Darmstadt, Germany), vortexed (Reax control, Heidolph) for 30 s at 1000 rpm, and allowed to stand for 10 min. The methanol suspension was withdrawn and injected into the titration vessel filled with solvent (Aquastar®, Merck). The titration was performed with Hydranal Titrant 2 (Honeywell, Seelze, Germany).

2.10. cryoTEM

The morphology of LNPs was analyzed by cryo transmission electron microscopy (cryoTEM). Therefore, freshly prepared LNPs and reconstituted LNPs were concentrated using Amicon Ultra centrifugation units (Merck, Darmstadt, Germany). 4 µL sample was adsorbed onto holey carbon-coated grid (Lacey, Ted Pella, USA), blotted with Whatman 1 filter paper and vitrified into liquid ethane at −178 °C using a Leica GP2 plunger (Leica, Austria). Frozen grids were transferred onto a Talos electron microscope (Thermofisher Scientific, USA) using a Gatan 626 cryo-holder. Electron micrographs were recorded at an accelerating voltage of 200 kV and a nominal magnification of 73000 x, using a low-dose system (20 $e^-/Å^2$) and keeping the sample at low temperature. Micrographs were recorded on a CETA camera.

2.11. Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was conducted using GraphPad Prism 10 software. Statistical significance of differences between means of samples before and after lyophilization was evaluated with a two-tailed, unpaired *t*-test. Differences between formulations were analyzed by one-way ANOVA with Tukey's multiple comparisons test. The following terminology was used: * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; n.s. not significant $p > 0.05$.

3. Results

3.1. Formulation attributes

LNPs were prepared by rapid T-mixing based on the lipid composition of the Moderna vaccine, comprising SM-102, cholesterol, DSPC, and DMG-PEG2000 at a molar ratio of 50 : 38.5 : 10 : 1.5, respectively. PolyA was used as a surrogate for mRNA. After dialysis into a 20 mM Tris buffer pH 7.4, the lyo-/cryo-protectants and T_g-modifying excipients were added according to Table 1 resulting in a nucleic acid concentration of 10–15 µg/mL.

T_g' is a crucial parameter for designing the lyophilization cycle, as it defines initial process parameters during primary drying. T_g' of the liquid formulations were tested and are summarized in Table 1. The formulation with 10 % Suc showed the lowest value at –33.0 °C. In line with literature, trehalose samples exhibited higher T_g' temperatures, ranging from −29.5 °C (formulation 1 + 3) to −29.1 °C (formulation 10). The addition of 1 % T_g-modifier to 9 % Trh further increased T_g'. Among the T_g-modifiers PVP reached the highest T_g' at −27.3 °C, followed by CD at −27.9 °C, and Dex at −28.9 °C. Increasing the ratio of the T_g-modifier lead to higher T_g' values, which was a more pronounced effect for CD (3 %, −24.7 °C) compared to PVP (3 %, −26.8 °C; 5 %, −25.5 °C), potentially enabling more aggressive lyophilization parameters for these excipients.

The osmolality of the reconstituted formulations ranged from 196 mOsmol/kg for 5 % Trh + 5 % PVP to 345 mOsmol/kg for 10 % Suc, which is primarily influenced by the content of the protectants and T_g-

modifiers. The total solid content of 10 % (w/V) was maintained across formulations to ensure comparability, while still targeting physiologically tolerable conditions.

The reconstitution time is given in Table 1 as the average of non-collapsed samples from each formulation analyzed during the study, with low variation indicated by the standard deviation. Generally, samples showed fast reconstitution times from 15 to 49 s with sucrose samples having the shortest reconstitution time. Longest reconstitution times were measured for 7 % Trh + 3 % CD, which was caused by small bubbles/foam created when water was added. PVP showed the best reconstitution behavior among the three T_g-modifiers. Reconstitution time remained stable throughout the stability study. For collapsed sucrose samples, reconstitution exceeded 60 s.

The pH values of all formulations were verified between 7.1 and 7.3, which did not change during the stability study, as reported below.

The storage temperature of the drug product should be chosen sufficiently below the product's T_g to ensure minimal mobility and maintain stability (Trenkenschuh and Friess, 2021). T_g of the lyophilized formulations were tested and are summarized in Table 1. An exemplary DSC profile for formulation 13 (9 % trehalose + 1 % PVP, present both inside and outside the polyA-LNPs) is provided in Fig. S-1. The formulation with 10 % Suc showed the lowest value of 42.2 °C, consistent with literature (Hinrichs et al., 2005). Trehalose samples exhibited higher T_g values of 97.3 °C (formulation 3), whilst the addition of 1 % T_g-modifier to 9 % Trh further increased T_g. The addition of 1 % CD reached the highest T_g at 101.2 °C, followed by Dex at 99.4 °C, and PVP at 98.1 °C. Increasing the ratio of the T_g-modifier led to higher T_g values, potentially enabling storage at higher temperatures for these formulations.

Residual moisture is an important quality attribute of lyophilized formulations as it might impact product stability. As expected, the sucrose samples had the highest residual moisture after lyophilization (2.5 %), while trehalose samples (formulations 3, 10) had residual moisture levels from 0.7 % to 1.0 %, and T_g-modifiers resulted in even lower levels from 0.3 % to 0.6 %. We re-evaluated moisture levels after 6 months storage at 25 °C and 40 °C and observed an increase in moisture across all samples, reaching 2.9 % – 3.5 % at 25 °C and 3.6 % – 3.9 % at 40 °C. A control study indicated, that for the tested formulations and the detected moisture levels, there was no impact on LNP's size, PDI, or EE. Please refer to the supporting information (Fig. S-2) for detailed information on this control study.

We further investigated the morphology of polyA-LNPs formulated with with 9 % Trh (w/V) and 1 % PVP (w/V) (formulation 8) before and after lyophilization (Fig. 1). Freshly prepared LNPs showed mainly a spherical structure with an electron-dense core, while lyophilized LNPs showed bleb structures. Some blebs even separated from the LNPs, forming vesicles (data not shown).

3.2. LNPs before vs. after lyophilization

Besides several formulation attributes, we measured the CQAs size, PDI, and EE before lyophilization (liquid) and after reconstitution with RNase-free water (lyo) (Fig. 2).

First, we investigated the stabilizing behavior of conventionally used cryo-/lyo-protectants, trehalose and sucrose, during the lyophilization process compared to combinations with 1 % of various T_g-modifiers. While trehalose resulted in an increase in size and PDI for the LNPs and a decrease in EE after lyophilization, the sucrose formulation better stabilized the LNPs during lyophilization. Notably, while trehalose exhibits a higher PDI after lyophilization, only a small increase in size was detected for sucrose, and EE and PDI did not change significantly (Fig. 2A). The addition of 1 % T_g-modifiers in combination with 9 % trehalose led to a slight decrease in size and no change in PDI for the CD samples. However, the corresponding liquid sample already showed a lower EE compared to the other formulations, which further decreased after lyophilization. PVP samples generally performed better than trehalose and sucrose alone, as well as better than the other T_g-

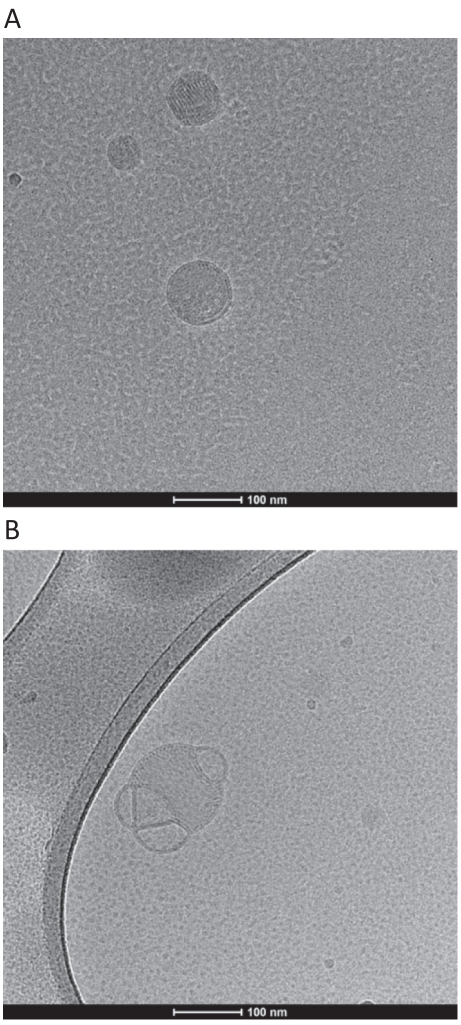

Fig. 1. Morphology of polyA-LNPs (A) before and (B) after lyophilization. Representative cryoTEM images of polyA-LNPs formulated with 9 % Trh (w/V) and 1 % PVP (w/V) (formulation 8) before lyophilization (liquid) and after lyophilization (lyo). Scale bars represent 100 nm.

modifiers. We observed no significant loss in EE and only slight changes in PDI or size. The dextran sample showed the highest increase in size and PDI, resulting in a polydisperse distribution and a loss in EE (Fig. 2A).

In a next step, to investigate the effect of the ratio between trehalose and T_g-modifier, we prepared three different ratios of Trh + PVP (9+1, 7+3, 5+5) and two different ratios of Trh + CD (9+1, 7+3). The PVP samples showed similar behavior for EE and PDI across all ratios. While the size for the 9 % Trh + 1 % PVP samples increased only slightly, the increase was more pronounced at higher PVP ratios (Fig. 2B). All CD samples were stable regarding size and PDI. As described above, 9 % Trh + 1 % CD had a comparatively low EE in the liquid state (79 % EE). This phenomenon was even more pronounced for 7 % Trh + 3 % CD (50 % EE). After lyophilization, there was a significant drop in EE for 1 % CD, while no significant change was observed for the sample with 3 % CD (Fig. 2B).

Formulation excipients, such as the lyo/cryo-protectants and T_g-modifiers, are typically added after dialysis and are therefore only present outside the LNPs. To explore another formulation optimization principle, we further tested if it makes a difference when the protectants are added before the mixing step, which results in the presence of protectants both inside and outside the LNPs (Formulation 10–12). As seen in Fig. 2C, the formulations containing protectants inside the LNPs are slightly larger after manufacturing in the liquid state before

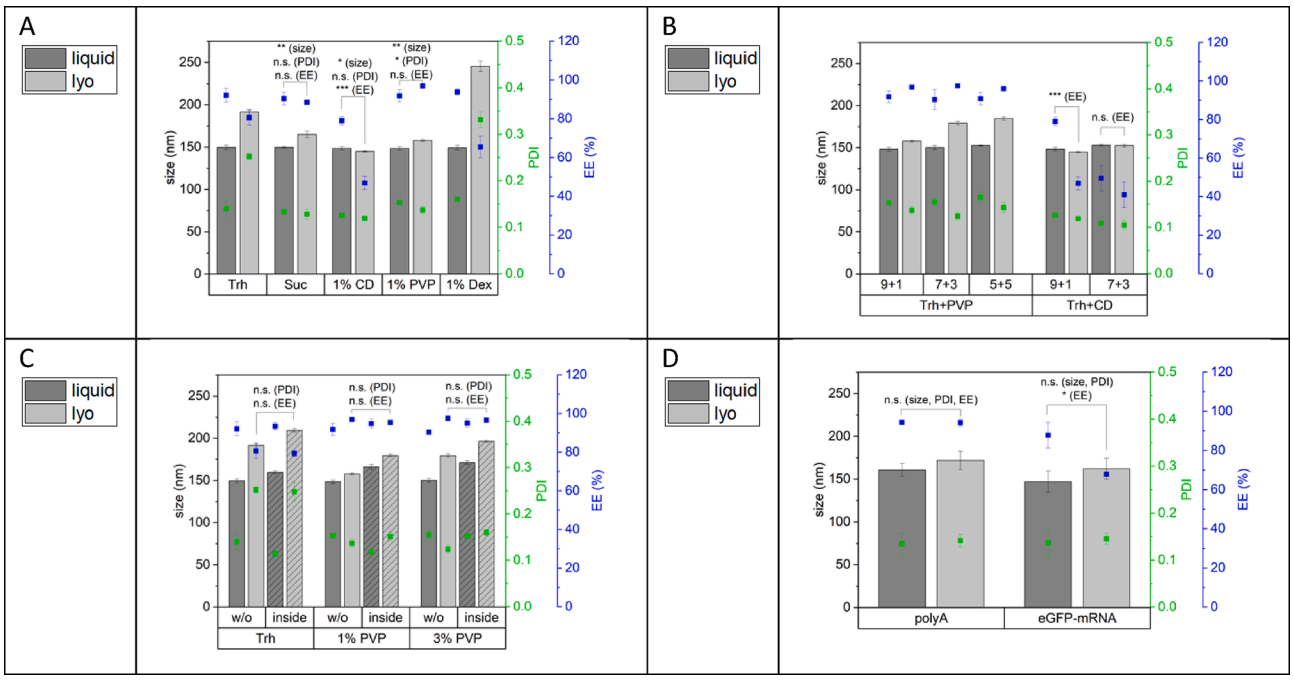

Fig. 2. Size, PDI, and encapsulation efficiency (EE) of LNPs before and after lyophilization. LNPs were formulated with 10 % (w/V) total solid content. **(A)** Different protectants and T_g-modifiers. Trehalose (Trh), sucrose (Suc), and 9 % (w/V) trehalose with 1 % (w/V) T_g-modifier: HP-beta-CD (CD), Kollidon 12PF (PVP), or dextran 40 (Dex). **(B)** Different ratios of trehalose + T_g-modifier. **(C)** Protectants outside of the LNPs (w/o) and inside and outside of the LNPs (inside) for the formulations trehalose (Trh), 9 % trehalose + 1 % Kollidon 12PF (1 % PVP), and 7 % trehalose + 3 % Kollidon 12PF (3 % PVP) **(D)** polyA-LNPs vs. eGFP-mRNA-LNPs with 9 % Trh + 1 % PVP present inside and outside of the LNP.

lyophilization, which may be optimized through process development. More importantly, they show the same behavior during the lyophilization process, with a small increase in size for the Trh + PVP formulations and a more pronounced increase for pure trehalose. While we detected no differences for EE and PDI after lyophilization (and also not in the attributes studied after storage, as elaborated further below), the impact of the presence of the sugar inside on RNA integrity, especially during the lyophilization process, remains to be studied.

Performing most of the experiments with the surrogate polyA, we confirmed the obtained results using a formulation containing eGFP-mRNA. Both polyA-LNPs and eGFP-mRNA-LNPs are in the same size range and PDI before and after lyophilization; however, they differ in EE (Fig. 2D). For liquid polyA-LNPs, the EE was 94 % while the EE of mRNA-LNPs was 88 %. PolyA-LNPs maintained this level, whereas the EE for mRNA-LNPs slightly decreased to 68 % after lyophilization. A decrease in EE may be mitigated through process development and further formulation optimization.

3.3. Stability data over 6 months

To eliminate the need for a cold chain for LNPs, we tested the stability of polyA-LNPs at 25 °C/60 % r.h. and 40 °C/75 % r.h. over six months. The temperature of 40 °C is typically tested to account for potential temperature excursions during shelf-life. The CQAs, including size, PDI, and EE, were measured after lyophilization (t_0) and after 1, 3, and 6 months of storage.

The lyophilized trehalose formulation showed a larger size and PDI compared to the liquid control dosage form, which may be optimized through manufacturing process development. However, these characteristics were maintained during storage at both temperatures. This indicates that colloidal stability is not problematic during storage at higher temperatures (Fig. 3A). We observed a marginal increase in size over time for the lyophilized trehalose formulation, with a more pronounced increase at 40 °C. For the EE, the liquid control formulation showed a dramatic loss in EE, decreasing from 96 % to 88 % at 25 °C and to 43 % at 40 °C after 6 months. In contrast, the lyophilized formulation maintained its initial EE of 80 % for both temperatures.

Next, we compared formulations with disaccharides sucrose and trehalose (Fig. 3B). While sucrose lyo cakes collapsed after only one month at 40 °C and after six months at 25 °C (Fig. S-3A), with shrinkage already observed after 3 months at 25 °C, trehalose lyo cakes remained intact throughout the entire stability study (Fig. S-3B). Before the collapse of the lyo cakes, sucrose exhibited comparable stability to trehalose formulations in terms of size, PDI, and EE but had a smaller initial size and PDI and a higher EE. Therefore, sucrose may be superior to trehalose for storage at 2–8 °C but cannot be stored at 25 °C or higher to achieve sufficient shelf-life.

The stability of formulations with 9 % trehalose + 1 % T_g-modifier in comparison to pure trehalose is shown in Fig. 3C. The addition of CD resulted in the smallest LNP sizes with no significant increase in size after 6 months at 40 °C. PVP yielded similar results at 25 °C, with only a small increase in size over time, which was more pronounced at 40 °C. Dextran was unable to maintain the size of the LNPs at 40 °C. PVP and CD kept the PDI below 0.2 over 6 months, whereas trehalose and dextran already started with a PDI higher than 0.2 after lyophilization. The EE indicated that adding PVP is superior to pure trehalose and to the addition of other T_g-modifiers. The EE of 9 % Trh + 1 % PVP was maintained > 90 % over six months, while pure trehalose showed values of 80 % EE CD and dextran displayed even lower EE.

We further compared different CD ratios and found that good stabilization of size and PDI was achieved by both ratios. However, the increase from 1 % (w/V) CD to 3 % (w/V) CD was detrimental to the EE (Fig. 3D). In contrast, the ratio of PVP/trehalose did not influence the EE (Fig. 3E). Also for the PDI, all samples behaved similarly except of the 5 % Trh + 5 % PVP sample stored at 40 °C, which exhibited an increased PDI. The size of the LNPs showed a slight increase over time, more pronounced at 40 °C and higher PVP ratios. The most stable formulation was identified as 9 % Trh + 1 % PVP (at 25 °C).

The effect of protectants and T_g-modifiers inside the LNPs is exemplified using the 9 % Trh + 1 % PVP formulation (Fig. 3F). The initial

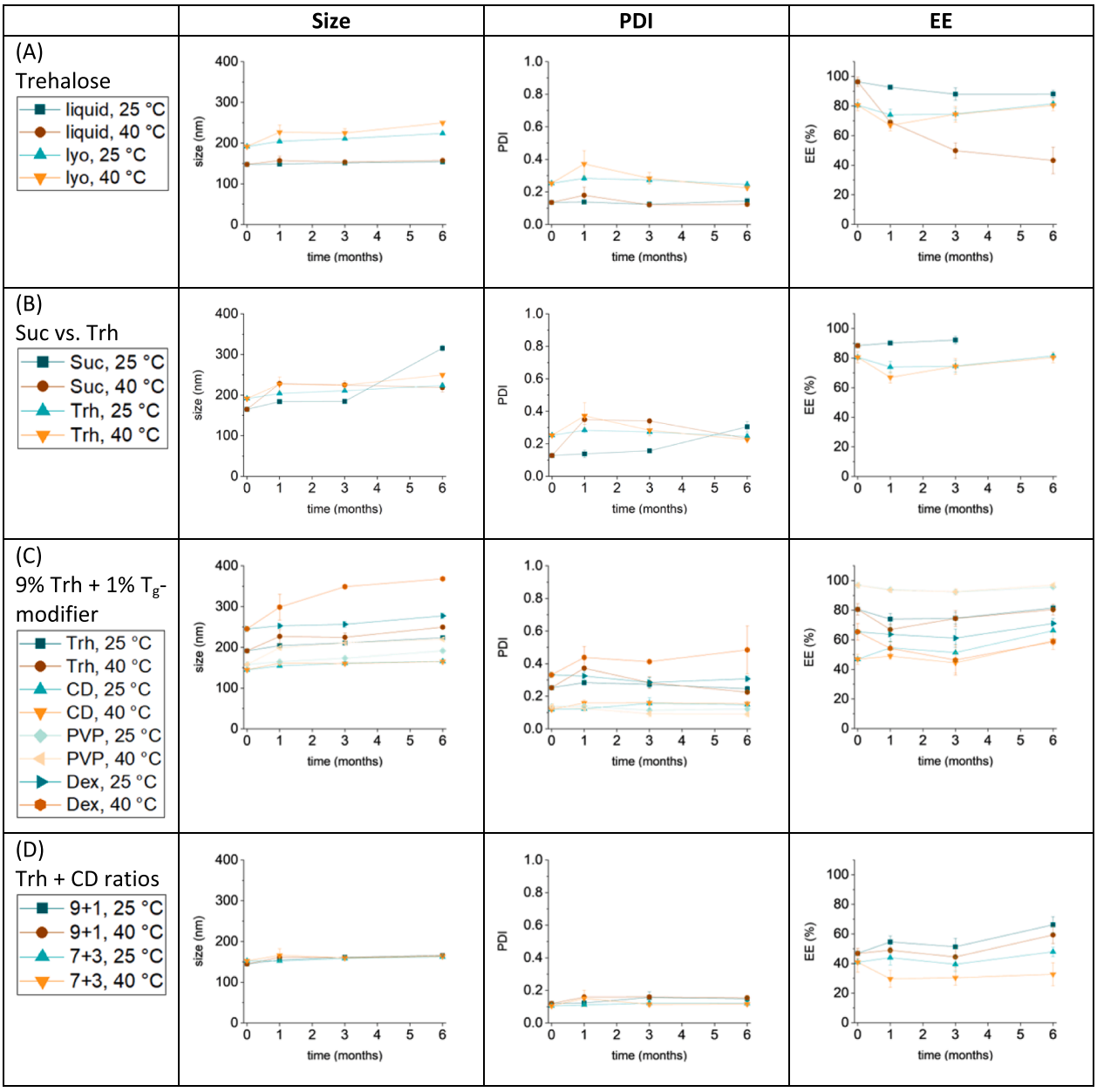

Fig. 3. Size, PDI, and encapsulation efficiency (EE) for LNPs before and after lyophilization and after 1, 3, and 6 months of storage at 2–8 °C, 25 °C/60 % r.h., and 40 °C/75 % r.h.. LNPs were formulated with 10 % (w/V) total solid content. (A) Trehalose liquid vs. lyo. (B) Sucrose (Suc) vs. trehalose (Trh). (C) 9 % trehalose (Trh) + 1 % T_g-modifier: HP-beta-CD (CD), Kollidon 12PF (PVP), or dextran 40 (Dex). (D) Different ratios of trehalose (Trh) + HP-beta-CD (CD). (E) Different ratios of trehalose (Trh) + Kollidon 12 PF (PVP). (F) Protectants only outside of the LNPs (out) and inside and outside of the LNPs (in + out) for the formulation trehalose 9 % trehalose (Trh) + 1 % Kollidon 12PF (PVP). (G) PolyA-LNPs vs. eGFP-mRNA-LNPs, 9 % Trh + 1 % PVP inside&outside. The data points at the initial time point after lyophilization are identical to those shown in Fig. 2. * Sucrose samples were collapsed after 6 months at 25 °C and 1 month at 40 °C.

bigger size of the LNPs with sugars present inside and outside increased slightly over storage, similar to the LNPs with only external sugar. Additionally, LNPs with sugar inside and outside did not differ in PDI and EE from those with sugar only outside. The same observations were made for 10 % Trh and 7 % Trh + 3 % PVP (data not shown).

To verify polyA results, the most promising formulation, 9 % Trh + 1 % PVP inside and outside of the LNPs, was also tested with eGFP-mRNA. Both formulations had similar sizes after lyophilization and exhibited similar trends during storage. At 2–8 °C (added as an additional storage temperature for these configurations), the size remained unchanged over the course of 6 months. At 25 °C, the size of the LNPs increased slightly, with a more pronounced increase at 40 °C. The PDI remained stable below 0.2 throughout the study. However, differences were observed between polyA-LNPs and mRNA-LNPs for EE. While the EE of lyophilized polyA-LNPs and liquid eGFP-mRNA-LNPs was maintained at > 90 %, the EE of lyophilized eGFP-mRNA-LNPs was lower. This could potentially be optimized through lyophilization process development. Nevertheless, both polyA-LNPs and eGFP-mRNA-LNPs maintained their initial EE over 6 months at 5 °C, 25 °C, and 40 °C. Notably, even the EE of the liquid eGFP-mRNA-LNPs in the proposed formulation was maintained at 2–8 °C (Fig. 3G).

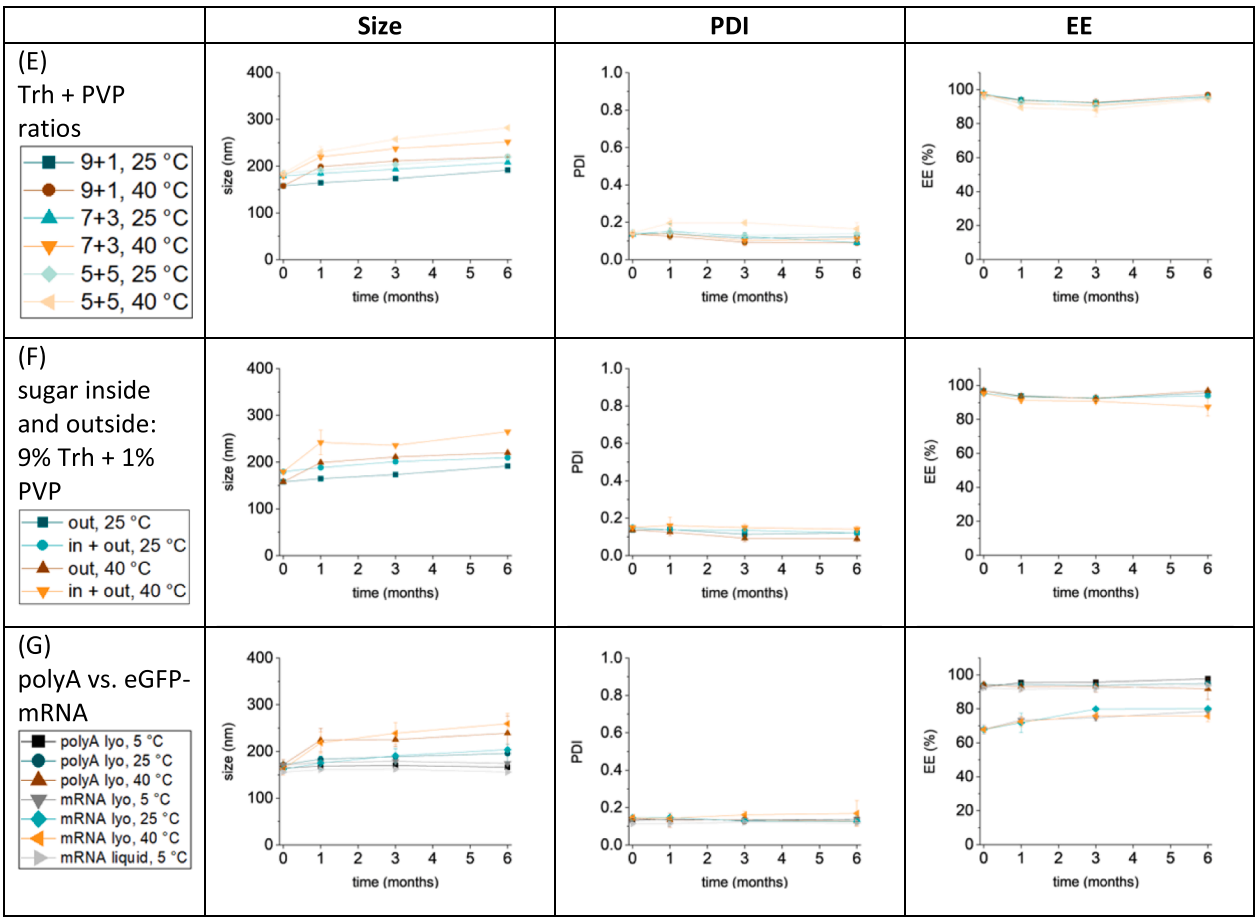

Fig. 3. (*continued*).

3.4. Cell culture experiments

To confirm the functionality of the eGFP-LNP formulations, we tested the mRNA-induced eGFP-expression in HeLa cells after storage at 2–8 °C, 25 °C, and 40 °C for up to 6 months, comparing them to the liquid control formulation (Fig. 4). The applied sample volume was kept constant. The reduced EE of eGFP-mRNA-LNPs compared to the polyA formulations due to the lyophilization process (Fig. 2D) is reflected in a decrease in median fluorescence intensity (MFI), whereas the percentage of GFP-positive cells, which is less sensitive, remained unchanged. However, we cannot finally conclude whether the decreased MFI is solely due to a drop in EE or also influenced by a reduction in mRNA integrity or an increase in particle size. We measured a high percentage of eGFP-positive cells (>95 %) for the liquid sample stored at 2–8 °C after one month, as well as for the lyophilized samples stored at 2–8 °C and 25 °C/60 % r.h.. While storage of the liquid LNPs at 2–8 °C beyond 1 month drastically reduced the percentage of eGFP-positive cells, the percentage of eGFP-positive cells for the lyophilized formulation was maintained at 2–8 °C. For the lyophilized formulations, the percentage of eGFP-positive cells decreased over time and with increasing temperature (25 °C and 40 °C). The MFI, being a more sensitive parameter, decreased for all samples following the same trends and reaching very low levels for samples showing a decrease in eGFP-positive cells.

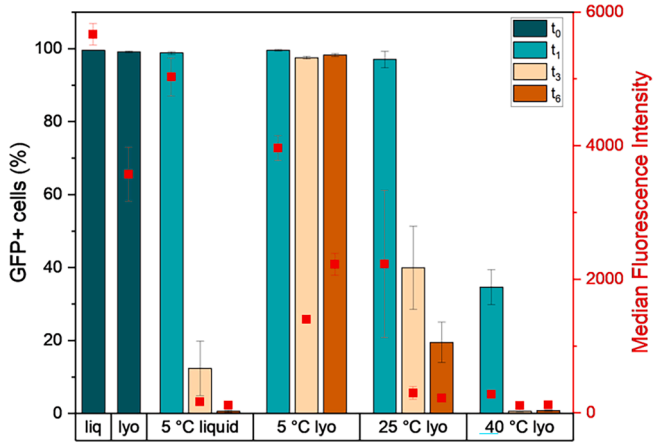

Fig. 4. Median fluorescence intensity and eGFP-positive HeLa cells. eGFP-expression after manufacturing (liq, t_0), after lyophilization (lyo, t_0), and storage of liquid (liquid) or lyophilized (lyo) LNPs at 2–8 °C, 25 °C, or 40 °C after 1, 3, and 6 months (t_1, t_3, t_6). eGFP-mRNA-LNPs were added to HeLa cells and measured after 24 h of incubation with flow cytometry.

4. Discussion

We performed a formulation screening for lyophilized LNPs with the aim of guiding future formulation development for freeze-dried LNPs, enabling storage temperatures at 2–8 °C and beyond. While the focus of this manuscript is not on the LNP assembly process or on optimizing the lyophilization process – which can be further optimized for smaller particle sizes or higher encapsulation efficiency in the case of eGFP-mRNA-LNPs – we evaluate the selection of excipients to best stabilize mRNA-LNPs in freeze-dried dosage forms.

In previous literature, the term < formulation of LNPs > often refers to either the composition of the lipids, which majorly impacts drug targeting, or the settings of the mixing process (Miao, 2023; Chen,

2012). From a formulator's perspective, we consider the formulation as the environment of the LNPs, which may include the buffer systems and other stabilizing excipients such as salts or sugars.

While we did not investigate different buffers (e.g., Tris buffer, which has already shown good results in other publications and is also used in the mRNA COVID vaccines Comirnaty® and Spikevax®[7,10,11,14,15]), nor re-investigate the impact of ionic strength during assembly, which may disturb the interaction between mRNA and ionizable lipids leading to a decrease in EE (Meulewaeter, 2023; Shirane, 2023); we focused on the stabilizing effect of different cryo-/lyo-protectants and T_g-modifiers in freeze-dried formulations. T_g-modifiers are excipients that increase either the T_g' of the liquid, important for setting of initial process parameters during primary drying, i.e. allowing for more aggressive cycle parameters and shorter process times, or of the T_g of the lyophilized drug product, which primarily defines storage conditions allowing for storage at elevated temperatures.

There are two concepts explaining the stabilization mechanism of cryo-/lyo-protectants. The water replacement hypothesis assumes that the sugars replace hydrogen bonds of water at the particle surface and between phospholipid head groups. The vitrification theory assumes that particles are kinetically stabilized. The amorphous glassy sugar matrix immobilizes the particles and slows down processes like diffusion or aggregation. Therefore, lyophilized products should be stored at least 10–20 °C below Tg (Trenkenschuh and Friess, 2021).

In fact, the current study demonstrates that the right selection of T_g-modifiers, in combination with conventionally used sugars like trehalose, maintains product attributes such as size, PDI, and EE over a course of 6 months at 25 °C and 40 °C, as studied using a polyA surrogate formulation. While we show the potential of lyophilized formulations over liquid formulations, especially for storage temperatures beyond 2–8 °C in cell culture experiments, emphasis must be put on further formulation optimization and lyophilization process development, which are interlinked. A thorough understanding of the lyophilization process and related process parameters on the CQAs of the LNPs must be obtained to minimize the impact of the lyophilization process on product quality.

To understand how lyophilization affects the morphology of LNPs, we captured cryo-TEM images of polyA-LNPs with 9 % trehalose (w/V) and 1 % PVP (w/V) (formulation 8) both before and after lyophilization. Our findings are consistent with previous studies, which also observed increased size and more bleb structures post-lyophilization, likely due to the physical stress induced by the lyophilization process (Fan, 2024; Meulewaeter, 2023). A closer examination of the bleb compartments after lyophilization reveals the absence of polyA in the bleb, which is consistent with the current state of research. It is important to distinguish between bleb formation after LNP assembly and dialysis and bleb formation after lyophilization. While bleb formation after LNP assembly typically results in mRNA-containing blebs with improved stability and potency (Simonsen, 2024), it has been previously reported that bleb structures induced during freezing of the lyophilization process are usually mRNA-empty and associated with reduced transfection potency (Meulewaeter, 2023; Henderson et al., 2022). This reduction in transfection potency is also reflected in our data.

In comparison to previous studies, we did not use chemically modified mRNA containing N1-methylpseudouridine instead of uridine (Muramatsu, 2022; Suzuki, 2022; Meulewaeter, 2023; Shirane, 2023; Ai, 2023). N1-methylpseudouridine modification not only reduces the immunogenicity of mRNA and elevates the protein expression but results in enhanced stability (Ho et al., 2024). As a result, our unmodified eGFP-mRNA was sufficient to show improved stability due to lyophilization compared to the liquid formulation. To gain long-term stability at 2–8 °C and stability for several weeks at room temperature, the use of N1-methylpseudouridine may be beneficial.

For lyophilized formulations, we showed that pure sucrose stabilizes LNPs better than pure trehalose during lyophilization. This aligns with the findings of Li et al., who used ALC-0315 as ionizable lipid (Li, 2023). Similarly, Zhao et al. using TT3 as an ionizable lipid, observed a strong increase in size after lyophilization using 10 % trehalose but to a smaller extent for 10 % (w/V) sucrose, while there was no difference between the disaccharides at 20 % (w/V) (Zhao, 2020). In contrast, Meulewaeter et al. used C12-200 as an ionizable lipid and observed a slightly better performance with trehalose compared to sucrose at 12.5 % (w/V) total solid content (Meulewaeter, 2023). Most importantly, we show in the present study that while sucrose might be a promising excipient for storage at 2–8 °C and probably preferred due to trehalose's potential to crystallize in frozen state, sucrose formulations do not withstand long-term storage at 25 °C and related temperature deviations. As we aim to eliminate the cold chain for LNPs, we focused on storage at 25 °C and 40 °C. These storage conditions were too harsh for lyophilized sucrose-containing formulations, resulting in the collapse of the lyo cakes.

As a result, we combined trehalose with different T_g-modifiers to optimize stabilization and to raise T_g/T_g' of the formulations even further. The addition of CD allowed a very good stabilization of size and PDI during lyophilization and storage at elevated temperatures. However, as an undesired side effect, the EE of the liquid formulation dropped due to the addition of CD to the LNPs. This was even more pronounced at higher CD ratios. We hypothesize that this is a result of the complexation of cholesterol and phospholipids with beta-cyclodextrin, as previously observed in model membranes and calorimetric measurements, respectively (Anderson et al., 2004; López et al., 2011; Hatzi et al., 2007). In fact, in a side experiment (data not shown) we tried to saturate CD with cholesterol, which was not successful due to cholesterol's poor solubility in an aqueous solution.

Using PVP as a T_g-modifier, we achieved most promising results. 1 % PVP + 9 % trehalose was already sufficient to stabilize LNPs better than trehalose alone. The marginal size increase after lyophilization was reduced to approximately 10 nm, and PDI or EE remained unchanged after lyophilization as well as during storage. The size of the LNPs increased only by approximately 30 nm after 6 months of storage at 25 °C, and by approximately 60 nm at 40 °C. We initially expected that a higher PVP ratio, resulting in higher T_g, would possibly better stabilize LNP's size during storage; however, this was not confirmed in the experiments. The use of dextran did not bring any benefits regarding the stabilization of LNPs. The increase in size and PDI was stronger than with the pure trehalose sample. Additionally, the loss in EE was detrimental. Therefore, we conclude that dextran does not stabilize the LNPs and even prevents the stabilizing effect of trehalose.

Most of the experiments were performed with polyA-LNPs as a surrogate for mRNA-LNPs. The formulation with 9 % Trh + 1 % PVP inside and outside of the LNPs was manufactured with polyA and eGFP-mRNA to compare both cargos. After manufacturing, they were comparable in size and PDI, but eGFP-mRNA-LNPs had a lower EE, which may be further improved by optimization of the assembly process. Importantly, size and PDI were similarly maintained after lyophilization, but EE decreased for the eGFP-mRNA-LNP. The different behaviors may be explained by the inherent structure of the surrogate and the eGFP-mRNA. While eGFP-mRNA consists of 997 nucleotides of unmodified bases, the polyA used in this study, has a molecular weight of 100 – 500 kDa corresponding to 300 to 1500 nucleotides. The polyA exhibits a broad molecular mass distribution consisting only of the base adenine. Schober et al. showed that larger mRNA molecules are encapsulated with higher efficiency than smaller RNA molecules (Schober et al., 2024), which may or may not be one of the reasons for reduced EE. The additional 3 bases used in the eGFP-mRNA are less lipophilic compared to adenine (Azarani and Hecker, 2001), which may impact the hydrophobic interactions with the lipids. Another factor could be the secondary structure of mRNA (Mauger, 2019), which has not been described for polyA. Despite the differences, we identified polyA as a suitable surrogate for mRNA formulation pre-selection because formulations insufficient for polyA do not need to be repeated with expensive functional mRNA. Most importantly, polyA-LNPs and eGFP-mRNA-LNPs showed the same behavior during storage for all CQAs.

The exact mechanism of stabilizing mRNA-LNPs through lyophilization is still unclear. mRNA strand breakage typically occurs due to cleavage of RNA phosphodiester bonds through intramolecular transesterification (Wayment-Steele, 2021). and a higher degree of secondary structure has been proposed to be more favorable for protein expression (Oude Blenke, 2022). Flexibility to access the conformational space is likely a key to enabling transesterification reactions of mRNA (Oude Blenke, 2022). However, the influence of formulation properties or dosage form on the secondary structure of encapsulated mRNA as a result of environmental changes has not yet been studied and the removal of water and its impact on secondary structure conservation currently remains unclear.

Besides the T_g modifiers, we have investigated another formulation principle for stabilizing mRNA-LNPs, which was to study the influence of sugars/protectants also inside the LNPs specifically to protect the mRNA. This was achieved by the addition of protectants to the aqueous phase before the mixing step. LNPs with sugar inside and outside were slightly bigger after manufacturing but had the same PDI and EE as LNPs where sugars were only added after dialysis. We detected no difference during the lyophilization process or storage in the CQAs, which identifies this approach as promising formulation principle. As these experiments were only performed with polyA-LNPs, the impact on the mRNA integrity and functionality is not known yet, which is part of current research.

5. Conclusion

Formulation design for freeze-dried mRNA-LNPs, comprising the selection of excipients to best stabilize the assembled LNP with encapsulated mRNA, is crucial to achieve long-term storage of the drug product. Targeting to eliminate the cold chain for mRNA-therapeutics, we propose a lyophilized formulation dosage form consisting of a combination of conventionally used cryo/lyo-protectants, such as trehalose, and a T_g-modifier, namely 1 % PVP at a total solid content of 10 %. While sucrose may serve as an alternative to trehalose when storage at 2–8 °C is intended, storage at higher temperature cannot be achieved due to lyo cake collapse. In addition, we found that adding sugars before the mixing steps does not influence LNPs during lyophilization, but the potential beneficial effect on mRNA integrity remains to be studied. We further demonstrate the potential of the PVP formulation composition in cell culture experiments with eGFP-mRNA-LNPs showing the same stability behavior of size, PDI, and EE during storage as the polyA-LNPs, making them a suitable and cost-efficient surrogate for mRNA formulation screenings. To fully unleash the potential of the proposed formulation composition, modified mRNA, e.g., using N1-methylpseudouridines, along with optimized manufacturing assembly processes, are desirable. We are further investigating to understand the interplay of lyophilization process parameters on CQAs to best minimize the impact of the lyophilization process on the drug product. Ultimately, this research piece may serve as guiding document for formulation scientists for designing and optimizing freeze-dried formulations of mRNA-LNPs.

CRediT authorship contribution statement

Anna Ruppl: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Denis Kiesewetter:** Validation, Investigation. **Monika Koell-Weber:** Methodology, Investigation. **Thomas Lemazurier:** Methodology, Formal analysis. **Regine Süss:** Writing – review & editing, Supervision. **Andrea Allmendinger:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2025.125272.

Data availability

Data will be made available on request.

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