

Transfection efficiency of mRNA-LNPs after aerosolization with Rayleigh breakup based SMIs

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Summary

In this study, the suitability of Rayleigh based soft-mist devices for the delivery of mRNA-LNPs is ascertained. The devices with micro-nozzles of either 1.7 µm or 4.0 µm pore sizes were tested and compared to an impinging jet based soft-mist device.

Post-aerosolization characterization included particle size, polydispersity index (PDI), and zeta potential, alongside a fluorescence-based transfection assay to evaluate functional delivery of eGFP mRNA in Calu-3 epithelial cells. Both micro-nozzle pore sizes preserved the physicochemical characteristics of the LNPs within acceptable limits, with both nozzles showing higher transfection efficiencies compared to the impinging jet-based device and available literature.

This study highlighted the importance of device-specific design parameters in maintaining both structural and functional integrity of mRNA therapeutics during aerosol delivery, providing insight into future development of intranasal and inhaled nucleic acid-based therapies.

Key Message

Rayleigh-based soft mist technology enables high-yield delivery of intact and functional mRNA-LNPs compared to impinging jet-based systems.

Introduction

Messenger RNA (mRNA) therapeutics has had rapid advancement in vaccination, with the clinical success of mRNA-based vaccines in addressing COVID-19 [1]. The use of lipid nanoparticle (LNP) for mRNA delivery, as demonstrated in these vaccines, plays a vital role in protecting mRNA integrity during administration [2]. However, effective pulmonary and intranasal delivery of mRNA remains a significant challenge. The mechanical shear stress generated during aerosolization can deform the LNP structure, potentially exposing the mRNA to enzymatic degradation [3-5]. Soft Mist Inhalers (SMIs), particularly those leveraging Rayleigh jet technology, have gained traction as promising devices for delivering sensitive biologics, due to its low-velocity aerosol generation [6, 7]. However, the impact of different Rayleigh jet nozzle sizes is yet to be determined.

This study determines the performance of a Rayleigh-based SMI platform, the pre-filled syringe inhaler (PFSI), with two micro-nozzle pore sizes. Namely, a nozzle with 1.7 µm pores and a nozzle with 4.0 µm, with the aim of evaluating their suitability for the delivery of active mRNA. The 4.0 µm nozzle is hypothesised to exert lower mechanical shear stress on the formulation compared to the 1.7 µm nozzle. Both are theorized to exert lower shear stress compared to the impinging jet-based device. To investigate this, we performed physicochemical characterization of the post-aerosolized active mRNA-LNP formulation which include particle size, polydispersity index (PDI), surface charge, and functional activity assay using a fluorescence-based transfection assay in Calu-3 epithelial cells to assess the intact mRNA post-aerosolization. These findings will provide insight into the suitability of Rayleigh based soft-mist devices in delivery of aerosol-based nucleic acid therapeutics.

Materials and Method

mRNA-Lipid Nanoparticles Formulation

Active enhanced green fluorescent protein (eGFP) mRNA-LNP (ISO9001-standard, UNSW RNA institute, Sydney, Australia) were formulated using a microfluidic technique (NanoAssemblr IGNITE+) following the Moderna's SPIKEVAX vaccine composition at N/P ratio of 6. The mRNA-LNP formulation physical and functional properties adhered to predetermined acceptance criteria (Table 1) provided by the UNSW RNA institute. Before use, the mRNA-LNPs were diluted to 10 µg/mL working concentration in saline unless otherwise specified.

Table 1. mRNA-LNP characterization metrics and acceptance criteria, Mean $\pm$ SD. (n = 3)

Parameters	Product Quality Attributes	Acceptance Criteria
LNP Size (z-average diameter; nm)	139.9 $\pm$ 2.8	< 200
Polydispersity Index (PDI)	0.08 $\pm$ 0.03	< 0.4
RNA Encapsulation Efficiency %	86.99 $\pm$ 3.1	> 80 $\pm$ 10
RNA Concentration µg/mL	35.3 $\pm$ 0.8	$\geq$ 30 $\pm$ 20

mRNA-LNP Atomisation

Two Rayleigh-based soft mist inhaler (SMI) devices (Resyca, Enschede, Netherlands) were evaluated: a Pre-filled Syringe Inhaler (PFSI) with the 1.7 µm nozzle, and a soft mist atomiser with the 4.0 µm nozzle. For comparison of SMI technology, an impinging jet-based device (Boehringer Ingelheim; Biberach, Germany) was also included in the study. All devices were sterilised prior to use. The PFSI and atomiser were each loaded with 500 µL and 1 mL of formulation, respectively, while the impinging jet-based device was loaded with 4 mL. Aerosolized samples were collected into pre-chilled tubes under sterile conditions within a laminar flow hood and subsequently analysed for particle size, surface charge, and mRNA-LNP functional activity.

LNPs hydrodynamic size and zeta potential

The size and charge of the mRNA-LNPs were measured using dynamic light scattering (Zetasizer Nano ZS, Malvern, UK). LNPs in saline as control were compared to post-aerosolized LNPs collected in tubes, as described above. The LNP solutions were measured for particle size, surface charge, and polydispersity index (PDI).

Transfection efficiency using eGFP

To assess the biological activity of intact mRNA post-aerosolization using the devices, transfection efficiency assay looking at fluorescence of the eGFP mRNA-LNP on Calu-3 human lung epithelial cells. Calu-3 cells were seeded at 4.7×10^5 cells/mL in 12-well plates (1 mL) and incubated for 72 h at 37°C, 5% CO₂. Active eGFP mRNA-LNPs were diluted in Opti-MEM™ reduced-serum medium without phenol red to a working concentration of 10 µg/mL. Aerosolised mRNA-LNPs were collected in pre-chilled tubes as described in the mRNA-LNP atomisation section. Cells at ~70% confluency were then treated with either pre- or post-aerosolised mRNA-LNPs. The formulations were pipetted directly onto the cells and incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO₂. The medium was then replaced with complete media and incubated for 24 h at 37°C, 5% CO₂. Four images per well of bright-field and fluorescence images were taken to observe cell morphology and fluorescence using the time-lapse microscope.

Statistical Analysis

All results are presented as the mean $\pm$ standard deviation of three independent experiments. ImageJ analysis was performed for fluorescence measurement, following the formula: Calculated Total Fluorescence (CTF) = Integrated Density sample – (Area sample $\times$ Mean Gray Value background). Statistical analysis was by one-way of variance (ANOVA) with Dunnett's multiple comparisons using GraphPad Prism 10.4.2 (GraphPad Prism Inc., La Jolla, CA, USA), shown by * p < 0.05, *** p < 0.001).

Results

Following aerosolization of active mRNA-LNPs, no statistically significant changes were seen in the average LNP particle size for the Rayleigh nozzle devices, with a significant increase in average size seen for the impinging jet device. PDI remained almost unchanged for both Rayleigh nozzle sizes with a significant increase observed for the impinging jet device. However, all obtained values were still well within the acceptance criteria as shown in Table 1. Surface charge showed no significant differences

between pre- and post-aerosolization samples for either nozzle size or the impinging jet-based device (Figure 1C).

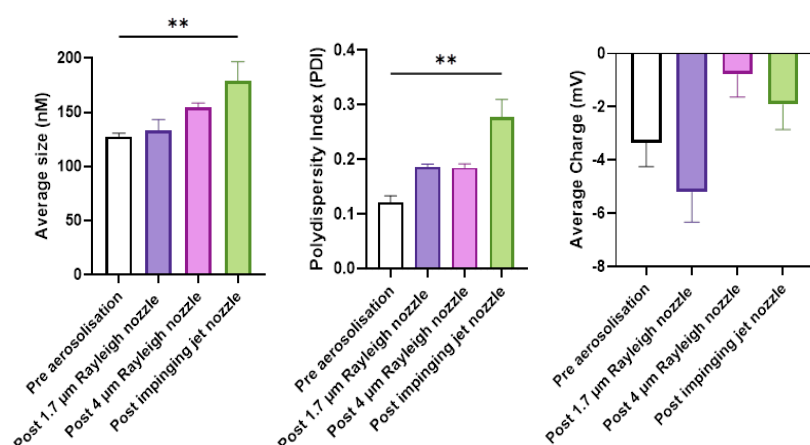


Figure 1. Changes in the physicochemical properties of mRNA-LNPs following aerosolization using the impinging jet nozzle and 1.7 μm and 4.0 μm Rayleigh nozzle. (A) Particle size of LNPs pre- and post-aerosolization, (B) PDI comparison, and (C) surface charge (zeta potential). Data are presented as mean $\pm$ SD ($n = 3$). One-way ANOVA with Dunnett's multiple comparisons test was performed. $*p < 0.05$.

Previous studies have reported a 40% reduction in mRNA biological activity with most intranasal devices, such as the Mucosal Atomisation and Laminar Fluid Ejection devices, while inhalation devices like the PARI eFlow and Aerogen Solo nebulisers often result in losses exceeding 60% [3, 8]. To ascertain the biological activity for the two Rayleigh SMI device nozzle pore sizes, and the impinging jet based device, activity of aerosolized functional mRNA-LNPs was assessed via a transfection efficiency assay based on eGFP fluorescence. Figure 2A, shows Calu-3 cell confluency across all conditions, as observed in the brightfield images. A significant reduction in fluorescence was observed following aerosolization via the 1.7 μm nozzle device compared to pre-aerosolized control, indicative of lower transfection efficiency and reduced delivery of intact mRNA-LNPs (Figure 2B). The impinging jet based device showed a further reduction in fluorescence, significantly lower compared to both Rayleigh nozzle pore sizes. Quantitative analysis showed a 81% transfection efficiency for the 4.0 μm Rayleigh nozzle equipped device. The 1.7 μm nozzle device has a transfection efficiency of 65%. The impinging jet based device had an transfection efficiency of 18%.

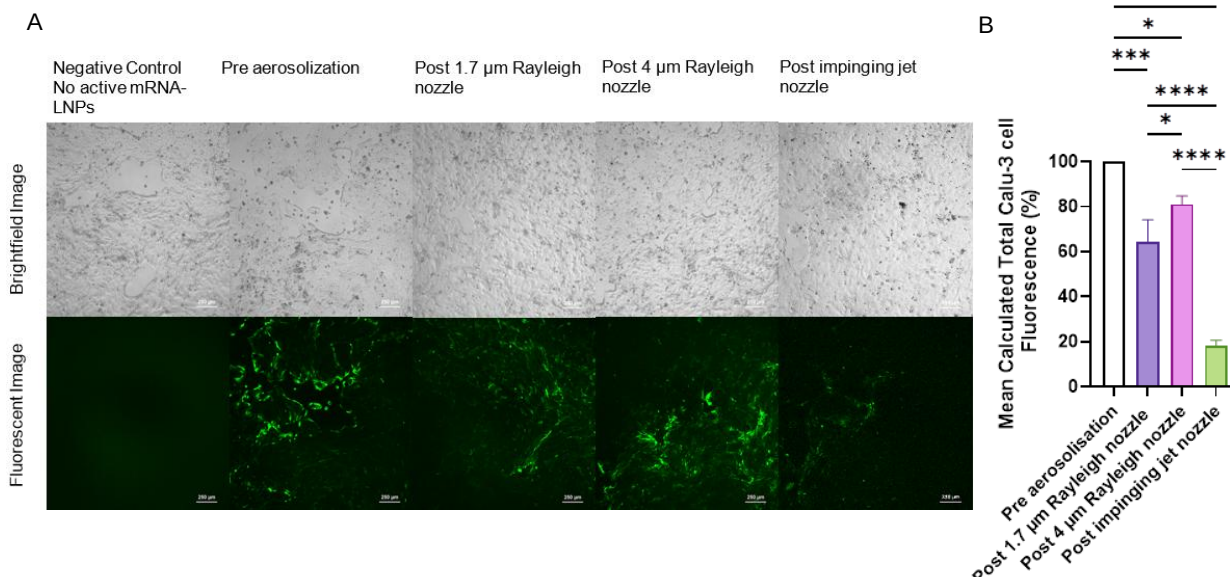


Figure 2. Functional mRNA integrity post-aerosolization using 1.7 μm Rayleigh nozzle, 4 μm Rayleigh nozzle, and impinging jet nozzle. (A) Brightfield and fluorescent images taken by time-lapse microscope (B) ImageJ analysis of the fluorescent images to show CTF. Data are presented as mean $\pm$ SD ($n = 3$). One-way ANOVA with Dunnett's multiple comparisons test was performed. $*p < 0.05$.

Discussion

A comparison between the 1.7 μm and 4.0 μm Rayleigh nozzle-based soft-mist devices revealed no substantial differences in post-aerosolized size or PDI, which is consistent with both devices utilising the same nozzle technology. Both nozzles were able to maintain size and PDI of the active mRNA-LNP formulation within acceptable physicochemical criteria. This was different for the impinging jet based device, where significant changes were observed. The surface charge of post-aerosolized mRNA-LNPs for both Rayleigh nozzles remained slightly negative to near neutral (0 to -10 mV), advantageous for

both inhaled and intranasal delivery. For pulmonary delivery via the PFSI (1.7 μm nozzle), a slightly negative to neutral zeta potential may reduce electrostatic repulsion from the negatively charged airway epithelium while avoiding the cytotoxicity associated with highly positively charged particles [9-12].

Both soft mist Rayleigh nozzle sizes were able to largely preserve mRNA-LNP integrity and functionality, achieving 65% and 81% of the biological activity observed in the pre-aerosolized LNPs, for the 1.7 μm and 4.0 μm nozzles respectively. The impinging jet base device substantially reduce functional mRNA activity with a transfection efficiency of 18%. Ho et al. recently investigated similar mRNA-LNP formulations to assess nanoparticle integrity and biological activity post-aerosolization [8]. Among the nasal devices tested, they observed the highest level of preserved luciferase protein expression, reaching approximately 60% relative to the non-aerosolized control, highlighting the vulnerability of mRNA-LNPs to mechanical stress during aerosol delivery [8]. Previous studies have similarly reported that most nasal devices result in more than a 40% reduction in mRNA biological activity, while inhalation devices often cause losses exceeding 60% [3, 8]. These findings highlight the 1.7 μm and 4.0 μm Rayleigh based SMIs as promising technologies for delivery of biologic therapeutics.

Overall, the impinging jet nozzle preserves significantly lower expression of mRNA-LNPs compared to 1.7 μm and 4.0 μm Rayleigh nozzle, potentially due to the higher shear forces generated by the impinging jet nozzle's colliding jet actuation mechanism, which is based on the Savart principle. The collision of two high-velocity liquid jets generated by the impinging jet nozzle to produce the droplet mist, applies greater mechanical stress on the mRNA-LNP formulation [7, 13]. In contrast, 1.7 μm and 4.0 μm Rayleigh nozzle using the Rayleigh break up principle allows a low energy liquid stream to passively passed through a nanotech membrane nozzle using a piston driven syringe, producing mono-disperse droplets [14, 15].

Conclusion

Despite differences in micro-nozzle pore size, the overall physicochemical properties of active mRNA LNPs remained comparable post-aerosolization between the 1.7 μm and 4.0 μm nozzles, utilising the same nozzle technology, compared to the impinging jet-based device which showed substantial changes post aerosolization. Importantly, the Rayleigh-based SMIs have demonstrated the capability to maintain mRNA LNP structural integrity and enable functional delivery, supporting the compatibility of these platforms with aerosolized nucleic acid therapeutics.

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