Inhalation Properties and Stability of Nebulized Naked siRNA Solution for Pulmonary Therapy

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The use of naked unmodified small interfering RNA (N-siRNA) without vector has previously been investigated as a pulmonary therapy. However, little is known regarding stabilities and aerodynamic particle sizes of N-siRNA-containing droplets; nebulizers have not yet been optimized for N-siRNA solutions. Thus, in this study, we investigated the feasibility of inhaled N-siRNA solutions for pulmonary therapy using nebulization. Various nebulizers and N-siRNA concentrations were assessed in terms of siRNA integrity after nebulization, and inhalation properties including aerodynamic particle size were examined. In comparison with ultrasonic-, air-jet-, and vibrating-mesh nebulizers, N-siRNA integrity was not affected by nebulization. Thus, in further experiments, performances of N-siRNA aerosols with different nebulizers and N-siRNA concentrations were evaluated and screened using an aerodynamic particle sizer (APS) which employed the time-of-flight principle or a cascade impactor. Mean mass aerodynamic diameters of N-siRNA-containing droplets from vibrating-mesh nebulizers tended to decrease with increasing N-siRNA concentrations, reflecting the influence of N-siRNA solutions on surface tension, as indicated by contact angles. These data indicate the utility of APS instruments for investigating the nebulized characteristics of expensive drugs including siRNAs and may facilitate the development of N-siRNA inhalation formulations.

Key words small interfering RNA (siRNA); vibrating-mesh nebulizer; aerodynamic particle size; surface tension; contact angle

Small interfering RNA (siRNA) has a great therapeutic potential as a tool for the post-transcriptional silencing of target gene expression. Recently, the field of siRNA delivery has rapidly progressed, and the local pulmonary delivery of siRNA has been investigated for various lung diseases. Inhalation is the most popular noninvasive method for delivering therapeutic siRNA agents to the lungs; local siRNA inhalation therapy reduced virus titers in the lung and attenuated local pulmonary chemokine production after acute lung injury and infection. However, naked (unmodified) siRNA (N-siRNA) is susceptible to nuclease degradation; siRNA delivery systems facilitating gene silencing efficiency, including those using cationic materials, can have cytotoxic effects. N-siRNA has been delivered with some success to the lung, although systemic delivery of N-siRNA generally fails to produce significant gene silencing effects. N-siRNA lacks delivery vectors that produce toxicity and inflammation; thus, N-siRNA formulation for pulmonary delivery offers advantages for safety and simplicity. However, few developments of N-siRNA formulations have been reported.

Direct pulmonary N-siRNA delivery has been achieved in humans following the inhalation of aerosols generated by inhalers or nebulizers. Three types of inhalation devices are currently available for pulmonary drug delivery, including metered dose inhalers, dry powder inhalers, and nebulizers. Liquid nebulization effectively generates aerosols for pulmonary drug delivery; atomization theories suggest that aerosol sizes and output characteristics are dependent on operating principles, conditions, and mechanical constructions of nebulizers as well as on physicochemical properties of nebulized fluids. Formulations for efficient nebulization are usually designed using empirical methods that consider pharmacological effects and physicochemical properties, including viscosity, surface tension, and drug concentrations. However, N-siRNA delivery by aerosol inhalation may result in marked losses in transfection efficiency because of the nebulization process. Higher concentrations of N-siRNA are necessary for the therapeutic application of aerosol inhalation techniques than for siRNA carrier systems, including cationic lipids and polymers. Stabilities and aerodynamic particle sizes of N-siRNA-containing droplets remain poorly characterized, and nebulizers have not yet been optimized for N-siRNA.

Here, the nebulization properties of N-siRNA solutions were investigated, and air-jet-, ultrasonic-, and vibrating-mesh nebulizers were compared in terms of the relationship between N-siRNA concentrations and aerodynamic particle sizes.

Experimental

Materials The sense and antisense siRNAs 5’-CACUGC AAGUGG ACA UCA AC G-3’ and 5’-ttGAUGUCC ACU GC AGUUG-3’, respectively, were purchased from Dharmacon Inc. (Lafayette, CO, U.S.A.) and were used as model siRNAs. Polyvinyl alcohol (PVA; molecular weight (MW)=25000; hydrolyzation=88.0%; polymerization=500) was purchased from Kuraray (Osaka, Japan). Low-molecular weight salmon sperm DNA and fluorescein isothiocyanate–dextran (FD; MW=10000) were purchased from Sigma (St. Louis, MO, U.S.A.).

Air-Jet-, Ultrasonic-, and Vibrating-Mesh Nebulization All nebulization experiments were performed using air-jet nebulizers (CompAir NE-C28, Omron; Kyoto, Japan), ultrasonic nebulizers (NE-U07, Omron), and passively vibrating-mesh nebulizers (MicroAir NE-U22, Omron). Experiments were performed with a starting reservoir volume of 5 mL, and...
samples were taken from collected aerosol mists and the reservoir. Technical nebulizer information from the manufacturer is presented in Table 1.

**Assessment of N-siRNA Stability Using Agarose Gel Electrophoresis after Nebulization** The stability of N-siRNA solutions in tris(hydroxymethyl)aminomethane–ethylenediaminetetraacetic acid (Tris–EDTA) buffer (TE buffer containing 10 mM Tris–HCl and 1 mM EDTA (pH 7.4); Sigma) during nebulization and sonication in a bath-type sonicator (35 kHz, UT-205S, Sharp Corp., Osaka, Japan) was investigated using electrophoresis with 3% (w/v) agarose gels (agarose 21; Nippon gene, Toyama, Japan) in Tris–acetate EDTA buffer (pH 8.3). Gel electrophoresis was performed at a constant voltage of 100 V for 0.5 h, and siRNA bands from 0.4 µg siRNA samples were visualized using ethidium bromide staining. Gel images were acquired using FLA-5100 (FUJIFILM, Tokyo, Japan).

**In Vitro Assessment of Inhalation Qualities of Nebulized Mists** Nebulizers were connected to an Andersen cascade impactor (ACI; Andersen nonviable sampler, Model AN-200; Tokyo Direc Co., Japan) via a throat comprising five connecting tubes. Subsequently, siRNA solutions were nebulized, and the system was vacuumed using air streams of 28.3 L/min for 5 min. After actuation, siRNAs were collected by from the nebulizer, the throat, and stages 0–7 of the cascade impactor by rinsing with Milli-Q water. Quantities of siRNA from each stage were then determined using Ribogreen assays (Molecular Probes, Eugene, OR, U.S.A.).

Nebulized aerosol performances of the formulations were determined using a TSI model 3306 Respirable Impactor Inlet (TSI Inc., Shoreview, MN, U.S.A.) coupled with a TSI model 3321 aerodynamic particle sizer (APS; TSI Inc.). Aerosol particle analyses were performed continuously every 30 s until the end of each run at a flow rate of 28.3 L/min. Subsequently, mean mass aerodynamic diameters (MMADs) and geometric standard deviations of aerosol particles generated by each nebulizer were determined, and comparisons of size distributions were made in terms of central tendencies and spread, respectively. Particle diameters in nebulized mists were also measured using a laser diffraction size analyzer (LDSA-SPR3500A; Nikkiso Co., Ltd., Tokyo, Japan).

**Measurement of Contact Angles as an Indicator of Surface Tension** Water droplets of 2.4 µL were deposited on glass slides, and contact angles were determined using a dropmaster DM100 (Kyowa Interface Science, Saitama, Japan).

**Results and Discussion**

**N-siRNA Stability after Nebulization** Nebulizers are widely used to generate liquid aerosols and can deliver large volumes of drug solutions or suspensions by inhalation. However, because almost all aerosol droplets are recycled into the reservoir during nebulization, repeated shear stress may lead

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**Table 1. Supplier Specifications of Nebulizers**

| Type        | NE-C28 air-jet | NE-U07 ultrasonic | NE-U22 passively vibrating mesh |
|-------------|----------------|-------------------|---------------------------------|
| Nebulization rate (mL/min) | 0.4           | 1                 | 0.25                            |
| Maximum volume (mL) | 7             | 10                | 7                               |
| Minimum volume (mL) | 2             | 2                 | 0.3                             |
| Residual volume (mL) | 0.5           | 0.5               | 0.3                             |
| MMAD\(^a\) (µm) | 5             | 1–8               | 5                               |
| Frequency (MHz) | 2.4           | 0.18              |                                 |
| Electricity consumption (W) | 58            | 20                | 1.5                             |

\(^a\) Mean mass aerodynamic diameter.

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**Fig. 1. Effects of Nebulizer Types and Nebulization Times on Small Interfering RNA (siRNA) Quality**

The integrity and stability of naked siRNA (N-siRNA) were determined after nebulization using agarose gel electrophoresis; effects of the vibrating-mesh nebulizer on N-siRNA integrity (A) in nebulized N-siRNA solutions and (B) in the reservoir; (C) effects of air-jet nebulization on N-siRNA integrity in the reservoir; (D) effects of duration of sonication in a bath-type sonicator and an ultrasonic nebulizer on N-siRNA integrity.
to degradation of nucleic acids in N-siRNA solutions. Hence, the integrity of N-siRNA remaining in nebulizer reservoirs and in collected aerosol mists after 10 min of nebulization was examined using agarose gel electrophoresis (Fig. 1). In these experiments, several N-siRNA solutions of differing concentrations were nebulized using air-jet- and vibrating-mesh nebulizers. However, intact N-siRNA bands indicated no N-siRNA degradation.

Collection of N-siRNA aerosols from ultrasonic nebulizers was difficult. Therefore, to examine N-siRNA stability during ultrasonic cavitation, N-siRNA solutions were treated in a bath-type ultrasonicator instead of ultrasonic nebulizers. Cavitation by ultrasonication may be more aggressive than that during nebulization, because bath-type ultrasonicators have a much lower frequency (35 kHz) than nebulizers (2.4 MHz). However, in the present experiments, N-siRNA was stable against sonication forces and ultrasonic nebulization did not degrade N-siRNA solutions.

Thus, gene silencing by nebulized siRNA was examined using A549 cells that stably expressed firefly luciferase (A549-Luc; Experimental S1 and Fig. S1). These experiments showed similar RNA interference (RNAi) effects of nebulized siRNA and residual siRNA in the vibrating-mesh nebulizer to those of the untreated original siRNA. Hence, nebulization did not affect the integrity and RNAi function of original N-siRNA (Figs. 1, S1).

Effects of N-siRNA Concentrations and Nebulizer Types on MMADs after Aerosolization

Particle sizes of 1–5 µm are considered optimal for delivery of therapeutic agents to alveolar regions, and nebulizers theoretically produce droplets within this range (Table 1). However, addition of drugs and excipients to nebulizing solutions may dramatically affect droplet formulations, necessitating careful selection of formulations. Accordingly, particle size analyses of aerosols from nebulizers are typically performed using multistage cascade impactors, including ACI. This technique directly determines weights and aerodynamic particle sizes of therapeutically active pharmaceutical ingredients (APIs) and indicates the likely location of deposition within the respiratory tract. However, assessments of the inhalation properties of small quantities of API using cascade impactors are labor intensive and difficult, and more efficient techniques are necessary for development, particularly for early-stage products. In the absence of more rapid multistage impactor-based techniques, so-called real-time APS is commonly used according to the time-of-flight principle. This instrument typically allows measurements of particle sizes within a minute and may therefore be suitable for screening the inhalation properties of expensive APIs, including siRNAs.

Delivery of inhaled aerosol N-siRNA to the lung is generally evaluated using in vitro methods. In the present experiments, N-siRNA solutions in TE buffer were aerosolized using three nebulizer types at N-siRNA concentrations of 0.1 and 3.0 mg/mL, and MMADs were all dependent on nebulizer types and N-siRNA concentrations (Table 2) and decreased

| Measurement apparatus | Nebulizer | siRNA concentration (mg/mL) | MMAD (µm) | GSD |
|------------------------|-----------|-----------------------------|------------|-----|
| APS®                   | Air-jet   | 0.1                         | 4.52±0.02  | 1.46±0.02 |
|                        |           | 3.0                         | 4.31±0.01  | 1.78±0.04 |
| Ultrasonic             | 0.1       | 5.32±0.03                   | 1.51±0.01  |
|                        | 3.0       | 5.23±0.10                   | 1.60±0.01  |
| Vibrating mesh         | 0.1       | 5.81±0.13                   | 1.49±0.01  |
|                        | 3.0       | 4.36±0.27                   | 1.87±0.06  |
| ACS®                   | Vibrating mesh | 0.1          | 4.44±0.14  | 2.24±0.38 |
|                        |           | 3.0                         | 3.24±0.59  | 5.94±3.89 |

Data are expressed as the mean±S.D., n=3. a) Mean mass aerodynamic diameter. b) Geometric standard deviation. c) Aerodynamic particle size.
with increasing N-siRNA concentrations in APS experiments. However, MMADs were greater when a vibrating-mesh nebulizer was used, indicating that N-siRNA concentrations play a role in aerosol generation from vibrating-mesh nebulizers. Similarly, ACI and LDSA measurements of aerosolized particle size distributions from vibrating-mesh nebulizers indicated decreasing MMADs with N-siRNA concentrations (Figs. 2A, B).

Patterns of size distribution varied with particle sizes and differed between analyzers. Twin peaks were detected using ACI, specifically on the nebulized 3.0 mg/mL solution (Fig. 2A), whereas size distributions with single large peaks were observed using LDSA (Fig. 2B) and APS (Fig. 2C). These observations indicate that the large ACI peak in the smaller size range did not exist. Both LDSA and APS employ lasers to directly measure aerosol particle sizes and the number of droplets; LDSA detects laser diffraction patterns of sprayed aerosols, whereas APS is based on the principles of time-of-flight, which is determined for each particle between two laser beams. Conversely, the number of aerosol droplets cannot be determined using ACI, and the captured aerosols in each impactor stage are collected by washing; quantitative determination of siRNA in each sample was performed. Therefore, droplets of smaller particles (<2.0 µm) detected by ACI may have higher concentrations than larger droplets.

The present interactions of inhaled N-siRNA with lung tissues and cells might have been limited by the absence of transfection vectors, including cationic liposomes. Moreover, pulmonary absorption is greater for small molecular compounds (MW <1000), and the absorption of high molecular weight substances including N-siRNA, from the lung into the systemic circulation may be limited. Therefore, after deep deposition in the lung following inhalation using nebulizers, N-siRNA may remain in the lung fluid.

Inhalation Properties of Polymer Solutions after Aerosolization: The effect of N-siRNA concentrations on aerosolized droplets was confirmed using various model polymers with hydrophilic properties and molecular weights that were similar to those of N-siRNA (nominal MW, 13000 g/mol). These included low-molecular weight salmon sperm DNA (MW, 50000–100000), PVA (MW, 20000), and FD (MW, 10000), and APS-MMADs were examined after nebulization using the vibrating-mesh nebulizer. Similar to nebulized N-siRNA solutions, MMADs of DNA and PVA solutions were dependent on polymer concentrations. However, MMADs of nebulized FD solutions varied little with polymer concentrations.

Contact Angles of Polymer Solutions: Following nebulization using a vibrating-mesh nebulizer, MMADs of polymer solutions increased with polymer concentrations (Table 3), reflecting relationships between surface tension, viscosity, and aerodynamic forces. In agreement, solute concentrations and aerosolized particle sizes after nebulization were related in a previous study.

In the present study, contact angles of various droplets of polymer solutions were measured to indicate surface tension (Fig. 3). Maximum concentrations of N-siRNA were limited to 8.3 mg/mL for contact angle experiment because our siRNA stock was insufficient to prepare 10 mg/mL solution. Contact angles decreased with increasing N-siRNA, PVA, and DNA concentrations (Fig. 3), potentially reflecting the effects of polymer concentrations on droplet size distributions. In contrast, neither contact angles nor MMADs of FD solutions varied with concentration (Table 3).

Polymer viscosities did not vary in the concentration range

Table 3. Effects of Polymers on Aerodynamic Particle Sizer Droplet Sizes of Polymer Solutions Nebulized Using a Vibrating-Mesh Nebulizer

| Polymer | Concentration (mg/mL) | MMAD$^a$ (µm) | GSD$^b$ |
|---------|-----------------------|----------------|--------|
| DNA | 0.1 | 5.60±0.12 | 1.88±0.01 |
| | 3.0 | 1.56±0.08 | 2.40±0.03 |
| | 10 | 1.40±0.10 | 2.03±0.12 |
| PVA$^c$ | 0.1 | 5.19±0.13 | 1.50±0.01 |
| | 3.0 | 5.03±0.03 | 1.58±0.02 |
| | 10 | 1.56±0.25 | 2.18±0.17 |
| FD$^d$ | 0.1 | 5.38±0.09 | 1.49±0.02 |
| | 3.0 | 4.54±0.07 | 1.65±0.01 |
| | 10 | 5.21±0.12 | 1.80±0.03 |

Data are expressed as mean±S.D., n=3. a) Mean mass aerodynamic diameter. b) Geometric standard deviation. c) Polyvinyl alcohol. d) Fluorescein isothiocyanate–dextran.
(0.1–10 mg/mL) used in this study (data not shown). Hence, although the influence of viscosity on droplet sizes may be limited, decreased contact angles of N-siRNA, PVA, and DNA solutions reflected decreased droplet sizes (Fig. S2). However, other physicochemical factors may also influence droplet sizes.

Conclusion
Here, the stabilities and particle sizes of nebulized N-siRNA solutions were evaluated to investigate the therapeutic potential of pulmonary N-siRNA delivery using nebulizers. Although N-siRNA stability was robust under conditions of nebulization, the present data indicate that polymer concentrations remain an important consideration during formulation development. Vibrating-mesh nebulizers generated aerosolized droplets with smaller MMADs from solutions with high N-siRNA concentrations, reflecting the influence of N-siRNA polymers on surface tension, as indicated by contact angles. Taken together, these data indicate the potential utility of N-siRNA solutions and vibrating-mesh nebulizers as pulmonary therapies for various lung diseases.

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Conflict of Interests
The authors declare no conflict of interest.

Supplementary Materials
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