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White Paper

Stabilization of RNA-loaded Lipid Nanoparticles by Lyophilization

Strict, ultra-cold chain processes are necessary to maintain the viability and quality of RNA-based vaccines and therapeutics. This cold-chain supply presents a significant challenge, including increased costs, difficulties with worldwide distribution, and inconvenience for end users. Effective management of cold-chain logistics requires precise coordination from start to finish, including temperature monitoring, real-time tracking for traceability, and well-trained and skilled logistics personnel to ensure retention of product quality (Figure 1).

Strategies to increase the stability of RNA-based medicines in relatively higher temperatures is essential. The lack of thermostability and ultra-cold storage requirements for the mRNA-based COVID-19 vaccines limited their global use, despite being the most effective modality against the virus. Most developing countries did not have the cold chain storage equipment and facilities to enable mass vaccination.[1]

This article describes how lyophilization of RNA-loaded lipid nanoparticles (LNPs) enables the storage and shipment of therapeutic formulations at logistically convenient temperatures while preserving their physical properties and biological performance.

The Process and Benefits of Lyophilization

The process of lyophilization removes water from the LNP formulation. Lyophilized formulations can be stored at temperatures above 0 °C which facilitates more simple shipping and distribution.

Lyophilization can be challenging, however, as it puts stress on the formulation which can affect physicochemical properties and, ultimately, biological performance. As such, expertise and state-of-the-art lyophilization equipment is required to design, optimize, and scale the storage matrix and lyophilization cycle. The lyophilized product must then be characterized in terms of LNP properties and *in vitro* and *in vivo* performance. Figure 2 outlines the efficient three-step LNP lyophilization process used by our service organization to produce stable, freeze dried RNA-loaded LNPs.

Screening to Select the Storage Matrix

The first step in developing a lyophilized product is a comprehensive screening to select the best excipients (Emprove® Excipients) to create a suitable storage matrix for lyophilization. Freeze-thaw studies are performed at this stage to evaluate the storage matrix.

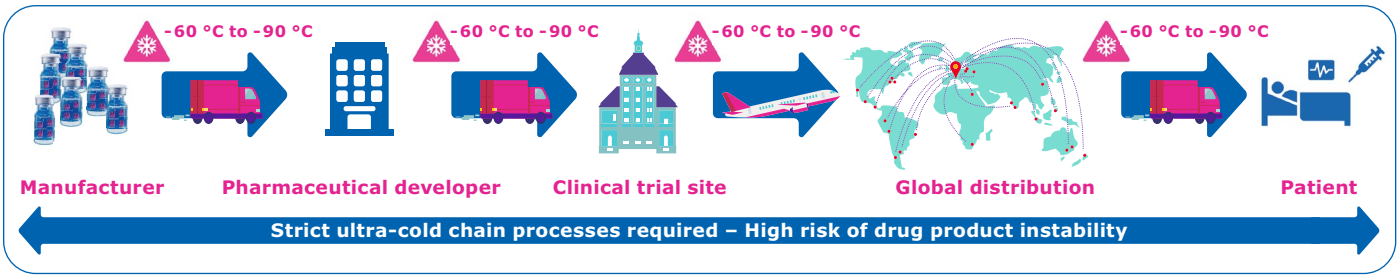

Figure 1.

Ultra-cold chain processes are required when there is a high risk of product instability.

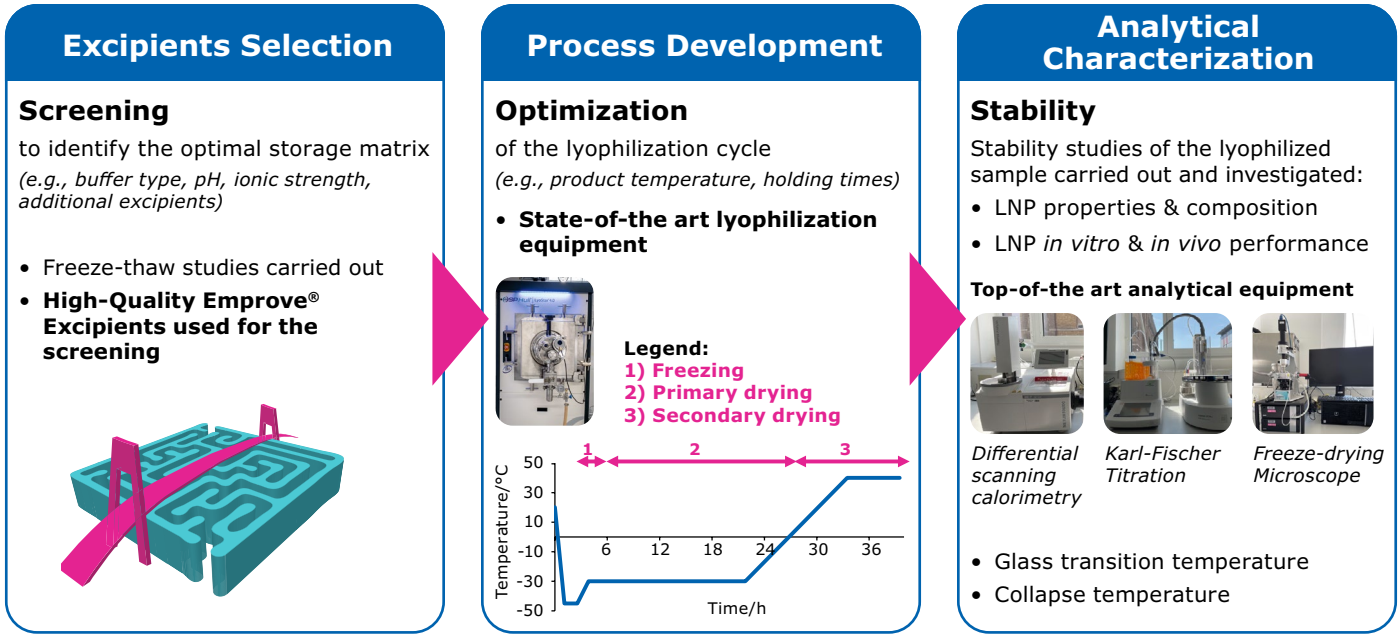

Figure 2.

A three-step process is used to customize the development cycle of a lyophilized LNP formulation.

Optimization of the Lyophilization Cycle

Once the storage matrix is defined, our existing, optimized lyophilization cycle is modified as needed to meet specific requirements presented by the formulation. As an example, parameters related to the freezing process, the primary and secondary drying phase can be optimized.

At this stage, it is also essential to ensure that the developed formulation composition and lyophilization process are scalable under GMP. This is ensured by state-of-the-art lyophilization and analytical equipment throughout our development and production facilities.

Analytical Characterization

State-of-the-art analytical equipment and techniques are used to further refine the lyophilization cycle, if needed. As an example, we use differential scanning calorimetry and freeze-drying microscopy to determine glass transition and collapse temperatures of the samples, while the water content of the lyophilized product is determined by Karl-Fischer titration.

Stability studies of the lyophilized samples are then performed to evaluate LNP properties and composition as well as *in vitro* and *in vivo* performance (Figure 3). At specific time points, analysis of the formulation stability includes (but not limited to) assessment of:

- Optical inspection (e.g., cake appearance)
- Water content
- Reconstitution time
- Particle size and polydispersity of LNP
- Encapsulation efficiency of the payload
- Total RNA payload content
- RNA purity and integrity
- Lipid identity and content
- Osmolality and pH
- Presence of subvisible (foreign) particles

In vitro performance is evaluated using assays for e.g., transfection efficiency and toxicity, while *in vivo* performance is measured by e.g., biodistribution and protein expression/gene silencing.

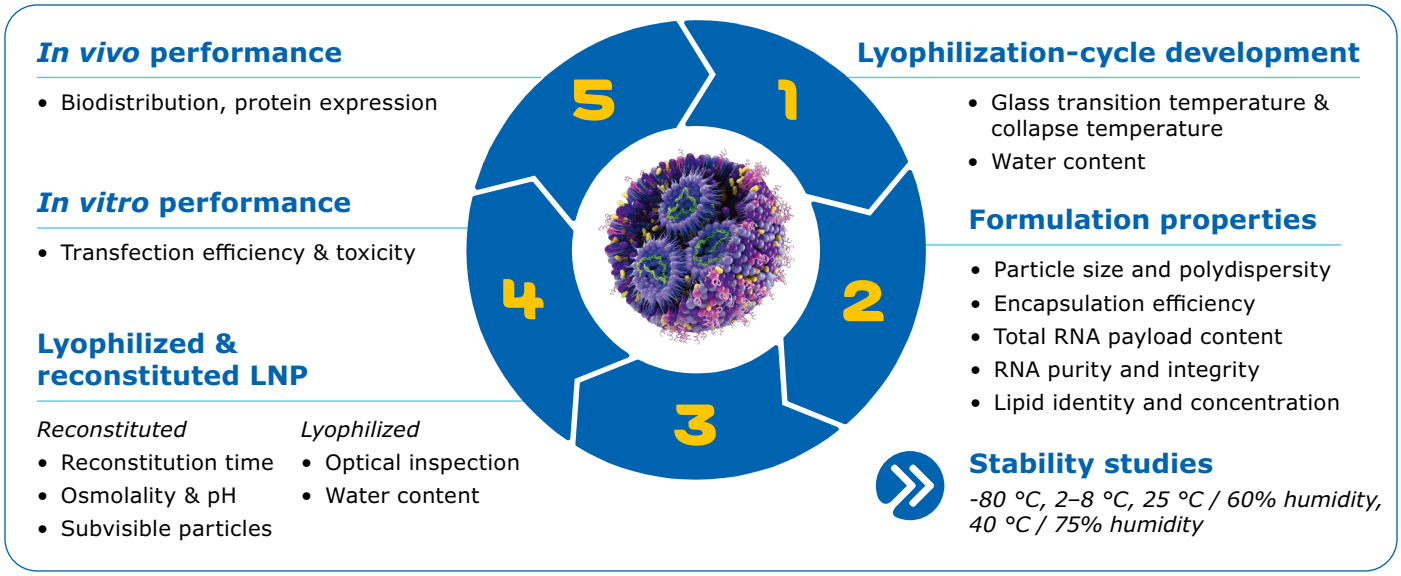

Figure 3.

State-of-the-art analytical equipment and techniques used to assess the lyophilization process and LNP stability.

Confirmation of Physico-Chemical Properties

Table 1 summarizes the physico-chemical properties of a mRNA- and a siRNA-loaded LNP formulation before and after freeze-drying. Particle size, polydispersity, and encapsulation efficiency were all maintained following our lyophilization process.

		Pre lyophilization	Post lyophilization
mRNA LNP	**Particle Size (nm)**	99 ± 1	99 ± 2
	Polydispersity Index (PDI)	0.18 ± 0.02	0.24 ± 0.04
	Encapsulation Efficiency (%)	93 ± 1	79 ± 1
siRNA LNP	**Particle Size (nm)**	80 ± 1	89 ± 1
	Polydispersity Index (PDI)	0.17 ± 0.03	0.25 ± 0.02
	Encapsulation Efficiency (%)	94 ± 1	79 ± 6

Table 1.

Physico-chemical properties of the mRNA- and siRNA-loaded LNP formulations before and after lyophilization.

Next, the *in vitro* biological performance of the formulation was tested by adding LNPs loaded with mRNA encoding for luciferase to muscle cells and LNPs loaded with siRNA targeting luciferase to cancer cells (Figure 4). In both cases, biological performance was maintained following lyophilization.

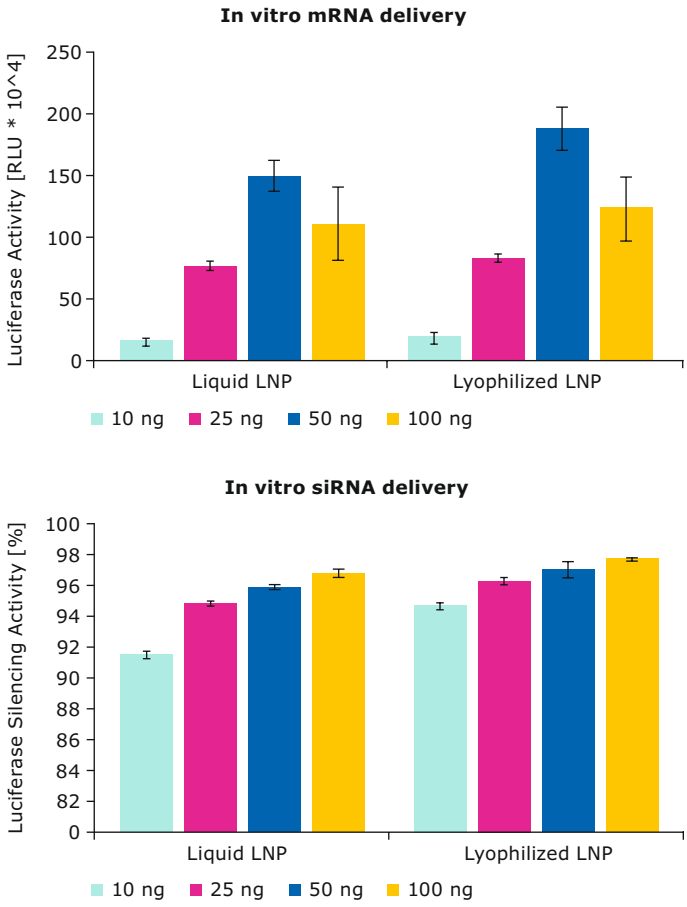

Figure 4.

In vitro efficacy of siRNA- and mRNA-LNP before and after lyophilization.

Confirmation of *In Vivo* Performance

In vivo performance of the mRNA-LNP formulation before and after lyophilization was studied upon intravenous injection of BALB/c mice. As shown by whole body bioluminescence imaging, biological performance was equivalent at all time points evaluated (Figure 5).

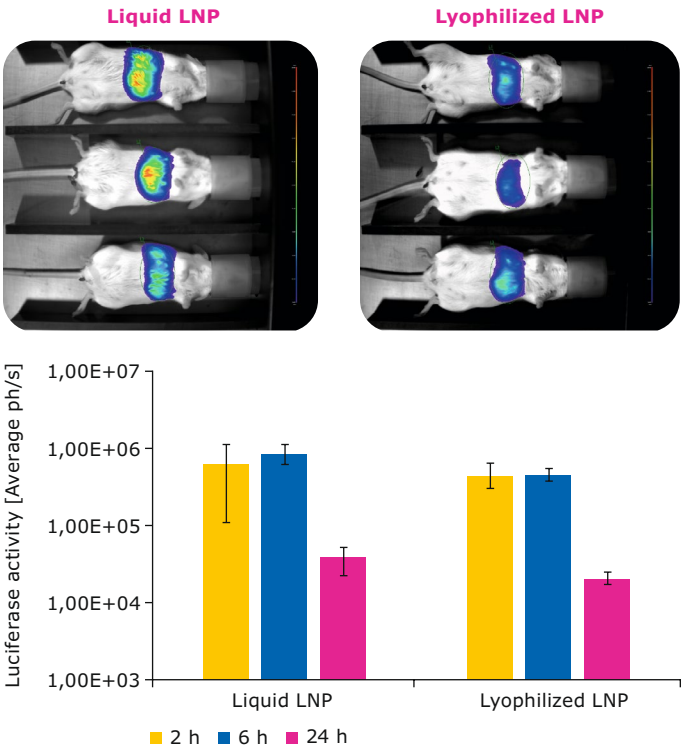

Figure 5.

In vivo efficacy and distribution of mRNA-LNP before and after lyophilization.

Confirmation of Storage Stability

A storage stability study was performed to assess the optimal storage conditions for the lyophilized RNA-loaded LNP. Storage of lyophilized mRNA-LNP at elevated temperatures for one month did not affect physico-chemical properties (Table 2 and Figure 6). Overall, *in vitro* performance was also maintained (tested in muscle and hepatocyte cell line) as measured by a luciferase assay; however, there was some loss of relative integrity in the accelerated condition (25 °C).

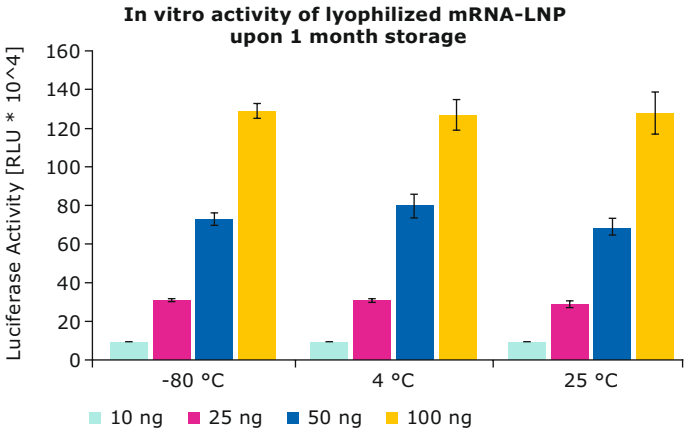

Figure 6.

In vitro efficacy of lyophilized mRNA-LNP stored at elevated temperatures for one month.

	Post lyophilization	-80 °C	4 °C	25 °C
	t = 0	t = 1 month		
Diameter (nm)	99 ± 2	99 ± 1	98 ± 2	102 ± 2
Polydispersity Index (PDI)	0.24 ± 0.02	0.22 ± 0.02	0.17 ± 0.01	0.22 ± 0.03
Encapsulation Efficiency (%)	79 ± 1	81 ± 1	82 ± 2	82 ± 2
Relative RNA Integrity (%)	91 ± 2	94 ± 1	91 ± 1	71 ± 4

Table 2.

Physico-chemical properties of the lyophilized mRNA-loaded LNP stored at elevated temperatures for one month.

Conclusion

RNA medicines are essential in fighting severe diseases and protecting the global population from pandemic events. However, their widespread availability is constrained by the challenges of ultra-cold chain storage and distribution, limiting access in many regions and to countless patients in need.

The results presented in this article demonstrate the ability to lyophilize RNA-loaded LNP formulations and to maintain their *in vitro* and *in vivo* biological performance. Lyophilization improves the storage stability of the investigated formulations at elevated temperatures, thus, facilitating a much more simple global distribution of these emerging therapeutic modalities.

References

1. Uddin MN, Roni MA. Challenges of Storage and Stability of mRNA-Based COVID-19 Vaccines. Vaccines (Basel). 2021 Sep 17; 9(9):1033. doi: 10.3390/vaccines9091033.

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