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ORIGINAL ARTICLE

Spray-Dried and Spray-Freeze-Dried Powder Formulations of an Anti-Interleukin-4Rα Antibody for Pulmonary Delivery

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Abstract

Objective The therapeutic options for severe asthma are limited, and the biological therapies are all parenterally administered. The purpose of this study was to formulate a monoclonal antibody that targets the receptor for IL-4, an interleukin implicated in the pathogenesis of severe asthma, into a dry powder intended for delivery via inhalation.

Methods Dehydration was achieved using either spray drying or spray freeze drying, which exposes the thermolabile biomacromolecules to stresses such as shear and adverse temperatures. 2-hydroxypropyl-beta-cyclodextrin was incorporated into the formulation as protein stabiliser and aerosol performance enhancer. The powder formulations were characterised in terms of physical and aerodynamic properties, while the antibody was assessed with regard to its structural stability, antigen-binding ability, and *in vitro* biological activity after drying.

Results The spray-freeze-dried formulations exhibited satisfactory aerosol performance, with emitted fraction exceeding 80% and fine particle fraction of around 50%. The aerosolisation of the spray-dried powders was hindered possibly by high residual moisture. Nevertheless, the antigen-binding ability and inhibitory potency were unaffected for the antibody in the selected spray-dried and spray-freeze-dried formulations, and the antibody was physically stable even after one-year storage at ambient conditions.

Conclusions The findings of this study establish the feasibility of developing an inhaled dry powder formulation of an anti-IL-4R antibody using spray drying and spray freeze drying techniques with potential for the treatment of severe asthma.

Keywords Asthma · inhalation · spray drying · spray freeze drying · therapeutic antibody

Introduction

Asthma is a major chronic disease of the airways affecting an estimated 262 million people worldwide in 2019 [1]. Of the adult patients, approximately 4% have severe asthma [2], which can be extremely debilitating if not well-managed [3]. Current treatment of severe asthma involves high-dose inhaled corticosteroids and long-acting bronchodilators or systemic corticosteroids [3, 4], and there are concerns about the long-term use of oral corticosteroids due to the associated adverse effects including osteoporosis, diabetes, adrenal suppression, and depression [5, 6]. Most patients with severe asthma fall under the type 2 inflammation phenotype [3] that is characterised by eosinophilia and immune cytokines such as interleukins (ILs) 4, 5, and 13 [7].

Biologic therapy offers an alternative to escalating corticosteroid regimen for patients with severe asthma that is poorly controlled. Some biologics that have been approved for this indication include the anti-immunoglobulin (Ig) E omalizumab, anti-IL-4 receptor alpha (IL-4Rα) dupilumab, anti-IL-5Rα benralizumab, and anti-IL-5 mepolizumab and reslizumab [3]. These monoclonal antibodies (mAb) are all administered parenterally,

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which expose non-target organs to potentially high levels of drugs, thereby increasing the risk of systemic adverse events [8]. Intravenous infusions (in the case of reslizumab) require trained medical personnel [9] and are associated with sharps injuries and related transmission of blood-borne infections [10]. Although subcutaneous injections may be self-administered, non-invasive methods of administration are by and large better accepted among patients and healthcare professionals, especially in the context of chronic conditions [11].

For a respiratory disease like asthma, local treatment by delivering drugs directly to the lungs by means of oral inhalation is accompanied with numerous advantages such as rapid onset of action, possible dose reduction, minimal systemic side effects, and higher bioavailability [12]. A once-daily dosing frequency of inhaled biotherapeutics that was undertaken in clinical trials of omalizumab [13] and abrezekimab [14] for asthma is convenient and allows self-administration. Nebulisers are existing inhalation devices that are often investigated for the non-invasive pulmonary administration of antibodies [15]. On the other hand, dry powder inhalers (DPIs) may present a more compatible platform given that they generate neither heat nor a large air-liquid interface during drug administration, and the dosage form is in the solid state, all of which bolster protein stability [16, 17]. Proteins in solution are more prone to chemical and physical degradation processes which are hydrolytically driven [18]. They also require the cold chain which is a formidable logistical challenge that adds to the high costs of producing mAbs [19]. Thus, another benefit of formulating thermosensitive proteins into dry powders is the ease of transport and storage.

Spray drying and spray freeze drying are two particle engineering technologies routinely utilised to manufacture inhalable powders of biologics [20]. Spray drying is a one-step process whereby a liquid drug formulation is atomised into a hot drying gas to produce particles by solvent evaporation [21]. Thermal stresses at high temperatures and shear forces during atomisation are the main factors that can affect stability of proteins [22]. In spray freeze drying, the drug solution is atomised directly above a cryogenic liquid; the droplets freeze instantaneously and are collected in the liquid. The lyophilisation process is completed after the frozen particles undergo sublimation to remove the solvent [23]. Although spray freeze drying produces dry powders without subjecting biomacromolecules to heat stress, this technique involves shear stress during atomisation, thermodynamic instability during lyophilisation, and protein adsorption at the air-liquid interface, all of which may promote aggregation [24, 25]. Due to the delicate nature of biologics, additional stabilising excipients are needed for their protective effects against the various stresses during these drying processes.

Carbohydrates are frequently used as excipients in solid-state biotherapeutics [26], and among them, cyclodextrins have emerged as a promising class of protein stabilisers [27, 28] that can be engineered to possess particle properties relevant to inhalation delivery [29]. Cyclodextrins stabilise proteins by several proposed mechanisms such as water replacement, vitrification, and surfactant-like effects [28]. Notably, 2-hydroxypropyl-beta-cyclodextrin (2HPβCD) is a hydroxyalkyl derivative of cyclodextrin reported to be effective in protecting proteins with its unique amphiphilic quality and availability of hydrogen bonds, while producing powders with good aerosol performance and longer shelf-life [28, 30, 31]. It is included as an excipient in a number of licensed drug products for the intravenous, intramuscular, and oral routes [32]. Notwithstanding limited data on its safety when delivered by the inhalation route [33], 2HPβCD is known to be well-tolerated in humans after short-term nasal administration [34].

In contrast to some lyophilised biologics that are reconstituted prior to administration via intravenous infusion, inhaled biologics require suitable aerodynamic properties in addition to protein stabilisation in the dry state [20, 35]. This extra assemblage of criteria complicates the formulation and manufacturing process, as well as demand more comprehensive characterisation of the powder aerosols. In this work, a series of solid-state anti-human IL-4Rα mAb was prepared using spray drying and spray freeze drying to produce inhalable powders intended for pulmonary delivery. To this end, the mAb was co-formulated with 2HPβCD as the protein stabiliser and aerosol performance enhancer. The aims of this study were (i) to develop and characterise the spray-dried (SD) and spray-freeze-dried (SFD) powder formulations of the anti-human IL-4Rα mAb, and (ii) to assess the protein stability and *in vitro* bioactivity of the mAb post-processing.

Materials and Methods

Materials

Anti-human IL-4Rα mAb (10 mg/mL) in phosphate-buffered saline (PBS) was received from Shanghai MabGeek Biotech. Co., Ltd. (Shanghai, China) and stored at -80°C. The anti-human IL-4Rα mAb is a humanised IgG4 developed by MabGeek, generated from mouse hybridoma and expressed by CHO-K1 cells (ATCC® CCL-61™, Manassas, VA, USA). 2HPβCD, bovine serum albumin (BSA), Tween® 20, Brilliant Blue R-250, and sodium phosphate (Na_3PO_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human (rh) IL-4Rα, rhIL-4, granulocyte-macrophage colony-stimulating factor (rhGM-CSF), and substrate reagent pack, which comprises colour

reagents A (stabilised hydrogen peroxide) and B (stabilised tetramethylbenzidine), were purchased from R&D Systems (Minneapolis, MN, USA). The detection antibody, a horseradish peroxidase (HRP)-conjugated polyclonal goat $F(ab')_2$ directed against human IgG, was procured from Abcam (Cambridge, UK). Bradford reagent was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Dithiothreitol (DTT), prestained protein ladder (PageRuler™ Plus), RPMI 1640 medium powder, foetal bovine serum (FBS), and antibiotic-antimycotic (Anti-Anti) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) was bought from MedChemExpress (Monmouth Junction, NJ, USA). Sulfuric acid (H_2SO_4) was acquired from BDH Chemicals (Poole, England) and sodium bicarbonate ($NaHCO_3$) from VWR Chemicals BDH® (Leuven, Belgium). The wash buffer (0.05% v/v Tween® 20 in PBS), stop solution (2N H_2SO_4), destaining solution (50% v/v methanol plus 10% v/v acetic acid in distilled water), and mobile phase (150 mM Na_3PO_4 buffer, pH 6.8) were prepared in-house. Ultrapure water used was obtained from a laboratory water purification system with pore size rating 0.2 µm (Barnstead NANOpure Diamond™, APS Water Services, Van Nuys, CA, USA).

Formulation and Drying

Preparation of Feed Solutions

The antibody solutions were thawed and desalted by ultrafiltration (Amicon® Ultra 30K, Millipore, Sigma-Aldrich) for two 20-minute cycles at 4000×g, consisting of a dilution with ultrapure water in-between. The concentrated antibodies were quantified by Bradford protein assay using bovine γ-globulin as the standard. For each formulation, 2HPβCD was weighed and dissolved in an appropriate volume of ultrapure water according to the composition shown in Table 1. Antibody solution was added to the 2HPβCD solution immediately prior to the drying procedure and the feed solution was mixed by gently swirling.

Spray Drying

A mini spray dryer (B-290, BÜCHI Labortechnik AG, Flawil, Switzerland) set to following operating conditions, which were adopted and modified from a previous study [36], was used: spray gas (nitrogen) flow 742 L/hour, inlet temperature 100°C, 3% peristaltic pump rate (approximately 0.9 mL/min), and 100% aspirator rate (gas flow rate of approximately 35 m^3/hour). The feed solutions were atomised by an integrated two-fluid nozzle of 0.7 mm internal diameter (BÜCHI) and dispersed into the spray cylinder. In addition to the two primary SD formulations, three extended SD formulations based on the composition of SD1 were prepared. These were spray-dried at inlet temperatures of 120°C (SD1a), 150°C (SD1b), and 200°C (SD1c) while keeping all other parameters the same as the primary SD formulations.

Spray Freeze Drying

The feed solution was first drawn into a 10-mL syringe which was then connected via a silicone feeding tube to the same two-fluid nozzle used for spray drying. The spraying and freezing parameters were adopted from a previous study [37]. Using a syringe pump, the solution was driven through the nozzle at a controlled rate of 2 mL/min. Nitrogen gas flow rate was set at 670 L/hour. Since the nozzle tip was positioned above liquid nitrogen, the atomised liquid droplets froze instantaneously as they descended onto the liquid nitrogen. The stainless-steel vessels containing the frozen particles suspended in liquid nitrogen were transferred into a freeze-dryer (FreeZone® 6 Litre benchtop freeze-dry system with stoppering tray dryer, Labconco®, Kansas City, MO, USA). Primary drying was carried out for 20 hours at -25°C, followed by a gradual increase in the temperature over 4 hours at a constant ramp rate of 0.19°C per minute. Secondary drying continued for the remaining 48 hours at 20°C. The chamber pressure was kept below 0.021 mbar throughout.

Table 1 Composition of feed solutions for spray drying and spray freeze drying. Feed solutions for the three extended SD formulations (SD1a, SD1b, and SD1c) were identical in composition to SD1

Formulation	Drying method	Antibody content (% w/w)	2HPβCD content (% w/w)	Solute concentration
SD1	Spray drying	25	75	2% w/v
SD2		50	50	
SFD1	Spray freeze drying	25	75	5% w/v
SFD2		50	50	

2HPβCD: 2-hydroxypropyl-beta-cyclodextrin; SD: spray-dried; SFD: spray-freeze-dried

Scanning Electron Microscopy (SEM)

The particle morphology and geometric size were investigated by SEM. Samples were first mounted onto aluminium specimen tubs with adhesive carbon tape. To enhance sample conductivity and prevent overheating, the surface of the mounted samples was then coated with approximately 13 nm of gold-palladium for 120 seconds at 30 mA using argon gas (Q150R ES Plus, Quorum Technologies, East Sussex, UK). Subsequently, the samples were imaged using a field emission scanning electron microscope (Hitachi S-4800, Tokyo, Japan) at 5,000× and 10,000× magnifications at an accelerating voltage of 5 kV and 4.8–6.3 mm working distance.

Differential Scanning Calorimetry (DSC)

The thermal behaviour of the powder formulations was studied by DSC. Approximately 1–3 mg of SD formulations and 0.3–0.4 mg of SFD formulations were each weighed into a 5.4 × 2.0 mm aluminium hermetic pan (Jingyi Chemical Materials, Shanghai, China) encapsulated with a needle-pierced lid. The pans were sealed using a sample press and loaded onto an indium-calibrated differential scanning calorimeter (DSC 250, TA Instruments, New Castle, DE, USA) and kept isothermal at 0°C for 10 minutes before being heated at a rate of 10°C/min to 300°C. The DSC thermograms were plotted using Origin® software (OriginLab®, Northampton, MA, USA).

Thermogravimetric Analysis (TGA)

The water content of the powder formulations was determined by TGA. Approximately 0.3–4 mg of each powder formulation was heated from ambient temperature to 105°C at a constant rate of 10°C/min in a thermogravimetric analyser (TGA 5500, TA Instruments, New Castle, DE, USA). The weight loss would account for the residual moisture that evaporated from the sample.

Aerosol Performance

A Next Generation Impactor (NGI; Copley Scientific, Nottingham, UK) was used to evaluate the aerosolisation efficacy of the powder formulations. A pressure drop of 4 kPa was achieved by an airflow rate adjusted to approximately 54 L/min using a high-resistance handheld osmohale™ DPI (Pharmaxis, Frenchs Forest, NSW, Australia). At this flow rate, the flow duration was fixed at 4.4 seconds to allow 4 L of air to be withdrawn per run. The impaction surfaces of the NGI collection cups were sprayed with a thin layer of silicone lubricant to reduce particle bounce [38]. Each sample was weighed (10±0.1 mg for SD powders; 3.5±0.1 mg for SFD powders) and loaded into a size 3 gelatin capsule (Capsugel®, Morristown, NJ, USA), and placed in the inhaler. For each assayed element of the NGI assembly, 5 mL of ultrapure water was used to dissolve the powder. The solution was drawn into a 1-mL syringe and filtered through a nylon syringe filter of 0.45 µm pore size (Membrane Solutions®, Auburn, WA, USA) into an amber glass vial. The vials were capped and refrigerated at 4°C before further analysis. Each formulation was tested in triplicate. The concentrations of 2HPβCD were determined by high-performance liquid chromatography (HPLC) coupled to a refractive index detector (RID) with two conjoined Hi-Plex H guard columns (Agilent Technologies, Santa Clara, CA, USA) ran using ultrapure water as the mobile phase at 65°C. The flow rate was 0.6 mL/min, and the injection volume was 50 µL with a stoptime of 8 minutes. The peaks were integrated using Agilent Technologies OpenLab CDS ChemStation Edition (version C.01.06) software and the peak areas compared to a calibration curve.

The deposition profile was defined by the following parameters: recovered dose (RD), emitted dose (ED), emitted fraction (EF), fine particle dose (FPD), fine particle fraction (FPF), mass median aerodynamic diameter (MMAD), and geometric standard deviation (GSD). With reference to 2HPβCD, the RD is the mass recovered from all the twelve elements of the NGI assembly; the ED is the mass discharged from the inhaler; and the FPD is the assayed mass with aerodynamic diameters less than 5 µm. The formulae for EF and FPF are given below. The MMAD and GSD were calculated according to the methods described in *USP on Compounding* [39]. The MMAD is the diameter at which half of the aerosolised particles by mass are larger and the other half smaller, while the GSD reflects the spread of the particle aerodynamic diameters [40].

$$EF = \frac{ED}{RD} \quad FPF = \frac{FPD}{RD}$$

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was applied to verify the molecular mass and fragmentation of the antibody after the drying process. The powder formulations were reconstituted with ultrapure water and the unprocessed monoclonal antibody (mAb-up) was included as reference. Two sets of sample solutions were prepared, with one treated with 5 mM DTT to produce reducing conditions, and the other without. The reduced samples were boiled at 95°C for 5 minutes in a dry bath. After each well was loaded with 2 µg of antibody, the 10% acrylamide gels were run in an electrophoresis system (Mini-PROTEAN® Tetra System, Bio-Rad) at an applied voltage of 80 V for 40 mins, then at 120 V for a further 60 mins. After electrophoresis, the gels were stained

in 0.1% w/v Coomassie Brilliant blue R-250 for 2 hours at room temperature on an orbital shaker. Destaining was accomplished after two rounds of hourly washing with fresh destaining solution, followed by an overnight wash. Images of the protein bands were visualised and captured using a G:BOX Chemi XR5 gel documentation system (Syngene, Cambridge, UK) controlled by the GeneSys software (version 1.6.9.0, Syngene).

Size-Exclusion Chromatography (SEC)

Given that aggregation is a predominant concern in the development of antibody-based biotherapeutics [41], the monomer content was monitored as a gauge of physical stability and product homogeneity during storage. SEC was employed to quantify monomer levels of the primary formulations at three time points (1 week, 4 months, and 1 year post-drying) and the extended formulations at two time points (1 week and 6 months post-drying). The SEC system encompassed HPLC coupled to a diode array detector (Agilent Technologies) and was performed on a Yarra™ 3 µm SEC-3000 column (phenomenex®, Torrance, CA, USA) at 25°C. The flow rate of the mobile phase (aqueous Na_3PO_4) was 0.8 mL/min and the detection UV wavelength was set at 214 nm. Fifty microlitres of buffer-reconstituted sample solutions, adjusted to a concentration of 200 µg/mL antibody, was injected with a stoptime of 16 minutes. The monomer peaks were integrated using Agilent Technologies OpenLab CDS ChemStation Edition (version C.01.03) software and the percent monomer content was calculated.

Enzyme-Linked Immunosorbent Assay (ELISA)

Ninety-six-well microplates were coated overnight at 4°C with 50 ng capture antigen (rhIL-4Rα) per well. The wells were washed with wash buffer and blocked with reagent diluent (2% w/v BSA in PBS) for at least one hour at room temperature, before being washed again. The selected formulations and mAb-up were adjusted to 100 and 10 µg/mL with reagent diluent and added in triplicate to the wells. After incubation for 90 minutes at room temperature, the plates were washed and added with HRP-conjugated detection antibody, diluted 80,000-fold in reagent diluent (6.25 ng/mL). The plates were left to incubate for 1 hour at room temperature and washed thereafter. Substrate solution consisting of colour reagents A and B mixed in equal portions was added to the wells. The plates were placed in a black resealable bag to avoid direct light and incubated for 20 minutes at room temperature. Stop solution was then added and the plates were gently tapped to ensure thorough mixing. Absorbance at wavelengths of 450 nm and 570 nm was measured by a microplate spectrophotometer (Thermo Scientific Multiskan GO) and subtracted from each other to correct for optical inaccuracies. The ELISA experiment was repeated thrice. GraphPad Prism (version 8.2.1, San Diego, CA, USA) was used to plot the bar chart showing the mean optical density values.

Cell Anti-Proliferation Assay

Human erythroleukaemic TF-1 (ATCC® CRL-2003™) cells were used for the anti-proliferation assay because they have an absolute dependence on growth factors such as GM-CSF, IL-4, and IL-13 for long-term proliferation and survival [42]. The anti-human IL-4Rα antibody competes with IL-4 for binding to IL-4Rα, thereby suppressing proliferation of the TF-1 cells in the absence of other growth factors. The cells were grown in complete growth medium (CGM) that was composed of RPMI-1640 as the base medium, supplemented with 2.0 g/L $NaHCO_3$, 2 ng/mL rhGM-CSF, 10% heat-inactivated FBS, and 1% Anti-Anti (a mixture of penicillin, streptomycin, and amphotericin B). The cells were harvested by centrifugation at 100×g for 10 minutes, and resuspended in the assay medium (CGM without rhGM-CSF) at a concentration of 6.25×10^5 cells/mL. The cells were added to flat-bottom 96-well microplates (TPP®, Trasadingen, Switzerland) at a density of 7.5×10^4 cells/120 µL per well. After 24 hours of starvation at 37°C and 5% CO_2, 30 µL of test solution was added to each well in duplicate. The test solutions were prepared by 3-fold serial dilutions of reconstituted antibody solutions with the assay medium, followed by the addition of rhIL-4. A series of 10 different concentrations of antibody, beginning with 100 µL/mL, in the final reaction volume of 150 µL was produced. All wells contained a fixed concentration of rhIL-4 (8 ng/mL). The plate was incubated for 2 days under the same conditions. At the 45th hour, 10 µL of CCK-8 solution was added into each well, and incubation was continued for a further 3 hours. After the incubation, the plate was mixed gently on a microplate mixer to ensure homogeneous distribution of dye, and the absorbance was read at 450 nm. Using GraphPad Prism, the optical density (*y*-axis) was plotted against the log antibody concentration (*x*-axis) to obtain concentration-response curves and IC_{50} values. The assays were run in quadruplicate.

Statistical Analysis

All data are reported as mean ± standard deviation. Two-tailed Student's *t*-test was performed using GraphPad Prism (version 8.2.1) to evaluate differences in optical density and IC_{50} values, where P values < 0.05 were deemed statistically significant.

Results

Spray Drying and Spray Freeze Drying

After the drying processes, the recovered powders were transferred into transparent glass vials and maintained at around 25% relative humidity in an auto dry box (Eureka Dry Tech, Taipei, Taiwan) at air-conditioned temperature (around 22°C). The inlet and outlet temperatures for the SD formulations, processing yield, and residual water content are displayed in Table 2. The processing yield is defined as the percentage of powder weight in the product collection vessel to the total solute mass in the feed solution. Among the primary formulations, spray freeze drying produced higher process yields (> 60%) and lower residual water content (~7%) than spray drying. Despite being spray-dried at higher temperatures, the extended formulations did not have an apparent reduction in residual moisture content.

Morphology

SEM of the powder formulations from the primary and extended formulations (Fig. 1) reveals generally globular particles with multiple dents on the smooth surface, creating a shrunken appearance. This morphology increases the surface-area-to-volume ratio relative to a perfect sphere. The majority of the SD particles are less than 5 µm in diameter. For the two SFD formulations, the particles are spherical and porous. The SFD particles are visibly larger than the SD particles, although the aerodynamic diameter would be smaller when the density is lower [43].

Thermal Behaviour

The DSC thermograms of the formulations are shown in Fig. 2. Observed in every sample is a broad endothermic peak before 100°C, correlating to dehydration of the powders. The dehydration peak is smaller for the SFD formulations, suggesting a lower water content, which is in agreement with the TGA results. Crucially, the absence of distinct sharp peaks across all the thermograms indicates the amorphous nature of the powder formulations.

Aerosol Performance

The key parameters describing the aerodynamic properties of the powder formulations, namely, EF, FPF, MMAD, and GSD, are shown in Table 3. These parameters represent the dispersibility, respirable concentration, particle size, and particle size distribution of the powders, respectively. The EF was considerably higher (> 82%) for the SFD formulations compared with the SD formulations (~53 to 62%), suggesting that lower moisture content is critical to achieve greater powder dispersion. The differences in FPF were less remarkable, with average values ranging from ~36% to 56%. The MMAD of all the formulations were within the desirable range of 0.5–5 µm for deep lung penetration [44]. The GSD values were all higher than 1.22, indicating that the aerosols were poly- or hetero-disperse [45], typical of particles emitted by most atomisers [46].

Antibody Stability

The absence of artifact bands on the SDS-PAGE images (Fig. 3) demonstrate that the structural integrity of the SD antibody in the powder formulations was preserved after the drying processes. There are some faint low molecular weight bands in the SFD formulations, which suggests a small degree of fragmentation might have occurred. In the non-reduced samples, the bands at around 150 kDa coincide with the molecular weight of intact IgG. Under reducing conditions, the disulfide bonds linking the various chains of the IgG were cleaved, giving rise to bands at 50 and 25 kDa, which correspond to the molecular weight of the heavy and light chains, respectively [47].

Table 2 Drying outcomes: outlet temperature, processing yield, water content

Formulation	Inlet temperature	Outlet temperature	Processing yield	Water content
		Primary formulations		
SD1	100°C	63°C	50.2%	9.3%
SD2	100°C	65°C	23.7%	8.6%
SFD1	–	–	69.1%	6.9%
SFD2	–	–	60.8%	6.9%
		Extended formulations		
SD1a	120°C	78°C	80.1%	8.7%
SD1b	150°C	99°C	67.9%	9.2%
SD1c	200°C	129°C	72.6%	7.6%

SD: spray-dried; SFD: spray-freeze-dried

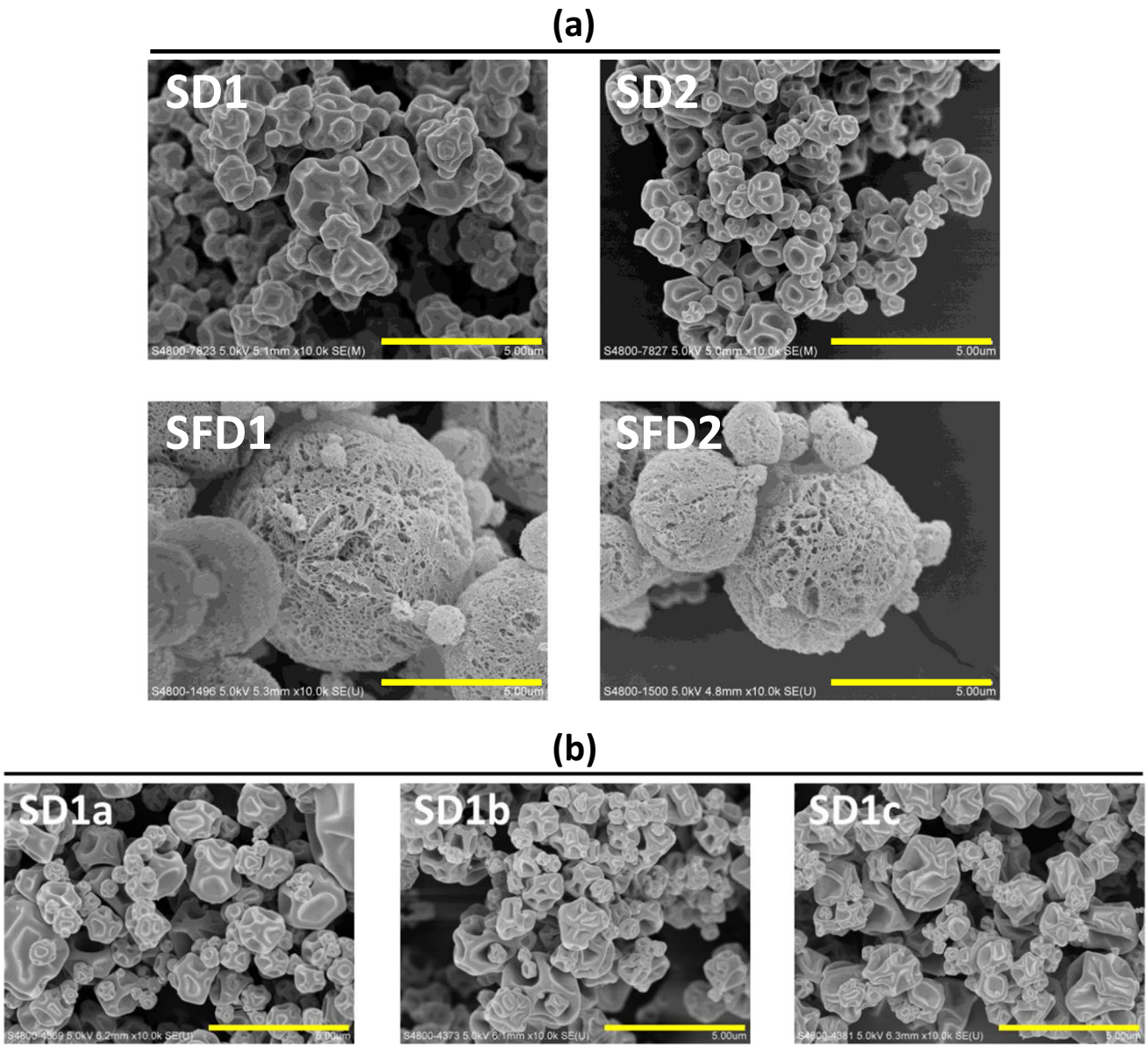

Fig. 1 SEM images of the (**a**) primary formulations and (**b**) extended formulations. Scale bar=5 µm.

The antibody monomer content of the primary formulations was quantified at three time points over the duration of a year using SEC (Fig. 4). Even though the samples were not stored refrigerated or frozen, the decrement in monomer content was modest, especially for the formulations containing less antibody (i.e., SD1 & SFD1, 1.1–2.8% vs. SD2 & SFD2, 7.2–7.5%). This suggests that protein concentration may play role in exacerbating aggregation [48]. For the extended formulations, the decrease in monomer content of the formulations spray-dried at lower temperatures, SD1a and b, was minimal, i.e., 0.1% and 1.1% respectively, after half a year. However, for SD1c which was spray-dried at 200°C, the monomer content was substantially lower compared with the unprocessed antibody (40% reduction), followed by a further drop of 16.1% after 6 months. This highlights that extreme temperatures can induce protein aggregation [49], even in the presence of a protein stabiliser.

Antigen-Binding and Inhibitory Potency

The capacity of the antibody to bind to its antigen (IL-4Rα) after the drying processes was assessed by ELISA (Fig. 5). At the two selected antibody concentrations, there was no statistically significant difference in the optical density values between the dried formulations and the unprocessed mAb (100 µg/mL: SD2 vs. mAb-up, p=0.0696; SFD2 vs. mAb-up, p=0.3281; 10 µg/mL: SD2 vs. mAb-up, p=0.9572; SFD2 vs. mAb-up, p=0.9661).

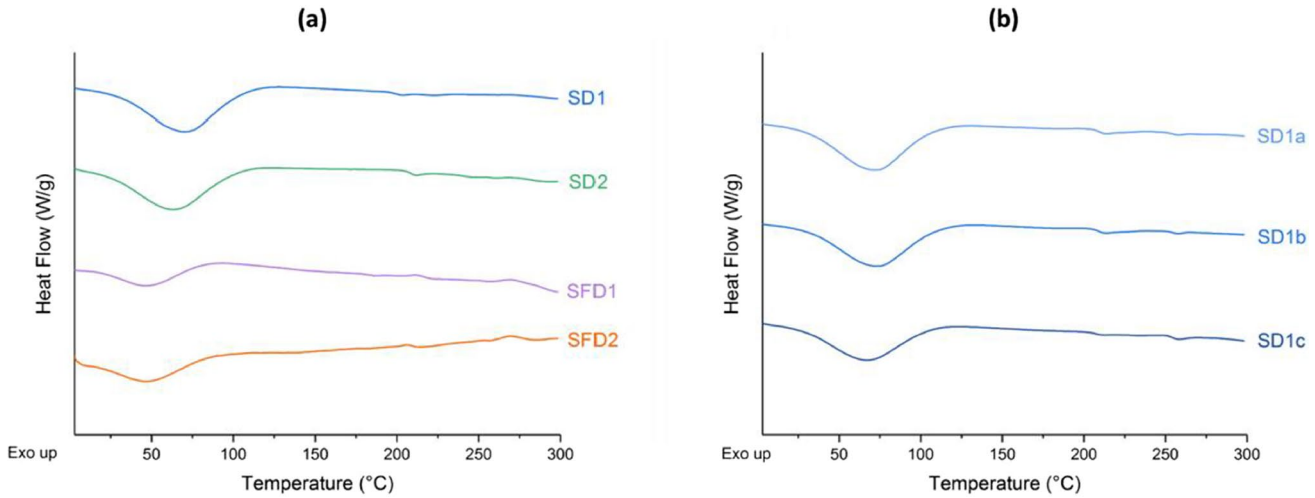

Fig. 2 DSC thermograms of the (**a**) primary formulations and (**b**) extended formulations.

Table 3 Aerodynamic properties of the primary formulations and extended formulations

Formulation	EF	FPF	MMAD	GSD
Primary formulations				
SD1	60.4 ± 5.1%	48.5 ± 6.3%	2.13 µm	2.05
SD2	52.5 ± 0.8%	46.6 ± 0.8%	1.57 µm	2.11
SFD1	84.1 ± 1.9%	56.0 ± 9.5%	1.53 µm	3.58
SFD2	81.5 ± 3.0%	48.7 ± 4.7%	1.60 µm	3.48
Extended formulations				
SD1a	54.9 ± 4.7%	36.2 ± 4.0%	2.51 µm	2.02
SD1b	53.2 ± 1.0%	39.4 ± 1.2%	2.43 µm	1.92
SD1c	62.4 ± 1.6%	49.7 ± 1.1%	2.23 µm	1.88

EF: emitted fraction; FPF: fine particle fraction; GSD: geometric standard deviation; MMAD: mass median aerodynamic diameter

The concentration-response curve in Fig. 6 was obtained by first depriving TF-1 cells of the essential growth factors required for survival, followed by salvaging the cells with rhIL-4, but also in the presence of a range of anti-human IL-4 antibody concentrations. Increasing the antibody concentration led to a reduction in cell proliferation, hence the IC_{50} values for the formulations could be compared with that for the mAb-up (mAb-up: 0.566±0.106 µg/mL; SD2: 0.632±0.120 µg/mL; SFD2: 0.837±0.208 µg/mL). The differences in IC_{50} values between the antibody in the dried formulations and mAb-up were not statistically significant (SD2 vs. mAb-up, p=0.3269; SFD2 vs. mAb-up, p=0.1392). This illustrates that after the drying processes, the antibody still retained its inhibitory potency relative to its initial state.

Discussion

The experimental design of this study allows comparisons to be made between spray drying and spray freeze drying as practicable methods of producing dry powders of a biomacromolecule that is intended for pulmonary delivery. The suitability of the powder formulations for the inhalation route was ascertained through an array of analytical methods. Processing yield is an important determinant when considering scaling up the manufacture of pharmaceuticals [50], and in this regard the yields reported here are modest at best. In spray drying, the product loss was probably attributed to the adhesion and accumulation of particles on the inner walls of the spray cylinder and cyclone [51]. In spray freeze drying, some product was lost likely due to the retention of residual feed solution in the syringe and feeding tube. Nevertheless, spray drying is a more established and popular drying technology than spray freeze drying for industrial scale production [19, 52, 53].

Residual water content was a key parameter in this study for two reasons. Firstly, moisture can increase particle cohesion, causing agglomeration and drastically lower the FPF [54, 55]. The second reason relates to the stabilisation of proteins in the dry state. Water can effectively reduce the glass transition temperature of sugar glasses and enhance local mobility of the biomacromolecules which is detrimental to protein stability [26]. Hence, for the sake of aerosol performance and protein stability, there was a dire need to minimise water content. The water content measurements were all higher than the target range of 3 to 4% that is ideal for inhaled powder formulations containing biologics [56]. This was not attained even with spray freeze drying, a method known to produce low residual moisture content [57], hypothetically due to the presence of 2HPβCD, since it was the common excipient across all the formulations.

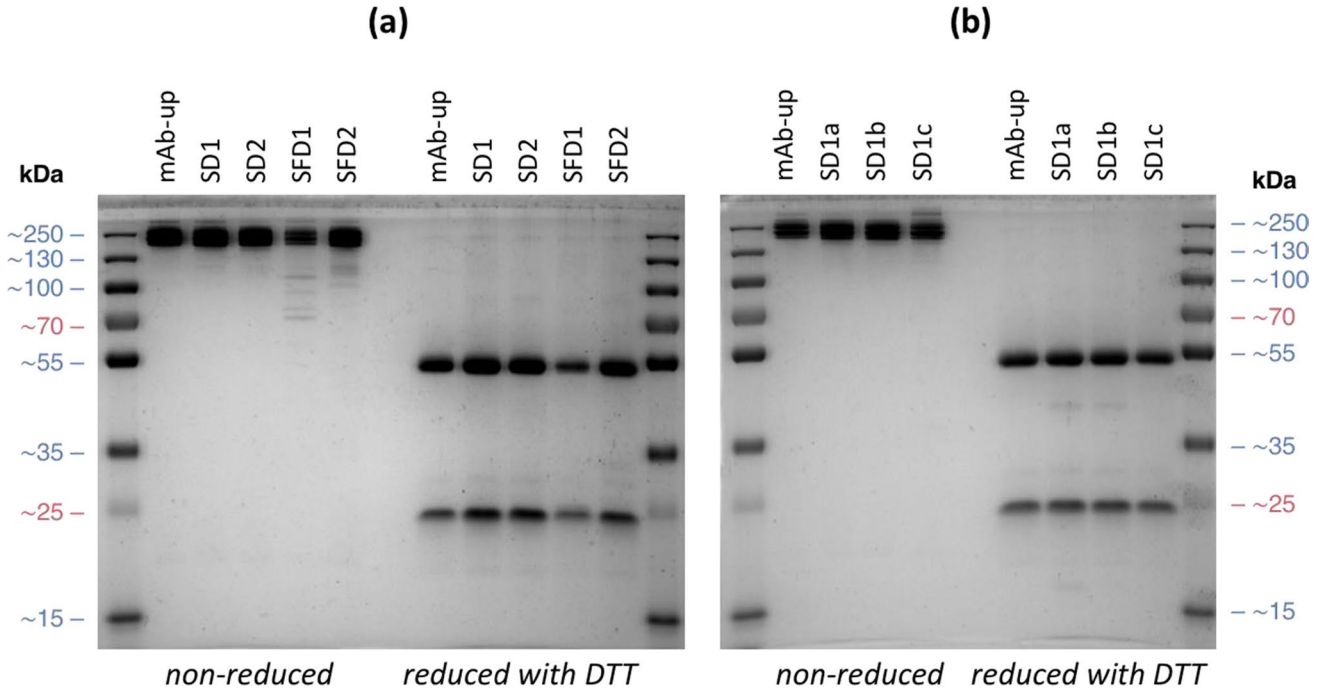

Fig. 3 SDS-PAGE images of the (**a**) primary formulations and (**b**) extended formulations. DTT: dithiothreitol; mAb-up: unprocessed monoclonal antibody.

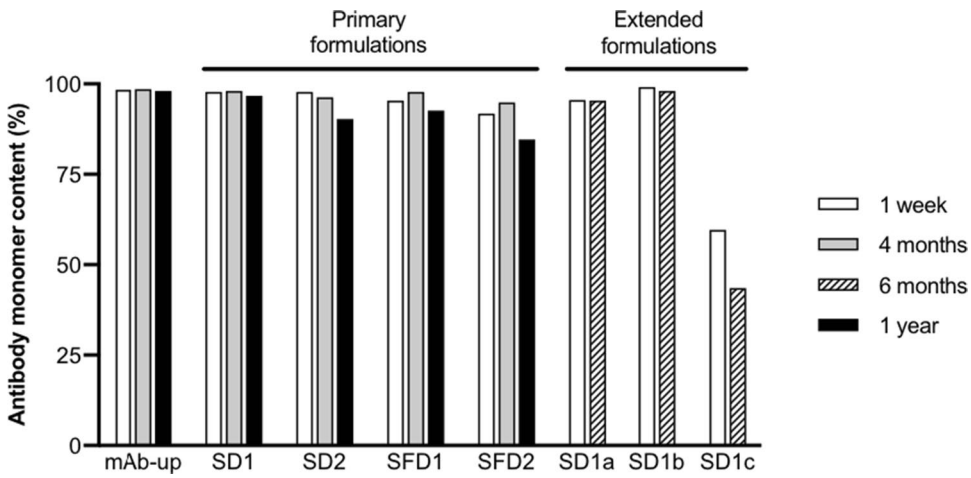

Fig. 4 Antibody monomer content of the primary and extended formulations quantified by SEC. mAb-up: unprocessed monoclonal antibody.

2HPβCD was chosen as the excipient in this study for partly because of its non-reducing nature [58] and presumed low hygroscopicity [59]. The hygroscopicity of cyclodextrins, despite their use in medicinal research, are not well-understood [60], particularly when complexed with biological molecules. The extended formulations were indeed prepared in an attempt to reduce the water content by increasing the spray-drying temperature. Another concern with water content, although less relevant here, is that it drives protein degradation via the Maillard reaction [26] associated with reducing sugars such as lactose, glucose, and maltose [61]. Nonetheless, over-drying should be avoided as electrostatic charges may affect aerosolisation performance [62].

Comparing spray freeze drying and spray drying, the former was superior in terms of producing marginally lower moisture content and more consequentially, better aerosol performance. The EF of the SFD powders (84.1±1.9% and 81.5±3.0%) was higher than all the EF generated by the SD powders (52.5–62.4%), while the FPF was largely comparable. The residual powder in the SD formulations was distributed between the capsule and inhaler approximately in a 4:7 mass ratio (15.7 ± 3.6% vs. 27.6 ± 2.9%). It is plausible that the favourable aerosol properties of the SFD powders could be ascribed to the particle morphology, water content, and MMAD (1.53 µm and 1.60 µm). As shown in the SEM images, the larger and more porous SFD particles

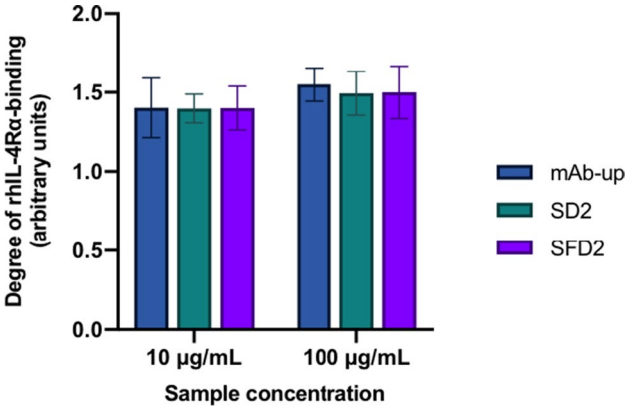

Fig. 5 Antigen-binding ability of the antibody in the selected SD and SFD formulations by ELISA (n=4). 96-well plates were coated with rhIL-4Rα. mAb-up: unprocessed monoclonal antibody.

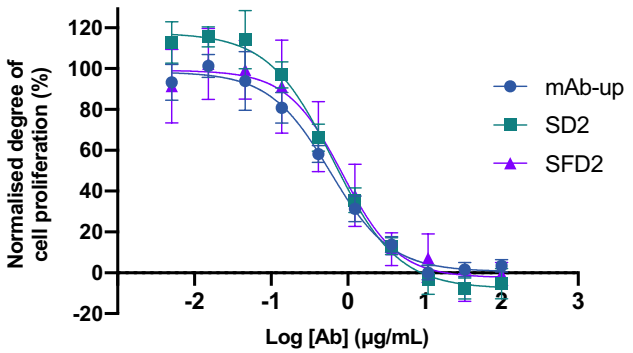

Fig. 6 Concentration-response curve of the inhibitory effect of the antibody in the selected SD and SFD formulations on the proliferation of TF-1 cells stimulated by rhIL-4 (n=4). The optical density is normalised with respect to the best-fit top (100%) and bottom (0%) values of the mAb-up curve in the individual runs. mAb-up: unprocessed monoclonal antibody.

were conceivably more flowable and dispersible, contributing to their desirable aerosol performance. The physical and aerodynamic characteristics of the SFD powders observed in this study were similar to those obtained by another research group, although their IgG formulations included trehalose in combination with 2HPβCD [63, 64]. From the four primary formulations, it appears that increasing the protein concentration, which effectively reduced the 2HPβCD content, might have adverse effects on aerosol performance, as both the EF and FPF were slightly decreased when the antibody concentration was doubled from 25% to 50%.

Despite yielding powders with relatively high levels of water and moderate dispersibility, spray drying still holds promise as a means of creating respirable particles. Two pertinent outcomes have been achieved in the SD formulations here, the ideal aerodynamic diameter (0.5–5.0 µm) for deposition in the lower airways desirable for asthma management [40] and the amorphous solid-state structure. It is customary for spray drying to generate amorphous materials [52] and this physical state is fundamental to the vitrification mechanism of protein stabilisation by the sugar [26]. Furthermore, spray drying has several advantages over spray freeze drying including operational simplicity, easy scalability, lower production costs [52]. Most pivotally, there is huge potential for the water content to be reduced and aerosol performance improved in spray drying. In a study involving SD powder formulations of infliximab (using trehalose and cysteine as excipients) for respiratory administration, the EF spanned 70 to 93%, with a few FPF values exceeding 60% [65]. Another study prepared SD bevacizumab with trehalose and leucine for delivery by inhalation which attained a 3–4% water content and a FPF of 82% [56]. Even though these SD formulations that produced encouraging results, albeit under different experimental conditions, did not incorporate any cyclodextrins, 2HPβCD has demonstrated the capacity to yield microparticles with satisfactory aerodynamic behaviour and stabilise antibody during spray drying and long-term storage [29]. It is also common for cyclodextrins to form an amorphous glassy matrix [28].

With the exception of SFD2, the antibody in the other primary formulations were adequately stabilised. There was an approximate 10% reduction in the monomer content of SFD2 measured one week after spray freeze drying, compared with the unprocessed antibody stock used to prepare the feed solutions. Considering the monomer content between the two particle engineering techniques, it seems that spray freeze drying was more damaging to the antibody than spray drying, particularly when the concentration of the antibody was increased from 25% in SFD1 to 50% in SFD2, with a reciprocal decrease in 2HPβCD concentration. This underscores the necessity to optimise the protein-to-excipient ratio for individual proteins. Aggregation of antibodies not only lowers the bioavailability of pharmacologically active monomers, but could also induce immunogenicity [15]. In a randomised clinical study involving adults patients with mild allergic asthma, one participant was found to have developed neutralising antibodies to the nebulised omalizumab [13]. In spite of the higher amount of antibody aggregates in SFD2, the antigen-binding ability and *in vitro* inhibitory potency were retained relative to SD2 and mAb-up. IL-4 is one of the cytokines that mediates type 2 inflammation in asthma and promotes eosinophil recruitment, leading to airway hyperresponsiveness and remodelling [66]. By inhibiting IL-4Rα, the signalling pathway for type 2 inflammation can be blocked for the treatment of asthma [67]. The stability conferred to the antibody justifies the utility of 2HPβCD as lyoprotectant [28]. A limitation of this study was the omission of pure SD and SFD antibody to elucidate the protective effects of 2HPβCD to mitigate protein denaturation.

The three extended formulations were included to examine the effects of increasing the inlet temperature during

spray drying on aerosol performance and protein stability. The resultant outlet temperature, which is measured at a point prior to the entrance of the cyclone where particles are separated from the gas stream, represents the highest temperature that the product may be subjected to [68]. Although increasing the inlet temperature from 100°C to 200°C led to a parallel increase in the outlet temperature, the water content did not reduce, except when the inlet temperature was raised to 200°C. This suggests that relying on the spray-drying temperature to lower moisture content is an ineffective approach, unless an excessively high inlet temperature is applied. A study on SD anti-IgE mAb and mannitol came to a similar conclusion [69]. Other work that also increased inlet temperature in SD formulations of proteins and carbohydrates reported only marginal variations in the moisture content. In one study, the moisture content was high (7.4–9.7%) at inlet temperatures between 80 and 140°C for SD anti-IgE mAb and lactose [70]. Another study noted a slight decrease in residual moisture (0.5–1.5%) when the inlet temperature was increased from 90 to 130°C for SD formulations of IgG1 and mannitol, but observed a direct relationship between residual moisture and protein content [71]. In these studies, as well as ours, the increase in the outlet temperature did not translate into substantial reductions in the residual moisture content, a phenomenon that could be attributed to the intrinsic binding between the antibody and water molecules [72]. Nonetheless, such high temperatures did not affect the morphology of the particles formed or the structural integrity of the antibody. Congruent with the water content, the differences in aerosol performance between the extended formulations were likewise unremarkable.

With regard to the physical stability of the antibody in the extended formulations, there is a distinction between SD1c (inlet temperature of 200°C) and the other two formulations that were spray-dried at lower temperatures. Even at inlet temperatures of 120°C and 150°C, the monomer content was unaltered relative to mAb-up, indicating the efficacy of 2HPβCD to stabilise the labile biomacromolecules against thermal stress. However, this protection was surmounted when an enormous quantity of heat was introduced. Since the antibody was in the liquid state before solvent removal, it is imperative to keep the temperature well below the melting temperature in order to prevent conformational changes and loss of functionality [26, 49]. During the early phases of solvent evaporation in spray drying, the thermal stress is attenuated by the "web-bulb" effect [52], which presents the lowest temperature reached through evaporative cooling that the atomised droplets encounter [73].

A major obstacle identified in this work for the development of inhaled biologics was the undesirably high levels of water in the SD powder formulations. Antibody destabilisation during storage in an unduly moist microenvironment may potentially be more detrimental than the physical and thermal stresses experienced amid processing, since the reduction in monomer content was more pronounced at one year compared to one week after drying for most of the primary formulations. As such, future work should be channelled towards investigating alternative strategies to minimise moisture content in solid dosage forms. These could include using organic in lieu of aqueous solvents [74, 75], integrating supplementary vacuum drying steps [71, 76, 77], employing dehumidified air or dry nitrogen as the spray gas [72], and incorporating additional hydrophobic or non-hygroscopic excipients, for instance, cysteine [78] or leucine [79]. It will also be useful to conduct stress testing to evaluate the susceptibility of the excipient-stabilised antibody under conditions of elevated temperature and humidity.

Conclusions

This work corroborates the feasibility of using 2HPβCD as a protein stabiliser in SD and SFD powders of a mAb. Antibody monomer content in the solid state remained broadly unchanged over a storage period of a year at ambient conditions, confirming the physical stability of the thermolabile biomacromolecule. The structural integrity and antigen-binding ability of the dried mAb were preserved, while the *in vitro* biological activity was unaffected. The SD and SFD particles possessed morphological characteristics and aerodynamic diameter that are generally suited for pulmonary delivery. In particular, the SFD powder formulations exhibited satisfactory aerosol performance, although the optimal protein-excipient ratio needs to be determined to ensure adequate stability. The high water content is postulated as the primary cause for deficient dispersibility of the SD powder formulations. Efforts should be made to reduce the residual moisture through optimisation of the formulation and drying process. The successful development of an orally inhaled anti-IL-4Rα mAb is a tantalising prospect that is much needed for patients with severe asthma.

Conflict of Interest The authors declare no conflict of interest.

Author Contributions This study was conceptualised and designed by J.K.W.L., H.W.P., and J.C.K.L. The experiments and data analyses were performed by H.W.P. The manuscript was written by H.W.P. and edited by H.W.P., H.C.S., S.W.S.L., and J.K.W.L. The antibody was developed by J.G., L.Z., and C.Z. All authors reviewed the manuscript and approved the final version.

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