## Albumin as an alternative dispersion enhancer for inhalable siRNA spray dried powders

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### Summary

Respiratory diseases such as asthma or infections are often attributed to the (over)expression of disease-causing genes. Exploiting RNA interference (RNAi), local administration of RNAi molecules has become an attractive treatment strategy. Our previous study has shown that leucine could promote the aerosol performance of otherwise poorly dispersed siRNA powders to achieve a fine particle fraction (FPF) of 44.4%. However, the need of a large amount of leucine (50% w/w) and its hydrophobic nature posed limitations such as restricting siRNA load and solubility issues. In this study, we investigated human serum albumin (HSA) as an alternative dispersion enhancer to leucine in improving the aerosol performance of spray dried siRNA powders. At 2% w/w siRNA, the highest siRNA concentration in an inhalable solid formulation ever reported, we prepared and characterised siRNA powders co-spray dried with HSA (5% or 35% w/w) and mannitol as the bulking agent. The solutions were prepared at 1% or 2% w/v solute concentrations. The result of cascade impactor assay showed that at 35% HSA and at 1% solute concentration, the resultant powder exhibited a FPF of 68.9%. Scanning electron microscopy images revealed that particles with higher HSA composition exhibited less regular shape with wrinkled surfaces. The median physical size of the particles was between 1.5 to 2.2 μm as measured by laser diffraction. The structure of siRNA was also preserved as shown in gel retardation assay. This study demonstrated that HSA could serve as an effective dispersion enhancer of spray dried siRNA powders.

### Key Message

Human serum albumin was shown to serve as an effective dispersion enhancer to improve spray dried powders of siRNA for inhalation. The spray dried formulation containing 1% w/w siRNA and 35% w/w human serum albumin exhibited the highest FPF of 68.9%.

### Introduction

Many respiratory diseases are associated with the expression of disease-causing genes, and/or the expression of foreign genes from pathogens, with examples including the overexpression of inflammatory regulators in chronic asthma and chronic obstructive pulmonary disease (COPD), as well as the expression of viral proteins during influenza infection. RNA interference (RNAi) represents an effective mechanism to transiently silence the expression of genes as targeted by RNAi mediators such as small interfering RNA (siRNA) and micro RNA (miRNA). Local administration of the RNAi molecules has become an attractive therapeutic strategy to combat these diseases [1-3]. Our group has previously reported the spray dried powder formulation of siRNA for inhalation using leucine as dispersion enhancer [4]. While the formulation exhibited satisfactory aerosol performance with FPF of 44.4%, the need of large amount (50% w/w) of hydrophobic leucine poses solubility issue and limits the siRNA load in the formulation. The current study explored the use of human serum albumin (HSA) as an alternative dispersion enhancer to prepare inhalable spray dried powder formulations of siRNA. It is hypothesized that during the drying process, the macromolecular albumin competed with siRNA to stay on the particle surface, modify the surface and reduce cohesiveness, leading to improved dispersion and delivery efficiency. Although HSA is yet to be approved as excipient for inhalation product, it has been frequently used in different studies as an excipient for inhalable formulations [5, 6]. It has also been inhaled in human subjects as a radiolabeled aerosol for the study of aerosol distribution in lungs [7, 8].

### Experimental Methods

#### Materials

Human serum albumin (HSA) (Sigma-Aldrich; Poole, UK), mannitol (Pearlitol 160C; Lestrem, France) and model siRNA targeting monocyte chemoattractant protein 1 (MCP-1; Integrated DNA Technologies; IL, US) (Antisense sequence: 5′- CCG UAA UCU GAA GCU AAU TT-3′) was dissolved in ultrapure water in the compositions as shown in Table 1.

#### Spray drying

A Büchi B-290 Mini spray dryer in tandem with a B-296 dehumidifier (Büchi Labortechnik AG; Postfach, Switzerland) was used to spray dry the solution using the conditions as stated in Table 2. The powder formulations were stored in glass vials inside a desiccator at ambient temperature. The mass of powder recovered from the collecting vial relative to the initial mass input constituted the production yield.

**Table 1 – Compositions of siRNA/HSA/mannitol formulations used for spray drying.**

| Formulations | Composition (% w/w) | | | Solute Concentration (% w/v) |
|---|---|---|---|---|
| | siRNA | HSA | Mannitol | |
| 2S5H-1 | 2 | 5 | 93 | 1 |
| 2S5H-2 | | | | 2 |
| 2S35H-1 | | 35 | 63 | 1 |
| 2S35H-2 | | | | 2 |

**Table 2 – Spray drying conditions used for the preparation of siRNA/HSA/mannitol dry powder formulations.**

| Parameters | Inlet temperature | Feed rate | Atomization | Aspiration | Configuration |
|---|---|---|---|---|---|
| | 80°C | 1.4 ml/min | 742 L/h; Air | 35 $m^3$/h | Closed loop; Suction mode |

Aerosol performance study and particle size measurement

To study the *in vitro* aerosol performances of the formulations, the Next Generation Impactor (NGI; Copley Scientific, UK) was used with a low resistance capsule-based inhaler as the dispersing device (Breezhaler; Novartis Pharmaceuticals, Hong Kong). Approximately 6 mg of powder was used for each dispersion. The dispersion flow rate was 90 L/min and duration was 2.7 seconds. Since mannitol was the most abundant component in all the formulations, the amount of powder deposited on each compartment of the NGI was quantified with mannitol using high performance liquid chromatography upon recovering the powders with ultrapure water. An ion-exchange ligand-exchange column (Agilent Hi-Plex Ca, 7.7 × 50 mm, 8µl; Agilent Technologies, CA, USA) was used to resolve mannitol which was detected with a refractive index detector (G1362A; Agilent Technologies). The emitted particle (powders that exited the inhalers) and the fine particle (particles with aerodynamic diameter under 5 µm) fraction, with respect to the total recovered powder from the assay, was calculated. The primary particle size of the powder formulations was determined using laser diffractometry (Mastersizer 2000; Malvern Instruments Ltd., Worcestershire, UK) and the dispersion pressure was 4 bar.

Gel retardation assay and morphology study

Gel retardation assay was performed to study the integrity of siRNA in spray dried powders. Particle morphology was visualized using scanning electron microscopy (Hitachi S-4800 FEG Scanning Electron Microscope; Hitachi; Tokyo, Japan).

**Results and Discussion**

From our previous studies, it was demonstrated that siRNA could remain intact at the present spray drying condition [4]. The present study aimed to investigate HSA as an alternative dispersion enhancer to leucine in spray dried powder, with siRNA load up to 2% by mass. This is the highest siRNA concentration in a solid formulation that has been reported to date [9]. It is anticipated that there is an enrichment of HSA on the particle surface during spray drying due to its large molecular size and low mobility, and hence modifying the particle surface properties. Two levels of HSA and two levels of solute concentration were selected. Under the current spray drying condition, the outlet temperature reached was 50°C. The highest production yield achieved was 69% (2S35H-2) and the lowest was 46.5% (2S35H-1) (Table 3). The EF and the FPF results are shown in Figure 1. All formulations achieved an EF of above 85%, suggesting the formulations could be readily dispersed. The use of a high level of HSA resulted in a higher FPF, despite only the difference between 2S5H-1 and 2S35H-1 was significant (one-way ANOVA, Tukey's post-hoc test, $p < 0.05$). The latter also gave the highest FPF value of 68.9%. The effect of solute concentration on aerosol performance was not conclusive. As a comparison, the FPF of siRNA dry powder using 50% w/w leucine as dispersion enhancer has been reported to be 44.4%. Here, a better aerosol performance could be achieved with a lower amount of dispersion enhancer, potentially allowing a higher siRNA load in the formulation.

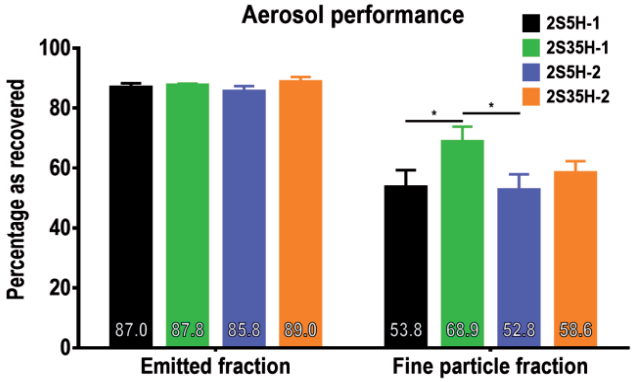


**Figure 1 – Emitted fraction (EF) and fine particle fraction (FPF) expressed as percentage relative to recovered dose. EF was similar among all formulations. A higher FPF was achieved for formulations with higher HSA composition. Data were presented as mean ± standard deviation (*n* = 3). The results were analysed by one-way ANOVA followed by Tukey's post-hoc test (* represents $p < 0.05$).**

The image of gel retardation assay was shown in Figure 2. The integrity of siRNA in spray dried powders was preserved. Our preliminary data of *in vitro* transfection study also showed that the bioactivity of the siRNA was successfully maintained (data now shown). Furthermore, it was confirmed that siRNA remains in its free form without complexing with HSA. Although the integrity of HSA in the spray dried powder was not evaluated here, it has been reported that proteins could be spray dried at high temperature (100-130 ºC) without compromising their structures and bioactivity[10, 11]. The particle surface morphology of the formulations was visualised by SEM and was shown in Figure 3. At low HSA level, the particles were spherical in shape with minor ridges on the surface. As HSA level increased to 35% w/w, the particles transformed from spherical to irregular shape with wrinkled surface. It is hypothesised that the wrinkles reduced the effective surface area between particles for interparticle interactions, thus reducing particle aggregation and promoting powder dispersibility.

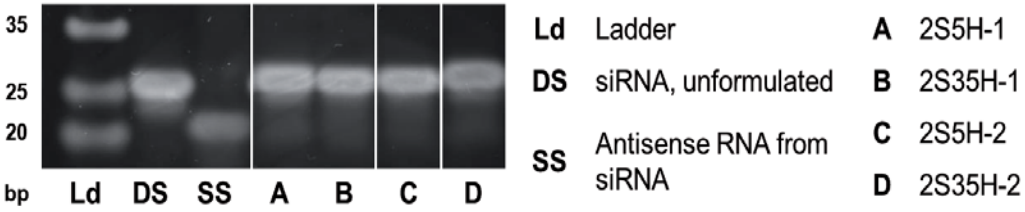


**Figure 2 – Gel retardation assay (non-denaturing 15% PAGE) of reconstituted samples after spray drying. DS and SS represent unformulated siRNA and the antisense RNA (a single strand RNA) from siRNA as control, respectively. The gel image was sliced and reordered from the same retardation assay.**

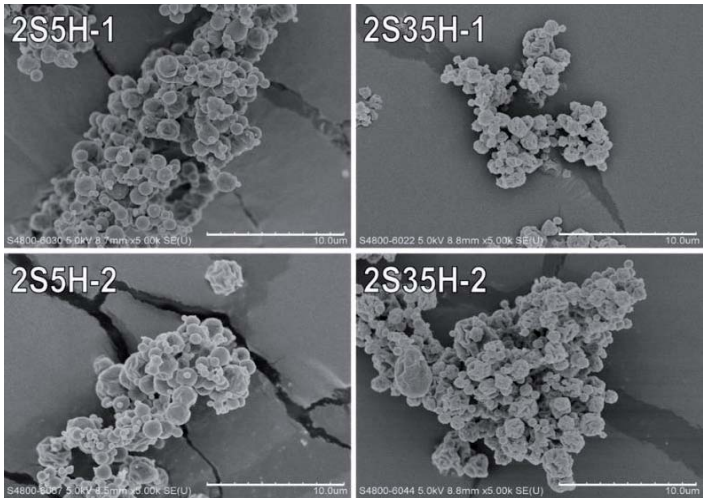


**Figure 3 – Scanning electron microscope images of the formulations at 5,000× magnification. Scale bar equals 10 µm.**

The 10th, 50th (median) and 90th percentile of the particle size distribution is shown in Table 3. Overall, all the powder formulations prepared had a median diameter between 1.5 to 2.2 µm, and all their 90th percentile were under 4 µm. It was noticed that particles prepared from feed solution with higher feed solute concentration were larger in size, because a higher solute concentration implied a higher solid content and thus a larger particle for droplets with the same volume. The span measured the dispersity in size distribution and is defined as $(D_{90} – D_{10}) / D_{50}$. All four formulations exhibited a narrow distribution as indicated by the small span value.

**Table 3 – Production yield and particle size distribution of the powder formulations (unit: µm). Values in brackets refer to standard deviation. *n* = 3**

| Sample | Yield (%) | $D_{10}$ | $D_{50}$ | $D_{90}$ | Span |
|---|---|---|---|---|---|
| 2S5H-1 | 65.0 | 0.93 (0.008) | 1.77 (0.023) | 3.20 (0.041) | 1.29 (0.003) |
| 2S35H-1 | 46.5 | 0.77 (0.012) | 1.52 (0.002) | 2.80 (0.024) | 1.34 (0.022) |
| 2S5H-2 | 68.2 | 0.96 (0.002) | 2.00 (0.017) | 3.78 (0.030) | 1.41 (0.005) |
| 2S35H-2 | 69.0 | 1.05 (0.003) | 2.17 (0.011) | 3.99 (0.018) | 1.35 (0.004) |

## Conclusions

The present study investigated the use of HSA as alternative dispersion enhancer to improve the aerosol performance of (naked) siRNA spray dried powders for inhalation. Compared to previous reports, where a substantial amount of leucine was used, it was shown here that powders with higher FPF could be achieved with HSA despite a lower amount was employed. The best performing formulation was 2S35H-1, with FPF value of 68.9%. Future study direction includes further increasing siRNA load in the formulations to clinical relevant levels, demonstrating bioactivity of siRNA using *in vitro* or *in vivo* transfection experiment, and long term stability of the formulations.

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