HUMAN VACCINES & IMMUNOTHERAPEUTICS
2025, VOL. 21, NO. 1, 2477384
https://doi.org/10.1080/21645515.2025.2477384

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RESEARCH ARTICLE

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Lyophilized monkeypox mRNA lipid nanoparticle vaccines with long-term stability and robust immune responses in mice

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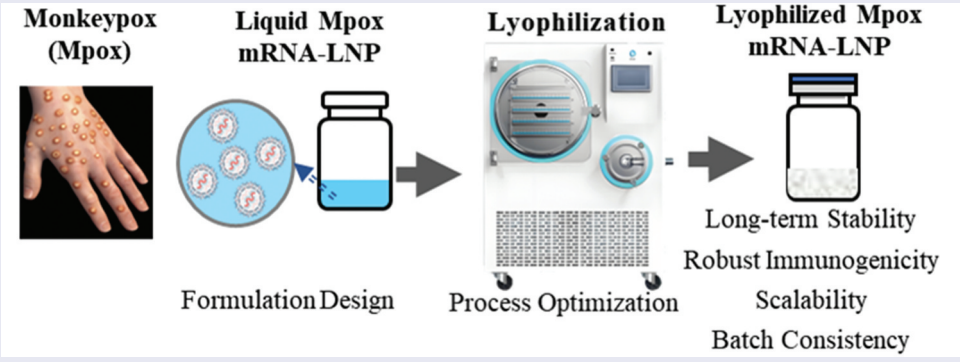
ABSTRACT

The World Health Organization (WHO) has recently declared another global health emergency due to the rapidly spreading monkeypox (Mpox) outbreak in numerous African countries. To address the unmet need to contain the outbreak using the existing vaccines, this study developed a lyophilization process for an effective, scalable and affordable Mpox mRNA-LNP vaccine candidate to address the global health crisis. A comprehensive evaluation and optimization of the vaccine formulation (the type/concentration of cryoprotectants, the type/concentration of buffer system, as well as the mRNA concentration and reconstitution solvent) and the freeze-drying process parameters (freezing method, temperature, cooling rate and primary/secondary drying conditions) were conducted. The freeze-dried product exhibits a uniform appearance and a moisture content of less than 1%. Reconstitution of the lyophilized mRNA-LNP resulted in equivalent particle size/polydispersity index, encapsulation efficiency and mRNA integrity compared to that of freshly prepared mRNA-LNP. Furthermore, the lyophilization process can be scaled up 100-fold to 2000 vials/batch. Notably, the lyophilized mRNA-LNP demonstrated a storage stability of at least 12 months at 4°C, and at ambient temperature for a minimum of 8 h post-reconstitution, exhibiting minimal deterioration in product quality. The in vitro biological activity and in vivo immunogenicity of the lyophilized mRNA-LNP was comparable to that of the freshly prepared mRNA-LNP. These results provide a compelling rationale for the utilization of lyophilization technology in enhancing the accessibility of the Mpox mRNA vaccine in developing countries, a strategy that is crucial for containing the global epidemic of Mpox infection.



ARTICLE HISTORY
Received 3 December 2024
Revised 10 February 2025
Accepted 5 March 2025

KEYWORDS
Monkeypox; lyophilized mRNA vaccines; scalability; batch consistency; long-term stability

Introduction

The global outbreak of the 2022 monkeypox virus (Mpox) infection of humans has raised public health concerns, particularly with regard to the potential for human-to-human transmission of zoonotic diseases and the recent escalating outbreak of the deadly clade 1b Mpox in many African countries.[1–7] This development led to the declaration of another global public health emergency by the World Health Organization (WHO) in August 2024.[8] The present moment is characterized by an unmet need to contain the outbreak, owing to the limitations of the existing vaccines. These limitations include the need to ascertain the efficacy of the vaccines against Mpox in at-risk populations and constrained vaccine supply, particularly in developing countries where the epidemic is ongoing.[9,10] The development of an effective, scalable and affordable vaccine against Mpox is imminent to address this continental and global health crisis as quickly and equitably as possible.[10]

The mRNA vaccines has garnered significant attention due to their notable immunogenicity, expeditious preparation, substantial yield, and substantiated safety data, as evidenced by the extensive utilization of the two commercially available mRNA

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/21645515.2025.2477384.

vaccines for the treatment of SARS-CoV-2.[11–13] These attributes position mRNA vaccines as a promising vaccine modality for expeditious containment of the Mpox outbreak. Recent efforts have have focused on the development of mRNA vaccines against Mpox, utilizing lipid nanoparticles (LNP) for delivery.[14–18] However, the current mRNA-LNP vaccines have been demonstrated to be stable at <-60°C, a storage and transportation condition that increases the cost, reduces the availability and affordability and limits the use of the vaccines, particularly in developing countries.[13,19,20] The development of a better formulation for a mRNA-LNP vaccine is important to contain the Mpox epidemic in Africa as quickly as possible.

Lyophilization technology has been demonstrated to effectively preserve the biological activity of heat- and water-sensitive biological products.[21–25] The feasibility of lyophilizing mRNA-LNP has been investigated. For instance, firefly luciferase encoded by a lyophilizing mRNA-LNP has been shown to maintain mRNA expression efficiency in mice.[26] A nucleoside-modified mRNA-LNP formulated prepared as a freeze-dried cake exhibited stable physicochemical properties after 12 weeks of storage at room temperature or at least 24 weeks of storage at 4°C.[24] A continuous freeze-drying technique based on spin freezing enabled the storage of mRNA-LNP at 4°C for 12 weeks.[25] In addition to the stable physiochemical properties, a lyophilized mRNA-LNP vaccine candidate against SARS-CoV-2 induced robust immune responses in mice after storage at 25°C for 6 months.[27] The significance of cationic lipids in freeze-dried mRNA vaccines has also been documented. Yuta Suzuki et al. have developed a group of ionizable lipids and elucidated the importance of branched-tail lipid structure for strong immunogenicity and optimized lyophilization conditions through buffer optimization technique.[28] The efficacy of lyophilizing mRNA-LNP formulations comprising a reduction-sensitive ionizable lipid in the presence of 20% (w/v) sucrose has been demonstrated, through conventional batch freeze-drying or an innovative continuous spin lyophilization process.[29] However, it should be noted that the formulation and lyophilization process for the mRNA vaccines remain to be well defined and scaled up.

In a previous study, we have developed a quadrivalent mRNA-LNP vaccine candidate against Mpox in a frozen liquid formulation.[14] In this work, we developed a lyophilization process to improve the formulation of the Mpox mRNA-LNP vaccine. The optimization the formulation for lyophilizing mRNA vaccine candidate entailed a comprehensive evaluation of various formulation parameters, including the type and the concentration of cryoprotectants, the type and the concentration of the buffer system as well as mRNA concentration and reconstitute solvent (Figure 1). Additionally, the freeze-drying process parameters, such as the freezing method, temperature, cooling rate, and primary/secondary drying conditions were thoroughly investigated. The quality attributes studied for the process optimization included hydrodynamic particle size, polydispersity index (PDI), encapsulation efficiency (EE%) and mRNA molecule integrity. The long-term, accelerated- and high-temperature stability as well as the in-use stability, in-vitro biological activity and in-vivo immunogenicity of lyophilized products were examined to verify the formulation and lyophilization process. Furthermore, the lyophilization process was scaled up to a capacity of 2000 vials/batch.

Material and methods

Materials

ATP, GTP, CTP, UTP, Cap analogue, T7 RNA polymerase, RNase enzyme inhibitor, pyrophosphatase, and OligoDT beads were purchased from Vazyme. DNaseI enzyme was purchased from NEB. Cationic lipid and PEG-lipid were purchased from Xiamen Sinopeg Biotech Co., Ltd. Cholesterol was purchased from Sigma-Aldrich. DSPC was purchased from AVT (Shanghai) Pharmaceutical Tech Co., Ltd. Tris was

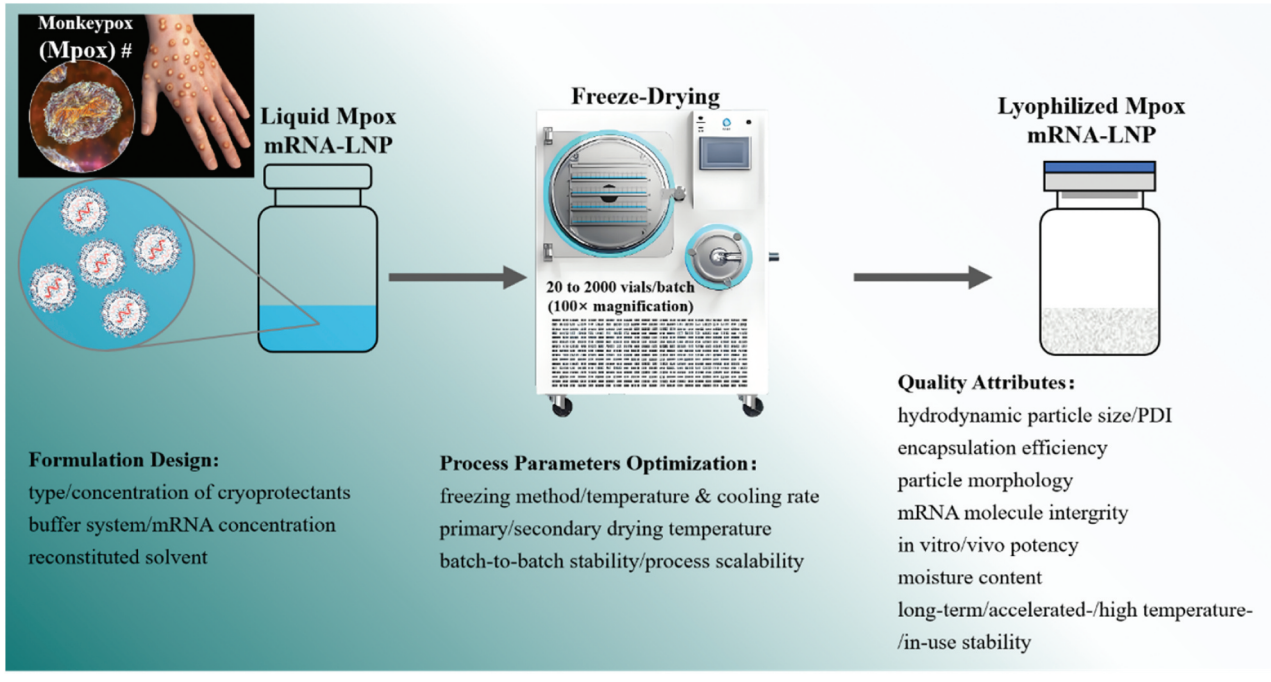

Figure 1. Scheme of the lyophilization of mpox mRNA-LNP. #: image of mpox copies from internet.

purchased from Sangon Biotech (Shanghai) Co., Ltd. Sucrose was purchased from Pfanstiehl, Inc. Trehalose dihydrate (TREHA®) was purchased from Nagase Viita Co., Ltd. Citric acid, sodium citrate, and maltose were purchased from Sinopharm Group Chemical Reagent Co., Ltd. 100 kDa ultrafiltration tube (UFC9100), and ELISPOT plate (MSIPS4W10) were purchased from Millipore. DNase/RNase-free water (DEPC treated, AM9922), RiboGreen assay kit (R11490), PBS buffer (10010031), fixable aqua dead cell stain kit (L34966A), IC fixation buffer (00-8222-49), permeabilized buffer (008333–56), CD16/CD32 monoclonal antibody (14-0161-85), goat anti-human IgG (A-11013), TMB buffer (002023) were purchased from Thermo Fisher Scientific. HEK-293T cell lines were purchased from iCell Bioscience Inc. (Shanghai, China). B5 monoclonal antibodies (RAB-2442) was purchased from Henxy Biotech. HRP-conjugated goat anti-mouse IgG (1031–05) was purchased from Southern Biotech. Terminating solution (E40500) was purchased from New Cell & Molecular Biotech. Mouse IFN-γ (HRP) ELISpot flex (3321-2 h) kit was purchased from MABTECH. Hoechst 33,342 and 20-mm confocal dish were purchased from Biosharp.

Methods

Construction of recombinant plasmid

The Mpox mRNA was synthesized and purified based on the published literature.[14] The mRNA of Mpox vaccine candidate was designed by inserting the vaccinia virus (VACV) A27, L1, A33, and B5 genes into the coding region of the plasmid, pUCYH, in tandem with a modified P2A linker between each gene. A T7 promoter sequence and a 5' UTR sequence including Kozak sequence were added to the upstream of the genes and a stop codon TGATAA, 3' UTR, PolyA, and BspQ I cleavage sites were added to the downstream. The target genes were next cloned into pUCYH plasmid to create plasmid Mpox-pUCYH, which was confirmed by Sanger sequencing. The sequences of the recombinant plasmid and mRNA (3100 nt) have been disclosed in a patent (CN116286913B).

Preparation of Mpox mRNA

The recombinant plasmids were extracted and linearized by restriction endonuclease BspQ I cleavage followed by plasmid recovery and purification. In vitro transcription reaction (IVT in 50 μL) was performed at 37°C for 3 h after vortexing of the mixture containing linearized plasmid template, ATP (100 mm), GTP (100 mm), CTP (100 mm), UTP (100 mm), Cap analog (100 mm), T7 RNA polymerase (200 U/μL), 10× T7 Reaction Solution, RNase enzyme inhibitor (40 U/μL), pyrophosphatase (0.1 U/μL), and RNase-free water. After the IVT, 170 μL of RNase-free water, 5 μL of DNaseI enzyme, and 25 μL of 10× DNaseI enzyme reaction solution were added to the mRNA and incubated at 37°C for 20 min. Purified mRNA was obtained using OligoDT beads. The mRNA integrity and purity were analyzed by agarose gel (Bio-Rad) and HPLC-SEC (Thermo Fisher Scientific).

Preparation of Mpox mRNA-LNP

The purified Mpox mRNA was diluted to 137 μg/mL with 50 mm citric acid buffer (pH 4.0), to obtain an aqueous solution. The organic phase solution was prepared by dissolving ionizable lipid, DSPC, cholesterol, and PEG2000-lipid in ethanol (Table S1). The aqueous and organic phase solutions were siphoned into a home-made two-inlet confined impinging jet microfluidic device with a staggered herringbone microfluidic mixer at a flow ratio of 3:1 with a total flow rate of 60 mL/min for mRNA encapsulation. The mRNA-LNP complex was next rapidly diluted in 10 mm Tris (pH 7.4) at room temperature and centrifuged at 1690×g at 4°C for 10 min in an ultrafiltration tube. The mRNA-LNP complex was concentrated to approximately 0.8 mg/mL and then filtered through a 0.22 μm filter to sterilize the mRNA-LNP vaccine. Finally, the appropriate concentration of cryoprotectants and Tris buffer were added to the mRNA-LNP solution to adjust the mRNA concentration to 0.1 mg/mL or 0.5 mg/mL. A similar procedure was conducted when using different concentrations of Tris and PBS.

The lyophilization of mRNA-LNP

The mRNA-LNP was dispensed into appropriate cryoprotectant in 16 mm vials at a dose of 0.5 mL per vial. The lyophilizer (LYO-0.5, Tofflon) was pre-cooled to −20°C at the freezing stage before the vials were placed on the lyophilizer shelf for the appropriate time, followed by cooling to the appropriate temperature at the appropriate slow cooling rate and held at that temperature for the appropriate time. During the primary drying step, the temperature of the lyophilizer shelf was increased to −30°C, the pressure was reduced to 3 Pa and lyophilization was maintained for 30 h. In the secondary drying step, the temperature of the shelf of the lyophilizer was increased to 25°C, with the pressure maintained at 3 Pa for 6 h. In the liquid nitrogen freezing, the lyophilizer was pre-cooled to the set freezing temperature followed by rapid immersion of the vials into liquid nitrogen to be frozen. The frozen vials were then placed on the shelves of the lyophilizer for 4 h. In ControLyo pre-freezing, the vials were placed on the lyophilizer shelf (at room temperature) followed by cooling the lyophilizer to −8°C and reducing the pressure in the lyophilizer to 30 Pa. The pressure was changed to atmospheric pressure after the solution in the vials was frozen followed by reducing the temperature to −50°C at a cooling rate of 0.45°C/min or 1°C/min and maintaining at −50°C for 4 h.

Analysis of particle size

Hydrodynamic particle sizes and PDI of the mRNA-LNP vaccines were recorded using a Malvern particle size analyzer (Zetasizer Pro). A 20 μL of samples was diluted to 1 mL with the appropriate buffer solution and analyzed at 25°C. Each measurement was repeated three times.

Determination of mRNA encapsulation efficiency (EE%)

The concentration of unencapsulated mRNA (C_{free}) and the total mRNA concentration (C_{total}) in mRNA-LNP formulations were determined according to the product instructions of RiboGreen nucleic acid assay kit. To determine the C_{total}, the mRNA-LNP preparations were demulsified with 1% Triton X-100 for 10 min at 40°C. The EE% was calculated using the following equation:

$$EE\% = \frac{C_{total} - C_{free}}{C_{total}} \times 100$$

Determination of mRNA integrity

The mRNA integrity was measured using the Fragment Analyzer 5200 (Agilent). After being demulsified with 1% Triton X-100 for 10 min, the mRNA-LNP was incubated at 70°C for 2 min and treated with an ice bath to denature the mRNA molecule. RNA marker (15 nt) was used as an internal standard, with the RNA marker mixed with the sample at a ratio of 11:1. RNA ladder (200, 500, 1000, 1500, 2000, 3000, 4000, 6000 nt) was used as an external standard. RNA isolation gel containing Intercalating dye was used as isolation medium. The injection conditions were as follows: the injection voltage was set at −5 kV, and the injection time was 4 s. The separation conditions were as follows: the separation voltage was set at −8 kV, and the separation time was 40 min. The quantification of mRNA length was performed using ProSize Data Analysis Software (Agilent). The percentage of mRNA integrity was quantified by smear analysis of ProSize Data Analysis Software: the area under the curve (AUC) in the interval defined by the mRNA length (3100 ± 20%, 2480–3720 nt) was determined and expressed as a percentage of AUC the total mRNA.

Analysis of moisture content

A 0.05 g of lyophilized powder was taken for the measurement of moisture content using Coulometric KF Titrator (C30S, METTLER TOLEDO).

Cryo-transmission electron microscopy

The freshly prepared liquid mRNA-LNP was lyophilized and 4 μL of each reconstituted sample was applied to a grid (R2/1 Cu, 300 mesh, Quantifoil, Germany) followed by dabbing the grid in liquid ethane at 4°C with 100% humidity for 3 s using a Vitrobot Mark IV System (Thermo Fisher Scientific). Images were captured using a Talos F200C G2 transmission electron microscope (Thermo Fisher Scientific) operated at 200 kV. The magnification was set at 36,000 with a pixel size of 5.75 Å to visualize nanoparticle dimensions.

Detection of in-vitro activity of mRNA-LNP using flow cytometry

Antigen expression by Mpox mRNA-LNP vaccine candidate[14] was analyzed using flow cytometry with detection of one of the four encoded antigens, B5 as an example. Briefly, 1×10^6 of HEK-293T cells were transfected with 5 μg of freshly prepared Mpox mRNA-LNP (Mpox-C5, 0.1 mg/mL), or lyophilized Mpox-C5 (Lyo-Mpox-C5 (6 M), 0.1 mg/mL) that was freeze-dried and stored at 4°C for 6 months. The cells were treated with an equal volume of empty LNP as a negative control. After culturing of the cells at 37°C for 24 h, cells were detached from the surface of six-well plate with PBS containing 3% FBS, followed by staining with aqua fluorescent reactive dye (1:1000) to differentiate live and dead cells. Cells were fixed with IC fixation buffer, permeabilized buffer, and blocked with CD16/CD32 monoclonal antibody (1:1000). Finally, the cells were stained with monoclonal antibody that specifically recognizes B5 (1:200), followed by adding anti-human AF488 (1:500) as secondary antibody. Cells were acquired on a FACS Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) and data were analyzed using FlowJo 10.8.1.

Immunofluorescence imaging analysis

Briefly, 1×10^6 of HEK-293T cells were seeded on a 20-mm confocal dish and cultured for 24 h. Then, the cells were transfected with 2.5 μg of freshly prepared Mpox mRNA-LNP (Mpox-C5, 0.1 mg/mL), or lyophilized Mpox-C5 (Lyo-Mpox-C5, 0.1 mg/mL). The cells were treated with an equal volume of empty LNP as a negative control. After culturing of the cells for 24 h, the cells were fixed with IC fixation buffer, permeabilized buffer, and blocked with CD16/CD32 monoclonal antibody (1:1000). The cells were then stained with monoclonal antibody that specifically recognizes B5 (1:200), followed by adding anti-human AF488 (1:500) as secondary antibody. Finally, the nucleus was labeled in blue with Hoechst 33,342. Confocal microscopy analysis of those cells was carried out with an inverted laser-scanning confocal microscope (Leica STELLARIS).

Immunization of mice

All experiments were performed strictly in accordance with the Guidelines of Care and Use of Laboratory Animals by the Ministry of Science and Technology of the People's Republic of China. Animal protocols were approved by the Animal Care and Use Committee of PLL (IACUC number PL23-0696-2; Approval Date: 15 January 2023).

Female BALB/c mice aged 6–8 weeks were grown under specific pathogen-free conditions at PharmaLegacy Laboratories (PLL, Shanghai, China) Co., Ltd. To evaluate the immunogenicity of the Mpox mRNA-LNP encoding four vaccinia antigens (A27, A33, L1 and B5), animals were randomly divided into three groups (5 mice in each group): (1) saline group, (2) freshly prepared Mpox-C5 group, (3) Lyo-Mpox-C5 (6 M) group. Animals were immunized twice by intramuscular injection at an interval of 4 weeks. The vaccination volume is 50 μL per mouse and the Mpox concentration is 0.1 mg/mL, i.e. 5 μg of Mpox molecules was injected into mouse per vaccination. Sera were collected on day 21 and day 42 post-primary immunization for detection of antigen-specific antibodies. On day 56 post-primary immunization, spleens were harvested for evaluation of cellular immunity.

Detection of antigen-specific binding antibody. ELISA was used to measure the titers of antigen-specific IgG antibodies in mouse sera. Each antigen (A27, A33, L1, and B5) was diluted to 1 μg/mL with coating buffer followed by coating the 96-well plates with each antigen overnight at 4°C, respectively. The serum was serially diluted 3-fold and added to the plate coated with 5% milk at 37°C for 1 h. After washing with PBST, the plate was incubated with HRP-conjugated goat anti-mouse IgG (1:400) at 37°C for 1 h and color reaction was developed by adding 100 μL TMB for 5 min followed by adding 50 μL of terminating solution to stop the reaction. The absorbance was measured at 450/620 nm using an ELISA plate reader (Thermo Fisher Scientific). The mean OD (optical density) of serum from naive mice was multiplied by 2.1 to define the positive cutoff point. The lower limit of detection for antibody titers was 100.

Detection of neutralizing antibody. Briefly, mouse serum was inactivated at 56°C for 30 min, diluted in a 3-fold gradient with

DMEM medium with 20% baby rabbit complement, then mixed with VACV virus and incubated at 37°C for 2 h. The mixture was added to the VERO E6 cells and incubated for 2 h before replacing media with DMEM containing 2% FBS (v/v) and 0.3% (w/v) methylcellulose. After incubation for 28 h, the cells were fixed with 4% PFA, stained with crystal violet solution and counted the plaque. Finally, the NT_{50} was calculated following Reed and Muench method. The lower limit of detection for neutralizing antibody titer was 50.

Detection of cellular immune responses using ELISPOT. The ELISPOT plate was filled with 35% ethanol at 50 µL/well for 1 min. The liquid was discarded and the plate was washed with sterile deionized water at 200 µL/well 5 times. IFN-γ capturing antibody was diluted to 15 µg/mL with PBS and added to the above plates at 100 µL/well, and the plates were incubated overnight at 4°C. On the next day, the plates were washed with PBS and RPMI 1640 medium (containing 10% FBS) was added followed by incubation at room temperature for 30 min. Single-cell suspension was obtained from a mouse spleen and cell numbers were counted after lysis of erythrocyte. The culture medium in the plate was discarded and appropriate numbers of cells were added. Next, peptides (Peptides pool of A27, L1–1, L1–2, A33, B5–1, and B5–2) were added at a final concentration of 2.5 µg/mL each. The plates were incubated at 37°C for 36 h followed by discarding the cells and washing with PBS. The diluted detection antibody (1:1000) was added and the plate was incubated at room temperature for 2 h. The diluted streptavidin-HRP (1:1000) was added and the plate was incubated at room temperature for 1 h. Finally, TMB solution was added followed by washing with deionized water when spots appeared. After the plates were dried at room temperature, photographs were taken and the spots in the wells were counted using an enzyme-linked immune-spot analyzer (Cellular Technology Ltd., Shaker Heights, USA, S6 Universal).

Statistical analysis

For the data of cell activity and animal immunogenicity, statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, SanDiego, CA, USA). Differences between the two groups were analyzed with unpaired t-tests. Significance level was marked in figures and annotated in the figure legends.

Results and discussion

Evaluation of process parameters and optimization of a lyophilization process for a mpox mRNA-LNP vaccine candidate

Effect of cryoprotectants on the lyophilization of mpox mRNA-LNP

A study was conducted in which three types of cryoprotectants (sucrose, trehalose and maltose) were evaluated individually or in combination for their effects on lyophilization. The Mpox mRNA was loaded by LNP (0.1 mg mRNA/mL LNP) using microfluidics, and the resulting hydrodynamic particle size, PDI and EE% were 73.4 nm, 0.07 and 97.18%, respectively. An appropriate cryoprotectant was added to the mRNA-LNP at 10% (w/v), after which the mixture was frozen at −80°C. Following a freezing period of 12 h, the sample was thawed, and the particle size, PDI, and EE% were measured. As shown in Table S2, the particle size of Mpox mRNA-LNP increased by approximately 20 nm and the EE% decreased by approximately 2% after freezing when trehalose or maltose was used alone as a cryoprotectant. In contrast, there were minimal changes of particle size and EE% before and after freezing when sucrose was used as a cryoprotectant. This may be due to the fact that sucrose is capable of effectively replacing water molecules during lyophilization, forming hydrogen bonds with LNP lipids and thereby protecting the lipid membrane structure.[30] Meanwhile, the sucrose can undergo freezing and concentration to form a highly viscous glass matrix that prevents osmotic changes in lipid membranes.[31,32] Based on these observations, subsequent studies on the impact of cryoprotectants have primarily focused on sucrose and sucrose-containing cryoprotectants.

A total of ten formulations were designed, each containing various amounts of sucrose in combinations with different amounts of trehalose or maltose, as illustrated in Table 1. The glass transition temperature (Tg') and collapse temperature (Tc) of each formulation were initially analyzed, resulting in Tg' ranging from −31.8°C to −34.1°C and Tc ranging from −27.0°C to −32.0°C. The preliminary trial lyophilization conditions (20 vials/batch) for the selection of cryoprotectant was determined based on the principle that the freezing temperature should be less than Tg' and the primary drying temperature should be less than Tc.[33,34] The freezing temperature was set at −60°C (< Tg') and the primary drying temperature was set at −40°C (< Tc) with the freeze-drying process was conducted as follows: Samples enter the plate at 20°C and undergo a freezing process to −60°C at a cooling rate of 1°C/min and maintained at −60°C for 4 h, followed by primary drying at −40°C for 40 h (pressure at 3 Pa) and secondary drying at 25°C for 6 h (pressure at 3 Pa). A comparison of the physical characteristics revealed that the mRNA-LNP lacking cryoprotectants (C0) exhibited a modest quantity of white powder at the base of the bottle following freeze-drying, and the solution appeared turbid with numerous insoluble visible particles after reconstitution with water (Figure S1a). The particle size increased to over 300 nm and the EE% decreased to about 20% (Table 1), indicating that the original Mpox mRNA-LNP structure was destroyed in the absence of cryoprotectants. In contrast, all cryoprotectant-containing groups exhibited characteristic white solid cakes following freeze-drying, which rapidly redissolved upon water addition, yielding the translucent and pale blue suspension (Figure S1b). Among the ten formulations, the particle size, PDI and EE% of samples in groups C1, C4 and C5 exhibited minimal variation, i.e., the particle size increased from 77 nm to approximately 103–107 nm, PDI increased from 0.07 to approximately 0.1–0.13 and the EE% decreased from 96% to approximately 83% (Table 1 and Figure S1c-e), indicating that single cryoprotectant of 10% or 15% sucrose, or 10% sucrose in combination with 5% trehalose better maintained the physical characteristics of mRNA-LNP than other cryoprotectant groups. Consequently, subsequent studies were conducted using the three cryo-formulations defined in C1, C4 and C5, respectively.

Table 1. Comparison of the particle size, PDI and EE% of mpox mRNA-lnp formulated with different formulations before and after lyophilization.

	Cryoprotectants (%, w/v)				Tg′	Tc	Before lyophilization			After lyophilization		
Group	Total	Sucrose	Trehalose	Maltose	(°C)	(°C)	Size (nm)	PDI	EE (%)	Size (nm)	PDI	EE (%)
C0	0	0	0	0	N/A	N/A	76.31	0.08	95.60	323.45	0.36	20.53
C1	10	10	0	0	−34.1	−29.5	76.68	0.07	96.06	106.95	0.12	83.33
C2	10	5	5	0	−32.5	−28.3	76.83	0.06	95.72	103.77	0.14	83.53
C3	10	5	0	5	−32.5	−28.9	76.94	0.05	96.39	104.60	0.20	81.46
C4	15	15	0	0	−33.4	−29.8	76.97	0.08	96.44	104.15	0.10	82.36
C5	15	10	5	0	−31.8	−27.0	77.10	0.07	95.74	103.47	0.13	83.68
C6	15	10	0	5	−32.8	−28.9	76.51	0.06	95.74	111.93	0.12	77.23
C7	20	20	0	0	−33.0	−31.7	77.28	0.08	96.34	111.58	0.11	69.13
C8	20	15	5	0	−32.7	−29.7	77.27	0.05	96.10	100.23	0.11	79.04
C9	20	15	0	5	−32.9	−31.3	77.52	0.06	96.54	119.82	0.12	59.13
C10	20	10	10	0	−32.1	−32.0	77.73	0.06	96.21	103.95	0.12	76.29

Impact of the buffer system and the mRNA concentration on lyophilization of Mpox-LNP

Two buffer systems (PBS and Tris) have been utilized in the manufacturing of the mRNA vaccine for the treatment of SARS-CoV-2.[13] In this study, the investigation focused on the potential of PBS and Tris with a pH of 7.4 for the lyophilization process of Mpox mRNA-LNP. When PBS was used as the lyophilization buffer, compared with the freshly prepared liquid mRNA-LNP (with particle size of 79 nm, PDI of 0.11 and EE% of 94%), the particle size, PDI and EE% were 125 nm, 0.16 and 71%, respectively, of C4 group, 136 nm, 0.28 and 68%, respectively, of C1 group, and 133 nm, 0.27 and 66%, respectively, of C5 group after lyophilization (Figure 2a–c), demonstrated that group C4 exhibited the least variation in mRNA-LNP characterization. When 10 mm Tris was used for lyophilization, the particle size, PDI and EE% in all groups (C1, C4 and C5) were all found to be less than 105 nm, less than 0.15, and higher than 80%, respectively, indicating superior physicochemical properties in comparison to the PBS group. The suboptimal performance of PBS system in lyophilization was likely attributable to the relatively high salt concentration and pH shift in PBS, which compromised the stability of LNP during lyophilization.[25,35,36] Therefore, the Tris system was selected for the subsequent investigation.

The present study investigated the effects of Tris concentration (10, 20, 30, 50 and 100 mm) on the physicochemical properties of Mpox mRNA-LNP before and after lyophilization, utilizing the C5 formulation. It was found that the particle size and EE% of freshly prepared liquid Mpox-LNP had little changes with the increase of Tris concentration from 10 mm to 100 mm, meanwhile PDI was below 0.1 for all formulations (Figure 2d–f). The particle size of lyophilized Mpox-LNP exhibited a slight decrease with increasing Tris concentration, with a 5 nm decrease observed at the highest concentration (100 mm Tris) (Figure 2d). Concurrently, the PDI exhibited an increase, surpassing 0.1, though the impact of Tris concentration on the PDI remained ambiguous (Figure 2e). Furthermore, an increase in Tris concentration from 20 to 50 mm resulted in a decline in EE% of approximately 2–3% (Figure 2f) compared to the 10 mm Tris condition. A notable decrease to 65% of EE% was observed when 100 mm Tris was employed (Figure 2f). Given the negligible change in the particle size of the LNP and the observed decrease in EE% with increasing Tris concentration, a lower concentration of 10 mm Tris was selected for subsequent research.

Mpox mRNA-LNP with two different concentrations (0.1 mg/mL and 0.5 mg/mL) were prepared using C1, C4, and C5 as cryoprotectants in a 10 mm Tris buffer system. After freeze-drying, the white cakes obtained at both high and low concentrations of Mpox mRNA-LNP exhibited rapid dissolution upon the addition of water. The reconstituted suspension appeared translucent and pale blue for the low concentration group (0.1 mg/mL), while those of the high concentration group (0.5 mg/mL) appeared milky (Figure S2). It was observed that the appearance of the reconstituted suspension with different cryoprotectants remained indistinguishable at equivalent concentrations. As shown in Figure 2g, following reconstitution of lyophilized samples, the particle sizes of the low mRNA-LNP concentration group in the three cryoprotectants (95–97 nm) were smaller than those of the high concentration group (108–112 nm). For the groups of C1 and C4, there was little change on the PDI when mRNA concentration was increased from 0.1 mg/mL to 0.5 mg/mL, but the PDI increased slightly from 0.08 to about 0.13 for the group of C5 (Figure 2h). The EE% remained virtually unchanged in the high-concentration mRNA-LNP groups after freeze-drying (approximately 95%), yet a decline in EE% was observed in the low-concentration mRNA-LNP groups (approximately 86%) (Figure 2i). Therefore, an increase in mRNA concentration might result in an enhancement of particle size of lyophilized products but no effect on EE%. The increased susceptibility of the higher concentration of LNP particles to fusion when subjected to stress during the lyophilization process may be attributed to the larger particle size.

The preceding results indicate that the buffer system/concentration and the formulation concentration exert an influence on the characteristics of the lyophilized product. Therefore, it is essential to comprehensively examine the impact of Tris and mRNA concentration on the characteristics of lyophilized products, taking into account their impact on immunogenicity, as well as on the osmotic pressure, administration dose/volume and filling volume.[37]

Impact of freeze-drying process parameters on the lyophilization of Mpox mRNA-LNP

The freeze-drying process is comprised of three distinct stages: freezing, primary drying and secondary drying stages. The present study evaluated three freezing methods (liquid nitrogen freezing (Liquid N_2), controlled nucleation freezing (ControLyo), and conventional classical plate freezing

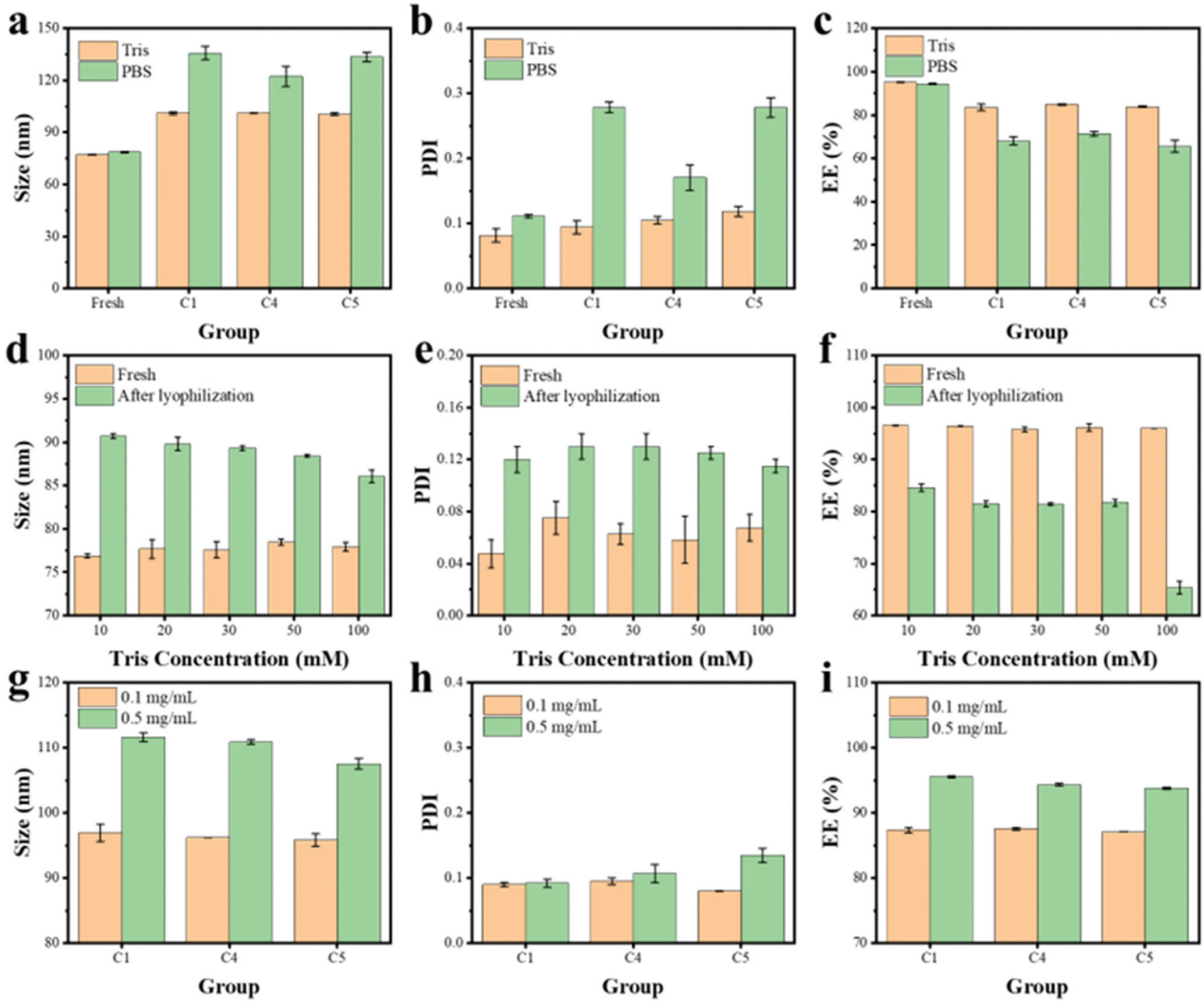

Figure 2. Formulation study of lyophilized mpox mRNA-lnp. (a) Size, (b) PDI, and (c) EE% of mpox-lnp with C1, C4, C5 cryoprotectant before and after lyophilization in different buffer systems (PBS and 10 mm tris); (d) size, (e) PDI, and (f) EE% of mpox-lnp with C5 cytoprotectant before and after lyophilization in different concentration of tris buffer (10 mm, 20 mm, 30 mm, 50 mm, and 100 mm); (g) size, (h) PDI, and (i) EE% of lyophilized mpox-lnp (C1, C4, and C5, 10 mm tris) at different mpox mRNA concentrations (0.1 mg/mL and 0.5 mg/mL).

(Classic-Fast and Classic-Slow)) for their impact on the freeze-drying of mRNA-LNP. As demonstrated in Figure 3a, the freeze-dried mRNA-LNP (in 10 mm Tris, C5, at 0.1 mg mRNA/mL) cake showed suboptimal physical properties, fragmenting into small pieces when subjected to freezing using Liquid N_2 or ControLyo methods, respectively, while the cake remained intact using Classical-Fast or Classic-Slow (Figure 3a). Furthermore, the Liquid N_2 group exhibited a greatest variation in particle size and EE% compared to the other groups. While the increase in particle size was less pronounced in the ControLyo group, it exhibited a lower EE % compared to Classic-Fast and Classic-Slow groups (Figure 3b,c).

Subsequently, the impacts of cooling rates at the freezing stage were compared on the product's performance in relation to conventional plate freezing, i.e., comparing freezing at temperature drop at 1°C/min vs. 0.45°C/min to −60°C. A gradual decrease in the cooling rate at 0.45°C/min likely resulted in a smaller particle size (Classical Fast-103 nm *vs*. Classical Slow-100 nm) and higher EE% (Classical Fast-84% *vs*. Classical Slow-86%) (Figure 3b,c), and therefore the cooling rate of 0.45°C/min was selected for further studies.

During lyophilization process, many unstable stresses arise, particularly during the freezing step. Compared with conventional plate freezing, although liquid nitrogen freezes the water molecules to form fine ice crystals quickly (−196°C) to significantly shorten the freezing time,[38] and controlled nucleation technology also reduces the freezing time,[39] these two above freezing techniques does not perform well in lyophilization of mRNA-LNP in terms of product appearance, particle size, PDI and EE%, which limited their application in freeze-drying. Conversely, conventional plate freezing has been shown to decrease the degree of supercooling of the solution, the osmotic pressure difference, and mechanical forces between the LNP lipid membrane, which minimizes the formation of ice crystals in the cavity and prevents mRNA molecules leakage,[40,41] resulting in a uniform freeze-dried cake.

In the next study, the influence of temperature control during freezing process was investigated, i.e., pre-cooling at −20°C before gradually decreasing the temperature to −60°C

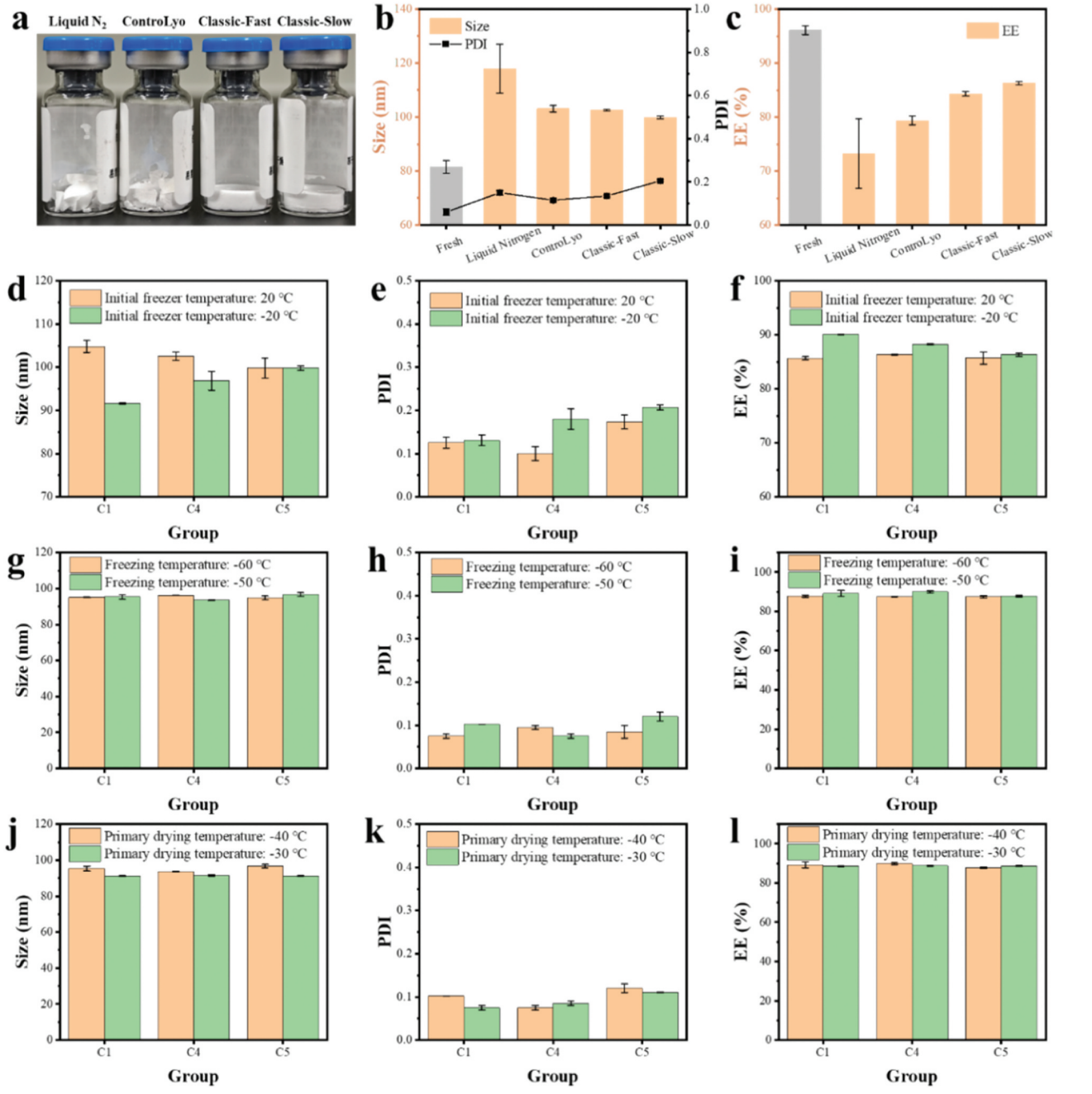

Figure 3. Lyophilization process study of lyophilized mpox mRNA-lnp. (a) Appearance, (b) size/pdi and (c) EE% of lyophilized mpox-lnp (C5, 10 mm tris, 0.1 mg/mL) using different freezing methods (liquid nitrogen, ControLyo, classic-fast, and classic-slow); (d) size, (e) PDI, and (f) EE% of lyophilized mpox-lnp (C1, C4, C5, 10 mm tris, 0.1 mg/mL, freezing temperature – 60°C, cooling rate 0.45 °C/min) prepared at different initial freezer temperatures (20°C, and – 20 °C); (g) size, (h) PDI, and (i) EE% of lyophilized mpox-lnp (C1, C4, C5, 10 mm tris, 0.1 mg/mL, plate temperature – 20°C, cooling rate 0.45 °C/min) at different freezing temperatures (–60°C, and – 50 °C); (j) size, (k) PDI, and (l) EE% of lyophilized mpox-lnp (C1, C4, C5, 10 mm tris, 0.1 mg/mL, freezer temperature – 20°C, freezing temperature – 50°C, cooling rate 0.45 °C/min) at primary drying temperatures (–40°C, –30 °C).

vs. decreasing the freezer temperature from room temperature to −60°C. As illustrated in Figure 3d–f, when the freezer was pre-cooled to −20°C before the samples were placed in and maintained at −20°C for 20 min followed by gradually reducing the temperature from −20°C to −60°C at a cooling rate of 0.45°C/min and kept at −60°C for 4 h, the particle size of mRNA-LNP in C1, C4 or C5 formulations maintained, but the EE% was higher compared to samples frozen from room temperature to −60°C without a pre-cooling at −20°C, indicating that pre-cooling at a lower temperature may help to maintain better key physical and chemical properties of mRNA-LNP. Furthermore, in order to ensure compatibility with the cooling capacity of an industrial freeze-dryer, the freezing temperature was increased from −60°C to −50°C. Lyophilized LNP was subjected to both freezing temperatures and a comparison of the data revealed minimal change in particle size, PDI, and EE% at a freezing temperature of −50°C (Figure 3g–i). Therefore, the proposed range of freezing temperatures can be set between −60°C and −50°C.

A more thorough investigation was conducted into the drying temperatures, encompassing both primary and secondary drying processes. The investigation revealed that the particle

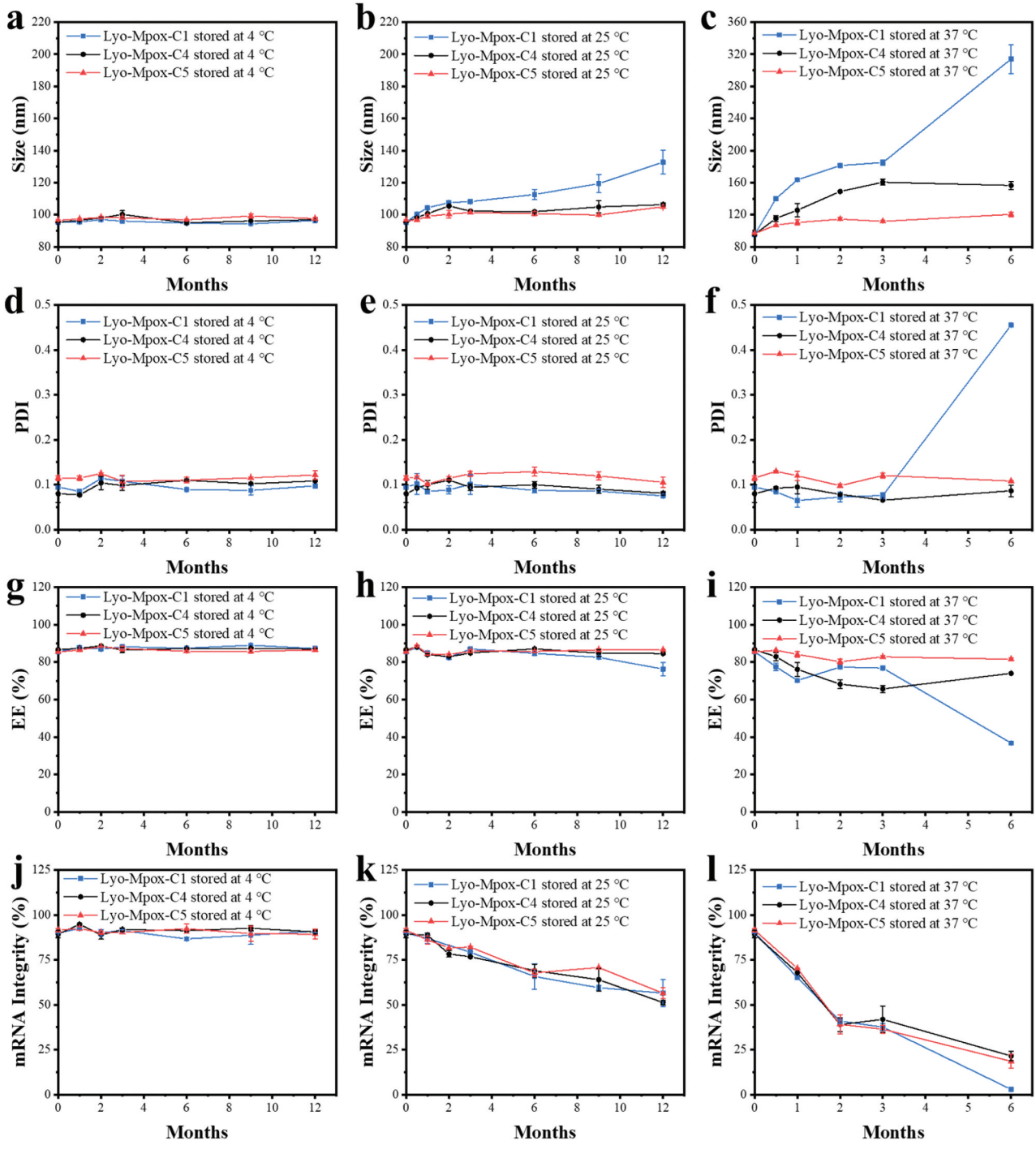

Figure 4. Stability studies of lyophilized mpox mRNA-lnp. Size (a-c), PDI (d-f), EE% (g-i) and mRNA integrity (j-k) of long-term, accelerated, and high-temperature stability of the lyophilized mpox-lnp (C1, C4, C5, 10 mm tris, 0.1 mg/mL) after 12 months of storage at 4°C (a, d, g, j) and 25°C (b, e, h, k), and after 6 months of storage at 37°C (c, f, i, l), respectively.

size, PDI and EE% of lyophilized Mpox-LNPs remained constant when the primary drying temperature was adjusted from −40 to −30°C (Figure 3j–l). In this instance, the lyophilized Mpox-LNP retained its mRNA integrity after secondary drying at 25°C, exhibiting a moisture residue as low as 0.6% (data not shown). The primary drying stage is known to remove the majority of the frozen crystal water in the product, while the secondary drying stage is responsible for the removal of the residual-free water. A low drying temperature may lead to an extended drying time or incomplete water removal from the product, which can potentially compromise the storage stability of the product. Conversely, high temperatures can lead to the collapse of the freeze-dried sample or compromise the integrity of the mRNA.[34] In light of the critical factors such as Tc and the operational duration of the freeze-drying process, this study has opted for a primary drying temperature of −30°C, which is approximately 0–3°C lower than Tc. This approach has been found to markedly reduce the primary drying time (30 h *vs.* 40 h) while maintaining the quality of the product. From the standpoint of thermal stability and moisture residue of the

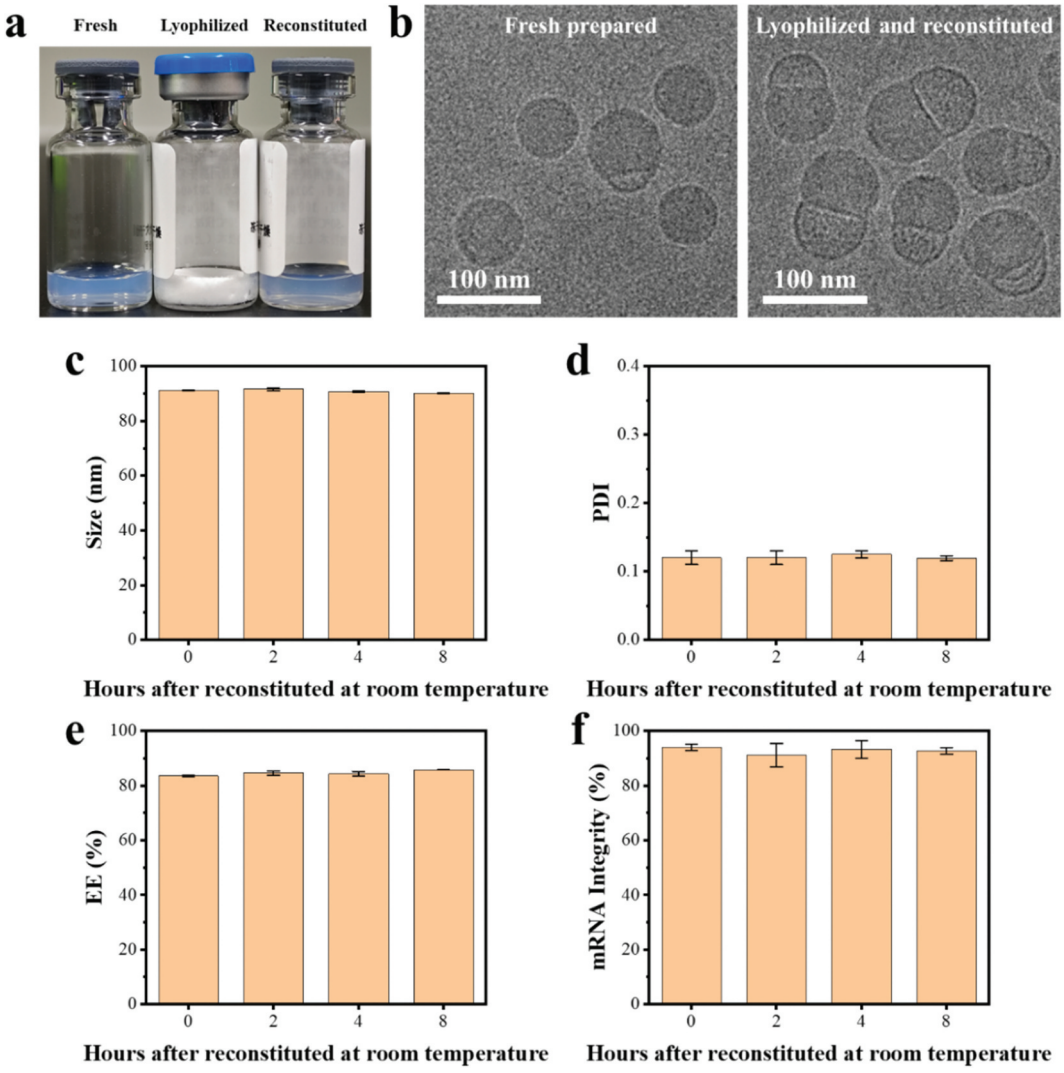

Figure 5. In-use stability study of lyophilized mpox mRNA-lnp. (a) Appearance and (b) cryo-tem images of mpox-lnp (C5, 10 mm tris, 0.1 mg/mL) before and after lyophilization. (c) Size, (d) PDI, (e) EE%, and (f) mRNA integrity of lyophilized mpox-lnp (C5, 10 mm tris, 0.1 mg/mL) after reconstitution with water and standing for 8 h at room temperature.

lyophilized preparation, 25°C was selected as the secondary drying temperature, which can expeditiously remove the residual moisture (less than 1%) within 6 h.

Stability study of lyophilized Mpox mRNA-LNP

Long-term and accelerated stability and stability under high-temperature. In order to evaluate the prolonged, accelerated stability, and stability under high-temperature conditions, a series of lyophilized Mpox mRNA-LNP formulations comprising various cryoprotective agents were prepared (C1, C4, and C5), and subsequently maintained at temperatures of 4°C, 25°C (60% relative humidity (RH)), and 37°C (75% RH), correspondingly.

Following a 12-month storage period at 4°C, all samples formulated using C1, C4 or C5 formulations showed minimal alterations in the appearance, particle size, PDI, EE% and mRNA integrity, indicating an excellent stability (Figure 4a,d, g,j). These findings suggested that the Mpox mRNA-LNPs maintain stability at 4°C for a minimum of 12 months. Under the accelerated condition (25°C, 60% RH), there was no noticeable change in the PDI and EE% but the particle size increased to approximately 106 nm and 105 nm from 97 nm for C4 and C5 groups, respectively, after a 12-month storage period. The particle size and EE% of group C1 changed to approximately 133 nm and 76%, respectively. The molecular integrity of mRNA gradually decreased to approximately 56%, 51% and 56% respective, for C1, C4 and C5 group after a 12-month storage period (Figure 4b,e,h,k). Following a 6-month storage period at 37°C (75% RH), significant alterations in particle size and EE% were observed among the C1 and C4 groups. Specifically, the particle size in the C1, C4 and C5 groups increased to >300 nm, 160 nm and 120 nm, respectively, from 97 nm. Concurrently, the EE% of mRNA-LNP in the C1, C4, and C5 groups exhibited a decline, reaching 36%, 70% and 81%, respectively. Notably, after a month, the mRNA integrity of samples in all groups experienced significant deterioration, with a reduction of approximately 20% (Figure 4c,f,i, l). Additionally, the lyophilized samples of C1 and C4 groups exhibited signs of shrinkage after 1 month, and after 6 months, the cakes in all the C1 group showed severe atrophy. In

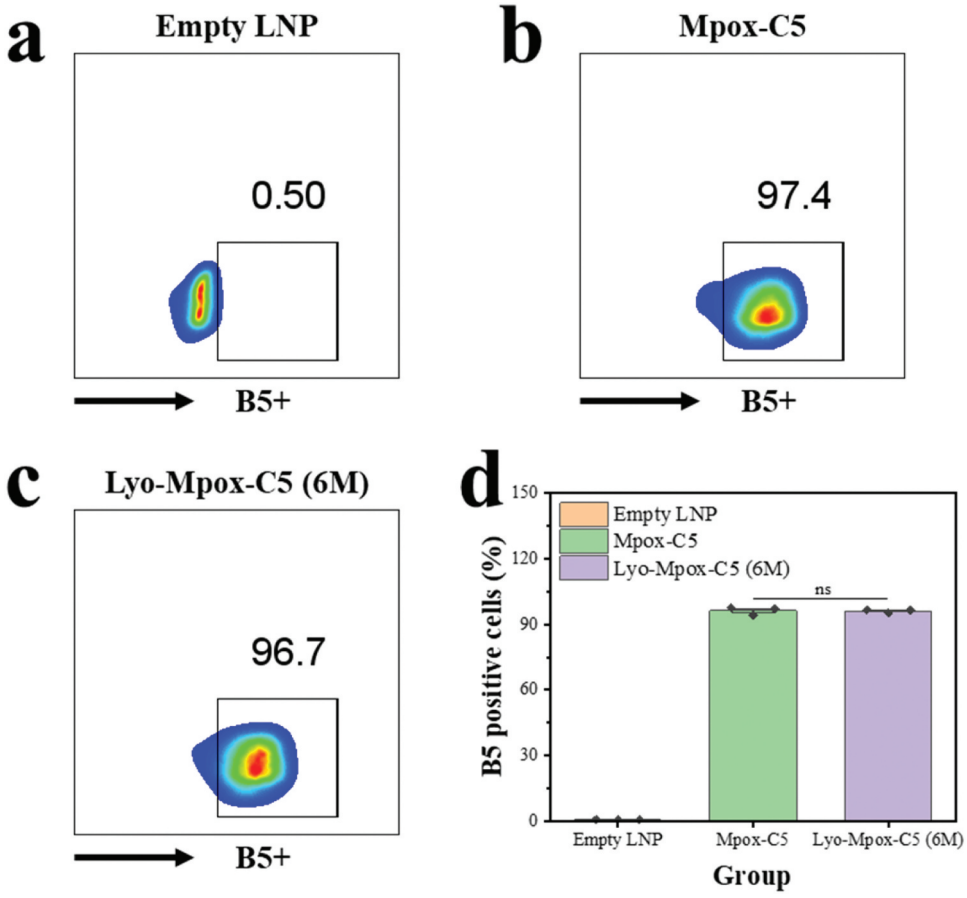

Figure 6. In vitro transfection efficiency study of lyophilized mpox mRNA-lnp. Flow cytogram of B5 protein after transfection of HEK-293T cells with samples of (a) empty LNP, (b) mpox-C5, and (c) lyo-mpox-C5 (6 M) vaccine. (d) Summary of B5 protein positive cells percentage of empty LNP, mpox-C5 and lyo-mpox-C5 (6 M) vaccine.

contrast, the cake in C5 group showed no discernible alterations in appearance after 6 months of storage (Figure S3).

While the formulation of C1, C4, and C5 has been demonstrated to safeguard mRNA-LNP against the adverse effects of freeze-drying, these three cryoprotectants have been observed to exert varying effects on the storage stability of the lyophilized product. Under storage conditions at 4°C, the lyophilized products containing one of these three protectants showed excellent long-term stability. However, with increasing storage temperature and humidity, i.e., 25°C (60% RH) or 37°C (75% RH), samples in the C5 group demonstrated superior performance compared to those in the C1 and C4 groups. The higher Tg of trehalose (121°C) contained in the C5 group (Tg of sucrose: 77°C) might contribute to the enhanced storage stability.[42,43] Under the conditions of high temperature and humidity, trehalose can undergo hydration by combining with water, thereby protecting the freeze-dried products from the deleterious effects of water.[35] Furthermore, the incorporation of trehalose has been proved to effectively retard the crystallization of sucrose in freeze-dried cakes, a process that can compromise the efficacy of cryoprotectants.[44,45] This observation suggested that a modest amount of trehalose might be essential to enhance the high-temperature stability of these systems. As a result, based on the stability data from long-term and accelerated stability studies, the formulation of 10% sucrose with 5% trehalose in 10 mm Tris was identified as the optimal choice for lyophilization of Mpox mRNA-LNP.

Resolubilization and in-use stability of lyophilized Mpox mRNA-LNP. A comparative analysis was conducted between water and a Tris buffer (10 mm) as solvents for the reconstitution of the lyophilized Mpox mRNA-LNP. The impact of the two solvents on the particle size and EE% of the lyophilized Mpox mRNA-LNP was observed to be more significant at a mRNA concentration of 0.5 mg/mL compared to 0.1 mg/mL (Figure S4). Compared to freshly prepared liquid samples, the lyophilized Mpox mRNA-LNP (in C5, 0.1 mg mRNA/mL) showed a similar translucent bluish state after reconstitution with water (Figure 5a). Cryogenic transmission electron microscopy (cryo-TEM) analysis revealed that the lyophilized mRNA-LNP reconstituted in water exhibited bleb-like structures, while the freshly prepared liquid mRNA-LNP had an amorphous solid core-like morphology with fewer blebs (Figure 5b). The alterations in particle morphology may be associated with the variations in particle size, PDI, and EE% of the lyophilized products, as evidenced by the data presented in *Section 3.1.2*. However, the bleb-like structure has demonstrated contradictory effects in disparate research endeavors, rendering it challenging to predict whether the activity of LNP is influenced by lyophilization.[46]

To evaluate the in-use stability of the lyophilized Mpox-LNP vaccine, lyophilized mRNA-LNP were reconstituted

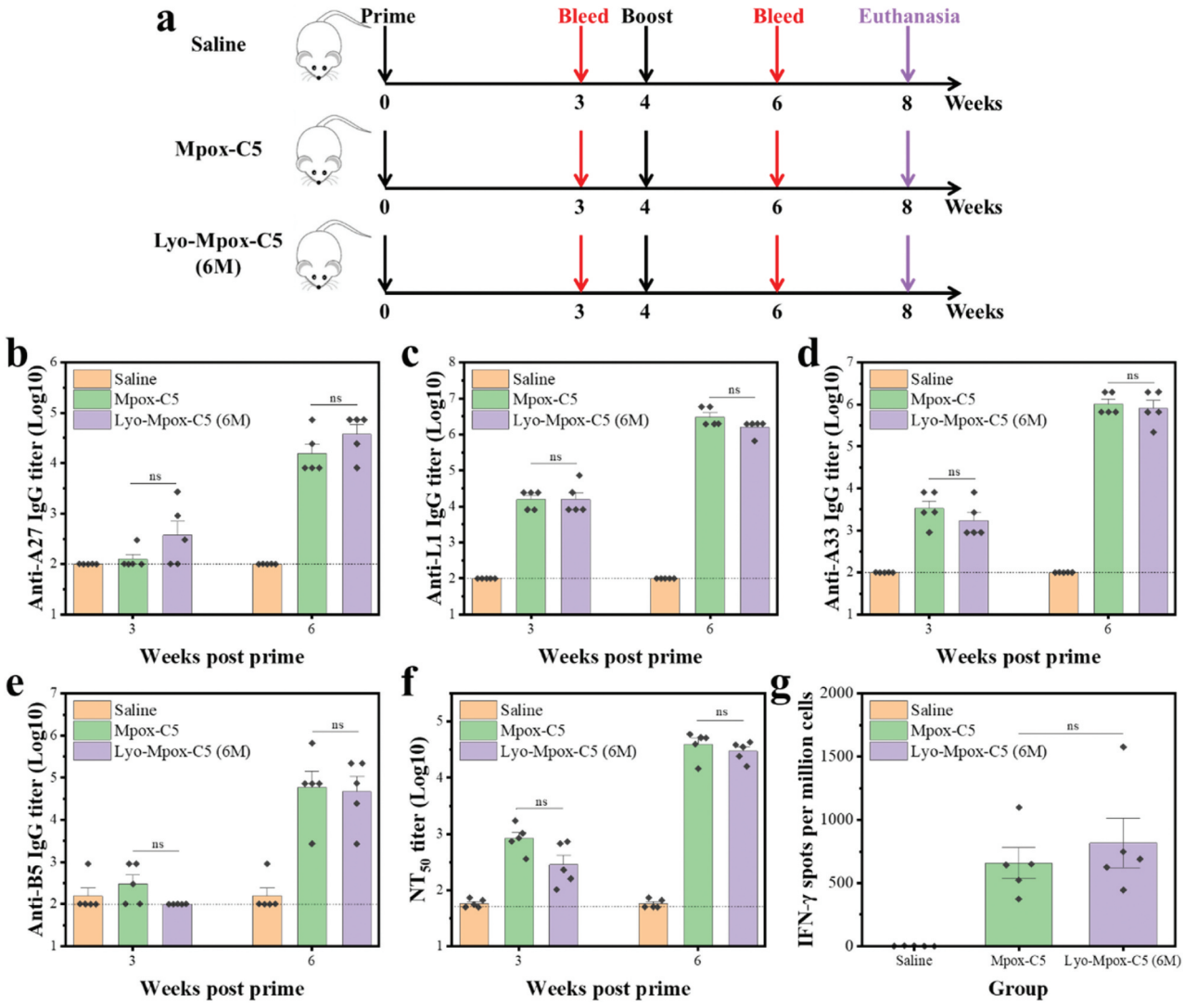

Figure 7. In vivo potency of mpox mRNA-lnp vaccine. (a) Immunization schedule of mice (mice were divided into 3 groups: saline, mpox-C5, and lyo-mpox-C5 (6 M). $n = 5$ for each group). The black, red, and purple arrows represent time points for immunizations, blood collection, and euthanasia. (b-e) serum binding antibody titer against (b) A27, (c) L1, (d) A33, and (e) B5 at 3- or 6-weeks post-prime. The dashed line indicates the limit of detection (LOD). (f) Serum VACV neutralization titer of mice at 3- or 6-weeks post-prime. The dashed line indicates the LOD. (g) Cellular immune response of ifn-γ in mice at 8-weeks post-prime.

using water and stored at room temperature. As illustrated in Figure 5c–f, the particle size/PDI, EE% and mRNA integrity of the reconstituted Mpox mRNA-LNP remained relatively unchanged following an 8-hour period of storage under ambient conditions. These findings suggested that the lyophilized Mpox-LNP could be maintained at room temperature for at least 8 h as a usage window. However, the impact of the structural change on biological activity and immunogenicity of the mRNA-LNP will be investigated in the next section.

Define a lyophilization process for a Mpox mRNA vaccine candidate

As outlined in the preceding sections of this study, the lyophilization process for the Mpox mRNA-LNP (20 vials/batch) has been defined. The mRNA-LNP was formulated in 10 mm Tris, 10% sucrose and 5% trehalose to reach a mRNA-LNP concentration of 0.1 mg/mL. The lyophilization process was delineated as such: samples were placed into the freezer which was pre-cooled to −20°C for 20 min before the temperature was decreased to −50°C at a cooling rate of 0.45°C/min and kept −50°C for 4 h. The primary drying process was next performed at a temperature of −30°C under pressure of 3 Pa for 30 h, and followed by the secondary drying process at 25°C under pressure of 3 Pa for 6 h, before storage at 4°C.

Evaluation of potency of lyophilized Mpox mRNA vaccine candidate

In vitro expression of lyophilized Mpox mRNA

To evaluate the in vitro biological activity, the quadrivalent Mpox mRNA-LNP was transfected into HEK-293T cells and the expression of one of the four antigens, B5 was selected for examination of transfection efficiency using flow cytometry. The following groups of samples were included in the study: Empty LNP, the freshly prepared liquid Mpox mRNA-LNP in C5 cryoprotectant (Mpox-C5), and the lyophilized Mpox-C5

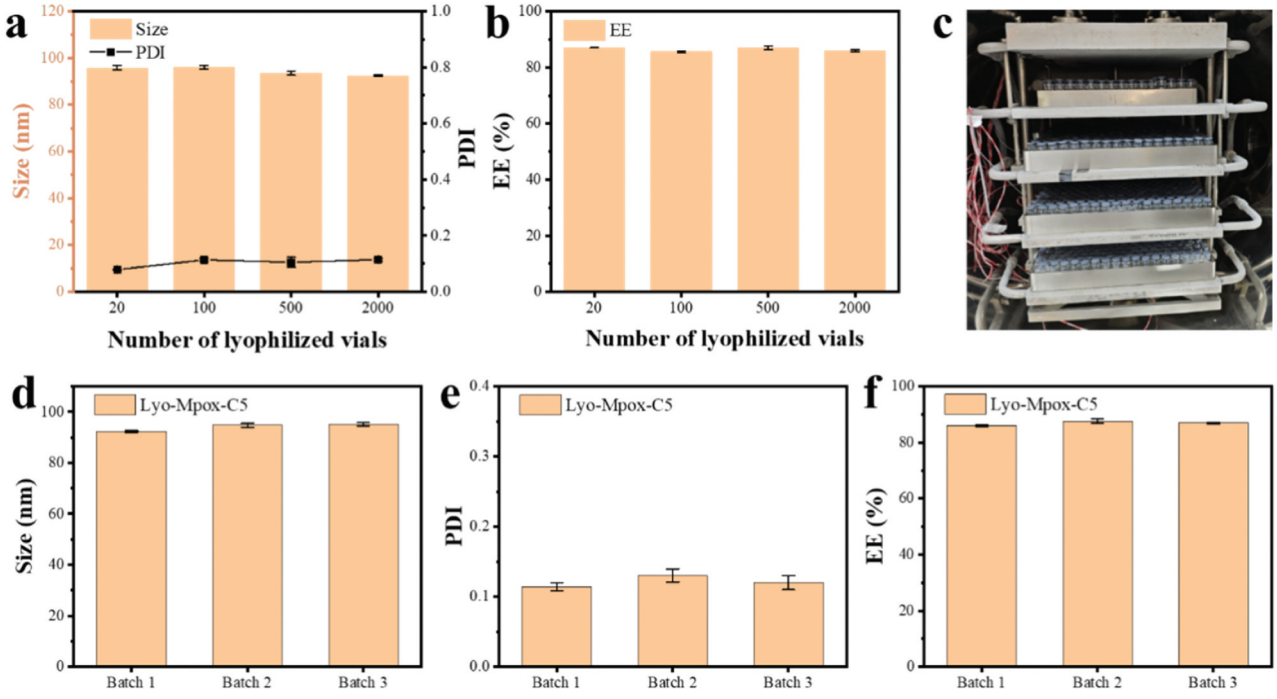

Figure 8. Pilot scale-up of lyophilized mpox-lnp. (a) Size/PDI,/PDI, and (b) EE% of lyophilized mpox-lnp (C5, 10 mm tris, 0.1 mg/mL mpox) at different batch scales (20 vials, 100 vials, 500 vials, and 2000 vials). (c) Lyophilizer loading image at 2000 vials/batch scale. (d) Size, (e) PDI, and (f) EE% of three batches of mpox-lnp (C5 cryoprotectant, 10 mm tris, 0.1 mg/mL, 2000 vials/batch) after lyophilization.

after 6-month storage at 4°C (Lyo-Mpox-C5 (6 M)). Cells that had been transfected with Mpox-C5 or Lyo-Mpox-C5 (6 M) demonstrated the expression of B5 protein, and the proportion of B5-positive cells was found to be comparable between the two groups, with values of 97.4% and 96.7%, respectively (Figure 6a–c). The equivalent expression level of B5 in HEK-293T cells after transfection with freshly prepared liquid Mpox-C5 and Lyo-Mpox-C5 (6 M) (Figure 6d) suggested that lyophilization and a 6-month storage of the lyophilized mRNA-LNP at 4°C did not alter the in vitro biological activity of Mpox mRNA-LNP vaccine. Additionally, immunofluorescence imaging revealed that nearly all cells in the Mpox-C5 and Lyo-Mpox-C5 groups showed expression of the B5 protein, with comparable fluorescence intensity (Figure S5).

Immunogenicity of the lyophilized Mpox mRNA-LNP in mice

To investigate the potency of a lyophilized mRNA vaccine, a lyophilized quadrivalent Mpox vaccine candidate was compared with a freshly prepared mRNA-LNP in BALB/c mice based on the vaccination procedure illustrated in Figure 7a. At 3 weeks post the prime immunization, there are no significant differences in A27-, L1-, A33- and B5-specific binding antibody responses between mice immunized with Mpox-C5 and Lyo-Mpox-C5 (Figure 7b–e). Comparable immune responses pattern was observed in mice of the two groups at 6 weeks post the prime immunization. It was noteworthy that mouse sera demonstrated substantial neutralizing activity against VACV virus in both the Mpox-C5 and Lyo-Mpox-C5 groups, with no statistically significant differences observed (Figure 7f). Similarly, the analysis of cellular immune response on day 56 post prime immunization showed that Mpox-C5 and Lyo-Mpox-C5 (6 M) induced equivalent IFN-γ-secreting cells (Figure 7g). In conclusion, lyophilized Mpox mRNA-LNP stored at 4°C for 6 months maintained the in vitro biological activity and immunogenicity in animals equivalent to that induced by freshly prepared Mpox mRNA-LNP.

Scale up of the lyophilization process for Mpox mRNA-LNP

The feasibility of the scale-up of the lyophilization process defined above (*Section 3.2*) was next investigated. The batch scale was gradually increased from 20 vials, 100 vials, 500 vials to 2000 vials and the primary drying time was changed accordingly, i.e., the primary drying time was increased from 30 h (20- and 100 vials/batch) to 40 h for 500 vials/batch, and 55 h for 2000 vials/batch, respectively. As shown in Figure 8a,b, the particle size, PDI and EE% of lyophilized Mpox mRNA-LNP at different manufacturing scales showed little difference. More importantly, at the largest scale (2000 vials/batch, Figure 8c), samples dried at multiple positions on the plate were tested, and consistent particle size, PDI and EE% were found (Table S3). The residue moisture was tested and a low moisture of 0.8% (data not shown) suggested indicated high drying efficiency. Additionally, the batch consistency of the lyophilization process was demonstrated on a scale of 2000 vials/batch (Figure 8d–f).

Conclusion

In this work, the process parameters of a lyophilization process for a Mpox mRNA-LNP vaccine candidate were evaluated and optimized, and the lyophilization process (20 vials/batch) was defined as follows: 10 mm Tris buffer containing 10% sucrose

and 5% trehalose was used as the lyophilization formulation. Samples were pre-cooled in the freezer with the plate temperature of −20°C for 20 min, followed by freezing at a cooling rate of 0.45°C/min to −50°C and keeping at −50°C for 4 h. The primary drying was conducted at temperature of −30°C (3 Pa) for 30 h and the secondary drying was performed at 25°C (3 Pa) for 6 h. The freeze-dried product has a uniform appearance and a low moisture content. Reconstitution of the lyophilized mRNA-LNP resulted in equivalent particle size, PDI, EE% and mRNA integrity compared to freshly prepared mRNA-LNP. Furthermore, the lyophilization process can be scaled up 100-fold to 2000 vials/batch. Importantly, the lyophilized mRNA-LNP could be stored at low temperature (4°C) for at least 12 months and at least 8 h after reconstitution at ambient temperature without significant change of product qualities. Moreover, the lyophilized Mpox mRNA-LNP maintained its in vitro biological activity and immunogenicity in mice in comparison to freshly prepared mRNA-LNP. Therefore, this work provides proof of concept for the use of lyophilization technology to improve the efficacy and availability of Mpox mRNA vaccine at reduced cost to help contain a global epidemic of Mpox infection.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported that there is no funding associated with the work featured in this article.

Notes on contributor

Dr ***Quanyi Yin*** is an employee of Yither Biotech Co., Ltd. His work focuses on the development of novel vaccine formulation and adjuvants.

Acknowledgments

The authors wish to acknowledge Drs Yizhi Wang and Haoran Huo from Tofflon Science and Technology Group Co., Ltd. for providing freeze dryer and for their helpful advice and guidance during this project.

Author contributions section

Wangbin: methodology, investigation, figures, writing-original draft; Quanyi Yin: conceptualization, methodology, project administration, writing original draft, review.

Li Yi: methodology, investigation; Caixia Su: investigation, animal study; Yang Wen: investigation, cell study; Man Qiao: analysis; Yingchen Ju & Zhihua Liu: methodology, review; Yelin Xiong & Zhilei Liu: supervision & review.

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