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Development of inhalable dry powder formulation of basic fibroblast growth factor

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ABSTRACT

Basic fibroblast growth factor (bFGF) is a promising agent for therapy of asthma or chronic obstructive pulmonary disease. We aim to develop an inhalable powder formulation of bFGF, which may provide a safe, effective, and convenient way of delivering bFGF to the disease-ridden lungs. Development of a bFGF dry powder formulation is constrained by the poor stability of bFGF and the uncertainty in compatibility of the protein with carrier excipients. With these constraints in mind, we prepared dry powders containing bFGF in combinations of albumin, phospholipid, lactose, and/or leucine, by spray drying, and evaluated the aerodynamic properties of the powders and the stability of bFGF loaded in the powders. While an ethanolic solution of phospholipid, albumin, and lactose produced dispersible powder, bFGF was unstable in ethanol. The stability of bFGF was preserved when spray-dried with lactose in an aqueous solution. Leucine was required to obtain dry powder with good dispersibility; however, increase in the leucine content more than 50% (w/w) negatively influenced the bFGF stability with no additional benefit to the aerodynamic properties of the powders. Dry powders containing 20% (w/w) leucine provided desirable aerodynamic properties (fine particle fraction of $25.2 \pm 5.4\%$ and mass median aerodynamic diameter of $4.7 \pm 0.9\ \mu m$) and $98.1 \pm 7\%$ recovery of bioactive bFGF. This result warrants further investigation of the biological activity of the inhaled bFGF in a disease model.

1. Introduction

Asthma is a chronic inflammatory disorder of the airways, whose severity and prevalence have significantly increased in recent decades (Wills-Karp et al., 1998). Allergic asthma is characterized by the airway hyper-responsiveness to various stimuli and excessive mucus production (Wills-Karp et al., 1998), and remodeling of the airway epithelium, which lead to varying degrees of airway obstruction (Holgate, 2002). Chronic obstructive pulmonary disease (COPD) is another obstructive lung disease, which involves chronic inflammation resulting in irreversible and progressive destruction of lung parenchyma and contraction of small airways (Barnes Peter and Kleinert, 2004). Due to the continuously increasing mortality, COPD is recognized as a major global health problem (Barnes Peter and Kleinert, 2004).

Conventional therapy for asthma and COPD is based on anti-inflammatory drugs and bronchodilators, such as corticosteroids (Osterman et al., 1997), leukotriene modifiers (Fanta, 2009), cromolyn (Fanta, 2009; Pleasants, 2006), β-adrenergic agonists (Pleasants, 2006; Fanta, 2009; Nathan, 1992), and anti-cholinergic agents (Pleasants, 2006). These drugs are typically administrated either systemically as oral dosage forms (Pleasants, 2006) or locally as inhalable dosage forms (Nathan, 1992), such as pressurized metered-dose inhalers (Pleasants, 2006; Fanta, 2009), dry powder inhalers (Osterman et al., 1997), or nebulizers (Fanta, 2009; Pleasants, 2006).

On the other hand, recent studies suggest beneficial effects of recombinant basic fibroblast growth factor (bFGF) for therapy of the obstructive lung diseases (Jeon et al., 2007; Morino et al., 2005). bFGF is one of the key growth factors that modulate lung morphogenesis, playing roles in proliferation and/or migration of vascular endothelial and smooth muscle cells, fibroblasts, and airway epithelial cells (Jeon et al., 2007; Nugent and Iozzo, 2000). bFGF treatment inhibited airway hyper-responsiveness, mucus production, and lung inflammation in an asthma mouse model (Jeon et al., 2007). Intratracheal administration of a controlled release formulation of bFGF ameliorated emphysema and improved the pulmonary gas exchange in a beagle dog model (Morino et al., 2005).

Given the potential therapeutic effects on the pulmonary functions of the bFGF, one may expect that an inhalable formulation

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Table 1
Compositions and properties of dry powders.

Code	Excipients (%)	Solvent	ED[a] (%)	FPF[b] of nominal dose (%)	MMAD (μm)	Yield[c] (%)	bFGF recovery[d] (%)
SD-1	DPPC, albumin, lactose (60, 20, 20)	70% ethanol	92.9 ± 4.7	34.3 ± 10.4	4.0 ± 0.3	10.8 ± 2.5	Not available
SD-2	DPPC, lactose (60, 40)	70% ethanol	85.9 ± 13.2	33.1 ± 7.0	4.6 ± 1.0	17.3 ± 3.1	41.7 (n = 1)
SD-3	Lactose (100)	Water	67.5 ± 8.3	14.2 ± 2.0	6.1 ± 0.2	<5	100.16 ± 6
SD-4	Lactose, leucine (80, 20)	Water	63.7 ± 5.7	25.2 ± 5.4	4.7 ± 0.9	15.0 ± 2.6	98.06 ± 7
SD-5	Lactose, leucine (50, 50)	Water	82.9 ± 5.3	22.7 ± 2.1	5.8 ± 0.1	24.5 ± 4.8	69.0 ± 6.6
SD-6	Leucine (100)	Water	92.1 ± 6.6	30.2 ± 6.7	5.3 ± 0.5	11.3 ± 2.5	44.0 ± 5.9

Data are expressed as averages and standard deviations of three independent batches unless specified otherwise.
[a] ED (Emitted dose): total powder mass exiting the inhaler.
[b] FPF (Fine particle fraction) of nominal dose: amount of powder with an aerodynamic size <4.7 μm (particles deposited at stage 3 and lower) divided by the initial total powder loaded in the Rotahaler (nominal dose: 10 mg).
[c] Yield = total powder recovery/total material input.
[d] bFGF recovery (%) = amount of recovered bFGF/amount of bFGF expected to be loaded in the F/SD-# powder.

would be an ideal way of delivering bFGF. First, it can deliver high drug concentrations directly to the disease site, thereby minimizing the risk of systemic side effects. Second, inhalable dosage forms may allow bypassing common challenges in oral administration of protein drugs, such as poor gastrointestinal absorption and the first-pass metabolism in the liver (Patton and Byron, 2007). Moreover, inhalable drugs are relatively convenient and well received by the patients as compared to other parenteral dosage forms (Brunton, 2008).

The objective of this study is to develop an inhalable powder formulation of bFGF. The main challenge in developing an inhalable formulation of bFGF is the requirement for carrier excipients that satisfy two distinct functions. Ideally, the carrier excipients should provide desirable aerodynamic properties and ensure that bFGF remains functionally and structurally intact during the formulation process and storage. Here, we used the spray-drying method to prepare inhalable dry powders containing bFGF and studied the effects of carrier excipients on the aerodynamic properties of the dry powder and the integrity of bFGF. The results of our study show that, albeit the poor stability profile (Caccia et al., 1992; Yu et al., 2007), bFGF can be successfully formulated as an inhalable powder when the carrier excipients are judiciously selected.

2. Materials and methods

2.1. Materials

Basic fibroblast growth factor (bFGF, DA-3201) was a gift of Dong-A Pharmaceutical Co., Ltd. (Yongin-si, Korea). Human serum albumin (96–99%) was obtained from Sigma. Lactose monohydrate was purchased from Mallinckrodt (Paris, Kentucky, USA), leucine (L-form, 99%) from Alfa Aesar (Ward Hill, MA, USA), and dipalmitoylphosphatidylcholine (DPPC) from Lipoid GmbH (Ludwigshafen, Germany). Dulbecco's modified Eagle's medium (DMEM), calf serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Production of spray-dried powders

Dry powders composed of excipients listed in Table 1 were produced by the LabPlant SD-05 spray dryer (Lab-Plant Ltd., Huddersfield, UK). Combinations of excipients were dissolved in 70% ethanol as a 0.2% (w/v) solution or in water as a 1% (w/v) solution. When ethanol was used, aqueous components (bFGF, albumin, or lactose) were dissolved in deionized water, DPPC in 95% ethanol, and the two solutions were mixed prior to spray drying. The solution was constantly stirred at 40 °C throughout the spray-drying process. For the water-based spray drying, all the components were dissolved in water and directly spray-dried. The solution was introduced to the spray-dryer at 17 mL/min (ethanol solution) or 4–6 mL/min (aqueous solution) and atomized through a 1-mm nozzle using compressed air. The inlet temperature was 150 °C. When bFGF was loaded in each platform powder, the protein was added to 3–4% (w/w) of the excipients, and the resulting powder was named as "F/SD-#," in which SD-# indicates the formulations in Table 1.

2.3. Anderson cascade impactor

Eight-stage Mark II Anderson Cascade Impactor (ACI) was used to evaluate the dry powder deposition in vitro. The dry powder (10 mg) was manually loaded in a hard gelatin capsule (size 3), put in a Rotahaler, and split-open to release the particles. Each set of dry powders was drawn through the ACI operated at a flow rate of 28.3 L/min for 10 s. The amount of particles deposited at each impaction stage was determined by measuring the difference in weight of the collection plate (for the filter stage, glass filter with pore size <1 μm, ThermoFisher). The effective cutoff aerodynamic diameters for each stage were: Stage 0, 9 μm; Stage 1, 5.8 μm; Stage 2, 4.7 μm; Stage 3, 3.3 μm; Stage 4, 2.1 μm; Stage 5, 1.1 μm; Stage 6, 0.65 μm; and Stage 7, 0.43 μm. The emitted dose (ED) was defined as the total powder mass exiting the inhaler. The fine particle fraction (FPF) was defined as the amount of powder with an aerodynamic size <4.7 μm (particles deposited at stage 3 and lower) divided by the initial total powder loaded in the Rotahaler (10 mg: nominal dose). The cumulative mass of powder less than effective cutoff diameter as percent of total mass recovered in the ACI was plotted against the effective cutoff diameter. The mass median aerodynamic diameter (MMAD) was defined on this graph as the particle size at which the line crossed the 50th percentile.

2.4. Scanning electron microscopy

The morphology of the prepared dry powder was examined using scanning electron microscopy. Dry powders were attached to specimen stubs using double-sided tape and sputter-coated with gold–palladium in the presence of argon gas using a Hummer I sputter coater (Anatech Ltd.). Dry powders were imaged with a JEOL JSM-840 scanning electron microscope (JEOL USA, Inc.) using a 5 kV accelerating voltage, a 10 mm working distance, a 70 μm objective aperture, and a probe current of 6×10^{-11} A.

2.5. Moisture content in dry powder

The moisture content in the dry powder was measured by the Karl Fischer titration method (Brinkmann–Metrohm Karl Fischer Coulometer). Samples were prepared as described in the literature (Zhou et al., 1998). Briefly, dry powder was accurately weighed and placed in a dry plastic tube. A portion of the Karl-Fischer reagent was withdrawn from the titration vessel using a dry syringe and added to the tube to resuspend the dry powder. The sample suspension was then returned to the titration vessel to measure the

water content. The data was presented as a weight percent of water in the powder.

2.6. Analysis of bFGF recovered from dry powder

To evaluate the stability of bFGF loaded in the dry powder, a known quantity of spray-dried powder was reconstituted in phosphate buffered saline (PBS, pH 7.4). The reconstituted solution was analyzed using reverse-phase high pressure liquid chromatography (RP-HPLC) and sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. When the protein recovery was incomplete, the dry powder was reconstituted in 5 M guanidine·HCl to disrupt potential hydrophobic interactions between proteins and excipients (Dunbar et al., 1997; Bhuyan, 2002) and compare with the recovery in PBS. RP-HPLC was conducted using an Agilent HPLC 1100 (USA), according to a condition in the literature (Astafieva et al., 1996) with slight modification (column: Vydac 214 TP C4 54; mobile phase: mixture of acetonitrile and water containing 0.01% trifloroacetic acid (linear gradient from 20:80 to 80:20, v/v, over 60 min); flow rate: 1 mL/min; detection: 220 nm; retention time: 21.8 min). SDS-PAGE was performed with samples prepared in a non-reducing condition (i.e., without using β-mercaptuethanol). The gels were stained with the silver staining kit (BioRad). Size exclusion chromatography was performed to compare with the SDS-PAGE (column: TSK G3000SWXL (300 mm × 7.8 mm); mobile phase: 100 mM sodium phosphate (pH 6.0) and 1 M NaCl; flow rate: 0.75 mL/min; detection: 220 nm; retention time: 15.2 min).

Bioactivity of the bFGF recovered from the dry powder (F/SD-4) was evaluated by the mitogenic activity assay according to a method in the literature (Wang et al., 2006) with modifications in cell density and the incubation time. Murine NIH/3T3 fibroblasts were seeded in 96-well plates (25,000 cells per cm^2) (7.5×10^3 per well) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. After 12 h, the medium was replaced with fresh DMEM with 0.4% calf serum. After 24 h of starvation, bFGF recovered from the F/SD-4 powder, whose concentrations were determined by RP-HPLC, was added to the medium to a final bFGF concentration of 10 or 50 ng/mL. For comparison, separate groups of cells were treated with equivalent amounts of reference bFGF, heat-denatured bFGF (containing the same concentration of bFGF as reference bFGF; boiled to complete evaporation of water followed by reconstitution in PBS), or a solution of SD-4 powder (blank dry powder omitting bFGF). As negative and positive controls, cells were treated with PBS or supplemented with 10% serum, respectively. The cell number was estimated by the MTT assay after 48 h of incubation. The formazan absorbance at 570 nm of each treatment group was normalized to the absorbance of cells grown in 10% serum.

2.7. Statistical analysis

ED, FPF, and MMAD were expressed as averages with standard deviations of 3 independent batches. One-way ANOVA was used to determine statistical difference among the groups, and pair-wise comparison was made using the Student *t*-test. A *p*-value of <0.05 on a two-tailed test was considered statistically significant.

3. Results and discussion

bFGF is a single-chain non-glycosylated protein made of 155 amino acids, with an isoelectric point at pH 9.6 and a molecular weight of 17.2 kDa (Nugent and Iozzo, 2000). Recent studies show that the bFGF has a positive effect in ameliorating symptoms of asthma (Jeon et al., 2007) and COPD (Morino et al., 2005). Therefore, it was envisioned that an inhalable dosage form of bFGF would provide an effective way of treating asthma and COPD locally. Among different types of inhalable dosage forms, a dry powder formulation was chosen, as it is portable, environmentally friendly, and relatively easy to use (Newman, 2004). However, the development of a dry powder formulation of bFGF is constrained by the relatively poor stability of bFGF and the uncertainty in its compatibility with carrier excipients. First, bFGF has four cysteine residues, which are highly reactive under oxidative conditions (Caccia et al., 1992). Its stability is also negatively affected by heat, organic solvent, and pH (Yu et al., 2007). Second, since the anticipated bFGF dose per inhalation is in the microgram range (Jeon et al., 2007), the use of inactive excipients is inevitable. The inactive excipients should satisfy two distinct requirements simultaneously: they should not only be able to provide desirable aerodynamic properties but also be compatible with bFGF. While there are a few known excipients suitable for formation of inhalable dry powders, such as disaccharides (Qian et al., 2009), phospholipids (Codrons et al., 2003), amino acids (Lucas et al., 1999; Li et al., 2003; Seville et al., 2007), or proteins (Codrons et al., 2003), it is unknown whether bFGF is stable in their presence, especially in the solid state.

With these constraints in mind, we prepared spray-dried powders containing bFGF in combinations of excipients, which have been used for inhalable dry powder formulations. Subsequently, we evaluated aerodynamic properties of the dry powder and stability of bFGF loaded in the powder. Here, the MMAD was aimed to be in the range of 0.5–5 μm (Lipworth, 2000), and the ED and FPF of the nominal dose to be comparable to those of "large porous particles" introduced by Edwards et al. (Ben-Jebria et al., 1999).

As a dry powder platform to load bFGF, we first chose a dry powder formulation with the known aerodynamic performance. Edwards et al. reported that highly dispersible microparticles can be prepared by spray drying a combination of phospholipid (e.g., DPPC), albumin, and lactose (Ben-Jebria et al., 1999; Bosquillon et al., 2001, 2004; Codrons et al., 2003). In this formulation, each component uniquely contributes to the aerodynamic properties of the resulting powder. For example, DPPC confers a sponge-like shape on the particles, lactose makes smaller particles, whereas albumin makes the particles lighter (Ben-Jebria et al., 1999) and less cohesive (Bosquillon et al., 2001). Moreover, these components are either approved by the U.S. Food and Drug Administration for inhalation (lactose) or endogenous to the lung (albumin, DPPC). Dry powders made of albumin, lactose, and DPPC (20/20/60, w/w/w) have high porosity and large size, providing a desirable MMAD in the range of 1–3 μm (Ben-Jebria et al., 1999; Bosquillon et al., 2001). For these reasons, the albumin/lactose/DPPC formulation has been exploited for inhalational delivery of a variety of drugs such as antibiotics (Tsifansky et al., 2008), albuterol (Ben-Jebria et al., 1999), a parathyroid hormone (Codrons et al., 2003), or a growth hormone (Bosquillon et al., 2004). To take advantage of the known effects of those excipients on aerodynamic properties of dry powders, we also produced dry powders consisting of albumin, lactose, and DPPC (SD-1) or lactose and DPPC (SD-2) by spray drying. In our hands, there was no significant difference between SD-1 and SD-2 in aerodynamic properties (Table 1; ED, FPF, and MMAD: $p > 0.05$ by *t*-test) and shape (Fig. 1). We thus opted to omit albumin in producing dry powder of bFGF, to avoid potential interactions between the two oppositely charged proteins at pH 7.4.

bFGF was loaded in SD-2, and the resulting powder (F/SD-2) was reconstituted in PBS to evaluate the bFGF stability. F/SD-2 formed a suspension when reconstituted in PBS due to the presence of DPPC. Interestingly, bFGF was not completely recovered from the F/SD-2 powder even after 3 days. Only 41.7% of the loaded bFGF was recovered from the F/SD-2 powder after 1 h of incubation in PBS, and no further protein recovery followed. It was speculated that three conditions might have negatively influenced the bFGF sta-

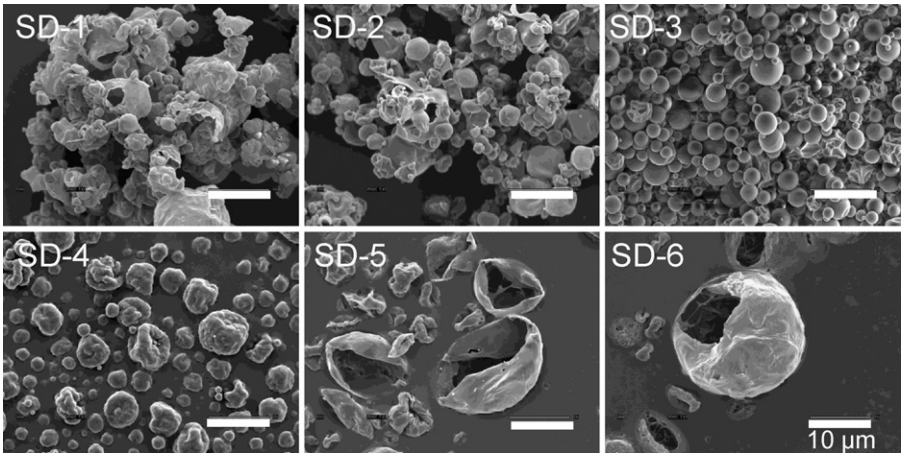

Fig. 1. Scanning electron micrographs of spray-dried powders. Magnification: 2500×. Scale bar = 10 μm.

bility leading to incomplete bFGF recovery: (i) DPPC, which may facilitate bFGF aggregation via hydrophobic interactions, (ii) the use of ethanol (included to dissolve DPPC and to facilitate evaporation of the spray) in preparation of the feed solution, and (iii) heating during the spray drying. To test this hypothesis, the F/SD-2 powder was incubated in 5 M guanidine·HCl, which disrupts non-covalent protein aggregation (Dunbar et al., 1997; Park et al., 1998), and compared with the same quantity of powder incubated in PBS using SDS-PAGE. As shown in Fig. 2, a significantly higher amount of bFGF was released in guanidine·HCl, verifying that the incomplete bFGF recovery was attributable to the protein aggregation. On the other hand, a powder omitting DPPC (i.e., a powder consisting of lactose and bFGF only, but prepared in 70% ethanol) was still unable to release bFGF in PBS completely (data not shown), suggesting that DPPC may not be the main reason for the incomplete release. Subsequently, we tested the stability of bFGF in 70% ethanol solution. A concentrated bFGF solution was diluted with 70% ethanol and immediately analyzed with RP-HPLC. Only 41% of the expected bFGF was detected by RP-HPLC. This result indicates that bFGF was unstable in 70% ethanol; thus, the feed solution should not be prepared in 70% ethanol. Finally, we tested the effect of heating by exposing an aqueous solution of bFGF and lactose to 150 °C (equivalent to the inlet temperature of spray drying) for 5 min or spray drying the solution and evaluating bFGF recovery from the resulting powder (F/SD-3) by RP-HPLC. While no bFGF was detected in the directly heated aqueous solution, the F/SD-3 powder reconstituted in PBS yielded 100.2 ± 6% (n = 3) recovery of the expected bFGF (Fig. 3). This result suggests that, despite the temperature-sensitivity of bFGF, the heat generated during the spray-drying process did not have a negative effect on the bFGF stability. This is because the actual heat to which the protein is exposed during the spray drying is much lower than the air temperature due to the concurrent solvent evaporation (Ameri and Maa, 2006). This result agrees with the earlier studies by Maa and Hsu (1996, 1997a,b) and Maa et al. (1996), which showed that the inlet temperature during spray drying did not significantly compromise the activity of the spray-dried drugs.

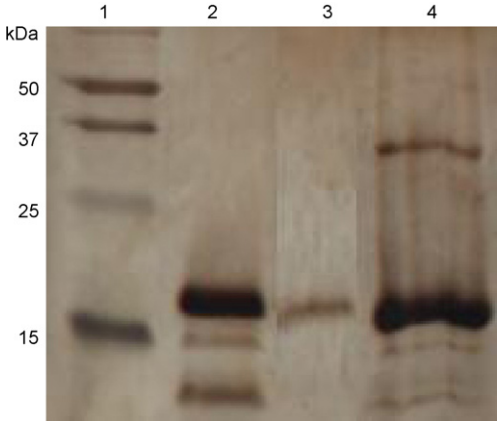

Fig. 2. Effect of guanidine·HCl on bFGF recovery from the powder of bFGF loaded in SD-2 (F/SD-2). Lane 1: Size marker; lane 2: reference bFGF (10 μg); lane 3: F/SD-2 powder (equivalent to 10 μg of bFGF) reconstituted in PBS; lane 4: F/SD-2 powder (equivalent to 10 μg of bFGF) reconstituted in 5 M guanidine·HCl.

Since ethanol had the main detrimental effect on bFGF stability, we explored alternative combinations of excipients, which could be prepared in an aqueous solution and would provide a dry powder with desirable aerodynamic properties when spray-dried. First, the dry powder was prepared using an aqueous solution of lactose (SD-3), in which bFGF survived the spray-drying process (Fig. 3). The aerodynamic properties of the SD-3 powder were far inferior to those of SD-1 (Table 1 and Fig. 4) (ED: $p < 0.01$; FPF: $p < 0.05$; and MMAD: $p < 0.01$ by t-test). Such difference can be related to the shape of the powders. Fig. 1 shows that SD-3 powders are relatively small and dense, which tend to be more prone to aggregation (Edwards et al., 1997), as compared to the SD-1 (large porous particles). The different particle shape is attributable to the composition of the SD-3 feed solution (aqueous solution of lactose), resulting in a relatively low Peclet number of the solute. The Peclet number is defined as: $P_e = \kappa/8D$, where κ is an evaporation rate of the spray droplets and D is a diffusion coefficient of the solute(s) (Vehring, 2008). Lactose in the SD-3 droplets are likely to have lower Peclet number than the components in SD-1 and SD-2 droplets, because κ of an aqueous solution is lower than that of 70% ethanol, and D (i.e., mobility of small molecular weight lactose) is higher than that of DPPC and/or albumin. Low Peclet number favors formation of dense spherical particles, while high Peclet number results in particles with shells that may deform in various ways (Vehring, 2008).

In an attempt to improve the aerodynamic properties of the powder, leucine was included in the formulation. Leucine has been frequently used in inhalable powders of a broad range of bioactive molecules, such as plasmid DNA (Li et al., 2003, 2005), salbutamol (Seville et al., 2007), and disodium cromoglycate (Chew et al., 2005), to increase dispersibility of the powder. In our study, the addition of leucine by 20% (w/w) (SD-4) enhanced aerodynamic proprieties of the powder significantly as compared to SD-3, resulting in FPF of 25.2 ± 5.4% ($p < 0.05$ by t-test; n = 3) and MMAD of 4.7 ± 0.9 μm ($p = 0.056$ by t-test; n = 3) (Table 1 and Fig. 4). Although ED was less than SD-1 ($p < 0.01$ by t-test), FPF (of nominal dose) and MMAD

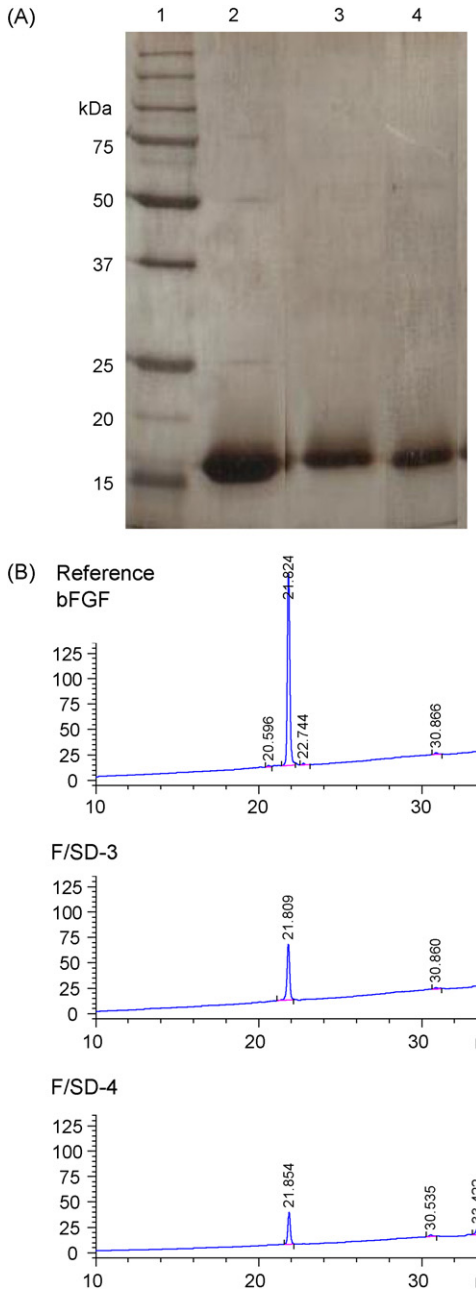

Fig. 3. (A) SDS-PAGE of bFGF recovered from spray-dried powders. Lane 1: Size marker; lane 2: reference bFGF (20 μg); lanes 3 and 4: F/SD-3 and F/SD-4 powder (equivalent to 5 μg of bFGF) reconstituted in PBS. (B) RP-HPLC chromatograms of F/SD-3 and F/SD-4 reconstituted in PBS. Note that concentrations of bFGF in the reference bFGF, F/SD-3, and F/SD-4 were 0.88, 0.35, and 0.16 mg/mL, respectively.

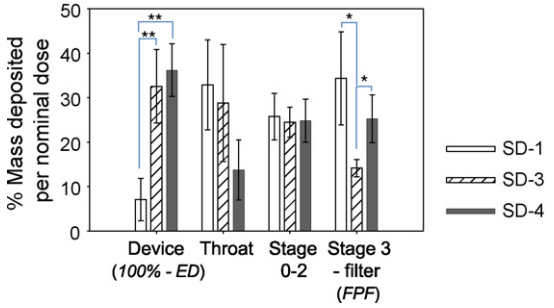

Fig. 4. ACI deposition profiles of SD-1, SD-3, and SD-4. Data are expressed as averages and standard deviations of three independent batches. $^{*}p < 0.05$, $^{**}p < 0.01$ by *t*-test.

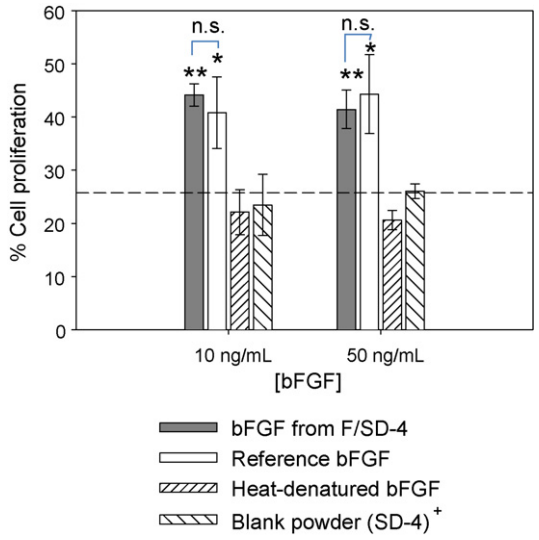

Fig. 5. Mitogenic activity of bFGF recovered from the F/SD-4. Blank powder† is a solution of SD-4 powder, which does not contain bFGF but the same amount of excipients (leucine and lactose) as the F/SD-4 powder. Percentage of cell proliferation = 100 × absorbance for cells grown with each treatment group/absorbance for cells grown in 10% serum. The dotted line indicates an average value for the cells treated with PBS after starvation. Data are expressed as averages and standard deviations of three measurements. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. PBS-treated cells, by *t*-test; n.s.: not significant.

were comparable to those of SD-1 (both $p > 0.05$ by *t*-test). Unlike F/SD-2 containing DPPC, F/SD-4 was completely dissolved in PBS and formed a solution. Importantly, bFGF loaded in SD-4 (F/SD-4) was completely recovered from the powder (98.1 ± 7%, $n = 3$), indicating that bFGF was compatible with lactose and leucine at this level. Structural integrity of bFGF recovered from the F/SD-4 was examined by comparing the recovered bFGF and reference bFGF in SDS-PAGE and RP-HPLC. As shown in Fig. 3, there was no significant difference between the recovered bFGF and reference bFGF in both SDS-PAGE and RP-HPLC. It is noted that the samples for the SDS-PAGE were prepared with a non-reducing sample buffer (without β-mercaptoethanol) to detect disulfide-crosslinked aggregates. The lack of aggregates in SDS-PAGE (lane 4 in Fig. 3) indicates that the bFGF in the F/SD-4 did not undergo thiol oxidation. On the size-exclusion chromatography, a small peak corresponding to dimeric bFGF was visible (data not shown), but it did not appear to influence the biological activity of bFGF. The biological activity of the spray-dried bFGF was verified by the mitogenic activity assay (Fig. 5). The spray-dried bFGF (F/SD-4) showed comparable mitogenic activity for the NIH/3T3 fibroblasts to that of reference bFGF at both concentration levels, while the heat-denatured bFGF or blank SD-4 powder (excipients only) did not show any activity at equivalent concentrations.

The effect of leucine on the aerodynamic properties is often attributed to its surfactant-like properties, which reduce the surface free energy of the dry powder and cohesive inter-particulate interactions (Chew et al., 2005; Shur et al., 2008). Alternatively (or additionally), it is thought that the hydrophobicity of leucine contributes to enhancing dispersibility of the powder (Vehring, 2008). Here, leucine confers a crumpled morphology to the powder by forming the leucine-rich surface, which cannot move as fast as receding droplets during spray drying (Vehring, 2008). In accordance with the latter explanation, the leucine-containing powder (SD-4) had irregular shape and rough surface, as opposed to the smooth and spherical lactose-only powder (SD-3), as shown in Fig. 1.

On the other hand, further increase in the leucine content (SD-5 and SD-6) did not bring about significant enhancement either

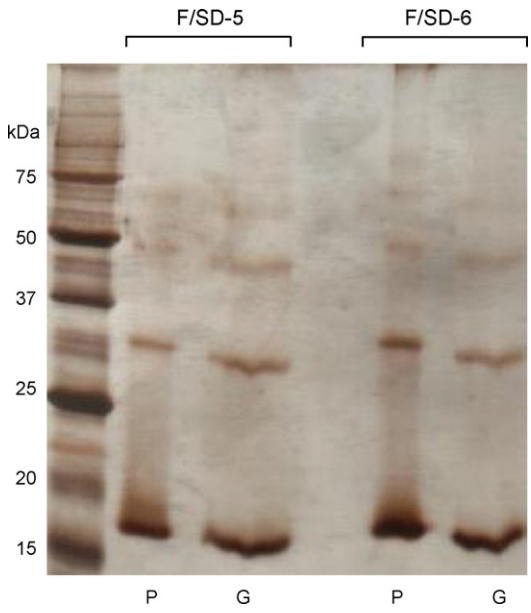

Fig. 6. SDS-PAGE of F/SD-5 and F/SD-6 reconstituted in PBS (P) or in 5 M guanidine·HCl (G). Both powders were equivalent to 4 μg of bFGF.

in FPF or MMAD as compared to SD-4 (Both $p > 0.05$ by ANOVA; $n = 3$). Interestingly, increase in the leucine content influenced the bFGF stability rather negatively. When bFGF was loaded in SD-5 or SD-6, the total bFGF recovery was far less than expected (Table 1). The presence of dimeric bFGF in the powder solution in PBS (Fig. 6) suggests that the incomplete release of bFGF from F/SD-5 and F/SD-6 was due to the protein aggregation mediated by the excessive leucine. On the other hand, unlike the F/SD-2 powder (Fig. 2), the protein release from F/SD-5 or F/SD-6 was not noticeably enhanced by guanidine·HCl, indicating that the protein aggregation due to the excessive leucine should be attributed to other unknown mechanisms than non-covalent aggregation. These results indicate that addition of an optimal amount of leucine is critical to achieving both desirable aerodynamic properties and stability of bFGF loaded in the powder. While the addition of leucine led to formation of increasingly bigger and hollow powders in proportion to the content, excessive leucine rather decreased the bFGF stability with no additional benefit to the aerodynamic properties of the resulting powders.

Not only was the SD-4 optimal for bFGF formulation with respect to the protein stability and aerodynamic properties, the SD-4 powder platform also showed an adequate moisture content, which critically influences the protein stability and powder performance. The moisture content in the SD-4 powder was $2.4 \pm 0.4\%$ (w/w), within the moisture level (1–3%, w/w) considered adequate for dry biological products (May et al., 1992; Towns, 1995). The moisture content did not significantly change after 2-week storage in dry atmosphere at room temperature ($2.9 \pm 0.6\%$, w/w, $p > 0.05$ vs. $2.4 \pm 0.4\%$, w/w). Given that the moisture content further decreased with the increase in the leucine content (SD-5: $1.6 \pm 0.4\%$, w/w; SD-6: $0.6 \pm 0.5\%$, w/w), the ability to maintain the low moisture content would be attributable to the hydrophobicity of leucine.

In addition, the SD-4 powder could be produced with a yield comparable to that of the SD-1 (SD-1: $10.8 \pm 2.5\%$ vs. SD-4: $15.0 \pm 2.6\%$; $p > 0.05$), the benchmark powder in this study. While the overall yields reported here are relatively low (although not unusual for the lab-scale spray-drying process), this result does not preclude that the yield can be improved with different equipment and at different scales.

4. Conclusions

bFGF was successfully loaded in inhalable dry powders maintaining its integrity. In achieving a stable bFGF formulation, it was necessary to use water-soluble excipients and spray-dry as an aqueous solution. The aerodynamic properties of the powder were significantly improved by addition of 20% (w/w) leucine. This result warrants further investigation of biological activity of inhaled bFGF in a disease model.

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