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Lyophilization Strategies Enhancing the Storage Stability of RNA-loaded Nanoparticles

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***Abstract*—Lipid nanoparticles (LNPs) have emerged as versatile nanocarriers for RNA therapeutics, protecting them from nuclease-mediated degradation and allowing efficient cell uptake. One main challenge of LNPs is represented by their limited stability in aqueous suspensions, due to RNA release and/or LNP collapse over time. Lyophilization has been investigated as an effective method to produce dry RNA-loaded LNPs (RNA-LNPs) that can be stored at low temperatures for months. Particularly, cryo- and lyo-protectants have been used as additives to preserve RNA-LNP integrity, prevent aggregation phenomena and maintain RNA bioactivity during freeze-drying and storage. In our research, we designed novel polymer-lipid hybrid nanoparticles combining the superior stability of synthetic polymers in physiological media and the high RNA loading efficiency of lipids. Herein, we investigated lyophilization as an optimal method to preserve the physicochemical and biological properties of RNA-based nanomedicines. To this purpose we analyzed state-of-the art storage methods for RNA-LNPs and, then, studied optimal storage conditions for microRNA (miRNA)-loaded hybrid nanoparticles developed in our laboratory. Results from this work highlight the key role of lyophilization in the presence of cryo-/lyo-protectants as a means to enhance long-term storage of RNA-nanotherapeutics, which is expected to widen their accessibility for large-scale clinical use.**

***Keywords*—Lipid nanoparticles, RNA therapies, lyophilization, long-term storage.**

I. Introduction

RNA-based therapies are innovative approaches in modern medicine, exploiting the versatility of RNA molecules to address a wide range of diseases, from genetic disorders to cancer and infectious diseases. To date, several RNA-based drugs have been approved, and many are in phase III trials [1]. The broad potential of this class of therapeutics is based on the precision and efficacy of their effect on the modulation of target protein expression [2].

However, safe and efficient *in vivo* delivery represents one main barrier to clinical translation of RNA-therapies. Indeed, the composition and physicochemical properties of nucleic acid-based therapeutics make them highly susceptible to nuclease-mediated degradation [3]. Additionally, RNAs are hydrophilic and negatively charged molecules unable to spontaneously cross the cell membrane. The loading of RNAs into nanocarriers can overcome short RNA half-life and limited cell internalization. Furthermore, nanocarriers may also facilitate cell-specific delivery of RNAs by recognising selective biomarkers on the cell surface [3].

Advances in nanotechnology and materials science have further enhanced the potentiality of nanomedicines, allowing customisation of nanoparticle (NP) physicochemical properties, such as size, shape, bulk and surface composition. The high degree of customisation provides effective solutions able to overcome biological barriers and achieve precise cell-targeted delivery.

Lipid NPs (LNPs) are currently the most used nanocarriers for the delivery of oligonucleotide-based drugs [1]. They are generally composed of ionisable cationic lipids, cholesterol, phospholipids and polyethylene glycol (PEG)-lipids (Fig. 1). Ionisable cationic lipids allow RNA loading by electrostatic interactions with negatively charged RNAs. Phospholipids, cholesterol and other helper lipids may further improve formulation stability and delivery efficiency [4]. PEG-lipids regulate particle size, prevent aggregation and prolong circulation time *in vivo* [5]. Numerous reviews have thoroughly explored the continuous advancements in the development of LNPs formulations with improved physicochemical and biological properties, by efficient manufacturing processes [6].

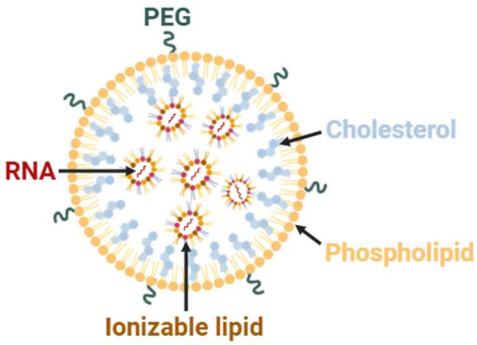


Fig. 1: Schematic illustration of RNA-LNPs. Created with BioRender.

LNPs have been first introduced into the clinics with the approval of Onpattro® in August 2018 [7]. Onpattro®, developed by Alnylam Pharmaceuticals and Sanofi Genzyme, was approved for intravenous (IV) administration of the siRNA drug Patisiran, used for the treatment of polyneuropathy in adults with hereditary transthyretin-mediated amyloidosis (hATTR). This marked the first clinical application of LNPs for RNA nanomedicine, demonstrating the potential of LNPs technology in delivering RNA-based therapies. During COVID-19 pandemics, LNPs were further exploited for the design of mRNA-based vaccines by Pfizer/BioNTech [8] and Moderna [9]. They been approved by

the Food and Drug Administration (FDA) for use even in children under the age of 5 [10]. Such new clinical application highlighted the versatility, safety and efficacy of RNA-LNPs. However, during the initial distribution phases of mRNA-LNPs based vaccines against COVID-19, their storage stability affected large scale clinical use. Due to the lack of complete knowledge on storage stability of RNA-LNPs, a low storage temperature (from -15 °C to -80 °C) was initially used as a cautious approach, causing logistical challenges and the wastage of vaccines [11]. Storage stability is now recognised as a key parameter for nanomedicine translation to clinical application once their efficacy and safety have been demonstrated at a preclinical stage [12]. However, despite many RNA-based formulations are approaching the final stages of market approval, the influence of storage conditions on their stability and efficacy remains largely unexplored [13]. In more detail, the term *nanoparticle stability* is employed to indicate the preservation of physicochemical characteristics (i.e., size, charge and polydispersity index, PDI) over a period of time. LNPs are usually prepared by self-assembly involving hydrophobic interactions, hydrogen bonding and electrostatic interactions between constituent components and drug cargo. Therefore, they generally suffer from inherent instability as they may disassemble or form larger aggregates over time [14]. Three main mechanisms contribute to the instability of RNA-LNPs. Firstly, mechanical stresses can damage LNPs. Secondly, chemical modifications, such as hydrolysis and oxidation of phospholipids, can alter the *in vivo* behavior of LNPs, thereby affecting their loading and release properties [15]. Thirdly, LNPs efficacy can be reduced by physical modifications *in vivo*, such as aggregation and fusion phenomena, or progressive permeabilization [16]. For these reasons, RNA-LNPs cannot be stored in aqueous suspension under mild temperature conditions (i.e., at 4 °C or room temperature) for a longer period than one week.

Storage stability of RNA-LNPs has been improved by freeze-thaw technique. To preserve NPs from damage during freezing, cryoprotectant agents, particularly sugars, have been used due to their biological safety and well-known ability to facilitate the cryopreservation of tissues and cells for extended time periods. Notably, COVID-19 mRNA vaccines need storage by freezing at low temperatures in the presence of sucrose (10% w/v), a well-known cryoprotectant [17]. Precisely, Pfizer/BioNtech vaccine is stored at −60 °C to −80 °C and after thawing needs dilution by saline before injection whereas Moderna vaccine is stored at −15 °C to −20 °C and can be directly administered after thawing [18]. Trehalose is another disaccharide widely exploited as cryoprotectant agent, which is present in the tissues of tardigrades (water bears) and helps them survive extreme temperature conditions [19]. Cryoprotectants can preserve RNA-LNPs by lowering the glass transition temperature of the aqueous suspension to prevent freezing, and by replacing water molecules to maintain the critical hydrogen bonding necessary for LNPs stability. However, the freeze-thaw method needs storage at very low temperatures, such as -20 °C or -70 °C, resulting in high storage and transport costs for RNA-therapeutics.

In order to enhance the long-term stability of RNA-LNPs, a better option is to convert them into dry liposomal formulations, via lyophilization (freeze-drying) with the addition of stabilizing agents, i.e., cryo- and lyo-protectants. Lyophilization is a technique commonly employed in the pharmaceutical industry to enhance the shelf-life and stability of various drugs through freezing and further drying by sublimation at low temperature and pressure. In their freeze-dried form, RNA-LNPs may be potentially transported on a global scale without the need to keep them at very low temperatures [20]. Indeed, freeze-drying is regarded as the most reliable method to preserve the biological activity of RNA-LNPs, enabling long-term storage at 4 °C or ambient temperature for 3 to 6 months [20].

However, the selection of an optimal type and concentration of cryoprotectant is fundamental for effective RNA-LNPs storage after lyophilization, without alterations in their physicochemical and biological properties after rehydration. The cryo- and lyo-protectants that have been mostly used for the freeze-drying of LNPs include: monosaccharides (e.g. glucose, fructose, mannitol, mannose, sorbitol, xylose), disaccharides (e.g., sucrose, lactose, trehalose), polysaccharides (e.g., raffinose), hydrophilic polymers (e.g., polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), gelatin), and hydrophilic chemicals (e.g., glycine) [20].

In a recent study, siRNA-LNPs were prepared combining 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG2000), cholesterol, and lipidoid $306O_{13}$ at a ratio of 10:1.5:38.5:50. This study evaluated the storage stability of siRNA-LNPs in different conditions: (i) in aqueous suspension at -20 °C, 2 °C and 25 °C; (ii) subjected to freeze-drying followed by rehydration; (iii) subjected to three freeze-thaw cycles at -80 °C. Conditions (ii) and (iii) were studied in the presence of varying concentrations of trehalose or sucrose (0, 1, 5, 10, or 20% (w/v)). siRNA-LNPs stored in aqueous solutions at 2 °C remained stable for over 150 days. Additionally, siRNA-LNPs demonstrated good stability at 2 °C across a range of pH values (3, 7.4, and 9). However, storage in aqueous suspension exposes RNA-LNPs to the risk of bacterial contamination. On the other hand, freeze-thaw cycles were found to cause siRNA-LNPs aggregation and to reduce siRNA silencing efficacy, although mitigated by using trehalose or sucrose [14]. Indeed, the freeze-thaw method caused a superior reduction in siRNA efficacy, compared to freeze-drying and storage in suspension. Finally, freeze-dried siRNA-LNPs with 20% (w/v) sucrose or trehalose appeared promising as they retained 80–87% of their gene silencing ability compared to freshly prepared siRNA-LNPs [14].

In another study, firefly luciferase-encoding mRNA-LNPs were first prepared. Then their stability and biological efficacy (evaluated by target mRNA protein expression) were studied upon storage by freezing or lyophilization, using three different cryoprotectants, such as mannitol, trehalose, and sucrose, at various concentrations (5, 10, 20 or 40 % (w/v)) [21]. *In vitro* and *in vivo* investigations showed that mRNA-LNPs, frozen and stored in liquid nitrogen in the presence of 5% (w/v) trehalose or sucrose, preserved mRNA efficacy after at least three months storage. However, they also found that the other rehydrated formulations of lyophilized mRNA-LNPs

containing 10, 20, and 40% (w/v) sucrose or trehalose exhibited similar mRNA efficiency *in vitro* compared to fresh mRNA-LNPs, after one week of storage at -80 °C [21].

In another study, mRNA-LNPs were prepared fromPEG lipid (PEG2000-C-DMA), cholesterol, DSPC, and an ionizable lipid ((6Z,16Z)-12-((Z)-dec-4-en-1-yl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate), at a molar ratio of 1.5:38.5:10:50. The mRNA-LNPs with 10% sucrose and 10% maltose as cryoprotectants were freeze-dried and stored at -80, -20, 4, 25 (room temperature), and 42 °C for 1, 3, and 6 months. The physicochemical properties remained largely unchanged after storage for up to 6 months at 4 °C or for 3 months at 25 °C. Firefly luciferase-encoding mRNA-LNPs demonstrated high bioluminescence imaging (indicating significant luciferase production) in mice, when injected intramuscularly or intradermally, even after storage at 4 °C for 6 months or at 25 °C for 3 months. Such findings suggest that freeze-dried mRNA-LNPs are a viable option for long-term storage under moderate temperature conditions such as 4 °C or room temperature [22].

In our research we designed novel polymer-lipid NPs for microRNA (miRNA) delivery with the aim of synergistically combining the high miRNA loading ability of lipids with the enhanced stability in physiological conditions of synthetic polymers [23]. Hybrid NPs are expected to show enhanced storage stability, longer *in vivo* half-life and controlled and sustained RNA delivery kinetics.

In this work, we applied an in-depth state of the art study of RNA-LNPs storage methods, to optimize storage conditions of hybrid NPs loaded with miRNA. Hybrid NPs subjected to freeze-drying, in the presence of 12% (w/v) trehalose as cryo- and lyo-protectant, were found to maintain their physicochemical properties and functional efficacy over different storage times.

II. Materials and Methods

Cationic and helper lipids were used to prepare liposomes via the thin lipid film hydration method [24]. Lipoplexes containing miRNAs (negmiR or miR-1) were prepared at an optimal amino to phosphate (N/P) molar ratio of 3, as previously reported [24]. Then, they were incubated at room temperature and subsequently diluted in RNase-free water. Hybrid NPs loaded with miRNAs were then prepared using lipoplexes and synthetic co-polymers, through nanoprecipitation, following a patented method.

The stability of hybrid NPs loaded with miRNAs was assessed upon analysis of various storage conditions: (i) incubation in suspension at 4 °C; (ii) freezing at -80 °C with/without cryoprotectant, followed by storage at -80 °C for different times and final thawing for reconstitution; (iii) freeze-drying with cryoprotectant, followed by storage at -20 °C for different times and final rehydration. Physical properties (hydrodynamic diameter, PDI, Z-potential) were analyzed via Dynamic Light Scattering (DLS). Functional properties were evaluated through *in vitro* cell transfection tests by treating adult human cardiac fibroblasts (AHCFs, CC-2903, Lonza) with miR-1 loaded hybrid NPs after storage for 0, 7 and 14 days under conditions (ii) and (iii). Twinfilin-1 (TWF-1) mRNA expression was analyzed at 48 h post-transfection.

III. Results

The long-term stability of hybrid NPs was evaluated by monitoring their hydrodynamic diameter, PDI, and Z-potential when stored in RNase-free water at 4 °C for up to 28 days. The hybrid NPs maintained stable hydrodynamic diameter, PDI, and Z-potential during this period, while their transfection efficiency was preserved only for 48 h, then significantly decreasing after 7 and 14 days. Such results suggested that storage of hybrid NPs suspensions at 4 °C appears suboptimal for their long-term preservation.

On the other hand, freeze-thaw at –80 °C caused a significant size increase (~ 800 nm), which was mitigated by adding trehalose as a cryoprotectant, reducing size to around 200 nm. Frozen hybrid NPs in the presence of 12% (w/v) trehalose showed similar physicochemical properties and bioactivity compared to freshly prepared hybrid NPs, after thawing.

Interestingly, freeze-drying with 7–12% (w/v) trehalose followed by rehydration ensured the preservation of hybrid NPs physicochemical properties (Fig. 2). Furthermore, after freeze-drying and storage for 14 days at -20 °C, hybrid NPs loaded with miR-1 showed similar TWF-1 mRNA downregulation compared to freshly prepared hybrid NPs.

Overall, results showed that freezing with 12% (w/v) trehalose, followed by storage at -80 °C and final thawing, and freeze-drying with 12% (w/v) trehalose followed by storage at -20 °C and final rehydration, are suitable storage methods for hybrid NPs loaded with miRNAs. Among them, freeze-drying will be further investigated, due to its higher versatility, allowing to test different storage temperatures (including room temperature), with the final aim to reduce storage costs and logistic issues in clinically approved RNA therapies.

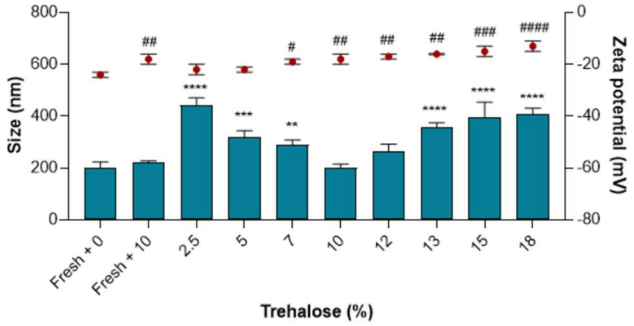


Fig. 2: Hydrodynamic diameter (blue bars) and Z-potential (red dots) of rehydrated hybrid NPs after freeze-drying in the presence of varying trehalose concentrations (2.5, 5, 7, 10, 12, 13, 15, and 18% (w/v)), compared to freshly prepared hybrid NPs (with 0% and 10% (w/v) trehalose).

IV. Conclusion

This study demonstrated that lyophilization is a reliable strategy to address the storage stability challenges of NPs loaded with RNAs. Lyophilized miRNA-loaded hybrid NPs with 12% (w/v) trehalose cryoprotectant preserved their physicochemical properties and transfection efficiency after storage at -20 °C for at least 14 days. Further tests are currently ongoing to ascertain the long-term storage stability of the hybrid NPs. Specifically, lyophilized hybrid NPs containing 10% and 12% (w/v) trehalose have been stored at -20 °C and will be analysed after 1, 4, and 6 months. The physicochemical characterisation of the samples will be conducted using DLS to assess size, PDI, and Z-potential. Concurrently, the biological functionality will be evaluated by assessing

cytocompatibility and the ability of the miRNAs loaded hybrid NPs to downregulate TWF-1 expression in AHCFs. Finally, sterility will be monitored throughout the storage period to confirm that the preservation method also ensures microbiological safety. Furthermore, the study will explore the potential for long-term storage under mild conditions by testing different storage temperatures, including 25 °C.

Our results support the adoption of lyophilization as an efficient method to overcome the storage constraints of RNA-based nanomedicines fostering their broader clinical application.

Future research will further optimise lyophilization protocols for diverse nanomedicine platforms, with the aim to allow long-term storage at still milder temperature conditions.

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