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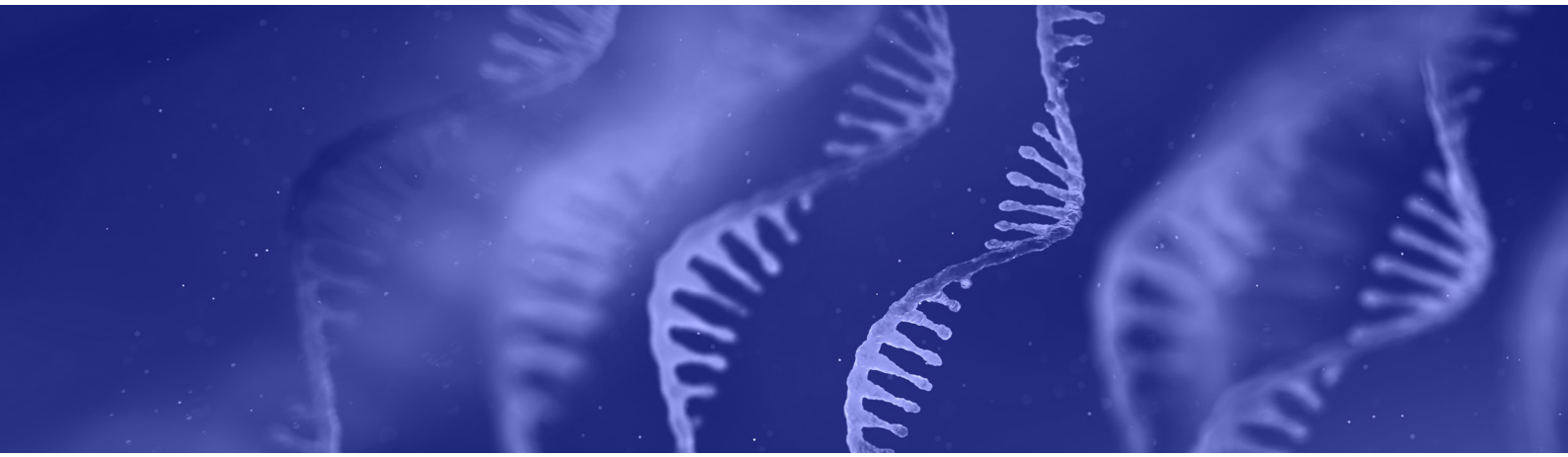
Stabilizing mRNA LNPs via controlled continuous freeze-drying

White Paper



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Introduction

After decades of extensive research, vaccines based on messenger RNA-lipid nanoparticles (mRNA-LNPs) emerge as a promising alternative to traditional vaccines, owing to their broad applicability, tailorable characteristics, expeditious and cell-free manufacturability. Nevertheless, the efficacy of mRNA-LNPs encounters impediments such as mRNA sequence design intricacies, optimal lipid nanoparticle (LNP) composition and the challenge of ensuring prolonged stability. The latter issue predominantly stems from mRNA vulnerability to oxidation and hydrolysis at refrigeration temperatures when in liquid state. Consequently, necessitating the preservation of mRNA-LNPs in a frozen state to guarantee an adequate shelf life. The stringent ultra-low temperature storage conditions impede the streamlined distribution of mRNA-LNP vaccines and exert a considerable influence on global public healthcare systems. Therefore, innovative solutions for enhancing the long-term stability of mRNA-LNPs have become imperative.

Lyophilization (freeze-drying) is considered to be one of the most promising approaches to stabilize mRNA-LNP formulations and enhance their stability at refrigerated or even room temperatures. Today, only a few case-studies exist where mRNA-LNPs were successfully dried. Ai et al. reported an enhanced thermostability of a SARS-CoV-2 mRNA-lipid nanoparticle vaccine where the physiochemical properties and bioactivities of the lyophilized vaccine showed no change at 25 °C for over 6 months. Furthermore, the dried vaccine could elicit potent humoral and cellular immunity whether in mice, rabbits, or rhesus macaques.

Moreover, research from Zhao et al. and Muramatsu et al. further support the evidence that lyophilization is a suitable technique to ensure sufficient thermostability of mRNA- LNP vaccines. One could argue that lyophilization will become the golden standard for mRNA-LNP vaccine production. Nonetheless, lyophilization is a slow, expensive, poorly controlled and labor-intensive process. Consequently, these disadvantages hamper the fast and cost-efficient development of mRNA-LNP vaccines.

In this White Paper the advantages associated with continuous and controlled lyophilization are described. This drying technology allows the R&D team and the MSAT team to save money and staffing. It opens a path for seamless scaling-out without the need to reformulate lyophilization protocols. All this may result in a faster time to the market for the drug or vaccine.

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Shortcomings and challenges of traditional lyophilization

Lyophilization is after more than 50 years of experience still a batch-wise process and is extremely time-consuming, inefficient and expensive. Drying cycles tend to last a couple of days to achieve the desired level of residual moisture. These long process times have a negative impact on the economic return. Therefore, the process scale is enlarged, leading to freeze-drying cycles where hundreds of thousands unit doses are dried per run. As a consequence, process failures during drying of such a huge amount of unit doses results in enormous losses. In addition, these colossal batch sizes are not compatible with changes in market demands.

Another highly underestimated challenge associated with lyophilization is the batch-to-batch and even vial-to-vial variability in quality due to inhomogeneity in heat transfer inside a freeze-dryer. These differences in energy input between the vials will induce variability in residual moisture content and consequently variation in critical quality attributes. To compensate for this intrinsic variability, researchers use very conservative temperature settings during formulation development. Consequently, some formulations may go through a non-optimal freeze-drying trajectory and may get rejected from the list of formulation options.

Up-scaling from initial drying cycles in the discovery phase to production scale poses significant challenges, primarily attributed to the variations in heat transfer inherent in transitioning from a laboratory-scale freeze-dryer to a fully-fledged production-scale freeze-dryer. Overall, the up-scaling process is perceived as time-intensive and material-consuming.

The variation in product quality and the material consumed during up-scaling phases are incompatible with the costly and valuable mRNA-LNP vaccines. The high value per unit of mRNA-LNPs used for customized gene therapies does not match well with the quality and variability constraints from batch lyophilization. Implementing continuous and controlled lyophilization addresses a lot of the above concerns.

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Continuous and controlled lyophilization

Continuous and controlled freeze-drying is a new drying technology where high product quality is ensured by process control at vial level. The drying technology is characterized by a short drying time (hours) enabled by spin freezing. During the spin freezing step, a vial containing the liquid formulation is rotated along its longitudinal axis at high velocity. Hence, the liquid formulation is spread out over the vial wall. The liquid content is subsequently solidified by the application of cooling gas, resulting in a thin layer of frozen material on the inner vial wall. Consequently, this thin product layer can be dried in hours instead of days.

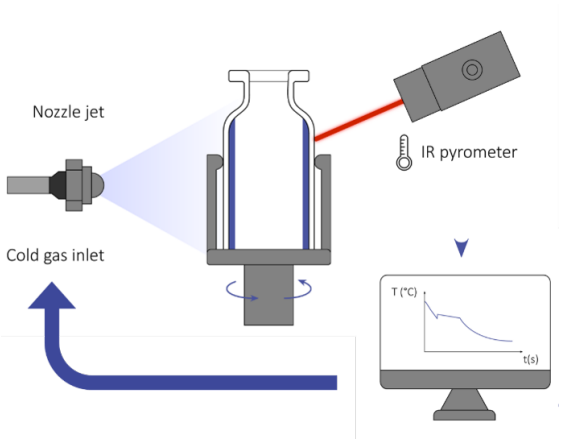

Figure 1: *Illustration of closed loop PID-algorithm during freezing and drying*

In the spin freezing process, precise temperature control of the vial is achieved through a feedback-control loop utilizing real-time thermal infrared measurements, as depicted in Figure 1. To elaborate, cooling is executed by directing cold gas towards the rotating vial, and the vial temperature is monitored by an infrared (IR) temperature sensor. During the initial liquid cooling phase, the predetermined cooling rate is achieved through closed-loop control. Based on continuous real-time IR temperature measurements, the cold gas flow rate is dynamically adjusted to ensure the vial is cooled at the specified rate. Once ice nucleation initiates, the freezing rate is regulated by varying the cold gas temperature while maintaining a constant mass flow, hence maintaining a consistent temperature difference between the cold gas and the vial. Following crystallization, further cooling of the formed ice layer is governed by the same closed-loop control procedure employed during liquid cooling. Ultimately, a thin frozen product layer is achieved with a temperature well below the glass transition temperature of the frozen product.

Next, the drying phase is initiated by lowering the pressure below equilibrium pressure of the frozen product. The key characteristics of the drying phase are the unique closed-loop control algorithm and the short drying times. The closed-loop control drying principle ensures a constant vial temperature by controlling the energy input during the drying phases. The energy used for drying originates from an infrared heater located outside the drying chamber unit and radiates the vial through a sapphire window and hence provides the required energy. The power input of the IR-heater is modified based on the vial temperature to keep the vial temperature constant. Note that the IR thermometer measures the temperature at the outside of the vial and not at the sublimation front. By using the temperature at the outside of the vial, an extra safety margin was built in as the product temperature at the inside of the vial is always colder than the temperature at the outside. Controlling the drying phase reduces the risk of collapse, which results in loss of product structure. This is a significant advantage in comparison to batch freeze-drying where the product temperature is only controlled in an indirect way. Moreover, no extra experiments for process optimization are required since the drying is performed immediately at the most optimal way from the start on. Hence, this drying technology results in high quality products with a minimum of experimental load and material consumption. Moreover, the control at vial level unlocks the potential for scaling out instead of scaling up, meaning that the process signature and settings remain identical in the initial drying cycle as in production.

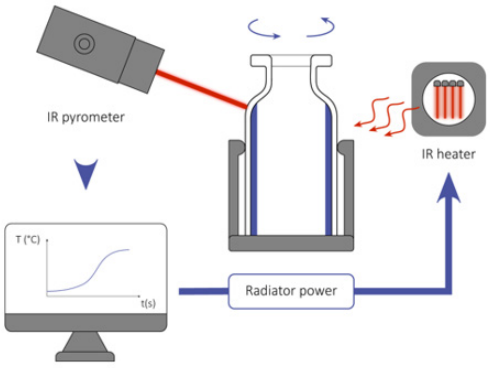

***Figure 2**: Illustration of closed loop PID-algorithm during freezing and drying*

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The importance of formulation composition for lyophilization success

The work by Meulewaeter et al., explored the opportunities of this new drying technology and compared it to classical batch freeze-drying. The researchers found that a sufficiently high ionizable lipid to mRNA weight ratio was necessary to prevent mRNA leakage during freeze-drying and that phosphate and Tris, but not PBS, were appropriate buffers for lyophilization of mRNA LNPs.

The data from this research suggests successful lyophilization of C12–200 mRNA LNPs in a formulation containing 12.5 m/V% sucrose. This was realized by increasing the C12–200:mRNA weight ratio from 10 to 20 and using Tris or phosphate buffer instead of PBS. Additionally, the freezing rate for LNPs with a C12–200:mRNA weight ratio of 10 suspended in Tris 20 mM was varied. The findings revealed that a fast-freezing rate exhibited superior efficacy in this context.

Cryo-electron microscopy (Cry-EM) analysis revealed that both the dialysis buffer and the freeze-drying process had an impact on the morphology of mRNA-LNPs, as depicted in figure 3. mRNA LNPs subjected to dialysis in Tris exhibited a solid amorphous core, consistent with previous descriptions of mRNA- LNPs.

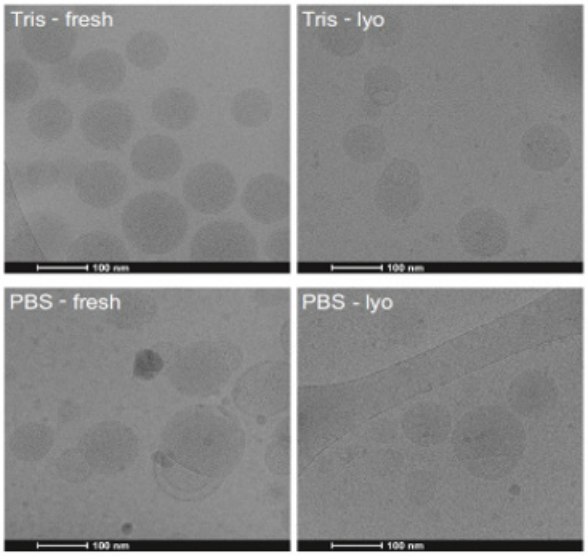

Figure 3: *Morphology of mRNA LNPs upon dialysis and lyophilization in Tris or PBS.*

Notably, dialysis in PBS led to mRNA-LNPs with diverse morphology, including LNPs containing aqueous compartments (labeled as ‘blebs’), which seemed to encapsulate mRNA. Additionally, some of these blebs separated entirely from the LNP and formed liposomal structures. The distinct morphologies responded differently to the lyophilization process. LNPs suspended in Tris formed blebs during lyophilization, likely due to phase separation induced by physical stress. Conversely, lyophilization of mRNA LNPs suspended in PBS resulted in multilamellar structures, mostly lacking the aqueous pockets observed in the fresh LNPs in PBS.

Enhanced thermostability by lyophilization preserves transfection efficiency

The study aimed to assess whether lyophilization could enhance the stability of mRNA-LNPs when stored at room temperature (RT, 22 °C) or elevated temperatures (37 °C). mRNA LNPs were formulated at a C12–200:mRNA weight ratio of 20 in Tris 20 mM (pH 7.4), using 12.5% sucrose as a lyoprotectant. Consequently, mRNA LNPs were stored in an aqueous state at 22 °C and in a lyophilized state at both 22 °C and 37 °C for 12 weeks.

For the aqueous mRNA-LNP dispersions stored at room temperature (RT, 22 °C), a notable reduction of 30% and 40% was observed in both the number of transfected cells and the mean fluorescence intensity (MFI) (Figure 4A & B). In contrast, lyophilized samples maintained their transfection efficiency over 12 weeks, whether stored at 22 °C or 37 °C, highlighting the evident advantage of lyophilization. However, it is noteworthy that the particle size increased from 80 to 150 nm upon storage of lyophilized mRNA LNPs at 37 °C (Figure 4E & F). Additionally, storage at 22 °C and 37 °C resulted in a slight reduction in the encapsulation efficiency of mRNA LNPs in both aqueous and lyophilized conditions (Figure 4C & D). Importantly, the encapsulation efficiency remained stable for 8 weeks for both lyophilized and aqueous mRNA LNPs.

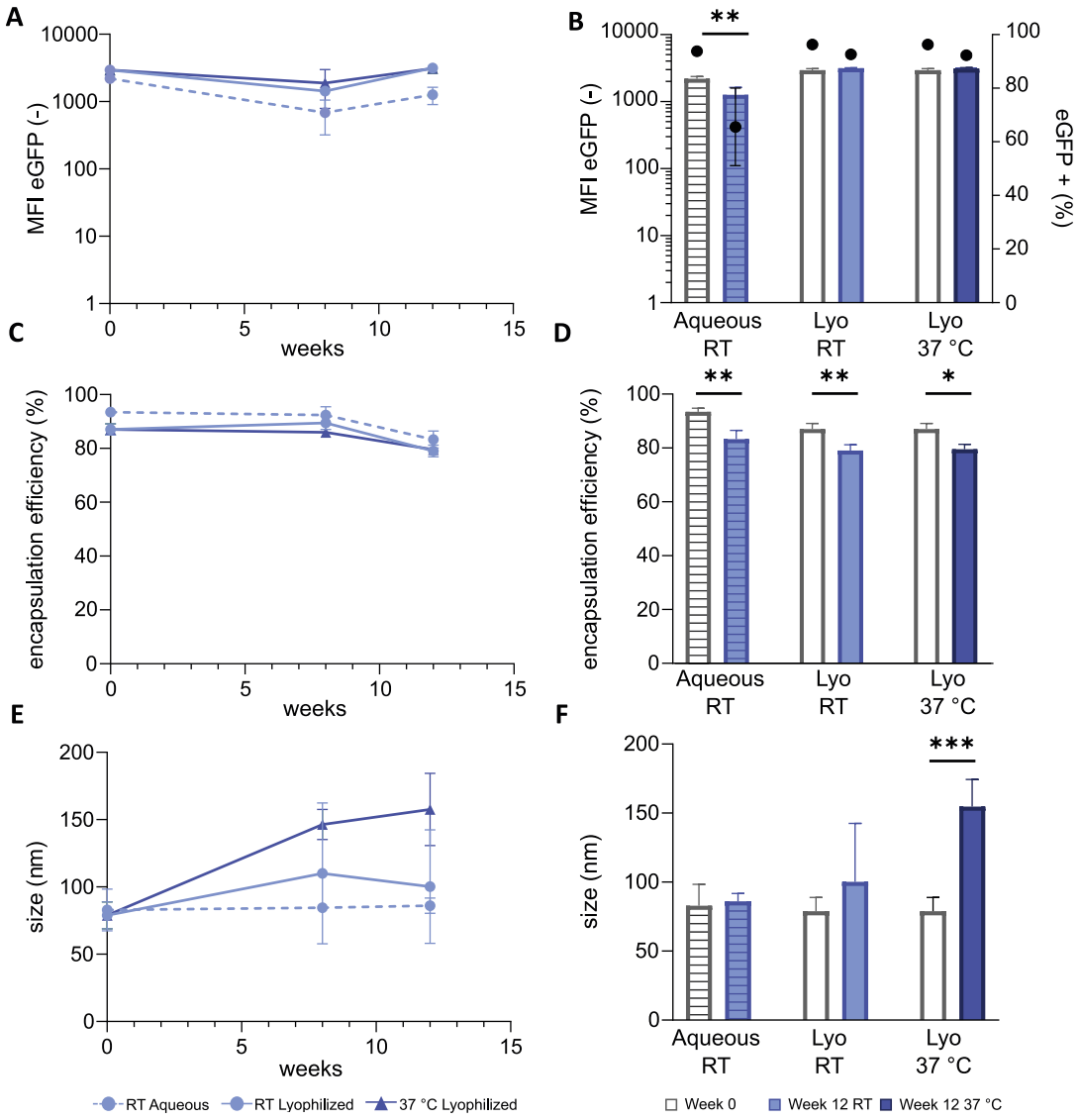

Figure 4: *Stability of mRNA LNPs in Aqueous and Lyophilized States at Room Temperature and 37 °C. mRNA LNPs containing eGFP encoding mRNA were prepared at a C12–200:mRNA weight ratio of 20 and subsequently subjected to dialysis in Tris 20 mM with a pH of 7.4. The mRNA LNPs were either stored in an aqueous state (represented by dashed lines and bars) or in a lyophilized state in the presence of 12.5 m/V% sucrose (indicated by full lines) at both 22 °C and 37 °C. (A, B) Transfection efficiency in HEK293T cells, expressed as Mean Fluorescence Intensity (MFI) of eGFP in viable cells, shown over time (A) and specifically at weeks 0 (grey) and 12 (color) only (B).(C, D) Encapsulation efficiency, determined using the RiboGreen assay, shown over time (C) and specifically at weeks 0 and 12 only (D). (E, F) Particle size, determined via Dynamic Light Scattering (DLS), shown over time (E) and specifically at weeks 0 and 12 only (F).*

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Conclusion

When optimal high ionizable lipid to mRNA weight ratio and optimal excipients were used, mRNA LNP characteristics and transfection efficiency were conserved after continuous and controlled lyophilization. Even after storing the lyophilized LNPs for 12 weeks at room temperature, the transfection efficiency was retrained. In conclusion, it was proved that continuous and controlled lyophilization offers the opportunity to dry mRNA LNPs in a fast and controlled way.

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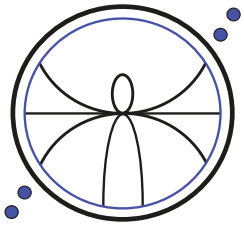

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