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The Development of Thin-film Freezing and Its Application to Improve Delivery of Biologics as Dry Powder Aerosols †

Stephanie Hufnagel, Sawittree Sahakijpijarn, Chaeho Moon, Zhengrong Cui and Robert O. Williams III *

Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy, The University of Texas at Austin, USA

Abstract

While the formulation of pharmaceuticals as liquids is common practice, powders are associated with enhanced stability, avoidance of the cold chain, lower dosing requirements, and more convenient administration. These are particularly critical for proteins, as they are expensive and complicated to manufacture. Powders also have improved aerosol properties for pulmonary delivery. Conventional techniques for formulating powders include spray-drying, shelf freeze-drying, spray freeze-drying, and spray freezing into liquid, but they produce powders with poor aerosol performance and/or activity due to suboptimal powder properties. Thin-film freezing (TFF) is a new cryogenic technique that can engineer highly porous, brittle, powder matrices with excellent aerosol performance properties and stability. Herein, we describe TFF in comparison to other cryogenic techniques. Physical properties of TFF powders such as morphology, moisture sorption, stability, solubility, and dissolution, as well as aerosol properties are discussed. In addition, factors that significantly affect the physical and aerosol properties of dry powders prepared by TFF, such as solids content, drug loading, solvent system, excipient, and dry powder delivery device, are analyzed. Finally, we provide evidence supporting the applicability of using TFF to prepare dry powder formulations of protein-based pharmaceuticals, enabling their cold chain-free storage as well as efficient pulmonary delivery.

Keywords: thin-film freezing, dry powder, cryogenic technique, protein, pulmonary delivery

1. Introduction

Therapeutics intended for pulmonary delivery via inhalation are supplied as liquids or powders. While a majority of them are developed as liquids, the preparation of drugs into powders affords several benefits, particularly for proteins and other biologics. Pharmaceutical dry powders are generally associated with enhanced stability, avoidance of the cold chain, improved aerosol properties, lower dosing requirements, and more convenient administration (Johnson, 1997). In particular, expensive biologics can benefit from being prepared as powders, as the dry powder state typically extends the shelf life of biologics and reduces the cost burden of their transportation and storage. Spray-drying (SD) and conventional shelf freeze-drying (shelf FD), as well as spray freeze-drying (SFD) and spray freezing into liquid (SFL) are techniques commonly employed to prepare powders. Unfortunately, in the context of biologics, all techniques cause aggregation, denaturation, and/or loss of activity of the biologics to some degree. In addition, these methods produce powders with low surface area, low yield, and/or a broad particle size distribution (Engstrom et al., 2008), which are not ideal for pulmonary delivery by inhalation, particularly of biologics.

Previously, we reported thin-film freezing (TFF) technology for pharmaceutical applications that can be applied to generate highly porous, brittle, powder matrices with excellent aerosol performance properties (Overhoff et al., 2007a). The resultant dry powder, upon sublimation of the solvent in the frozen thin films, is ideal for pulmonary delivery by inhalation. We have validated that the TFF technology is directly applicable to inhaled delivery of various small and large molecules including proteins, such as cytokines, enzymes (Engstrom et al., 2008), and more recently, antibodies. Currently, many therapeutics, including most biologics, for pulmonary delivery are administered by nebulization of a liquid. Unfortunately, nebulization exposes therapeutics, especially biologics such as proteins, to stressful conditions that can damage them from shear or elevated temperatures applied during delivery. In addition to the aforementioned limitations of liquids, the solution

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* Corresponding author: Robert O. Williams III;
Add: 2409 West University Avenue, PHR 4.214 Austin, TX 78712, USA
E-mail: bill.williams@austin.utexas.edu
TEL: +01-512-471-4681 FAX: +01-512-471-7474

for nebulization may contain excipients that are harmful to the lungs. An alternative to nebulization of a liquid is administration by dry powder inhalation, which addresses many of the limitations of nebulization. While some powders are already approved by the US FDA for inhalation, powders produced by TFF technology can minimize the flow-rate dependence of dry powder inhalers (DPIs), use of carriers (Ung et al., 2014), aggregation, denaturation, and size distribution issues of molecules while improving the surface area and yield. Overall, TFF offers an opportunity to improve powder properties and thus aerosol performance and therapeutic efficacy of therapeutics, which can be applied to biologics.

Biologic drugs continue to exist as a large proportion of FDA-approved products, with 25 % of all new chemical entities that were FDA-approved from 2015–2019 being biologics (de la Torre and Albericio, 2020). Additionally, biologics can be approved as biosimilars. Biologics are particularly challenging to formulate due to their large molecular size and sensitive nature. Unlike small molecules, biologics (e.g., proteins) must maintain primary, secondary, tertiary, and quaternary structures. They are expensive to produce and pulmonary delivery may minimize the required dose, and therefore cost, while still assuring efficacy and minimizing systemic toxicity, especially when they are delivered locally to the lung to treat pulmonary disease. For example, systemic delivery of antibodies, one of the largest and most complex biologics, overall carries a risk of side effects such as cytokine release syndrome (Guilleminault et al., 2014). Alternatively, inhaled antibodies are poorly systemically absorbed from the respiratory tract, providing an opportunity for local delivery to the lungs (Guilleminault et al., 2014). Of the thirty biologics that have undergone clinical trials for pulmonary delivery, only five have been delivered as a powder (Liang et al., 2020). Development of biologic formulations for pulmonary delivery has been reviewed in detail (Fröhlich and Salar-Behzadi, 2021; Liang et al., 2020). Herein we describe the development of the TFF technology, the process of TFF, and the general characteristics of powders prepared using TFF. Then, we discuss our experience in applying this technique to formulate protein dry powders for pulmonary delivery.

2. Thin-film freezing: the technique

2.1 The progression of cryogenic process development

Several techniques have been developed or transitioned from other industries and applied to pharmaceuticals, including SD, SFD, SFL, shelf FD, and TFF, in order to prepare powders. The latter four (i.e., SFD, SFL, shelf FD, and TFF) are considered cryogenic techniques and have the added benefit of avoidance of heat, which can directly and indirectly (e.g., evaporation of water) lead to denaturation or degradation of proteins (Overhoff et al., 2009). In cryogenic techniques, a liquid sample is frozen through the use of a cryogen and then lyophilized to remove the frozen solvent via sublimation.

Lyophilization alone, or shelf FD, is generally not sufficient to produce stable particles with high surface area (Engstrom et al., 2008) and submicron particle sizes, which are both necessary for optimal aerosol performance. Shelf FD involves placing the liquid formulation inside a lyophilizer, allowing the liquid to freeze as the shelf temperature is reduced, typically to around –40 °C to –55 °C. Afterwards, the pressure is reduced, sublimating the solvent until a powder is formed. In this method, the cooling rate is slow, around 0.017 K/s, which allows time for protein particles to grow, and achieving particles with a diameter less than a few microns and surface area greater than 1 m^2/g is difficult (Engstrom et al., 2008). The powder generated can be milled to improve the particle size and surface area, but this can be mechanically stressful for proteins and lead to heterogeneous particle sizes with limited yield. Therefore, the alternative processes SFD, SFL, and TFF have been utilized.

SFD involves spraying a liquid formulation into the gaseous vapor phase of a liquid cryogen. The cooling rate of SFD is high, about 10^6 K/s. Unfortunately, SFD has a large gas-liquid interface and shear stress, which can contribute significantly to protein aggregation (Engstrom et al., 2008). This technique produces porous powders with high surface area; however, the particle size is large (Overhoff et al., 2009). A benefit of SFD is that different atomization nozzles and spray rates can be chosen to optimize the droplet shape and size (Overhoff et al., 2009).

In order to minimize the gas-liquid interfacial area, the liquid formulation can be sprayed directly into the liquid cryogen, known as SFL. While the freezing rate is slower than that of SFD, about 10^3 K/s, SFL still adequately arrests the growth of crystals and has produced protein powders with less adsorption, aggregation, denaturation, and higher enzymatic activity than those prepared using SFD (Engstrom et al., 2008). SFL produces powders with improved activity compared to SFD and shelf FD powders due to the lower air-liquid interfacial area, slower cooling rate, and lower (but still adequately high) surface area. Generally, the slower cooling rate results in a lower surface area, which induces a lower chance of water adsorption, improving stability (Overhoff et al., 2009).

TFF was developed to find a cooling/freeze rate and surface area intermediate to that of shelf FD vs. SFD and SFL. In TFF, the liquid cryogen is filled in a rotating metal drum and a solution or suspension is dropped dropwise from above the drum, where the droplets (e.g., 2–4 mm in diameter) spread upon impact to form thin films

(~10–12 mm in diameter) that freeze at 10^2–10^3 K/s. A metal blade then releases the frozen films from the drum as it rotates, and the films are collected in a cryogen-cooled receptacle situated below the blade/drum. The frozen films are then transferred to a lyophilizer for solvent removal by sublimation. TFF is less complex than SFD and SFL because the liquid cryogen used to cool the metal drum does not need to be sterile and the cooling rate of TFF can be more easily controlled (Engstrom et al., 2008). Furthermore, spray characteristics in SFD or SFL are more complicated to control than drop characteristics during TFF. TFF allows for the processing of more concentrated, viscous protein solutions because they do not need to be atomized. TFF has close to 100 % yield while that of SFD is about 80 % (Engstrom et al., 2008). Powders produced by TFF follow a similar surface area and stability trend as SFL powders due to their similar cooling rates and relatively low air-liquid interface (Engstrom et al., 2008). TFF is a promising technique for the formulation of biologics as powders.

2.2 Modeling the TFF process

The cooling rate of cryogenic techniques is one of the main parameters that contributes to the final powder characteristics. As water freezes, liquid channels are formed between frozen molecules, with thinner channels minimizing collisions between particles (e.g., proteins), therefore preventing particle growth. During freezing, the viscosity of these channels increases, also contributing to slowed particle growth. If sugars are included as carriers, the viscosity will be further increased (Engstrom et al., 2008). Generally speaking, the slower the cooling rate, the fewer the nucleated ice domains, leading to thicker channels and more collisions and protein aggregation. A slower cooling rate also leads to phase separation, which can cause crystallization of the active ingredient (Overhoff et al., 2009). However, there is a point at which loss of protein activity can be attributed to a fast cooling rate (Overhoff et al., 2009). Therefore, an intermediate cooling rate is favorable. The rate of cooling in SFD, SFL, and TFF was measured for comparison, showing that compared to SFD and SFL, TFF had a slower rate of cooling (i.e., 2×10^2 K/s vs 3.8×10^6 and 7.2×10^3 for SFD and SFL, respectively) (Engstrom et al., 2008). These are all considerably faster than that of shelf FD (~1.7×10^{-2} K/s) (Engstrom et al., 2008). The large difference between SFD and TFF cooling rates has been explained by a two-orders-of-magnitude smaller surface area/volume ratio and 20–30 times larger film thickness of TFF compared to SFD. TFF has a slower cooling rate than SFD and SFL but the cooling rate is sufficiently high to prevent or minimize particle growth. TFF and its intermediate freezing rate may be one of the keys to its optimal powder properties. The solute or suspension concentration and processing temperature are additional variables to consider in the context of cooling rate. A high cooling rate coupled with a supersaturated solute leads to a larger number of smaller and more uniform nuclei that are tightly packed, which helps to limit crystal growth. A lower processing temperature in TFF can lead to a higher degree of supercooling, therefore a larger number of nuclei (Engstrom et al., 2007). The higher degree of supercooling and the larger number of nuclei prevent particle growth during the freezing process, generating smaller ice channels. As shown in **Fig. 1**, smaller ice channels were produced when a lysozyme/mannitol (50:50 w/w) powder (TFF MAN-lysozyme (50:50 w/w)) was manufactured at a lower temperature of –100 °C as compared to at –50 °C. Crystalline TFF voriconazole (VCZ) nanoaggregates with smaller nanoparticle size were also generated by processing at a lower temperature than at a higher temperature (Moon et al., 2019a). In contrast, amorphous TFF tacrolimus brittle matrix powder (TFF TAC) showed larger nanoaggregate structures when the processing temperature was lower (–70 °C versus –130 °C), potentially due to the lack of droplet spreading and higher contact angle at the lower temperature (Sahakijpijarn et al., 2020b).

Engstrom et al. compared the specific surface area (SSA) of lysozyme powders prepared by SFD, SFL, and TFF. At the same lysozyme concentration, the SFD powder exhibited the highest SSA, while the TFF powder had the lowest SSA (**Table 1**) (Engstrom et al., 2008). The high SSA of the SFD powder is related to the extremely rapid cooling rate of SFD. The higher cooling rate results in more

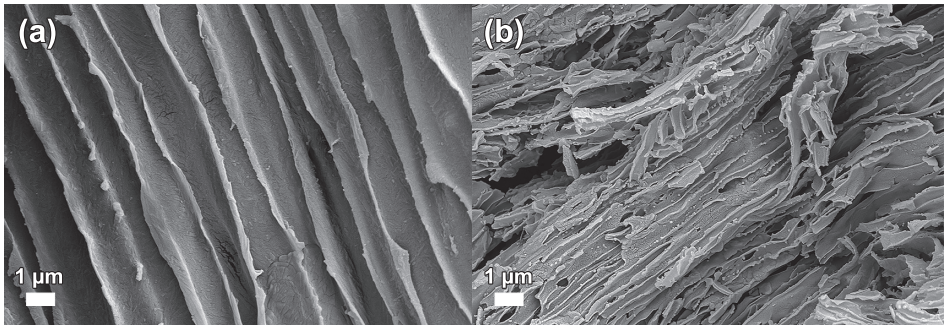

Fig. 1 Representative SEM images of TFF MAN-lysozyme (50:50 w/w) processed at **(a)** –50 °C or **(b)** –100 °C.

Table 1 Specific surface area (SSA) and bulk density of processed and unprocessed drug powders.

Formulation	Process	Specific surface area (m^2/g)	Bulk density (g/mL)	Reference
Tacrolimus unprocessed powder	Unprocessed	0.53	—	(Sinswat et al., 2008)
Tacrolimus	TFF (–70 °C)	42.22 ± 3.02	—	(Sahakijpijarn et al., 2020b)
Tacrolimus:lactose (50:50 w/w)	TFF (–70 °C)	143.13 ± 0.60	—	(Sahakijpijarn et al., 2020b)
Tacrolimus:lactose (95:5 w/w)	TFF (–70 °C)	73.58 ± 5.14	—	(Sahakijpijarn et al., 2020b)
Tacrolimus:mannitol (95:5 w/w)	TFF (–70 °C)	55.79 ± 6.49	—	(Sahakijpijarn et al., 2020b)
Tacrolimus:trehalose (95:5 w/w)	TFF (–70 °C)	57.16 ± 1.96	—	(Sahakijpijarn et al., 2020b)
Itraconazole unprocessed powder	Unprocessed	4.22	—	(Overhoff et al., 2007b)
Itraconazole:Hydroxypropylmethylcellulose phthalate (HP55) (1:4 w/w), 2 %w/v	TFF (–60 °C)	19.1	—	(Overhoff et al., 2007b)
Itraconazole:Hydroxypropylmethylcellulose phthalate (HP55) (4:1 w/w), 2 % w/v	TFF (–60 °C)	54.2	—	(Overhoff et al., 2007b)
Itraconazole:Hydroxypropylmethylcellulose phthalate (HP55) (1:4 w/w), 0.2 % w/v	TFF (–60 °C)	61.8	—	(Overhoff et al., 2007b)
Itraconazole:Hydroxypropylmethylcellulose phthalate (HP55) (4: w/w), 0.2 % w/v	TFF (–60 °C)	57.4	—	(Overhoff et al., 2007b)
Voriconazole unprocessed powder	Unprocessed	0.53 ± 0.08	7.86	(Beinborn et al., 2012a)
Micronized voriconazole	Micronization	3.46 ± 0.02	1.20	(Beinborn et al., 2012a)
Voriconazole	TFF (–40 °C)	9.38 ± 3.25	0.45	(Beinborn et al., 2012a)
Voriconazole-mannitol (50:50 w/w)	TFF (–70 °C)	16.3	—	(Moon et al., 2019b)
Voriconazole-PVP K25 (1:3 w/w)	TFF (–40 °C)	43.35 ± 5.67	0.05	(Beinborn et al., 2012a)
Physical mixture of Danazol: povidone K15 (1:2 w/w)	Unprocessed	0.69	—	(Overhoff et al., 2007a)
Danazol: povidone K15 (1:2 w/w)	TFF (–70 °C)	25.93	—	(Overhoff et al., 2007a)
Lysozyme 50 mg/mL	TFF (–100 °C)	31 ± 0.1	—	(Engstrom et al., 2008)
Lysozyme 50 mg/mL	SFL	34 ± 2	—	(Engstrom et al., 2008)
Lysozyme 50 mg/mL	SFD	126 ± 5	—	(Engstrom et al., 2008)
Lysozyme 5 mg/mL	TFF (–100 °C)	73 ± 0.8	—	(Engstrom et al., 2008)
Lysozyme 5 mg/mL	SFL	114 ± 11	—	(Engstrom et al., 2008)
Lysozyme 5 mg/mL	Shelf FD	4.4 ± 0.2	—	(Engstrom et al., 2008)

rapid nucleation and particle growth prevention during freezing. Although TFF produces particles with lower SSA compared to SFD and SFL, the cooling rate of TFF is high enough to prevent particle growth and induce rapid nucleation, thereby still forming particles with relatively high SSA (e.g., 31–73 m^2/g), especially when compared to shelf FD (e.g., 4.4 m^2/g) (**Table 1**, Engstrom et al., 2008). While the cooling rate can affect the SSA to a degree, SFL and SFD have produced lysozyme powders with similar SSAs > 100 m^2/g despite having cooling rates that differ by three orders of magnitude (Engstrom et al., 2007; 2008). The cooling rate had a higher impact on the particle size, as shown by SFL's larger particle size compared to SFD, particularly noticeable in high protein concentration situations (i.e., 50 mg/mL) (Engstrom et al., 2008). Even though TFF had a lower cooling rate, increasing the protein concentration could supersede the cooling rate's impact on the particle size, as TFF powders also had a large particle size at high concentrations. However, at a lower, more relevant protein concentration (i.e., 5 mg/mL), TFF allowed for thinner unfrozen channels with high viscosity, leading to a small particle size.

For TFF, the ability of the droplets to spread upon impact and form films influences the cooling rate, with the droplet

spreading time being shorter than the freezing time. Generally, the film thickness is on the scale of 200–400 μm and the diameter is around 10–12 mm (Engstrom et al., 2008). Because the thickness is substantially smaller than the diameter, the radial heat transfer is negligible and the heat transfer is considered to be one-dimensional (Engstrom et al., 2008). Under the assumption of constant thermal diffusivity, the heat transfer can be calculated using the thickness of the film, the temperature of the film, the processing temperature of the drum, and the distance from the top of the spread droplet (Engstrom et al., 2008). Using the heat transfer, the cooling rate can be determined. At 223 K, a 220 μm thick film cooled in 2×10^2 ms with a cooling rate of 3.9×10^2 K/s, for example (Engstrom et al., 2008).

Film diameter was proportional to the droplet diameter and the processing temperature (Engstrom et al., 2008). The processing temperature and parameters that affect the droplet size (e.g., solvent system, drop diameter, drop height) can be tailored for each application. As the processing temperature decreases, the droplet will freeze more quickly, allowing less time for spreading into a film before it becomes solid. While processing lactate dehydrogenase (LDH) protein with TFF, an average film thicknesses of 220 μm was formed at 223 K (–50 °C) and 320 μm was formed at 133 K (–140 °C), respectively. The film thickness was larger when the processing temperature was lower. For comparison, when the small molecule synthetic steroid danazol was prepared using TFF, the resultant film thickness ranged from 100–400 μm (Engstrom et al., 2008).

Infrared imaging was used to study the cooling of thin films prepared from an LDH protein solution. When a film was formed at 223 K, it had a diameter of 12 mm with a smooth edge. The IR camera revealed that the cooling front started at the edge of the film, moving inward. The film came to thermal equilibrium at 1.6 s. When the same film was processed at the lower temperature of 133 K, it had a smaller diameter of 10 mm and a jagged edge, the protrusions of which experiencing the coldest temperatures. In this case, after the cooling front moved inwards, it then reversed direction, causing a longer time of 3 s to reach thermal equilibration. While a low temperature is necessary to cause quick cooling, there is a limitation to how low is favorable. While the edge of the film was thinner and may cool at a different rate than the center of the film, this difference had no impact on the morphology throughout the film (Engstrom et al., 2008).

Thin films prepared by TFF demonstrated that the surface area/volume ratio decreased with an increasing droplet diameter. Once frozen, this ratio was 31–46 cm^{-1}. This is substantially smaller than SFD or SFL (6000 and 600 cm^{-1}, respectively). Loss of protein functionality has been attributed to a high surface area/volume ratio. Alternatively, SFL and TFF have a lower gas-liquid interfacial area than SFD and thus preserve protein functionality better due to their ability to reduce protein adsorption to the interface and thus aggregation (Engstrom et al., 2008).

Several variables describe the droplet spreading, including the density of the liquid (ρ), the impact velocity (V), the droplet diameter (D), and the interfacial tension of the droplet in air (γ). Together, these variables can be used to mathematically describe the droplet spreading, or Weber number (We), by Eqn (1):

$$We = \rho V^2 D/\gamma \tag{1}$$

When We is high (> 30), the droplets spread into a cylindrical film, while when We is low (< 1), the droplets undergo minimal spreading, and the droplets freeze as a spherical dome. These variables can be optimized to a degree: V can be controlled by the drop height, ρ can be controlled through choice of excipients and concentration of all components, and D can be controlled by the gauge of needle through which the droplets are generated. Engstrom et al. compared the typical drop height (H) of 10 cm to a shorter H of 1 cm (Engstrom et al., 2008). When H was 10 cm, the resultant films were cylindrical and the We was found to be 97, while an H of 1 cm yielded spherical domes and the We was 9.8 (Engstrom et al., 2008). A low H also reduces the film diameter (~4 mm) (Engstrom et al., 2008).

Overall, TFF is the optimal cryogenic technique due to its low gas-liquid interfacial area, intermediate cooling rate, sufficient surface area, small particle size, and controllable processing parameters such as temperature.

2.3 Characteristics of TFF powders

2.3.1 Particle morphology

Powder characteristics are generally described by their particle size and the morphology of their nanostructures. The particle size generally refers to the larger micron-ranged structure that is important for adequate aerosolization and lung deposition. The morphology of the nanostructures, however, provides more detailed information about the porosity, surface area, and freezing characteristics of the powder. The particle morphology of powders prepared by cryogenic bottom-up technologies (i.e., starting from drug in solution or suspension) is generally porous. In many cases, small molecule compounds are changed from crystalline to amorphous form after the TFF process, producing a nanostructured brittle matrix (Sahakijpijarn et al., 2020a; 2020b). In the brittle matrix, there are nanoparticles interconnected and agglomerated together as nanostructured aggregates (**Fig. 2(a)**) and are generally irregularly shaped (Sahakijpijarn et al., 2020a). The brittle matrix structure is not only observed in small molecule compounds, but also in the case of biologics, such as lysozyme (Engstrom et al., 2008). **Figs. 2(b)**, **2(c)**, and **2(d)** show the morphology of

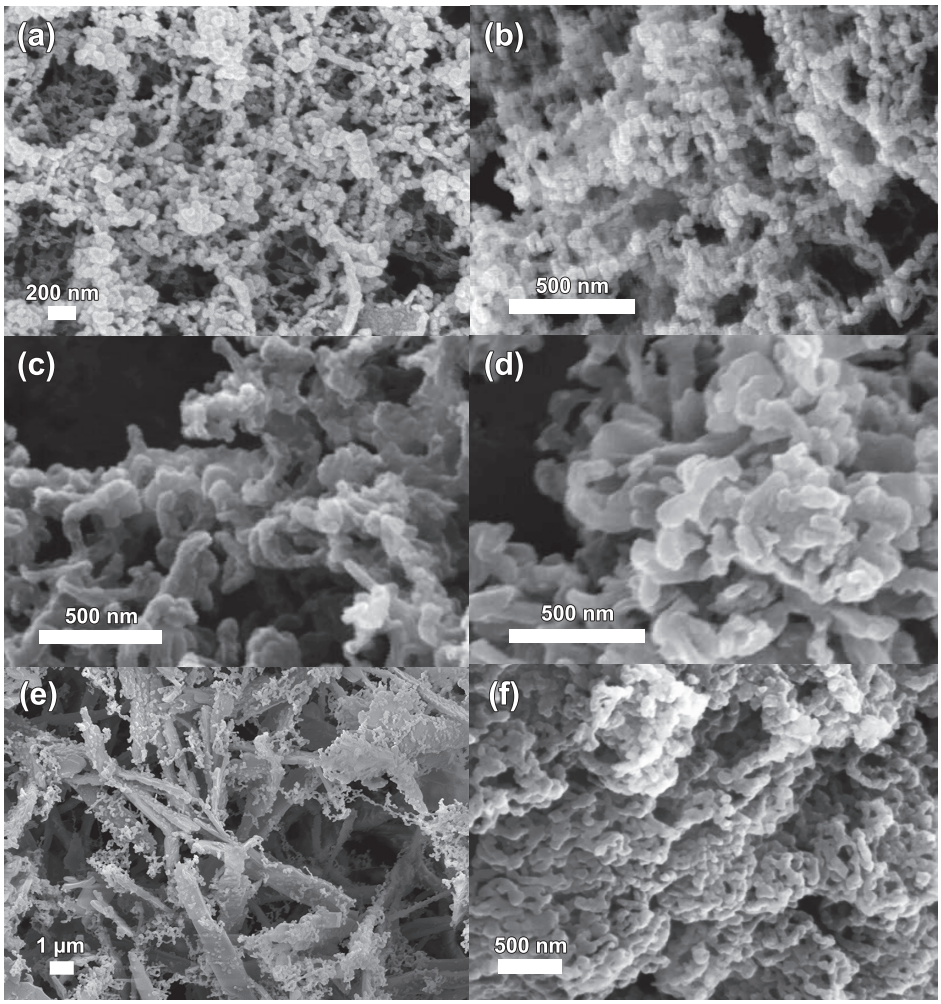

Fig. 2 Particle morphology of particles prepared by cryogenic methods. **(a)** TFF remdesivir with leucine (100 kx) **(b)** TFF lysozyme prepared at –50 °C (60 kx), **(c)** SFL lysozyme (60 kx), **(d)** SFD lysozyme (60 kx), **(e)** TFF VCZ with MAN (20 kx), **(f)** TFF VCZ with PVP K25 (10 kx). Reprinted with permission from **(a)** MDPI: Pharmaceutics (Sahakijpijarn et al., 2020a) **(b)** Springer Nature: Pharm. Res. (Engstrom et al., 2008) **(c–d)** Elsevier: Eur. J. Pharm. Sci. (Yu et al., 2006) **(e)** ACS: Mol. Pharm. (Moon et al., 2019b) and **(f)** Elsevier: Eur. J. Pharm. Sci. (Beinborn et al., 2012a).

lysozyme particles that were prepared by TFF, SFL, and SFD, respectively (Engstrom et al., 2008; Yu et al., 2006).

Among these three techniques, all particles showed similar morphologies. Particle morphology is related to several factors, such as formulation composition, solids content of solutions, solvent, and processing temperature (Moon et al., 2019a; Wang et al., 2014). In addition to the nanostructured brittle matrix, another particle morphology was observed in the case of VCZ. The particle morphology of TFF VCZ combined with mannitol (MAN) was a porous matrix of micron-sized flat irregular-shaped VCZ particles and irregular-shaped mannitol nanoparticles (**Fig. 2(e)**) (Moon et al., 2019b). Atomic force microscopy showed that the VCZ microparticles were packed by VCZ nanoaggregates (150–500 nm) (Moon et al., 2019b). Interestingly, the particle morphology of TFF VCZ combined with povidone K25 (PVP-K25) is a brittle matrix of nanostructured primary particles with a size of approximately 100 nm (**Fig. 1(f)**) (Beinborn et al., 2012a), which is a similar morphology to other brittle matrix powders. The difference in particle morphology of VCZ is related to the crystallinity of VCZ in the formulations. Due to the low glass transition temperature (T_g) of VCZ (1 °C), the inclusion of VCZ with a sufficient amount of a high T_g polymer (e.g., PVP) can inhibit the recrystallization during the drying cycle, resulting in an amorphous structure of VCZ (Beinborn et al., 2012a). In contrast, mannitol, a sugar alcohol, showed no interaction with VCZ, resulting in the crystalline structure of VCZ in TFF VCZ-MAN.

2.3.2 Moisture sorption

Generally, moisture/water sorption depends on the polarity of surface chemical groups and the available surface area (Newman et al., 2008). Although the highly porous nature of particles produced by TFF is beneficial with regard to dispersibility and aerosolization, the high surface area of porous particles can increase the tendency of water sorption compared to unprocessed powder. The hygroscopicity

of TFF formulations is also associated with the formulation composition. The formulations that contain a large amount of hydrophilic material can increase the hygroscopicity of the formulations (Sahakijpijarn et al., 2020b). Sahakijpijarn et al. investigated the effect of the amount of lactose (LAC) in the TFF formulations on the moisture sorption, which was determined gravimetrically by dynamic vapor sorption (Sahakijpijarn et al., 2020b). The higher amount of lactose resulted in higher moisture sorption. The moisture sorption of TFF tacrolimus (TAC) formulations containing 0 %, 5 %, and 20 % w/w lactose were 1 %, 2.5 %, and 9 % w/w at 25 °C/80 % relative humidity (RH) (Sahakijpijarn et al., 2020b). According to the hygroscopicity classification of the European Pharmacopoeia, TFF TAC formulations containing lactose are classified as moderately hygroscopic to very hygroscopic, while TFF neat TAC is classified as slightly hygroscopic (Newman et al., 2008). Moreover, mass loss due to surface moisture release during recrystallization was observed in a TAC formulation containing 50 % lactose, but not in other formulations (Sahakijpijarn et al., 2020b).

Another study compared the moisture sorption of different sugar excipients (Watts et al., 2013). The formulation containing lactose and raffinose had more weight gain at > 80 % RH than the formulations containing mannitol without other excipients. Mannitol was crystalline after the TFF process, while lactose and raffinose were amorphous (Watts et al., 2013). Amorphous materials generally tend to absorb moisture more than crystalline materials (Newman et al., 2008). Moreover, the effect of humidity on the aerosolization of low-density microparticles containing different excipients was also investigated. It was reported that water sorption to powder surface can both improve and reduce powder dispersibility. Capillary, electrostatic, and Van der Waals forces are used to describe the cohesive forces in powders, which play a major role in powder dispersibility (Watts et al., 2013). Capillary force is a predominant force at > 60 % RH, while electrostatic adhesion force is a predominant force at low humidity (Watts et al., 2013). In lactose-based formulations, high humidity increased the plasticity of the brittle matrix, thereby reducing the aerosol performance of the formulations. However, in mannitol-based formulations, moisture can improve the powder dispersibility due to a reduction in electrostatic charge (Watts et al., 2013).

2.3.3 Aerodynamic properties

TFF powders have been investigated for pulmonary drug delivery using dry powder inhalers (DPIs). The powder should have an appropriate mass median aerodynamic diameter (MMAD) in the range of 1–5 μm to maximize the delivery of the drug particles to the deep lungs. Powder dispersibility is controlled by the particle cohesive forces, which are related to contact area and separation distance between particles (Weers, 2000). The highly porous, low-density particles with a large geometric particle diameter, characteristics unique to the morphology of TFF powders, demonstrate superior powder dispersibility, as they have higher inter-particle separation distance, less contact area, and lower interparticle cohesive forces compared to the traditional micronized (e.g., milled) powder (Sahakijpijarn et al., 2020b). The highly porous and low-density brittle matrices can be aerosolized and dispersed to small particles of the same structure and density by the inhalation force through a passive DPI (Watts et al., 2013).

The aerodynamic properties of TFF formulations are dependent on the properties of the powder, which are influenced by formulation compositions and processing parameters. Excipients, such as sugars, are typically used to improve the dispersibility of dry powder formulations (Chen et al., 2016). Lactose is widely used as an excipient in FDA-approved inhaled products, while other sugars (e.g., trehalose, sucrose) and amino acids (e.g., leucine, trileucine) have recently gained more interest for pulmonary delivery (Pilcer and Amighi, 2010). Mannitol has been approved in ARIDOL™ for the assessment of bronchial hyperresponsiveness due to its induction of bronchoconstriction (Pharmaxis, 1964; Sahakijpijarn et al., 2020c). In many cases, TFF formulations containing an optimal amount of excipient exhibited high aerosol performance. Beinborn et al. compared the aerosol performance of TFF VCZ with micronized VCZ. The blended powder of inhalation grade, micronized VCZ and lactose (Inhalac® 70, 2 % w/w) exhibited 19.6 % fine particle fraction (FPF) and an MMAD of 2.7 μm when it was emitted from a Handihaler® at 60 L/min. Although TFF neat VCZ exhibited a larger MMAD (4.2 μm), the FPF was about two times higher compared to micronized VCZ and the MMAD was in the acceptable range (Beinborn et al., 2012a).

The effects of the type and amount of excipient and the drug loading on the aerosol performance were investigated in several studies. The drug loading and type of excipients were optimized to maximize the respirable dose for pulmonary delivery (Sahakijpijarn et al., 2020a). It was reported that TFF remdesivir combined with leucine had a larger FPF and smaller MMAD for the whole range of drug loading of remdesivir (20–80 % w/w) (Sahakijpijarn et al., 2020a). The formulations containing 80 % remdesivir with 20 % of different excipients (e.g., Captisol®, mannitol, lactose, leucine) showed good aerosol performance with FPFs ranging from 64.21–82.71 % and MMADs ranging from 2.17–2.53 μm (Sahakijpijarn et al., 2020a). The aerosol performances of formulations containing 95 % TAC with the combination of 5 % w/w lactose, mannitol, or trehalose were compared at 60 L/min using a Plastiape RS01 high resistance inhaler (Sahakijpijarn et al., 2020b). Lactose-based formulations exhibited higher FPFs and smaller MMADs compared to mannitol- and trehalose-based for-

mulations (Sahakijpijarn et al., 2020b). Additionally, it was reported that there was no significant difference in aerosol performance when the drug loading of TFF TAC formulations was increased from 60 % to 95 % w/w (Sahakijpijarn et al., 2020b). This indicates that the drug loading of TAC can be increased up to 95 % while maintaining optimal aerosol performance (69.31 % FPF, 2.61 μm MMAD) (Sahakijpijarn et al., 2020b). Another study from Moon et al. investigated the drug loading and the amount of mannitol in TFF formulations. When the drug loading of VCZ was increased from 50 % to 97 % w/w, the FPF increased from 28.5 % to 40.8 % (Moon et al., 2019b). The small amount of mannitol in TFF VCZ formulations functioned as a surface texture modifier, which can minimize the cohesive and adhesive forces between particles, and subsequently, improve the aerosol performance (Moon et al., 2019b). According to these cases, particles prepared by TFF are aerosolizable with a small amount of excipient needed in the formulation, providing the potential of TFF to prepare high drug loading formulations for pulmonary delivery of drugs requiring a high dose, which can also lower the powder burden for the patient. For large molecules such as nucleic acid-based molecules and proteins, however, increasing the drug loading in the final dry powders to a certain level can lead to a reduction of the aerosol performance properties of the dry powders (unpublished data), likely due to an increase in particle cohesive forces.

2.3.4 Stability, molecular interactions, and miscibility

The stability of TFF powders was investigated in several studies. It is well reported in literature that a crystalline form is more stable than its amorphous counterpart. For example, VCZ/MAN (95:5 w/w) crystalline nanoaggregates prepared by TFF were physically and chemically stable after storage at 25 °C/60 % RH for up to 13 months (Moon et al., 2019b). The MMAD and FPF of TFF VCZ/MAN (95:5 w/w) did not change statistically after the storage (Moon et al., 2019b). In the case of amorphous drugs, TFF TAC/LAC (95:5 w/w) was chemically and physically stable after storage at 25 °C/60 % RH and 40 °C/60 % RH for up to 6 months without the need of a stabilizer (Sahakijpijarn et al., 2020b). The moisture content of powder affected the aerodynamic properties and physical properties of formulations after storage. For example, when TFF TAC/LAC (95:5 w/w) powder was encapsulated in #3 hydroxypropyl methylcellulose (HPMC) capsules and stored in a borosilicate glass vial without desiccant, there was an increase in moisture content during storage, which led to a decrease in SSA and MMAD (Sahakijpijarn et al., 2020b). Since amorphous materials are generally susceptible to water plasticization, moisture on the particle surface resulted in a reduced brittle fracture and increased particle density due to the collapse of the brittle matrix structure (Watts et al., 2013). The changes in particle morphology and powder properties had detrimental effects on the aerodynamic properties, as the powder showed a significant decrease in FPF and emitted fraction (EF) and increase in MMAD (Sahakijpijarn et al., 2020b). However, the stability results suggested that a tightly closed container with desiccant helps to maintain and minimize the changes in the aerodynamic and physical properties of TFF powder (Sahakijpijarn et al., 2020b). When encapsulated powder of TAC/LAC (95:5 w/w) was dried to remove additional moisture absorbed during capsule filling and packing and then stored in a sealed glass container with desiccant, the physical and aerodynamic properties of encapsulated powder did not significantly change over 6 months stored at 25 °C/60 % RH and only slightly changed over 6 months stored at 40 °C/60 % RH (Sahakijpijarn et al., 2020b).

In the case of biologics, Thakkar et al. investigated the stability of a protein subunit vaccine dry powder prepared by TFF (Thakkar et al., 2017). It was reported that the particle size distribution of reconstituted ovalbumin-absorbed aluminum hydroxide dry powder prepared with 2 % w/v trehalose did not significantly change after one month of storage at RT, 30 °C, and 40 °C. After 3 months of storage at 40 °C, particle aggregation was slightly detected, but was not found at other storage temperatures. However, noticeable amounts of particle aggregates were detected after 6 months of storage at RT, 30 °C, and 40 °C, due to the visible buildup of moisture in the vials. The ovalbumin-aluminum hydroxide vaccine powders were reconstituted after storage at various temperatures for 1 or 3 months and were used to immunize mice. Importantly, there was no significant difference in the anti-ovalbumin IgG levels among mice that were immunized with freshly prepared ovalbumin-adsorbed aluminum hydroxide liquid vaccine or with the TFF vaccine powders after 3 months of storage at the different temperatures. The T_g (~120 °C) of the ovalbumin-adsorbed aluminum hydroxide dry powder prepared by TFF was higher than the storage temperatures (i.e., RT, 30 °C, and 40 °C), allowing the vaccine powder to remain chemically, physically, and immunogenically stable at all temperatures up to 3 months (Thakkar et al., 2017). These samples were stored in a desiccator, but not individually packaged in sealed pouches. With optimal storage conditions, they would remain stable for a longer period of time. This study showed the advantage of TFF in producing a biologic powder that is stable when stored for an extended period of time without a requirement for cold chain (Thakkar et al., 2017).

The interaction between a drug and an excipient with a high T_g generally improves the physical stability of amorphous solid dispersions of small molecule drugs, as well as the stability of biologics. The interaction between drug and excipient can increase the T_g of the system, resulting in a less molecularly mobile glassy state. In some

cases, however, the interaction is not necessary to stabilize the drug. For example, TAC, a high glass-forming ability drug (GFA class III), showed no interaction with LAC analyzed by ssNMR (Sahakijpijarn et al., 2020b). Although two T_gs were detected by DSC, indicating TAC and LAC were not molecularly dispersed, the analysis of domain size by ssNMR showed that the two compounds had good miscibility at a domain size of ~100 nm (Sahakijpijarn et al., 2020b). Despite phase separation and lack of interactions, TAC was physically stable, remaining amorphous after storage at 40 °C/75 % RH for 6 months in a sealed container and after exposure to 40 °C/75 %RH for 28 days in an open container (Sahakijpijarn et al., 2020b).

2.3.5 Solubility and dissolution

The enhanced dissolution of a drug in a TFF formulation is generally associated with the brittle matrix nature of TFF powders, and the matrix consists of the amorphous forms of the drug and the excipient. A dissolution test showed that TAC combined with lactose (5 % w/w) released a higher amount of drug within 6 hours compared to the physical mixture of unprocessed TAC and lactose (~85 % and ~30 % drug release, respectively) (Sahakijpijarn et al., 2020b). The drug loading and the amount of lactose in the formulation did not affect the rate and extent of drug release, as there was no difference in dissolution profiles between TFF TAC/LAC (50:50 w/w), TFF TAC/LAC (95:5 w/w), and TFF neat TAC (Sahakijpijarn et al., 2020b; Sinswat et al., 2008). The enhanced dissolution of TFF TAC formulations was associated with the enhanced solubility of the amorphous form of TAC. The amorphous form of a drug generally exists in a higher free energy state than its crystalline counterpart, thereby enhancing its solubility and dissolution rate (Trasi et al., 2017). It was reported that the solubility of amorphous TAC was 35-times higher than its crystalline solubility (Trasi et al., 2017). In another study, the dissolution of TFF remdesivir formulations containing different excipients (i.e., Captisol®, mannitol, lactose, and leucine) was compared with the dissolution of TFF neat remdesivir and unprocessed remdesivir (Sahakijpijarn et al., 2020a). All TFF remdesivir formulations exhibited faster and higher dissolution of remdesivir than unprocessed remdesivir (Sahakijpijarn et al., 2020a). Similar to the TAC case, the increased dissolution rate and extent of remdesivir release are also related to the higher solubility of amorphous remdesivir, compared to crystalline remdesivir (Sahakijpijarn et al., 2020a).

In several cases, the presence of a polymer as an excipient in a TFF formulation not only increases the rate and extent of dissolution, but also enhances and prolongs the supersaturated dissolution. The concentration (C) of drug dissolved in the media divided by the equilibrium solubility (C_{eq}) of the crystalline form yields the degree of supersaturation (C/C_{eq}) (Overhoff et al., 2008; Yang et al., 2008). Yang et al. reported that the colloidal dispersion of TFF itraconazole (ITZ)/MAN/lecithin (1:0.5:2 w/w/w) had a C/C_{eq} of 22-times at 5 min and the highest value of 27-times at 15 min in lung simulated fluid (Yang et al., 2008). After 3 h, the supersaturated ITZ concentration of TFF ITZ/MAN/lecithin started to decrease to about 7-times, while the physical mixture only reached 2-times at 15 mins and gradually decreased to a plateau of C_{eq} after 2 hours (Yang et al., 2008). For oral drug delivery, Overhoff et al. investigated the in vitro supersaturation dissolution of a TFF tacrolimus-sodium dodecyl sulfate formulation (TFF TAC-SDS) and the commercial tacrolimus capsule, Prograf®, in the two-stage dissolution test in acidic conditions. It was reported that TFF TAC-SDS exhibited a higher degree of supersaturation than Prograf® after 2 hours (17-times C/C_{eq} versus 14-times C/C_{eq}, respectively). Upon a pH shift from 1.2 to 6.8, the TFF TAC-SDS also exhibited an increase in supersaturation level from 17-times to 22-times at 15 min after the pH shift, while Prograf® did not maintain a high degree of supersaturation and started to precipitate out immediately after the pH shift. Although the degree of supersaturation gradually decreased to 2.5-times C/C_{eq} at 24 h after the pH shift, the area of under the dissolution curve (AUC) of the TFF TAC-SDS was higher than that of Prograf® under acidic and pH-shift conditions (Overhoff et al., 2008).

A benefit of formulating proteins as powders as opposed to liquids is that powders are not limited by the solubility of the protein in the dose (Johnson, 1997). The solubility of proteins is directly related to adsorption at the air-liquid interface and aggregation. Techniques that expose proteins to a high liquid interfacial area (e.g., SD, SFD), often require the use of additional excipients, such as cyclodextrins (Ramezani et al., 2017), surfactants (Bhatnagar B. and Tchessalov., 2020), ethanol (Johnson, 1997), or the utilization of acidic conditions (Liu et al., 1991). These excipients may also be necessary to modulate the dissolution of the protein. SFD bovine serum albumin (BSA) protein with or without trehalose was encapsulated in poly(D,L-lactide-*co*-glycolide) (PLG) or PLG/poloxamer microspheres (Carrasquillo et al., 2001). Within 4 h in phosphate-buffered saline (PBS) at 37 °C, SFD BSA/PLG and SFD BSA-trehalose/PLG released > 80 % of the protein, while the inclusion of poloxamer (1:20 and 1:1 PLG/poloxamer) extended the release over 1–2 days (Carrasquillo et al., 2001). Finally, due to their high porosity and high SSA, dry powders of biologics, such as vaccines, prepared by TFF can often rapidly dissolve upon contact with a diluent without any agitation, which is advantageous if there is a need to reconstitute a TFF dry powder. Of course, one can expect that TFF dry powders can readily dissolve in lung fluid once delivered into the lung.

2.4 Formulation and device considerations

2.4.1 Solids content

A few characteristics of TFF powders are affected by solids content, the total amount of solids dissolved or suspended in the liquid (e.g., active ingredient, carriers, other excipients). In terms of aerodynamic properties, the feeding solution with less solids content generally produces more aerosolizable powders. When a comparison of TFF VCZ nanoaggregates was made between 1 % and 3 % (w/v) solids content, 1 % was associated with a higher FPF of 67.5 % compared to 48.5 % at 3 % (Moon et al., 2019a). However, in some cases with an amorphous TFF brittle matrix powder, different solids content does not present significantly different aerodynamic properties (Sahakijpijarn et al., 2020a; 2020b) or a higher solids content actually produces improved aerosol properties (Beinborn et al., 2012b). The solids content can also influence the SSA. High-potency, amorphous TFF TAC powders resulted in about a 50 % larger SSA when the solids content was 0.75 % (w/v), as compared to 2.5 % (w/v) (Sahakijpijarn et al., 2020b). However, crystalline TFF VCZ powders did not show different SSAs when the solids content varied from 0.1 to 10 % (w/v) (Beinborn et al., 2012b).

In addition to aerodynamic properties and SSA, the solids content is also an important parameter for manufacturing with regard to scale up. The formulations developed by TFF are currently beginning Phase II clinical trials. When a large amount of TFF powders, on the scale of multi-kilograms, is necessary, higher solids content will reduce the total manufacture time of the powders.

2.4.2 Solvent system

Solvent systems used for TFF can significantly affect amorphicity, morphology, and aerosol performance. Beinborn et al. showed that TFF VCZ-PVP K12 (1:2 w/w) and TFF VCZ-PVP K30 (1:2 w/w) prepared with 1,4-dioxane as a solvent were amorphous (Beinborn et al., 2012b). However, when binary solvent systems of 1,4-dioxane and water (50:50 or 20:80 v/v) were used for these compositions, both formulations were crystalline (Beinborn et al., 2012b).

When a binary solvent system of water and acetonitrile is used for TFF, cryo-phase separation of the solvent system must be considered. A mixture of water and acetonitrile phase-separates during the freezing if 35–88 % (v/v) acetonitrile is included in the mixture (Zarzycki et al., 2006). When this cryo-phase separation occurs, a different morphology of powder formulations can be observed (Moon et al., 2019a). The mixture of water and acetonitrile not only impacts cryo-phase separation, but also affects the aerosol performance. In the case of TFF VCZ nanoaggregates, the higher portion of water included in the binary solvent system of water and acetonitrile enhanced aerodynamic properties with higher FPFs and smaller MMADs (Moon et al., 2019a).

2.4.3 Excipients

Carrier sugars are the most common excipients in the preparation of powders. Sugars raise the viscosity of the liquid thus slowing particle growth during rapid freezing. Sugars can also enhance the stability. Inclusion of a sugar in the formulation can help water vitrify at a lower cooling rate, meaning the faster cooling rate of SFD and SFL are not necessary.

In general, the characteristic porous particles prepared by TFF have high SSA and low bulk density. As shown in **Table 1**, the bulk density of TFF neat VCZ powder is 17-fold and 2.7-fold lower than that of unprocessed VCZ and micronized VCZ powder, respectively. Similarly, the SSAs of powders processed by TFF with or without an excipient were 4-times higher than unprocessed neat drug powder (**Table 1**). Without excipient, TFF neat VCZ showed lower SSA than TFF neat TAC (Beinborn et al., 2012a; Sinswat et al., 2008), which is related to the crystallinity and the unique morphology of VCZ nanoaggregates. The inclusion of sugars can further improve the SSA of TFF powders and is variable depending on the type and amount of sugar used. At 95 % drug loading, TFF TAC formulations that contained 5 % lactose (w/w) exhibited higher SSA compared to other formulations that contained 5 % w/w mannitol or 5 % trehalose (Sahakijpijarn et al., 2020b). A TFF TAC formulation containing 50 % w/w lactose showed a higher SSA than a TFF TAC formulation containing 5 % w/w lactose (Sahakijpijarn et al., 2020b).

In contrast, the effect of the amount of polymer as an excipient on SSA is not the same as that of sugar excipients (Overhoff et al., 2007b). At 2 % w/w solids content, TFF ITZ formulations containing 80 % w/w hydroxypropyl methylcellulose phthalate (HP55) resulted in lower SSA than the formulation containing 20 % HP55 (Overhoff et al., 2007b). However, at lower solids content (0.2 % w/w), there was no significant difference in SSA between these two formulations (Overhoff et al., 2007b). The higher level of polymer in the solution can prevent the formation of nanoparticles (Overhoff et al., 2007b), which produce a larger and less brittle structure. Overall, SSA is influenced by several parameters, including type of excipient and excipient loading, processing temperature, solids content, and solvent system composition.

2.4.4 Drug loading

The degree of drug loading in TFF powders can influence the particle morphology, but potentially less so if the powder is an amorphous brittle matrix (Sahakijpijarn et al., 2020a; Sahakijpijarn et al., 2020b). However, in case of crystalline TFF VCZ nanoaggregates, different drug loadings significantly affected particle morphologies

(Moon et al., 2019b). TFF VCZ showed crystalline nanoaggregates when processed with mannitol as a single excipient, which was phase-separated from VCZ (Moon et al., 2019b). While VCZ exhibits as nanoaggregates that are a few micrometers in size, mannitol exhibits as a brittle matrix powder that consists of particles that are smaller than 150 nm (Moon et al., 2019b). As a result of the different morphologies of these two phase-separated crystalline ingredients, a different ratio of VCZ and mannitol resulted in significantly different particle morphologies (Moon et al., 2019b). More interestingly, due to generating small mannitol nanoparticles, when VCZ loading is high (i.e., 90–97 %), the phase-separated mannitol nanoparticles reside on the flat surface of VCZ nanoaggregates and act as a surface modifying agent (Moon et al., 2019b). This reduces cohesive and adhesive energy of VCZ particles and enhances the aerodynamic properties of the powder.

Amorphous, brittle matrix TFF powders do not show different morphologies with different levels of drug loading; however, the aerodynamic properties of these powders still depend on drug loading for certain excipients and drugs. For TFF TAC with lactose, 70–80 % (w/w) TAC loading resulted in the highest FPF at the solids content of 0.75 % (w/v) (Sahakijpijarn et al., 2020b). In the case of TFF-remdesivir with Captisol®, the powders with higher drug loading aerosolized better than those with lower drug loading (Sahakijpijarn et al., 2020a). In contrast, the higher drug loading resulted in less aerosolizable TFF-remdesivir powders with leucine, showing larger MMADs and lower FPFs. However, when TFF-remdesivir was formulated with mannitol or lactose, 50 % and 80 % (w/w) drug loading exhibited similar FPF.

2.4.5 Loading dose and delivery device

The loading dose can also affect the aerosol performance. The aerosol properties of TFF VCZ nanoaggregates were tested and compared with high resistance RS00 and RS01 Plastiape devices (Moon et al., 2019a). When TFF-VCZ nanoaggregates were filled into #3 HPMC capsules with 10, 15, or 20 mg, the high resistance RS00 Plastiape device provided more consistent and enhanced aerosol performance within these filling ranges. While FPF and MMAD were not significantly different between the three doses, the 20 mg dose, with the capsule completely full, presented slightly lower aerosol properties than either the 10 or 15 mg dose.

The type of device used to deliver dry powder formulations is selected for its influence on its aerosol performance. While TFF powder formulations exhibit aerodynamic properties that are independent of flow rate, they are still affected by the type of resistance and device (Moon et al., 2019a; Sahakijpijarn et al., 2020b). The high dose TFF TAC brittle matrix powder demonstrated consistent MMAD and FPF in the range of flow rates between 30 and 60 L/min, which represented 1 and 4 kPa pressure drop, respectively, with a high resistance RS01 Plastiape device. However, when the same TFF TAC was aerosolized using a low resistance RS01 Plastiape device, it was less aerosolizable and relied more on flow rate compared to high resistance RS01 Plastiape device (Sahakijpijarn et al., 2020b). Interestingly, in the case of TFF VCZ nanoaggregates, both low resistance RS00 and RS01 Plastiape devices exhibit excellent aerosol properties (Moon et al., 2019a). Low and high resistance RS00 Plastiape devices perform better than the corresponding low and high resistance RS01 Plastiape devices. The most flow rate independent device for TFF-VCZ nanoaggregates was the high resistance RS00 Plastiape device, which creates smaller holes in the capsule shell wall, likely facilitating deaggregation the TFF nanoaggregates.

3. Applications of TFF: formulation of proteins as powders for pulmonary delivery

3.1 The need for pulmonary delivery of proteins into the lungs

Within the class of proteins, there are various types that have their own unique characteristics. Several proteins have been studied for delivery via the pulmonary route as dry powders: proteins (e.g., insulin, calcitonin, BSA), enzymes (e.g., dornase alpha, LDH, lysozyme), and antibodies (e.g., anti-IgE). The category of antibodies also includes antibody fragments and nanobodies. Antibody fragments typically consist of either the antigen binding fragment (Fab) or crystallization fragment (Fc). One type of antibody fragment is a domain antibody fragment (dAb), which is the smallest functional antigen-binding region of antibodies and are derived from a single variable region of either the light (V_L) or heavy (V_H) chain (Holt et al., 2003; Proudfoot et al., 2018). Antibody fragments can be more susceptible to aggregation after freeze-thawing than full length antibodies (Wang et al., 2007). However, due to their small size, they may have enhanced tissue penetration and are relatively easier to manufacture (Nelson, 2010). Nanobodies are proteins derived from heavy-chain only antibodies (HCAb) found in Camelidae and are about one tenth the size of an antibody. Finally, many vaccines are protein-based. Protein therapeutics continue to become more and more complex (e.g., antibody-drug conjugates) and will require much care when developing them as more stable powders (Bodier-Montagutelli et al., 2018) .

Protein-based therapeutics are often administered by parenteral routes, such as intravenous, subcutaneous, or intramuscular injection. There is a need to deliver some protein-based therapeutics directly to the lungs, largely due to their low distribution to the lung when administered

systemically, which means high doses are required to achieve adequate lung distribution. For example, Guilleminault et al. explored the delivery of cetuximab, an anti-epidermal growth factor receptor (EGFR) antibody, via aerosolization into the lungs for the treatment of lung cancer. In mice with orthotopic lung tumors, aerosolization of cetuximab in solution achieved substantially better delivery of cetuximab to the lung tissues as compared to intravenous administration, which led to a higher dose of cetuximab distributing to the tumors in the lung (i.e., up to 4-fold higher as compared to intravenous injection at 2 h) (Guilleminault et al., 2014). Similar studies and results were produced by Maillet et al. (Maillet et al., 2011). In fact, two proteins have been approved for delivery via the pulmonary route, including insulin for the treatment of diabetes (i.e., for systemic distribution) and dornase alpha, or Pulmozyme, a solution of recombinant human deoxyribonuclease indicated for reducing lung infections and improving lung function in patients with cystic fibrosis (i.e., for local lung distribution). Lungs have a high degree of vasculature, high surface area, thin epithelium, high permeability for large molecules, and fewer enzymes and higher pH compared to the gastrointestinal tract (Liang et al., 2020). The respiratory route is also less invasive (i.e., does not require a needle puncture or a trained health professional), has a faster onset of action, and minimizes systemic toxicity compared to the parenteral routes (Johnson, 1997).

3.2 The need for dry powder formulation of proteins for pulmonary delivery

Many medications are delivered to the lungs via nebulization, which is the aerosolization of liquid preparations and direct inhalation of the mist/aerosols into the lungs. Formulations for nebulization are prepared and stored in a liquid state, which is not ideal for proteins. Proteins are generally less stable in a liquid formulation than in a dry powder, and nebulization exposes proteins to harsh conditions, including shear stress and high temperatures exiting the device (Bodier-Montagutelli et al., 2018) and a high air-liquid interfacial area, causing aggregation (Wang et al., 2007). The percent of functional LDH was measured to be < 6 % after nebulization and could only be improved to 62 % with the use of a protectant (e.g., Tween 80, chitosan) (Albasarah et al., 2010). The most common nebulization device is the vibrating-mesh nebulizer because of its ability to deliver relatively high doses while being less destructive than ultrasonic or jet nebulizers (Lightwood et al., 2013; Maillet et al., 2008). Liquid formulations for nebulization can, and typically must, be customized for the specific molecule and often require the use of a surfactant (e.g., polysorbate, polyethylene glycol) or other stabilizer (Bodier-Montagutelli et al., 2018) and organic solvents, which can be toxic to the lungs. Solubility of the formulation excipients also limits how concentrated the dose can be, meaning a large dose may be necessary (Johnson, 1997). Furthermore, nebulizer devices are less convenient for the patient, and they often cannot be as easily transported compared to DPI devices. Administration of a nebulized protein usually requires several minutes of inhaling the aerosol. For example, Pulmozyme, when administered with the eRapid® Nebulizer system, should be inhaled for 1–5 minutes (Genentech, 2018). This is in contrast to a DPI, which can deliver a full dose in a few seconds as a bolus. There are benefits to nebulization, however, such as fewer manufacturing steps (e.g., no drying) (Bodier-Montagutelli et al., 2018), but the overall aerosol performance of nebulizers is worse than DPI devices. For example, tobramycin PulmoSphere dry powder administered via a DPI exhibited an emitted dose of 78.3 %, delivering 34.3 % of the total dose to the lungs, while the nebulized tobramycin product emitted 39.4 % of the dose, delivering only 5.0 % of the dose to the lungs (Newhouse et al., 2003). The nebulized dose had to be administered for 15 min (Newhouse et al., 2003). Preparing proteins as powders for delivery to the lungs can enhance aerosol performance as well as the storage stability of them. Aggregation also occurs in lyophilized powders, however, it can be minimized by inclusion of protectants (Yu et al., 2006).

Only a few protein-based therapeutics developed for pulmonary delivery have been formulated as powders. Human growth hormone (22 kDa protein) SD powder, infliximab (149 kDa antibody) SD powder, and influenza subunit vaccine (225 kDa protein) SD and SFD powders are in preclinical development (Liang et al., 2020). The influenza subunit vaccine SD and SFD powders were found to have FPFs of 37 % and 23 %, respectively (Saluja et al., 2010). A few are in the clinical development stage. For example, Novartis is developing CSJ117, anti-thymic stromal lymphopoietin (46 kDa antibody fragment), as a dry powder for inhalation. They report having completed a phase I trial in 2019, administering CSJ117 with a Concept1 single dose DPI to adult patients with mild, atopic asthma (NCT03138811). CSJ117 was well tolerated and reduced allergen-induced bronchoconstriction compared to placebo (Gauvreau et al., 2020). Recruitment for a phase II trial in adult patients with severe, uncontrolled asthma began in 2020 (NCT04410523). UCB Pharma designed VR942, a dry powder formulation of an anti-IL-13 antibody fragment, also known as CDP7766. A phase I clinical trial was conducted in 2018 with a Multidose F1P DPI showing that it was well tolerated for up to 10 days and achieved inhibition of fractional exhaled nitric oxide (FeNO) as a secondary outcome (Burgess et al., 2018). Patents can be found detailing the Novartis and UCB Pharma products, but it is difficult to determine which formulations were used in clinical trials and their corresponding aerosol

properties (Morgan et al., 2019; Rondeau et al., 2017). DAS181, or Fludase® is a 46 kDa sialidase fusion protein in phase 1/II trials for parainfluenza by Ansun BioPharma (Zenilman et al., 2015). It is a dry powder delivered by the Cyclohaler® DPI and its desired site of action is the central/upper respiratory tract and thus is designed to have an FPF of around < 10 % and MMAD 3–8 μm (Malakhov and Li, 2014). Insulin is a small protein (5.7 kDa) that is used to treat diabetes mellitus. Two insulin dry powders for inhalation products, Afrezza® by MannKind and Exubera® by Pfizer, were approved and commercialized. Exubera® was ultimately discontinued due to patient and provider dissatisfaction (Heinemann, 2008).

We have experimental evidence that TFF can be applied to prepare dry powders of proteins or protein-containing products while maintaining the activity of the proteins, and the resultant dry powders have excellent aerosol properties for lung delivery and can be potentially stored without cold chain.

3.3 Protein dry powders engineered using TFF technology

Over the years, we have successfully applied TFF technology to prepare dry powders of proteins and protein-containing products such as vaccines (Engstrom et al., 2008; Li et al., 2015; Moon et al., 2016; Thakkar et al., 2017). We have shown that protein dry powders prepared by TFF maintain their functional activity, have good aerosol performance properties, and have improved thermostability for potential cold chain-free storage.

For example, LDH was formulated into powders with trehalose (1:120, w/w) as a carrier using shelf FD, SFD, SFL, and TFF. For all except SFD, the activity of LDH in the dry powders was 97–100 %, whereas SFD produced an LDH powder with only 74–85 % activity (Engstrom et al., 2008). Around 20–30 % of protein was adsorbed to the gas-liquid interface in SFD, explaining the decrease in the activity of the LDH (Engstrom et al., 2008). Lysozyme at 5 mg/ml or 50 mg/ml was also formulated into powders using SFD, SFL, and TFF. Lysozyme powder prepared by shelf FD at 5 mg/ml showed an SSA of 4.4 ± 0.3 m^2/g (**Table 1**). Lysozyme powder produced via TFF at 5 mg/ml had a high SSA (i.e., 45–73 m^2/g) (**Table 1**, Engstrom et al., 2008). SFD can produce a powder with an even larger SSA (i.e., 126 m^2/g vs. 31–55 m^2/g for TFF at 50 mg/ml) (**Table 1**, Engstrom et al., 2008). As mentioned earlier, **Figs. 2(b)**, **2(c)**, and **2(d)** show the morphology of the lysozyme particles that were prepared by TFF, SFL, and SFD, respectively (Engstrom et al., 2008; Yu et al., 2006). All particles showed similar morphologies. Increasing the protein concentration from 5 to 50 mg/mL deceased the submicron particle fraction from 81–92 % to 62–66 %. The high concentration also produced more micron-sized particles, likely due to a higher degree of collisions contributing to particle growth, but 5 mg/mL is more clinically relevant (Engstrom et al., 2008). TFF and SFL produced similar morphologies when powders were examined by SEM, with the particle size increasing with decreasing freezing temperature. Again, the particle size was increased when the protein concentration was increased. SFD was able to form smaller particles than SFL and TFF.

Lysozyme dry powders were also prepared by TFF with mannitol as the excipient (50:50, w/w), and the resultant dry powders showed excellent aerosol properties, with MMAD values within the range of 1–5 μm and FPF up to 65 % (**Table 2**). As mentioned earlier, increasing the solids content in the lysozyme solution, from 1 % to 10 %, led to a reduction in the FPF.

Finally, dry powders of proteins or protein-containing products prepared by TFF also have excellent thermostability. As mentioned earlier, Li et al. reported that vaccines containing protein as antigens and an insoluble aluminum salt as an adjuvant can be readily transformed from liquid suspension into a dry powder without causing aggregation of the protein-adsorbed aluminum salt microparticles or a decrease in the immunogenicity of the antigen proteins following reconstitution of the dry powders (Li et al., 2015). Due to the presence of the aluminum salt particles in the vaccines, the vaccines may not be subjected to accidental slow freezing during distribution and storage, because slow freezing causes particle aggregation and loss of vaccine activity. Also, the protein antigens in the vaccines are generally unstable at high temperatures (e.g., RT). Therefore, vaccines containing aluminum salts as adjuvants must be kept in cold chain conditions (2–8 °C). The vaccine dry powders prepared by TFF are not sensitive to repeated slow freezing and thawing anymore. More importantly, a model protein antigen-based vaccine adjuvanted with aluminum oxyhydroxide that lost nearly all its immunogenicity after 3 months of storage at even 4 °C as a liquid did not show any significant immunogenicity decrease after it was stored as a TFF powder at 40 °C for 3 months (Thakkar et al., 2017).

TFF is a technology that enables the engineering of dry powder formulations of protein-based therapeutic products and the resultant dry powders have good aerosol

Table 2 Aerosol performance properties of MAN/lysozyme TFF powders (Moon et al., 2016).

Formulation	MMAD (μm)	GSD (μm)	FPF (%)
MAN/Lysozyme 50:50 (w/w) TFF 1 % (w/v)	2.820	3.864	65.029
MAN/Lysozyme 50:50 (w/w) TFF 5 % (w/v)	3.913	3.552	40.478
MAN/Lysozyme 50:50 (w/w) TFF 10 % (w/v)	3.546	2.825	34.150

properties and thermostability. Of course, even within dry powder formulations, challenges that must continue to be addressed are potential immunogenicity and toxicity to lungs due to concentrated doses. As previously described, due to the acceptable aerosol properties of dry powders prepared by TFF, dose and powder burden can be reduced. TFF can also play a role in minimizing the immunogenicity of protein-based products as the ultra-rapid freezing during TFF helps to minimize protein aggregation.

4. Conclusion

Thin-film freezing is an ultra-rapid cryogenic freezing technique. It can be used to prepare powders with tunable properties. Its intermediate cooling rate allows for the production of powders with high SSA, low aggregation, and submicron size particles. TFF is also a viable processing method for creating protein dry powders for pulmonary delivery. Proteins that are subjected to TFF and then sublimation to remove frozen solvent are able to maintain their structure and functionality. Dry powders of proteins prepared by TFF also have good thermostability as well as aerosol properties for pulmonary delivery.

Conflict of Interest Disclosure

Hufnagel, Moon, Sahakijpijarn, Cui, and Williams are co-inventors on IP related to this review paper. The University of Texas System has licensed this IP to TFF Pharmaceuticals, Inc. Moon, Sahakijpijarn, and Williams acknowledge consulting for TFF Pharmaceuticals, Inc. Williams and Cui own equity in TFF Pharmaceuticals, Inc.

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Authors' Short Biographies

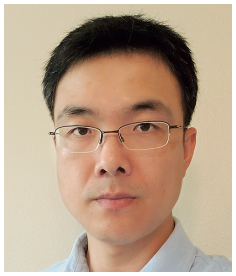
Stephanie Hufnagel

Stephanie Hufnagel received her Pharm.D. degree in 2016 and her Ph.D. degree in Pharmaceutical Sciences in 2021, both from The University of Texas at Austin, College of Pharmacy. Her primary focus is the formulation of immuno-oncology agents, including both large and small molecules. She currently works on the formulation of antibodies as dry powders.

Sawittree Sahakijpijarn

Sawittree Sahakijpijarn received her B.Sc. degree in Pharmaceutical Sciences from Chulalongkorn University in 2012 and her Ph.D. degree in Pharmaceutical Sciences from The University of Texas at Austin in 2020. She currently works as a postdoctoral research fellow in the Division of Molecular Pharmaceutics and Drug Delivery at The University of Texas at Austin. Her research focuses on pulmonary delivery of small molecule and biological drugs.

Authors' Short Biographies



Chaeho Moon

Chaeho Moon was born in South Korea and earned his Ph.D. degree in Molecular Pharmaceutics and Drug Delivery at The University of Texas at Austin. He is currently working as a Postdoc at UT Austin to develop powder formulations for dry powder inhalations, as well as TFF powder formulations of vaccine, mAb, protein, peptide, and mRNA/LNP products.

Zhengrong Cui

Zhengrong Cui is a Professor at The University of Texas at Austin, College of Pharmacy. His research interests lie in pharmaceutical drug and vaccine delivery. He also has extensive experience in thin-film freeze-drying of biologics and their needle-free delivery. Dr. Cui has published extensively in the fields of pharmaceutics, vaccinology, and nanotechnology. He is an inventor on many patents and patent applications. He serves as an editor to International Journal of Pharmaceutics. Dr. Cui holds a Ph.D. degree in Pharmaceutical Sciences from the University of Kentucky, College of Pharmacy. He is a Fellow of the American Association for Pharmaceutical Scientists.

Robert O. (Bill) Williams III

Robert O. (Bill) Williams III is the Johnson & Johnson Centennial Chair, Professor and Division Head of Pharmaceutics at the College of Pharmacy, University of Texas at Austin. Dr. Williams is Fellow of the American Association of Pharmaceutical Scientists, American Institute of Medical and Biological Engineering, and National Academy of Inventors. He was named Inventor of the Year by the University of Texas at Austin. He has founded several pharmaceutical companies. He has published over 220 peer-reviewed papers and co-edited books in pharmaceutical technology and drug delivery. He is an inventor on over 60 patents. He is Editor-in-chief of AAPS PharmSciTech.