



Long-term storage of lipid-like nanoparticles for mRNA delivery

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ABSTRACT

Lipid-like nanoparticles (LLNs) have been extensively explored for messenger RNA (mRNA) delivery in various biomedical applications. However, the long-term storage of these nanoparticles is still a challenge for their clinical translation. In this study, we investigated a series of conditions for the long-term storage of LLNs with encapsulation of mRNA. We evaluated the stability of LLNs with different concentrations of cryoprotectants (sucrose, trehalose or mannitol) under the conditions of freezing or lyophilization processes. Through *in vitro* and *in vivo* mRNA delivery studies, we identified the optimal storage condition, and found that the addition with 5% (w/v) sucrose or trehalose to LLNs could remain their mRNA delivery efficiency for at least three months in the liquid nitrogen storage condition.

1. Introduction

Messenger RNA (mRNA) represents a new class of therapeutics with the promise to prevent and treat various diseases [1–4]. As an alternative to plasmid DNA, mRNA exerts its function in the cell cytosol, thus avoiding the potential risks of genome mutagenesis [5,6]. Although the stability and immunogenicity of mRNA have become more controllable with the advances of *in vitro* transcription (IVT) technology [7], carriers are essential for efficient mRNA delivery *in vivo* [8–14]. Among different types of delivery carriers, lipid-like nanoparticles (LLNs), formulated by ionizable lipids, cholesterol, helper lipids, and polyethylene glycol (PEG), are one of the most developed mRNA delivery systems and are able to deliver a wide range of mRNAs both *in vitro* and *in vivo* [1,6,15–18]. However, little is known about the physical stability of these nanomaterials for mRNA delivery. Therefore, a systematic study of various conditions, such as temperature and physical states (aqueous, freezing, or lyophilized), for long-term storage of LLNs-mRNA, is urgently needed in order to provide a basis for future

clinical applications.

Freezing and lyophilization are the most commonly used methods for long-term storage of many types of nanoparticles [19–25]. However, macromolecules may be damaged due to stresses generated by crystallization and vacuum dehydration, thus decreasing the stability of nanoparticles unless appropriate cryoprotectants are used [26,27]. In this study, in order to identify the optimal storage conditions for LLNs-mRNA, we prepared these LLNs using an ionizable lipid, N^1 , N^3 , N^5 -tris(3-(didodecylamino)propyl) benzene-1,3,5-tricarboxamide derivative, TT3¹⁶. Next, we screened different conditions, including cryoprotectants (trehalose, glucose and mannitol) and physical states (aqueous, freezing, or lyophilized), and evaluated the properties such as nanoparticle size and mRNA expression *in vitro* and *in vivo*. Finally, we found that freezing LLNs-mRNA with 5% (w/v) sucrose or trehalose in liquid nitrogen was the optimal process for their long-term storage.

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2. Materials and methods

2.1. Materials

Bright-Glo luciferase assay substrate was obtained from Promega (Madison, WI). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG₂₀₀₀) were purchased from Avanti Polar Lipids. N^1 , N^3 , N^5 -tris(3-(didodecylamino)propyl) benzene-1,3,5-tricarboxamide derivative, TT3 was synthesized as reported before [16]. mRNAs encoding firefly luciferase (FLuc mRNA) were prepared as the reported method [17]. Cholesterol, trehalose, sucrose, mannitol, and other chemicals were purchased from Sigma-Aldrich. Hep3B cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Fetal bovine serum (FBS), Eagle's Minimum Essential Medium (EMEM), phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific.

2.2. Formulation and characterization of mRNA loaded LLNs

LLNs were prepared as previously described [16]. Briefly, TT3, DOPE, cholesterol, DMG-PEG₂₀₀₀ (molar ratio of 20/30/40/0.75) were mixed to form ethanol phase. FLuc mRNA solution and citrate buffer were used to form aqueous phase. Then the two phases were mixed together by pipetting to formulate mRNA loaded LLNs. Particle size and zeta potential of LLNs was measured using a NanoZS Zetasizer (Malvern, Worcestershire, U.K.) at a scattering angle of 173° and a temperature of 25 °C.

2.3. Luciferase assay *in vitro*

Hep3B cells were grown in EMEM supplemented with 100 mL/L of FBS. The cells were incubated at 37 °C in a 5% CO₂ environment and subcultured by partial digestion with 0.25% trypsin with EDTA.

Hep3B cells were seeded in white 96-well plates at the density of 2×10^4 cells per well, cultured overnight, and then treated with FLuc mRNA loaded LLNs at the concentration of 50 ng mRNA/well. After 18 h, 100 μ L luciferase substrate (Bright-Glo reagent, Promega, Madison, WI) was added to each well. After 5 min, the luminescence intensity was measured by the SpectraMax M5 microplate reader (Molecular Devices, LLC., Sunnyvale, CA).

2.4. Freeze–thaw studies

First, the PBS solution containing 0, 10, 20, 30, 40, or 60% (w/v) cryoprotectants (trehalose, sucrose, or mannitol) was prepared. Then, freshly formulated LLNs were diluted with the PBS solution (1:1, V/V). The final concentration of these cryoprotectants in LLNs solutions were 0, 5, 10, 15, 20, or 30% (w/v). After that, the LLNs were quickly frozen in liquid nitrogen and then were thawed at 37 °C for 3 cycles. Subsequently, the size of LLNs was measured using a NanoZS Zetasizer, and *in vitro* transfection efficiency was evaluated through a luciferase assay *in vitro*.

2.5. LLNs stored in liquid nitrogen

LLNs were formulated and diluted by PBS (1:1, V/V) containing different cryoprotectants (trehalose, sucrose, or mannitol). As mentioned above, the final concentration of these cryoprotectants in LLNs were 0, 2.5, 5, 10, or 15% (w/v). Then, the LLNs were frozen and stored in liquid nitrogen. At different time points, these LLNs were thawed and quantified with their size and luciferase expression. These LLNs were frozen and stored in liquid nitrogen until use.

2.6. Lyophilization of LLNs

LLNs were formulated and diluted by PBS (1:1, V/V) containing different cryoprotectants (trehalose, sucrose, or mannitol). Similarly, the final concentration of these cryoprotectants in LLNs were 5, 10, 20 or 40% (w/v). Then, the LLNs were quickly frozen in liquid nitrogen for 30 min. After that, the LLNs were performed lyophilization in a glass chamber for 12 h by a Virtis Unitrap II freeze dryer. The lyophilized LLNs were stored in –80 °C for 1 week. Then, the LLNs were reconstituted with double distilled (DI) water and further evaluated with size and luciferase expression.

2.7. Luciferase assay *in vivo*

All procedures of animal studies conducted at The Ohio State University were approved by the Institutional Animal Care and Use Committee (IACUC) and were also consistent with local, state, and federal regulations as applicable. C57BL/6 mice (6–8 weeks old from the Jackson Laboratory) were administered by a tail vein injection of a series of TT3 LLNs stored under different conditions at a dose of 0.25 mg/kg mRNA ($n = 3$). 6 h later, mice were intraperitoneally (i.p.) injected with the D-luciferin substrate (30 mg/mL). 8 min post injection, mice were euthanized in a CO₂ chamber. Next, major organs (the liver, spleen, kidneys, heart, and lung) were dissected and bioluminescence signals were immediately measured using a Xenogen IVIS imaging system (Caliper, Alameda, CA).

3. Results and discussion

3.1. Stability of LLNs in aqueous condition

In our study, we first evaluated the nanoparticles stability in aqueous condition. The LLNs were formulated and then stored at 4 °C. At different time points, the stability of LLNs were evaluated by measuring the size and luciferase mRNA delivery efficiency. Fresh LLNs were used for positive control. As shown in Fig. 1A, LLNs maintained size at 4 °C within a week, and then particle size increased slightly from 150 nm to nearly 190 nm. LLNs maintained zeta potential at 4 °C up to 5 months (Fig. S1). Meanwhile, the mRNA delivery efficiency reduced from 70.1% (one week) to 5.4% (five months) compared with that of fresh LLNs (Fig. 1B). These results suggested that aqueous condition is not a suitable way for long-term storage of LLNs-mRNA.

3.2. Stability of LLNs through three freeze–thaw cycles

Considering that LLNs cannot be stored for a long time in the aqueous condition, other methods were performed including freezing LLNs in liquid nitrogen and lyophilizing LLNs. Since both the freezing and drying processes may result in destabilization of the nanoparticles, we first examined cryoprotectants for the LLNs by the freeze–thaw assays. Trehalose, sucrose, and mannitol, extensively used as cryoprotectants for improving the stability of nanoparticles [24,28–33], were dissolved with PBS to prepare the stock solution. Then the stock solution was used to dilute the freshly prepared LLNs to afford a final concentration of 5, 10, 15, 20, or 30% (w/v). After three freeze–thaw cycles, the LLNs were characterized for their size and luciferases expression. As shown in Fig. 2A, the size of LLNs without cryoprotectant was significantly increased after freeze–thaw cycles, but most of the sizes of LLNs treated with cryoprotectant were similar to those of fresh LLNs. However, the types and concentration of cryoprotectants showed different ability to maintain mRNA delivery efficiency (Fig. 2B). The addition of 5% sucrose or trehalose maintained almost 100% luciferase expression efficiency compared with freshly prepared LLNs, while the addition of 5%

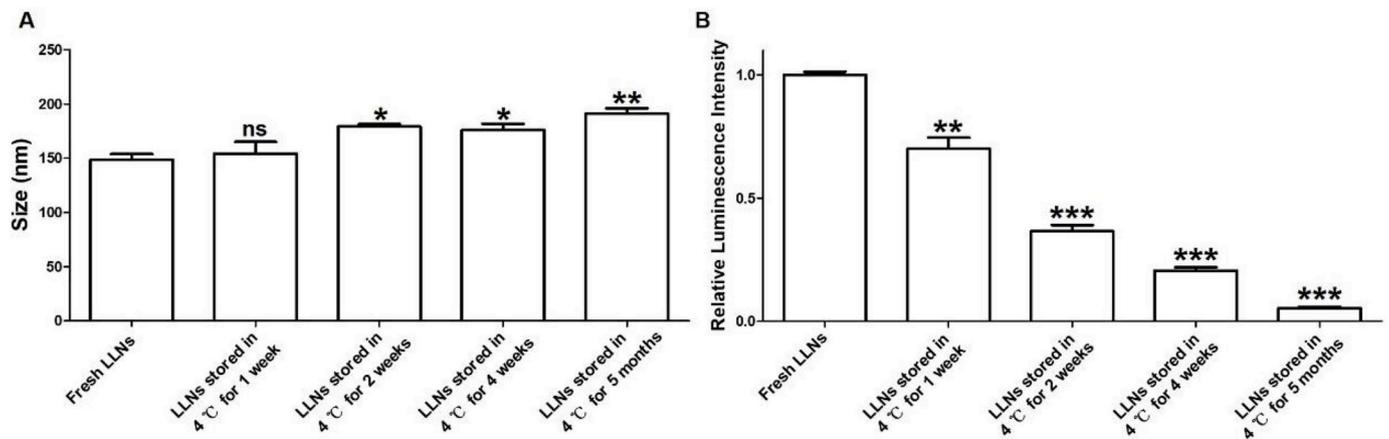


Fig. 1. The long-term stability of LLNs stored at 4 °C in aqueous condition. (A) Size of LLNs at different time points. (B) Relative luminescence intensity of LLNs. All data are presented as mean ± s.d (n = 3). Statistical significance was analyzed by the two-tailed Student's *t*-test. ***P* < 0.01, ****P* < 0.001. ns: not significant.

or 10% mannitol only maintained about 60% luciferase expression efficiency. These data showed that 5% sucrose or trehalose remained the mRNA delivery efficiency during the freeze–thaw cycles, indicating they may be appropriate cryoprotectants for freezing and lyophilization of LLNs-mRNA.

3.3. Stability of LLNs in liquid nitrogen

To optimize the condition for long-term storage of LLNs in liquid nitrogen, the amounts of cryoprotectants necessary to retain mRNA delivery efficiency were examined. Based on the freeze–thaw studies, 0, 2.5, 5, 10 or 15% (w/v) cryoprotectants were used to stabilize LLNs in liquid nitrogen. Similar to the results from the freeze–thaw studies, mannitol was not able to maintain the size or mRNA delivery efficiency in the freezing process (Fig. 3). Although LLNs supplemented with 2.5% or 5% sucrose showed similar luminescence intensity at different time points, the LLNs size in the 2.5% sucrose group significantly increased after 10 days. 5% sucrose seems to be a more appropriate concentration for stabilizing LLNs-mRNA in liquid nitrogen. LLNs supplemented with 5% sucrose or trehalose showed similar sizes, zeta potential and mRNA delivery efficiency compared with fresh LLNs (Fig. 3 and S1). Therefore,

5% sucrose and 5% trehalose were chosen to explore *in vivo* mRNA delivery efficiency.

3.4. Stability of lyophilized LLNs

Lyophilization is another method for long-term storage of nanoparticles. In order to obtain the optimal lyophilization condition, three different cryoprotectants were studied at four different concentrations, 5, 10, 20 or 40% (w/v). After obtaining the dry powders, LLNs were reconstituted with deionized water according to the original volume. The size and luciferase expression were measured pre- and post-lyophilization. As shown in Fig. 4A, in mannitol group, all the sizes of LLNs post-lyophilization significantly increased. In the sucrose and trehalose groups, the size of LLNs post-lyophilization was affected by the concentration of cryoprotectants. After lyophilization, the sizes of 5% sucrose and trehalose treated LLNs increased to 368 nm and 563 nm, respectively. Similarly, the sizes of 10% sucrose and trehalose treated LLNs significantly increased. However, 20 and 40% (w/v) sucrose and trehalose effectively maintained the sizes. As shown in Fig. 4B, lyophilized LLNs that contained 10, 20, 40% (w/v) sucrose or trehalose showed similar mRNA delivery efficiency compared to the fresh LLNs.

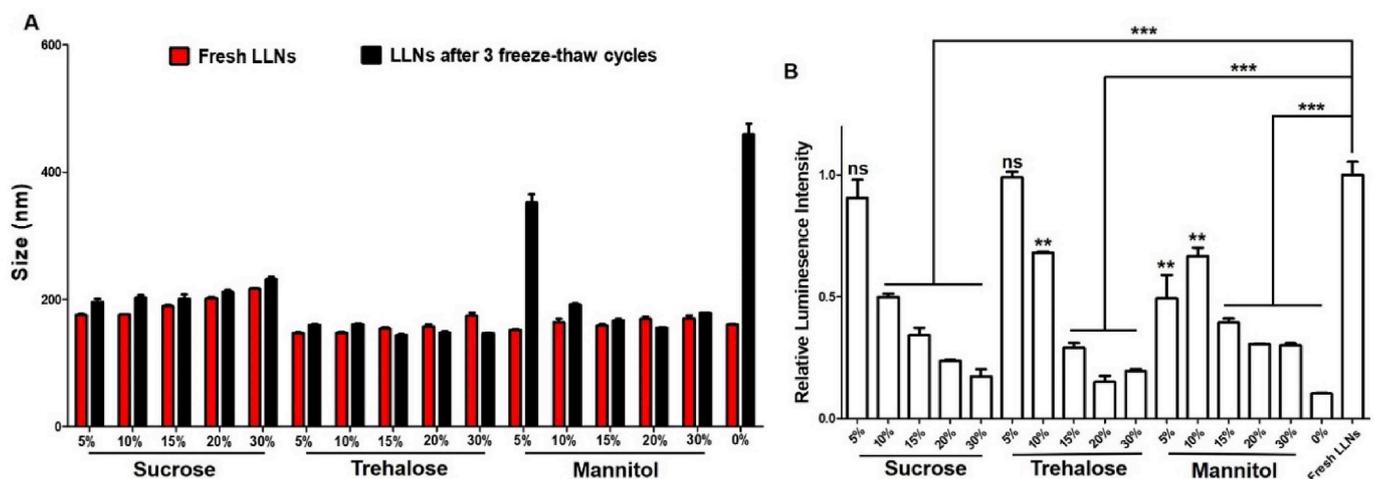


Fig. 2. The stability of LLNs containing cryoprotectants with different concentrations (w/v) after three freeze–thaw cycles. (A) Size of LLNs. (B) Relative luminescence intensity of LLNs. All data are presented as mean ± s.d (n = 3). Statistical significance was analyzed by the two-tailed Student's *t*-test. ***P* < 0.01, ****P* < 0.001.

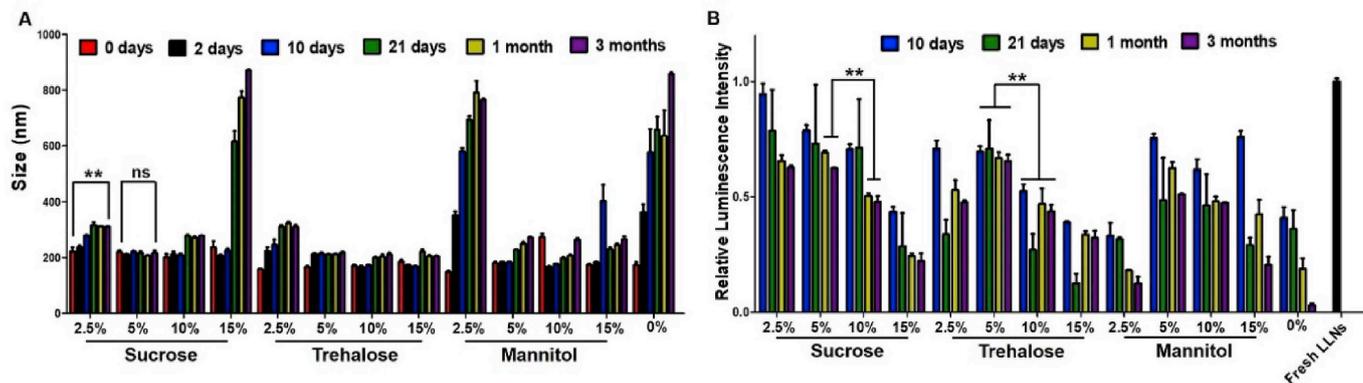


Fig. 3. The stability of LLNs containing cryoprotectants with different concentrations (w/v) stored in liquid nitrogen. (A) Size of LLNs. (B) Relative luminescence intensity of LLNs. All data are presented as mean ± s.d (n = 3). Statistical significance was analyzed by the two-tailed Student's *t*-test. **P < 0.01.

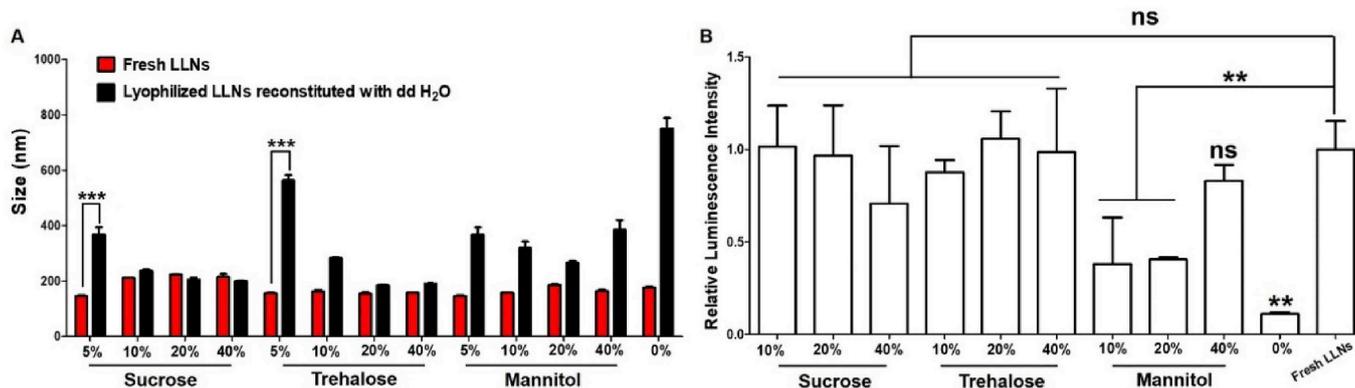


Fig. 4. The stability of lyophilized LLNs (A) Size of LLNs. (B) Relative luminescence intensity of LLNs. All data are presented as mean ± s.d (n = 3). Statistical significance was analyzed by the two-tailed Student's *t*-test. **P < 0.01, ***P < 0.001.

These data suggested that the addition of the cryoprotectants with suitable concentration could retain mRNA expression efficiency of lyophilized LLNs.

3.5. Effects of freezing or lyophilization on LLNs mRNA delivery *in vivo*

Based on the results *in vitro*, we identified the optimal conditions for storage of LLNs by liquid nitrogen or by lyophilization. In order to further confirm the condition, *in vivo* mRNA delivery efficiency was studied. 5% (w/v) sucrose or trehalose was used as cryoprotectants for the liquid nitrogen groups, while 20% (w/v) sucrose or trehalose was used for the lyophilization groups. After thaw or reconstitution, these LLNs were intravenously injected at an mRNA dose of 0.25 mg/kg. Fresh LLNs were formulated and served as a positive control. Six hours post injection, bioluminescence intensity of major organs was measured by the IVIS imaging system.

As shown in Fig. 5 and S2, liquid nitrogen groups (5% sucrose or trehalose LLNs) showed similar bioluminescence signal compared to fresh LLNs group up to 3 months. However, lyophilization groups showed significantly lower bioluminescence signal compared to fresh LLNs group. These *in vivo* results further confirmed that LLNs with 5% sucrose or trehalose stored in liquid nitrogen was a suitable method for long-term storage of LLNs-mRNA.

4. Conclusion

The long-term storage of LLNs-mRNA nanoparticles may facilitate clinical translation of mRNA-based therapeutics. In this study, we evaluated the stability of lipid-like nanoparticles (LLNs)-mRNA under the aqueous, freezing or lyophilized conditions. We also screened different cryoprotectants at various concentrations for protecting LLNs-mRNA under freezing and lyophilization. The results showed that LLNs-mRNA cannot maintain the stability through long-term storage in an aqueous condition. For lyophilization condition, although the addition of 20% (w/v) sucrose or trehalose stabilized the nanoparticles size and mRNA delivery efficiency *in vitro*, the lyophilized LLNs did not show efficiency *in vivo* delivery. LLNs-mediated mRNA delivery *in vivo* involves complex interactions with serum proteins [34]. We speculate that the nanostructure of LLNs-mRNA is changed during the lyophilization and reconstitution process, thereby affecting their interactions with serum proteins *in vivo*, and leading to the different mRNA delivery efficiency between *in vitro* and *in vivo* results. For the freezing conditions, the addition of 5% (w/v) sucrose or trehalose was necessary to maintain the mRNA expression efficiency both *in vitro* and *in vivo*. Overall, freezing LLNs-mRNA nanoparticles in liquid nitrogen with the addition of 5% (w/v) sucrose or trehalose may be an appropriate method for long-term mRNA nanoparticles storage.

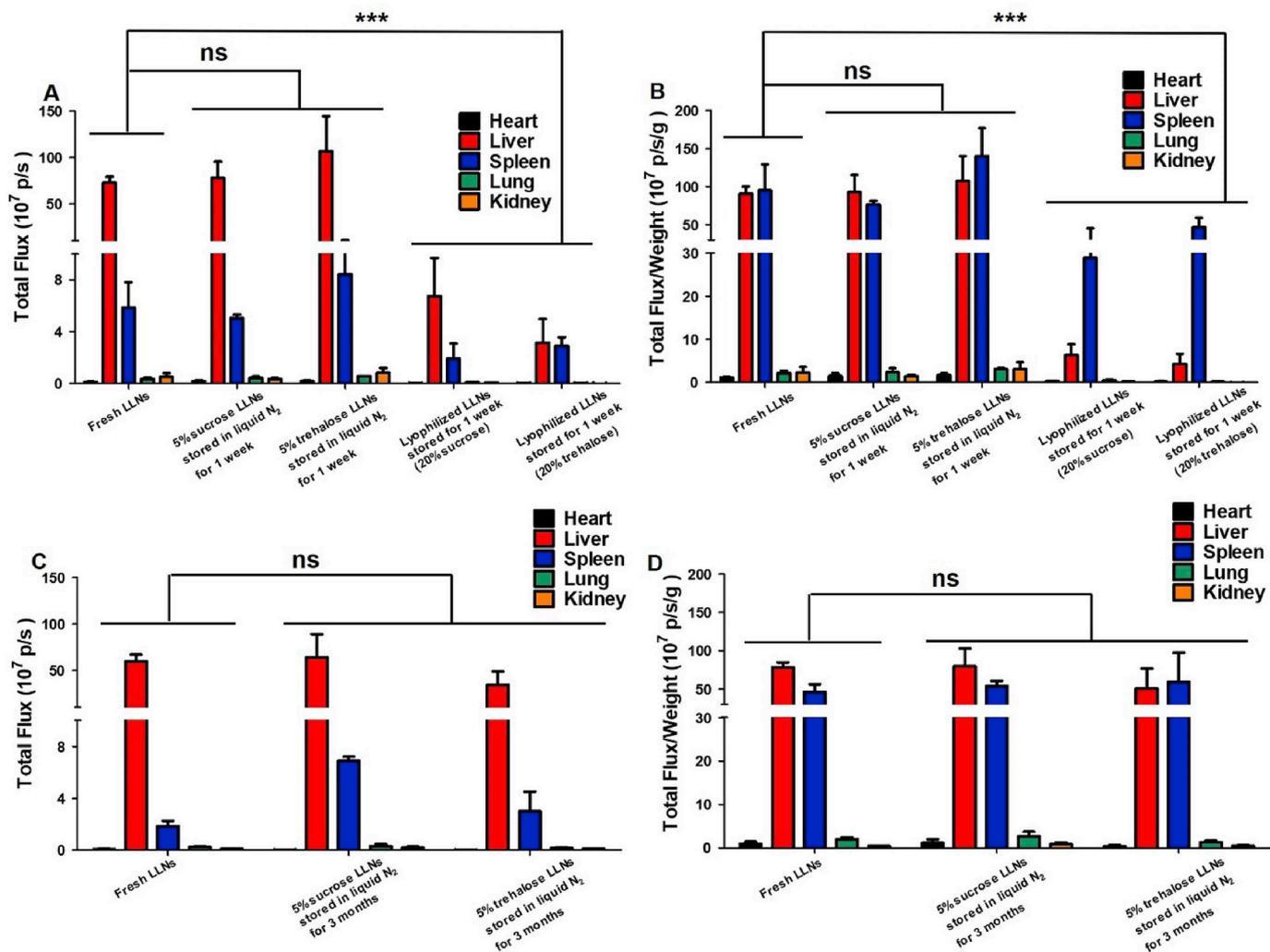


Fig. 5. *In vivo* mRNA delivery efficiency. Total bioluminescence signal (A, C) and normalized bioluminescence signal with tissue weight (B, D) of different LLNs. (A, B) were LLNs stored for 1 week, (C, D) were LLNs stored for 3 months. All data are presented as mean ± s.d (n = 3). Statistical significance was analyzed by the two-tailed Student's *t*-test. ***P < 0.001.

CRedit authorship contribution statement

Pengxuan Zhao: Conceptualization, Data curation, Formal analysis, Writing - original draft. **Xucheng Hou:** Formal analysis, Data curation, Writing - original draft. **Jingyue Yan:** Data curation, Methodology. **Shi Du:** Data curation, Methodology. **Yonger Xue:** Validation. **Wenqing Li:** Validation. **Guangya Xiang:** Writing - review & editing. **Yizhou Dong:** Funding acquisition, Supervision, Investigation, Writing - review & editing.

Declaration of competing interest

The authors disclose no conflicts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioactmat.2020.03.001>.

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