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Enhanced powder dispersion of dual-excipient spray-dried powder formulations of a monoclonal antibody and its fragment for local treatment of severe asthma

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ABSTRACT

The advent of biologics has brought renewed hope for patients with severe asthma, a condition notorious for being hampered by poor response to conventional therapies and adverse drug reactions owing to corticosteroid dependence. However, biologics are administered as injections, thereby precluding the benefits inhalation therapy could offer such as increased bioavailability at the site of action, minimal systemic side effects, non-invasiveness, and self-administration. Here, 2-hydroxypropyl-beta-cyclodextrin and L-leucine were co-spray-dried, as protein stabiliser and dispersion enhancer, respectively, at various weight ratios to produce a series of formulation platforms. Powder aerosolisation characteristics and particle morphology were assessed for suitability for pulmonary delivery. The selected platform with the best aerosol performance, a 1:1 ratio of the excipients, was then incorporated with a monoclonal antibody directed against IL-4 receptor alpha or its antigen-binding fragment. The dual-excipient antibody formulations exhibited emitted fraction of at least 80% and fine particle fraction exceeding 60% in cascade impactor study, while the residual moisture content was within a desirable range between 1% and 3%. The *in vitro* antigen-binding ability and inhibitory potency of the spray-dried antibody were satisfactorily preserved. The results from this study corroborate the viability of inhaled solid-state biomacromolecules as a promising treatment approach for asthma.

1. Introduction

The number of inflammatory diseases embracing the use of biologics in their arsenal of treatment modalities is expanding, and asthma is no exception. A major chronic disease of the respiratory system, asthma affected more than 260 million individuals across the world in 2019 (Institute for Health Metrics and Evaluation, 2020). Severe asthma, which accounts for around 3% to 10% of people with asthma, typically depends on a maximal high-dose inhaled corticosteroid with a long-acting beta$_2$-agonist (Chung et al., 2014; Global Initiative for Asthma, 2022). For patients who experience exacerbations or poor symptom control despite a high-dose inhaled therapy, or depend on maintenance oral corticosteroids, which are associated with severe long-term adverse effects (Lefebvre et al., 2015), biologic therapies are an option. Since severe asthma is often driven by type 2 inflammation that is characterised by airway eosinophilia and cytokines secreted by T-helper 2 cells such as interleukins (ILs) 4, 5, and 13 (Israel and Reddel, 2017), immunoglobulins (Ig) in clinical use and research strategically target these pathways.

There are a handful of monoclonal antibodies (mAbs) that have been approved by the U.S. FDA for the treatment of severe asthma, including omalizumab (IgE antagonist), mepolizumab and reslizumab (IL-5 antagonists), dupilumab (IL-4 receptor antagonist), benralizumab (IL-5 receptor antagonist), and tezepelumab (thymic stromal lymphopoietin antagonist). All of them are administered parenterally, which increases the risk of off-target systemic side effects and may require larger doses compared to local therapy (Borghardt et al., 2018; Irvine et al., 2013). A noninvasive route of delivery is no doubt more popular among patients and healthcare personnel, particularly in the management of a chronic condition like asthma. Exemplified by the array of inhaled products for

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asthma on the market, targeted delivery to the lungs through oral inhalation has advantages encompassing rapid onset of action, reduced systemic adverse reactions, high local bioavailability, and potential dose reduction (Liang et al., 2020). Although pulmonary delivery of antibodies has been investigated using dry powder inhalers (DPIs) or nebulisers (Hickey and Stewart, 2022; Matthews et al., 2020), the former group of inhalation devices are more compatible with labile biomacromolecules inasmuch as they do not generate heat (Shoyele and Slowey, 2006) or create a vast air–liquid interface (Fröhlich and Salar-Behzadi, 2021). In nebulisation, proteins are in the liquid state which necessitates the infamous cold chain that increases the manufacturing costs, negatively impacts the environment, and complicates the logistical operations (Sharma et al., 2021). On the other hand, proteins delivered through DPIs are in the solid state which, with proper formulation, can confer prolonged shelf-life and stability (Chang and Pikal, 2009), given that chemical and physical degradation reactions are driven hydrolytically (Lai and Topp, 1999). It has been proposed that a pressure drop of no less than 1 kPa, produced by the patient's inspiratory effort, is required to effectively fluidise and disperse the powder from a DPI (Clark et al., 2020). At the airflow resistances of many commercially available DPIs, the majority of patients with asthma, including those with severe asthma, are able to generate sufficient inspiratory flow rates necessary for adequate drug delivery to the lungs (Laube et al., 2011).

To produce biologics in powder form for inhalation, several particle-engineering technologies are available, including freeze drying-milling, spray freeze drying, and thin-film freeze drying (Chang et al., 2021). Spray drying was used in this study in view of its scalability on an industrial level, ability to generate particles with uniform size distribution, and the lower costs involved (Chow et al., 2007; Sharma et al., 2021). It is also an established drying technique to manufacture the now-defunct Exubera® (human insulin), one of the rare inhaled protein products that had received regulatory authorisation (White et al., 2005). A one-step process, spray drying involves the atomisation of a liquid formulation by means of a nozzle into a chamber of hot gas stream and the solvent in the atomised droplets is removed via evaporation (Ziaee et al., 2019). The high temperature of the drying gas subjects thermolabile molecules to heat stress while atomisation introduces mechanical shear forces and induces protein adsorption at an extensive air–liquid interface that may destabilise biomacromolecules (Chow et al., 2007). For these reasons, stabilising excipients are incorporated into protein formulations for their protective effects against the various stresses encountered during the dehydration stage (Chang et al., 2021).

Among the well-known classes of solid-state protein stabilisers, which include sugars, polyols, surfactants, and salts (Depreter et al., 2013), cyclodextrins, in particular, 2-hydroxypropyl-beta-cyclodextrin (2HPβCD) has gained attention as a promising excipient for both its protein-stabilising abilities (Serno et al., 2011) and capability to produce inhalable particles for pulmonary delivery (Ramezani et al., 2017). The mechanisms through which 2HPβCD stabilises proteins have been postulated to include water replacement effect, vitrification, and surface activity (Serno et al., 2011). Like some other cyclodextrin derivatives, 2HPβCD is found in commercialised pharmaceutical products as an excipient for the intramuscular, intravenous, and oral routes (Jansook et al., 2018). Even though it is not yet licenced for the respiratory route, presumably due to insufficient safety data, 2HPβCD administered by intranasal spray to healthy human volunteers was well-tolerated (al-Nakib et al., 1989) and short-term exposure in mice via nebulisation did not lead to overt lung toxicity (Evrard et al., 2004).

Our previous work characterised spray-dried (SD) and spray-freeze-dried (SFD) powder formulations of an anti-IL-4 receptor alpha (IL-4Rα) mAb with 2HPβCD in terms of physicochemical properties, aerosol performance, protein stability, and biological activity (Pan et al., 2022). Here, we sought to address the issues of high residual moisture content and suboptimal aerosolisation by the incorporation of leucine, which is a very hydrophobic amino acid. Leucine has been investigated as a dispersion enhancer in SD formulations of pharmaceuticals intended for inhalation, including biologics (Alhajj et al., 2021). We aimed to study the physical and aerodynamic characteristics of dual-excipient powder formulation platforms that comprised 2HPβCD and leucine in different weight ratios prepared by spray drying. The excipient ratio that achieved the most desirable outcomes was then applied to antibody-containing formulations. Besides the intact full-length mAb, the antigen-binding fragment (Fab) would be featured in the formulations as well. In addition, the stability and *in vitro* bioactivity of the SD antibody were evaluated.

2. Materials and methods

2.1. Materials

Humanised anti-IL-4Rα mAb (~18 mg/mL) and the Fab (~16 mg/mL) in phosphate-buffered saline (PBS) were developed and provided by Shanghai MabGeek Biotech Co. Ltd. (Shanghai, China), and stored at −80 °C. The antibody (IgG4) was generated from mouse hybridoma and expressed by stably-transfected CHO-K1 cells (ATCC® CCL-61™, Manassas, VA, USA). The Fab was also expressed by CHO-K1 cells, by transiently transfecting with the Fab sequence of the mAb. 2HPβCD, L-leucine, dithiothreitol (DTT), Coomassie Brilliant Blue R-250, trisodium phosphate (Na_3PO_4), bovine serum albumin (BSA), and Tween® 20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human (rh) IL-4Rα, rhIL-4, rhIL-13, granulocyte–macrophage colony-stimulating factor (rhGM-CSF), and substrate reagent pack (consisting of colour reagents A and B, stabilised hydrogen peroxide and stabilised tetramethylbenzidine, respectively) were purchased from R&D Systems (Minneapolis, MN, USA). Horseradish peroxidase-conjugated polyclonal goat $F(ab')_2$ directed against human IgG (detection antibody), was obtained from Abcam (Cambridge, UK). Prestained protein ladder (PageRuler™ Plus), RPMI-1640 medium powder, qualified foetal bovine serum (FBS), and Antibiotic-Antimycotic (penicillin–streptomycin-amphotericin B) were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) was procured from MedChemExpress (Monmouth Junction, NJ, USA) and Dual-Glo® Luciferase Assay System was obtained from Promega (Madison, WI, USA). Sulfuric acid (H_2SO_4) was bought from BDH Chemicals (Poole, England). Ultrapure water was retrieved from a laboratory water purification system with a 0.2-μm pore size rating (Barnstead NANOpure Diamond™, APS Water Services, Van Nuys, CA, USA).

2.2. Formulations and spray drying

Dual-excipient formulation platforms were prepared by weighing the appropriate amount of leucine and adding it to a concentrated solution

Table 1
Composition of the feed solutions for spray drying.

Formulation	2HPβCD content (% w/w)	L-leucine content (% w/w)	Antibody format	Antibody content (% w/w)
Excipient-only formulation platforms				
E0	100	0	–	–
E1	95	5	–	–
E2	90	10	–	–
E3	80	20	–	–
E4	50	50	–	–
Antibody-containing formulations				
F1	45	45	Fab	10
F2	37.5	37.5	Fab	25
F3	25	25	Fab	50
M1	45	45	mAb	10
M2	37.5	37.5	mAb	25
M3	25	25	mAb	50

2HPβCD: 2-hydroxypropyl-beta-cyclodextrin; Fab: antigen-binding fragment; mAb: full-length monoclonal antibody.

of 2HPβCD in ultrapure water according to the proportions stated in Table 1. Antibody concentration of the stock solutions was measured by UV absorbance at 280 nm. To prepare the antibody-containing formulations, the stock antibody solution was added to the excipient-only platform and the resultant mixture was swirled gently. The final solute concentration for all feed solutions was adjusted to 2% w/v with ultrapure water.

The feed solutions were pumped peristaltically at a 3% rate (~0.9 mL/min) into a mini spray dryer (B-290, BÜCHI Labortechnik AG, Flawil, Switzerland) operated at 100% aspiration rate (corresponding to a gas flow rate of about 35 m^3/hour) with nitrogen as the spray gas at a flow rate of 742 L/hour. These operating parameters were adopted from previous studies (Pan et al., 2022; Qiu et al., 2019). The inlet temperature was preset at 150 °C for the formulation platforms and 100 °C for the antibody-containing formulations. An integrated two-fluid nozzle of 0.7 mm internal diameter was used to atomise the solutions into the spray cylinder. The SD powder was transferred from the product collection vessel into a transparent glass vial and stored in an auto dry box (Eureka Dry Tech, Taipei, Taiwan) at air-conditioned temperature (around 22 °C) and a controlled relative humidity of approximately 25%.

2.3. Scanning electron microscopy (SEM)

Morphology and geometric size of the SD particles were studied using SEM. Powder samples were sprinkled onto aluminium specimen stubs using adhesive carbon tape. To enhance sample conductivity and prevent overheating, the surface of the mounted samples was coated with approximately 13 nm of gold–palladium for 120 seconds at 30 mA in an argon-rich environment (Q150T ES Plus, Quorum Technologies, East Sussex, UK). Photomicrographs of the particles were taken by a field emission scanning electron microscope (Hitachi S-4800, Tokyo, Japan) at 5,000 × and 10,000 × magnifications, an accelerating voltage of 5 kV, and 4.6–6.6 mm working distance.

2.4. Differential scanning calorimetry (DSC)

Thermal behaviour of the SD powders was investigated by DSC. Approximately 2–3 mg of each powder formulation was weighed into a 5.4 × 2.0 mm aluminium hermetic pan (Jingyi Chemical Materials, Shanghai, China) and covered with a perforated lid. The pans were sealed before loading onto an indium-calibrated differential scanning calorimeter (DSC 250, TA Instruments, New Castle, DE, USA). The DSC was programmed to hold isothermal for 10 min at 0 °C, followed by heating at a ramp rate of 10 °C/min to 300 °C. The thermograms were plotted using OriginPro® software (version 2022b, OriginLab®, Northampton, MA, USA).

2.5. Thermogravimetric analysis (TGA)

Moisture content of the SD antibody formulations was measured using a thermogravimetric analyser (TGA 550, TA Instruments, New Castle, DE, USA). An open platinum sample pan was loaded with 2 ± 0.5 mg of each powder formulation and heated at a constant rate of 10 °C/min from ambient temperature to 105 °C. The weight loss as monitored by a microbalance was deemed to account for the water that had evaporated from the solid sample during heating. Each measurement was done in triplicate.

2.6. Aerosol performance

The aerodynamic characteristics of the SD formulations were assessed by a Next Generation Impactor (NGI) attached to a right-angled induction port (Copley Scientific, Nottingham, UK). A size 3 gelatin capsule (Capsugel®, Morristown, NJ, USA) was filled with 10 ± 0.5 mg of powder and placed into a high-resistance handheld RS01 inhaler (Plastiape, Osnago, Italy). The air flow rate was adjusted to approximately 54 L/min to reach a pressure drop of 4 kPa across the inhaler. The powder was dispersed into the apparatus for 4.4 seconds to allow 4 litres of air to be withdrawn. The assessment was conducted thrice for each formulation. 2HPβCD concentration was quantified by high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA) and peak integration was performed on OpenLab CDS ChemStation Edition software (version C.01.06, Agilent Technologies) as previously described (Pan et al., 2022).

The aerosol performance is described by the following metrics: 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 regard to 2HPβCD, the RD is the total mass assayed from the whole NGI set-up; the ED is the mass discharged from the inhaler; and the FPD is the mass with aerodynamic diameter (d_{ae}) < 5 μm. The formulae for EF and FPF are furnished below. MMAD and GSD were calculated according to the methods stipulated in *USP on Compounding* (The United States Pharmacopeial Convention, 2014). MMAD is the d_{ae} at which half of the aerosolised particle mass lie under, while GSD signifies the width of the d_{ae} distribution (Finlay and Darquenne, 2020).

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

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was utilised to verify the molecular weight of the antibody and the Fab post-spray drying. Powder formulations of the antibody were reconstituted with ultrapure water and the protein concentration was adjusted to 0.2 mg/mL in both non-reducing and reducing (containing 5 mM DTT) SDS buffers. Unprocessed antibodies and aliquots of the feed solutions were included as reference. The sample solutions were boiled at 95 °C for 5 min in a dry bath, except for the non-reduced full-length mAb to minimise aggregation. Each well of the 10% Bis-Tris gels was loaded with 4 μg of antibody. The electrophoresis (Mini-PROTEAN® Tetra System, Bio-Rad), gel staining and destaining, and photography (G:BOX Chemi XR5 gel documentation system, Syngene, Cambridge, UK) steps have been outlined elsewhere (Pan et al., 2022).

2.8. Size-exclusion chromatography (SEC)

SEC separates proteins on the basis of molecular size and was employed to monitor the monomer content after spray drying. The chromatography system consisted of a Yarra™ 3 μm SEC-3000 column (phenomenex®, Torrance, CA, USA) connected to a diode array detector (Agilent Technologies) set at a UV wavelength of 214 nm. The flow rate of the mobile phase (150 mM aqueous Na_3PO_4, pH 6.8) was 0.8 mL/min and the stoptime was 18 min. Unprocessed antibodies were included as controls and 50 μL of each sample, adjusted with the buffer to a protein concentration of 0.2 mg/mL, was injected at 25 °C in triplicate. Integration of the peaks was performed using OpenLab CDS ChemStation Edition software (version C.01.03, Agilent Technologies) and the percent monomer content was calculated.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The binding capacity of the antibody of selected formulations was determined by labelled immunoassay. The ELISA protocol is detailed elsewhere (Pan et al., 2022). In brief, the capture antigen, rhIL-4Rα, was coated (50 ng/well) overnight and blocked with reagent diluent (2% w/v BSA in PBS). The protein concentration of the samples was adjusted to 100 and 1 μg/mL with the reagent diluent and the samples were added in duplicate to the wells. The peroxidase reaction was arrested by the

addition of 2 N H_2SO_4. Readings at 570 nm were subtracted from those at 450 nm to correct for optical imperfections in the plate. A total of three replicates were run for the ELISA experiment. The bar chart showing the optical density values was plotted using GraphPad Prism (version 8.2.1, San Diego, CA, USA).

2.10. Cellular antiproliferation assay

The inhibitory potency of the SD antibody was evaluated by an antiproliferation assay using human erythroleukaemic TF-1 cells (ATCC® CRL-2003™) in which the antibody competed with IL-4 or IL-13 for binding to IL-4R, a shared receptor target. Inhibition of constitutive growth factors, such as GM-CSF, IL-4, and IL-13, abates the long-term proliferation of TF-1 cells (Drexler et al., 1997). The cells were cultured in complete growth medium (CGM) composed of RPMI-1640 supplemented with 2.0 g/L $NaHCO_3$, 10% heat-inactivated FBS, 1% Antibiotic-Antimycotic, and 2 ng/mL rhGM-CSF. For the assay, cells were centrifuged at 100 × g for 10 min and resuspended in the assay medium (CGM less rhGM-CSF) at a concentration of 6.25×10^5 cells/mL. The harvested cells were added to a flat-bottom 96-well microplate (TPP®, Trasadingen, Switzerland) at a volume of 120 μL per well and starved for 24 hours at 37 °C and 5% CO_2. The selected antibody formulations were reconstituted in ultrapure water and the solutions, including the unprocessed antibody controls, were serially diluted 3-fold in the assay medium to produce 10 antibody concentrations. Each of these was supplemented with either 30 μL of rhIL-4 or rhIL-13 to salvage the cells, such that the final reaction mixture contained an interleukin at a fixed concentration of 8 ng/mL, with the antibody at a concentration that spanned from 5 ng/mL to 100 μg/mL. The test solutions were added in duplicate and the plate was incubated for a further 48 hours. Three hours prior to the end of this period, 10 μL of CCK-8 was added to each well. At the end of the entire incubation period, the plate was mixed gently on a microplate mixer and the absorbance was read at 450 nm using a microplate spectrophotometer (Thermo Scientific Multiskan GO). The assays were run in triplicate and data fitted with nonlinear regression to a sigmoidal equation using GraphPad Prism. The optical density values were normalised to the best-fit top (100%) and bottom (0%) values of the unprocessed antibody control for each run and plotted as a function of the common logarithm of the antibody concentration to obtain the half-maximal inhibitory concentration (IC_{50}).

2.11. Inhibition of STAT6 activation

TF-1 cells transfected with a STAT6-driven luciferase (Luc) reporter construct (designated TF-1/STAT6-Luc) were cultured in CGM. Between 12 and 16 hours prior to the experiment, the medium was changed to RPMI-1640 with 5% FBS and the cells were left to incubate overnight at 37 °C. On the day of the experiment, 12 different concentrations of antibody (Fab: 9.41 pg/mL–1.67 μg/mL; full-length antibody: 27.8 pg/mL–4.93 μg/mL), diluted 3-fold, were added at 50 μL per well to a 96-well microplate in duplicate. The cells were harvested and resuspended in RPMI-1640 with 5% FBS, and inoculated at 50 μL into each well. The plate was left in the incubator for 30 min. Thereafter, 50 μL of rhIL-4 or rhIL-13 was added to each well to achieve a final interleukin concentration of 2 (rhIL-4) or 20 ng/mL (rhIL-13) and cell density of 0.5×10^5 cells/mL. This was followed by a five-hour incubation and 50 μL of luciferase substrate was added to each well. After 10 min, the plate was read at 560 nm. The ratio of firefly:*Renilla*/TK luminescence in relative light units (RLU) for each well was calculated. A four-parameter regression was performed by GraphPad Prism, and the RLU-antibody concentration curves were plotted, from which the IC_{50} could be obtained. The experiment was performed three times.

2.12. Statistical analysis

All data are reported as mean ± standard deviation where applicable. Differences in optical density and IC_{50} values were assessed using paired two-tailed Student's *t*-test on GraphPad Prism. P values < 0.05 were considered statistically significant.

3. Results

3.1. Spray drying

The inlet and outlet temperatures for all 11 formulations and their processing yield are shown in Table 2. The processing yield is defined as the percentage of powder weight collected over the total solute mass in the feed solution. The inlet temperature was lowered for the antibody formulations to minimise heat stress. The outlet temperature ranged from 98 to 100 °C for the excipient platforms, and from 62 to 63 °C for the antibody formulations. Among the excipient platforms, the addition of leucine, even at 5% w/w, increased the production from approximately 46% to above 60%. The yields for the antibody formulations were higher, between 73% and 84%.

3.2. Particle morphology

Representative scanning electron photographs of the SD particles are shown in Fig. 1. The particle morphology exhibited striking variations as the leucine content is increased. At low leucine concentrations of 5% and 10% w/w (E1 and E2), the particles were similar to 100% SD 2HPβCD (E0). However, at higher leucine concentrations, E3 was highly creased with interparticle fibre-like features on a rough surface, whereas the globoid structure was largely absent in E4 and was instead replaced by hollow particles that were heterogeneously irregular in shape. At a high antibody concentration of 50% w/w (F3 and M3), the particles were more globular in shape, with multiple dimple-like features on a rough surface. In contrast, the formulations with lower antibody concentrations (F1,2 and M1,2) generally resembled E4; the microparticles manifested a collapsed hollow sphere morphology by enfolding upon themselves. The surface of these particles also appears more rugose. Most of the visualised particles were smaller than 5 μm in diameter.

3.3. Thermal analyses

The DSC thermograms of the leucine-containing powder formulations (all except E0) are distinct from that of the 2HPβCD-only formulation (E0) in respect of the additional endothermic peaks at temperatures between 230 and 270 °C, due to the presence of crystalline leucine (Fig. 2). These peaks shift rightwards, closer to the known melting point of leucine (~287 °C), and become more pronounced, as the leucine component begins to dominate in the formulations (Yalkowsky et al., 2010). The existence of residual water in the powders is evident in the other downward endothermic peaks below 100 °C that are much broader. Although this peak is present in all the formulations, it is slightly more prominent in F3 and M3, which contained a lower concentration of the hydrophobic leucine.

The residual water content of the antibody formulations was determined by TGA after spray drying (Table 2). The Fab formulations contained marginally more moisture compared with the mAb formulations, and the water content was highest in the formulations that had the lowest concentration of leucine (i.e., F3 and M3). Nevertheless, the mean water content of all the powders tested was low and ranged between 1.4 ± 0.2% and 2.6 ± 0.3% w/w, demonstrating the drying efficiency of the formulation and process to keep moisture at bay.

3.4. Aerosol performance

The EF and FPF obtained from the cascade impactor experiments, representing the dispersibility and respirable fraction of a powder formulation, respectively, are shown in Fig. 3. For the excipient platforms, the EF rises from around 54% to 81% as the relative leucine

Table 2
Spray-drying outcomes: outlet temperature, processing yield, mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD), and residual moisture content (RMC).

Formulation	Inlet temperature (°C)	Outlet temperature (°C)	Processing yield (%)	MMAD (μm)	GSD	RMC (% w/w)
Excipient-only formulation platforms						
E0	150	100	46.4	1.83	2.09	–
E1	150	99	60.4	2.04	2.26	–
E2	150	99	61.6	2.77	1.94	–
E3	150	98	71.9	4.19	2.07	–
E4	150	98	67.5	1.64	2.34	–
Antibody-containing formulations						
F1	100	62	73.0	1.46	2.33	2.1 ± 0.3
F2	100	62	78.0	1.68	2.24	1.9 ± 0.3
F3	100	62	83.3	1.95	2.16	2.6 ± 0.3
M1	100	63	73.7	1.50	2.30	1.4 ± 0.2
M2	100	63	76.4	1.59	2.24	1.8 ± 0.1
M3	100	63	84.1	2.38	2.17	2.0 ± 0.3

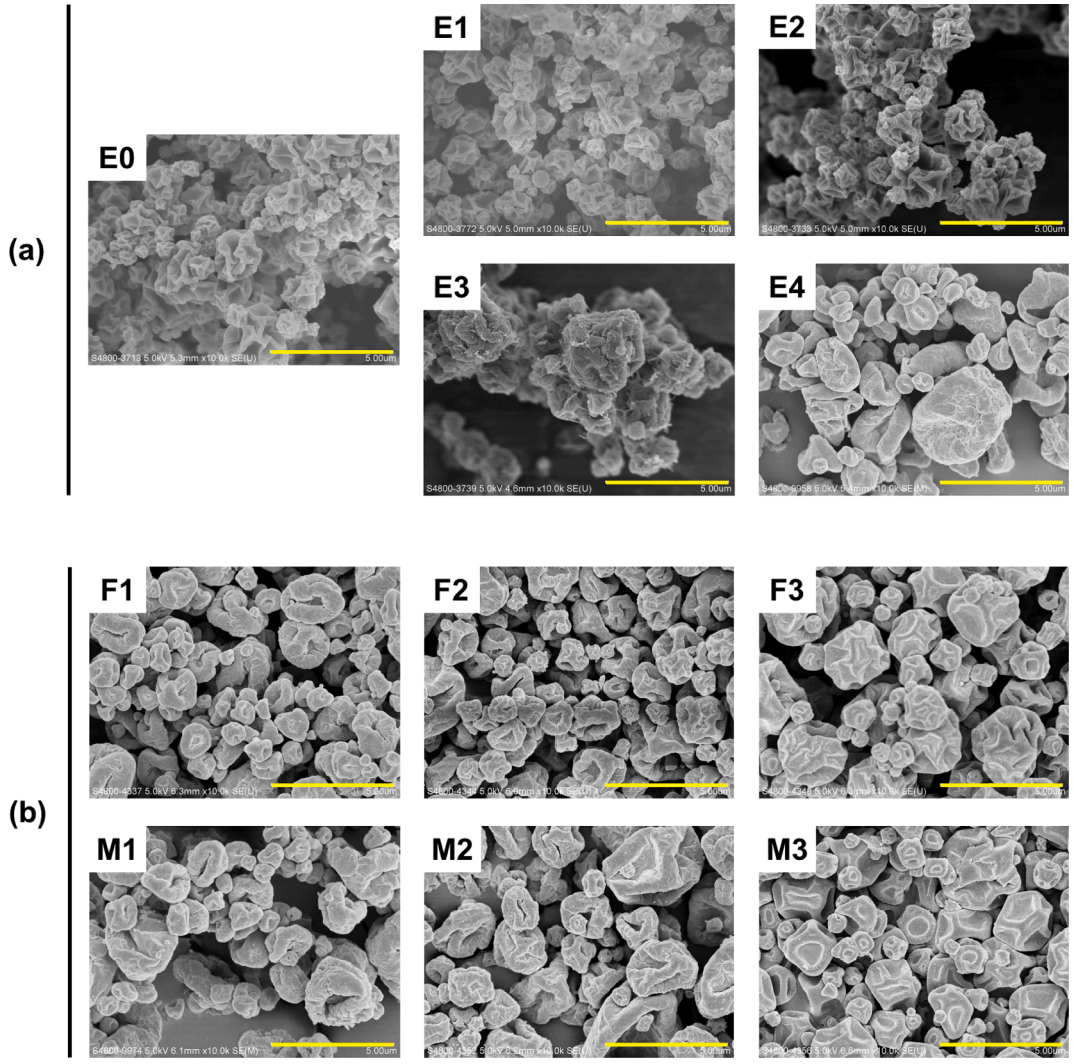

Fig. 1. Scanning electron microscopy (SEM) images of the (a) excipient-only formulations and (b) antibody-containing formulations. Scale bar = 5 μm.

content is gradually increased. Notably, formulation E4 had the highest EF as well as FPF of nearly 70%, therefore a 1:1 leucine-to-2HPβCD ratio was chosen as the excipient foundation for the antibody formulations. The mean EF of all six antibody formulations was above 80%. Apart from M3, which had an FPF of about 61%, the mean FPF of the rest of the antibody formulations was between 72% and 75%. The best aerosolisation characteristics belonged to the formulations containing 25% antibody and 75% excipients, i.e., F2 (EF, 86.5 ± 1.7%; FPF, 75.3 ± 2.7%) and M2 (EF, 85.6 ± 2.4%; FPF, 75.2 ± 4.4%).

The MMAD and GSD values calculated from the aerosolisation performance analysis are also displayed in Table 2. With the exception of formulation E3, the d_{ae} of the particles was within the critical size range of 1 to 3 μm that is optimal for pulmonary deposition (Chow et al., 2007; Malcolmson and Embleton, 1998), while the GSD values (≥ 1.22)

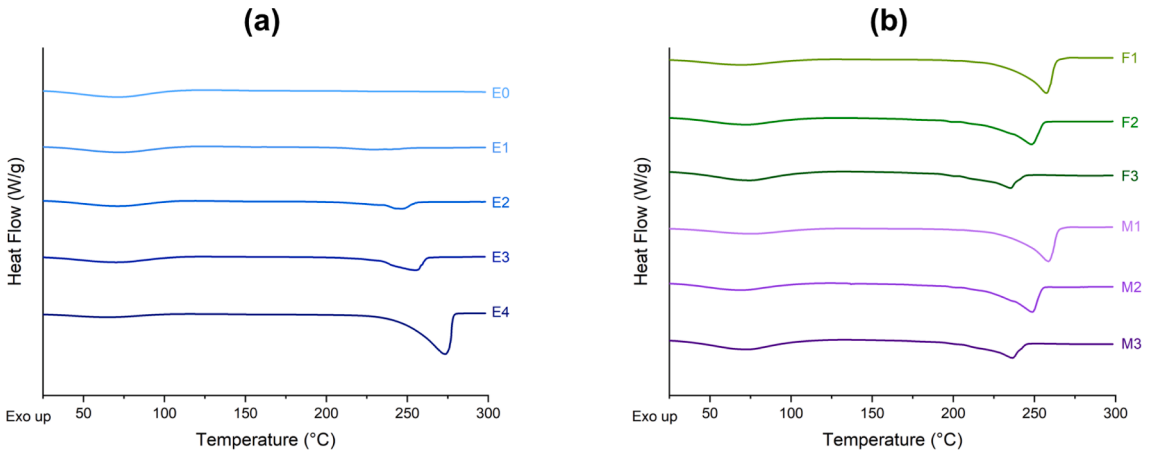

Fig. 2. Differential scanning calorimetry (DSC) thermograms of the (a) excipient platforms and (b) antibody formulations.

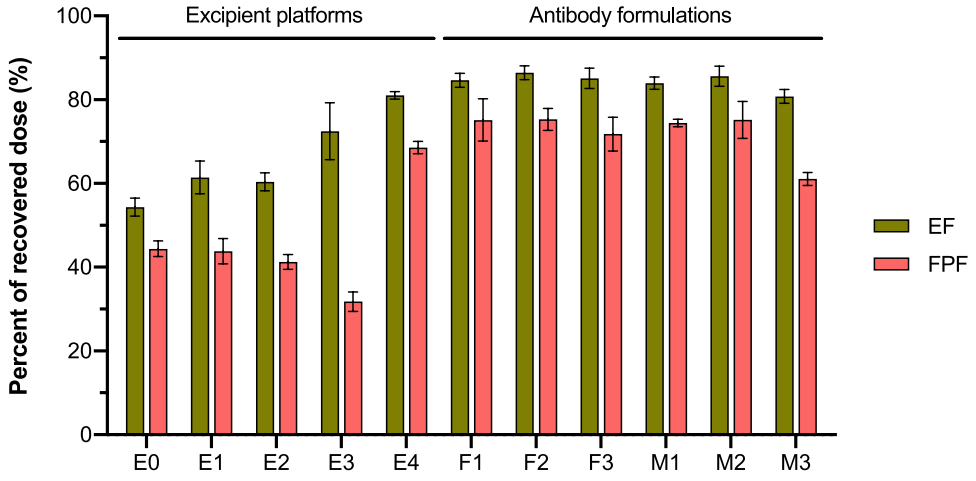

Fig. 3. Aerosolisation performance of the excipient platforms and antibody formulations assessed using a Next Generation Impactor (NGI), n = 3. EF: emitted fraction; FPF: fine particle fraction (cutoff aerodynamic diameter < 5 μm).

indicate a heterodisperse distribution, which is typical for therapeutic aerosols (Labiris and Dolovich, 2003; Laube et al., 2011). In the antibody formulations, raising the protein-to-excipient ratio increased the MMAD, which appears to have a moderate yield-enhancing effect. This is probably related to the separation efficiency of the cyclone, as smaller particles are more difficult to be segregated from the gas stream (Prinn et al., 2002).

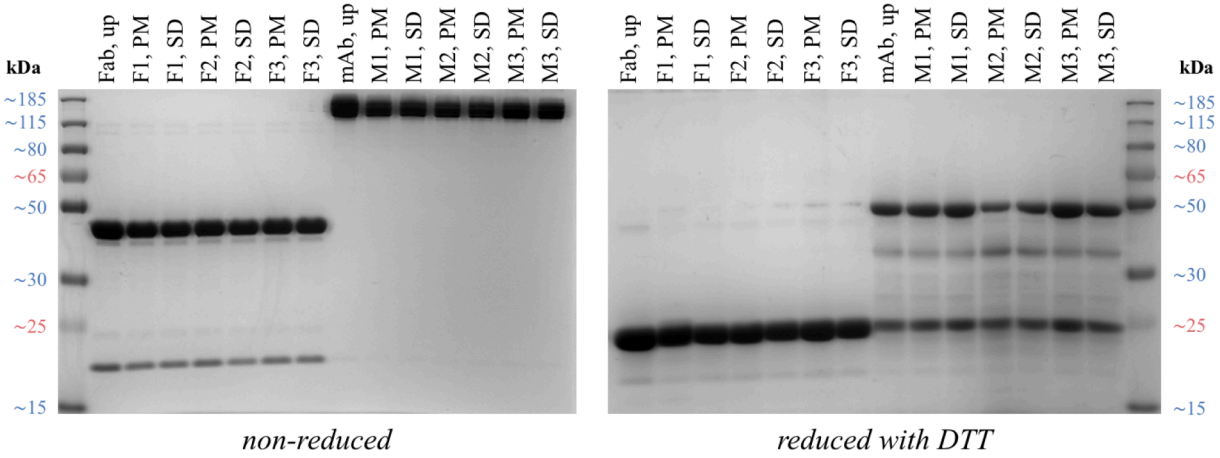

Fig. 4. SDS-PAGE gel images of the antibody formulations. DTT: dithiothreitol; Fab: antigen-binding fragment; mAb: full-length monoclonal antibody; PM: physical mixture (feed solution); SD: spray-dried; up: unprocessed.

3.5. Antibody stability

Electrophoretic protein separation was used to study the structural integrity of the antibody after spray drying, given that the high temperatures involved in the process could potentially cause thermal degradation, leading to fragmentation (Vlasak and Ionescu, 2011). The gel in the left panel of Fig. 4 shows that the Fab migrated under non-reducing conditions with an apparent molecular weight of ~45 kDa, while a thick band between 115 and 185 kDa was observed for the intact mAb samples. The additional lighter bands at around 20 kDa in the Fab samples were probably due to an extraneous protein contaminant in the stock antibody solution. The disulfide bonds of the antibodies on the gel in the right panel was reduced, which gave rise to a band at approximately 25 kDa for the Fab formulations (F1–3), and two primary bands at about 50 and 25 kDa for the mAb formulations (M1–3). Importantly, the patterns of the bands of the SD samples are essentially the same as those of the untreated antibody, indicating that there is no evidence of heat-induced fragmentation as a result of the spray-drying process (Nowak et al., 2017).

Stresses during spray drying and storage can destabilise proteins and induce aggregation, which remains a huge challenge in the development of biotechnology products as it can lead to immunogenicity and other adverse effects, batch-to-batch variability, and impaired efficacy (Lowe et al., 2011; Wang et al., 2010). To monitor antibody aggregation, the monomer content of the SD formulations was measured using SEC at two time points, 1 week and 10 months post-spray drying (Fig. 5). Monomer content is a crucial parameter of physical stability as unfolded proteins, even partially, are inherently aggregation-prone (Wu et al., 2014) and this has implications in the safety and efficacy of antibody-based pharmaceutical products (Hickey and Stewart, 2022). The unprocessed antibody was included at the first time point to discern aggregation attributable to the drying process. One week after spray drying, the average monomer content of the Fab formulations was close to 100% (99.8–99.9% vs. Fab-up 99.8%). For the full-length mAb formulations, the difference in monomer content between SD and unprocessed antibodies was small (86.0–99.0% vs. 93.6%, respectively). At 10 months post-spray drying, the largest decrement in monomer content was detected in F3 (99.9% to 90.0%), whereas the other formulations registered reductions of no more than 2.8%. This is fairly remarkable considering the samples were not frozen or stored under refrigeration throughout the study.

3.6. Antigen-binding ability and inhibitory potency

Two representative formulations, one each from the Fab and intact mAb formulations, advanced to *in vitro* experiments for assessment of the biological activity of the SD antibodies relative to their respective unprocessed (up) counterparts. Among the six antibody formulations, F2 and M2 were chosen in view of their better aerosol performance, since the other characteristics, such as particle morphology, residual water content, and protein structural integrity, did not demonstrate superiority of any particular formulation. The binding ability of the antibody to plate-coated rhIL-4Rα was assessed by ELISA (Fig. 6) at two empirically selected antibody concentrations, 1 and 100 μg/mL. There was no statistically significant difference between the Fab-up and F2 (1 μg/mL: $p = 0.4815$; 100 μg/mL: $p = 0.9904$) or between mAb-up and M2 (1 μg/mL: $p = 0.5810$; 100 μg/mL: $p = 0.1549$).

Given that the antibody interferes with the binding of IL-4 and IL-13 to IL-4R, the antiproliferation assay used TF-1 cells that have an absolute dependence on specific growth factors for survival (Drexler et al., 1997) to compare the inhibitory activity of the SD antibody as IC_{50} with that of the unprocessed (up) antibody. The TF-1 cells were separately exposed to rhIL-4 and rhIL-13, and the antibody concentration–response curves are shown respectively in the top and bottom panels of Fig. 7. In each of the four graphs, the two curves largely overlap each other, underscoring the almost identical inhibitory profiles of the tested antibody samples. In the presence of rhIL-4, the IC_{50} values were 0.534 ± 0.279 μg/mL (Fab-up), 0.535 ± 0.336 μg/mL (F2), 0.964 ± 0.458 μg/mL (mAb-up), and 1.262 ± 0.248 μg/mL (M2). There was no statistically significant difference in the IC_{50} between the SD antibody and the unprocessed antibody (F2 vs. Fab-up, $p = 0.9861$; M2 vs. mAb-up, $p = 0.1465$). With rhIL-13, the IC_{50} values were 0.081 ± 0.006 μg/mL (Fab-up), 0.082 ± 0.024 μg/mL (F2), 0.123 ± 0.055 μg/mL (mAb-up), and 0.134 ± 0.030 μg/mL

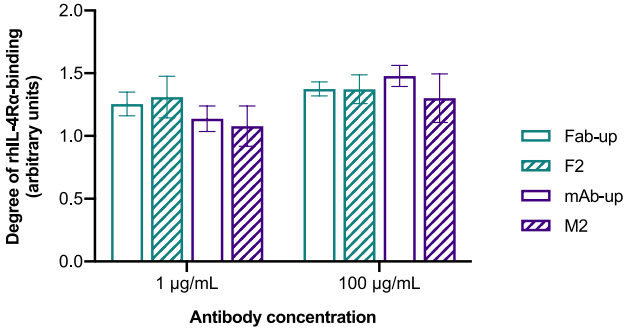

Fig. 6. Antigen-binding ability of the antibody in the selected Fab (F2) and mAb (M2) formulations determined by ELISA (n = 3). 96-well plates were coated with rhIL-4Rα. Fab: antigen-binding fragment; mAb: full-length monoclonal antibody; up: unprocessed.

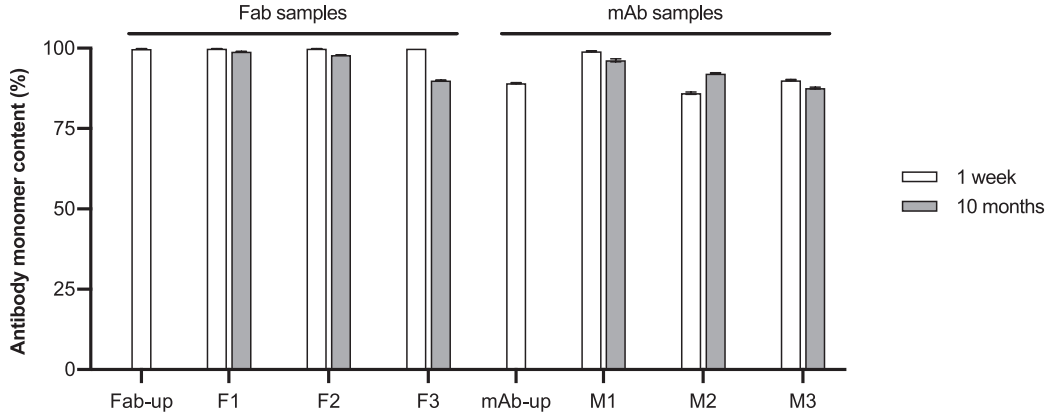

Fig. 5. Antibody monomer content of antibody formulations at 1 week and 10 months post-spray drying quantified by size-exclusion chromatography (SEC), n = 3. Fab: antigen-binding fragment; mAb: full-length monoclonal antibody; up: unprocessed.

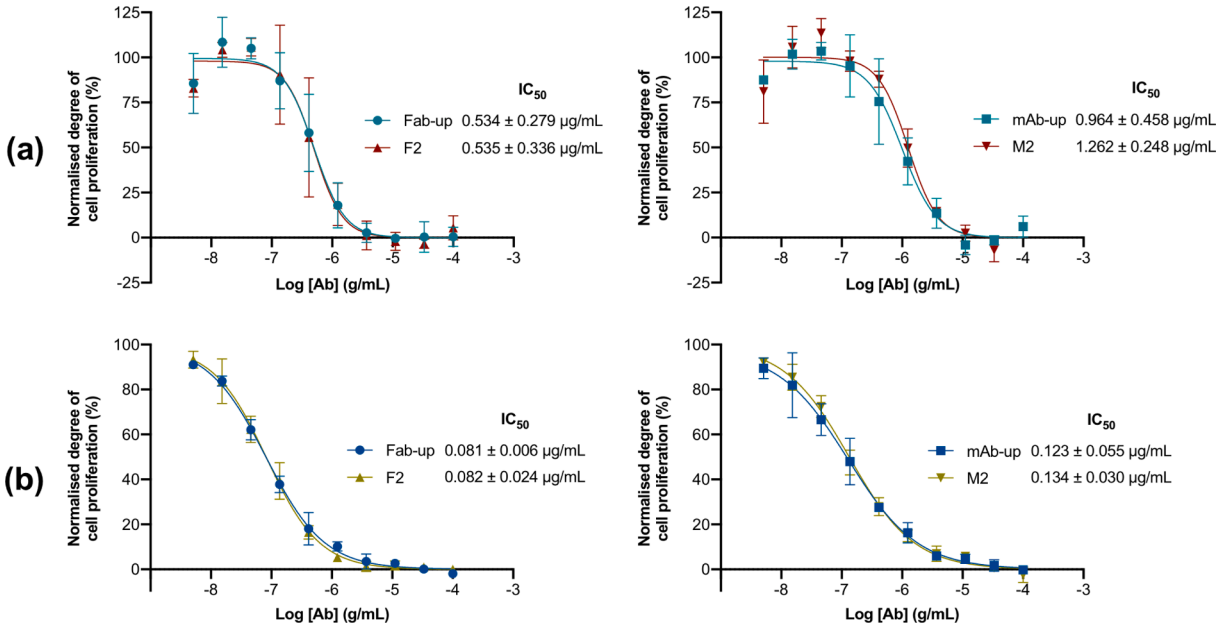

Fig. 7. Concentration-response curves of the inhibitory effect of the antibody in the selected Fab (F2) and intact mAb (M2) formulations on the proliferation of TF-1 cells (n = 3) stimulated by (a) rhIL-4 or (b) rhIL-13. The optical density is normalised with respect to the best-fit top (100%) and bottom (0%) values of the unprocessed antibody curve in the individual runs. [Ab]: concentration of antibody; Fab: antigen-binding fragment; mAb: full-length monoclonal antibody; up: unprocessed.

(M2). Again, no statistically significant difference was found between the formulated antibody and the untreated antibody control (F2 vs. Fab-up, $p = 0.9822$; M2 vs. mAb-up, $p = 0.5696$).

The TF-1 cells used to study the inhibitory effect of the antibody on STAT6 signalling pathway (Fig. 8) were stably transfected to express a luciferase reporter gene regulated by a STAT6-responsive promoter. With rhIL-4-induced STAT6 activation, the IC_{50} values were 9.79 ± 0.34 ng/mL (Fab-up), 8.75 ± 0.41 ng/mL (F2), 15.93 ± 1.89 ng/mL (mAb-up), and 14.97 ± 0.62 ng/mL (M2). The difference in IC_{50} between the formulated and untreated antibodies was not statistically significant (F2 vs. Fab-up, $p = 0.0589$; M2 vs. mAb-up, $p = 0.3229$). When induced by rhIL-13, the IC_{50} values were 9.79 ± 0.21 ng/mL (Fab-up), 9.60 ± 0.55 ng/mL (F2), 15.09 ± 3.28 ng/mL (mAb-up), and 12.32 ± 1.82 ng/mL (M2). Similarly, there was no statistically significant difference in the IC_{50} between the SD antibody and the unprocessed antibody (F2 vs. Fab-up, $p = 0.5707$; M2 vs. mAb-up, $p = 0.4350$). The ELISA results, cellular antiproliferation assay, and the inhibition of STAT6 activity effectively illustrate that the antigen-binding capacity and inhibitory potency of both SD Fab and full-length mAb were retained relative to their initial state before spray drying.

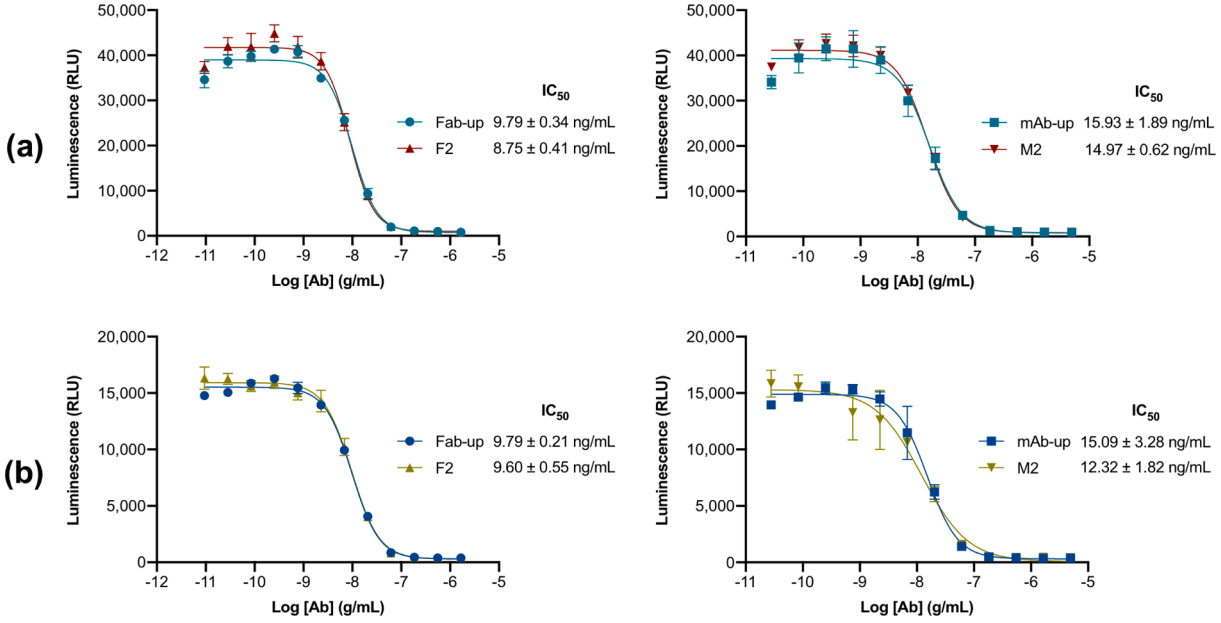

Fig. 8. Concentration-response curves of the inhibitory effect of the antibody in the selected Fab (F2) and intact mAb (M2) formulations on the activation of the STAT6 signalling pathway by (a) rhIL-4 or (b) rhIL-13 in TF-1/STAT6-Luc cells (n = 3). [Ab]: concentration of antibody; Fab: antigen-binding fragment; mAb: full-length monoclonal antibody; up: unprocessed.

4. Discussion

In our previous work, the anti-IL-4Rα mAb was spray-dried and spray-freeze-dried with 2HPβCD as the protein stabiliser (Pan et al., 2022). Although the antibody was decently stabilised and retained its antigen-binding ability and *in vitro* biological activity, powder dispersibility was suboptimal, plausibly impaired by the high residual water content (7–9% w/w) detected. Spray drying at a higher temperature might ameliorate this, since the relative humidity of the drying gas would be increased, leading to a dryer product. Particles with lower moisture content are less likely to be fused together by solid bridges to form larger agglomerates (Li et al., 2016). Nonetheless, excessively high temperatures should be avoided in preparations containing heat-sensitive biologics. Not only is moisture suspected to be a factor affecting the aerosolisation of powder formulations, water can also act as a plasticiser to potently lower the glass transition temperature of the excipient and increase local mobility of the embedded biomacromolecules, which destabilises the protein (Mensink et al., 2017). To enhance drying efficiency and circumvent moisture adsorption, leucine, a hydrophobic amino acid, was co-formulated at different weight ratios with 2HPβCD to investigate whether its incorporation would improve the aerodynamic characteristics of the powders compared with 2HPβCD alone.

Numerous studies have examined amino acid-based multi-component SD powder preparations for respiratory administration. They encompassed excipient-only platforms (Mangal et al., 2015; Sou et al., 2013) and formulations containing small-molecule drugs (Mah et al., 2019; Seville et al., 2007), small interfering RNA (Chow et al., 2017; Xu et al., 2022), antibodies (Faghihi et al., 2019; Shepard et al., 2021), and vaccines (Gomez et al., 2021; Lovalenti et al., 2016). However, in these studies, the co-excipients applied together with amino acids were primarily trehalose and mannitol, and none included any cyclodextrin. As such, 2HPβCD plus leucine was a novel combination in this regard. Mannitol crystallises easily (Eedara et al., 2021), which is detrimental to the biomacromolecule due to the combined effects of shear stresses and loss of protein–sugar interactions that are important for protein stabilisation (Izutsu et al., 1994; Mensink et al., 2017). Unlike mannitol, cyclodextrins tend to form amorphous glasses (Serno et al., 2011). Besides, it is usual for spray drying to generate amorphous glassy matrix (Chow et al., 2007), an essential aspect of the vitrification mechanism of protein stabilisation by sugars in the solid state (Mensink et al., 2017). The first recognisable benefit of including leucine in the formulations was the increase in processing yield, a vital consideration in the scale-up of biopharmaceutical manufacturing (Hernandez, 2016). Although on an industrial level spray drying can attain close to 100% yield, some powder adhesion and accumulation on the interior of the cyclone was visibly noted here, which could explain for the loss of product (Bowey et al., 2013).

The value of leucine in the formulations was clearly in enhancing the aerosolisation of the powder formulations. The mechanisms through which leucine promotes dispersion are multifaceted, from keeping water out to modifying the surface composition and morphology of the SD particles (Alhajj et al., 2021). It has been suggested that powder flowability or dispersibility is influenced by surface corrugation in two ways, altering the contact area between particles and mechanical interlocking or entrapment of adjacent particles (Chew and Chan, 2001; Walton and Mumford, 1999b). From the SEM images, leucine plus 2HPβCD yielded surface-corrugated microparticles, and the extent of corrugation and collapse of the outer shells increases with the leucine content. These morphological changes were consistent with observations in other studies that employed leucine as a dispersion enhancer (Mangal et al., 2015; Sou et al., 2013). The higher EF and FPF exhibited by E4 were perhaps ascribed to the high surface rugosity of the irregularly-shaped particles which reduced interparticle contact. Furthermore, the folded and hollow structure of the particles secondary to the inflation, rupture, and collapse of the crystalline crusts formed during the initial phase of drying lowered the density and thus the MMAD (Walton and Mumford, 1999a). However, overcorrugation can cause smaller particles to become embedded in the grooves of larger particles (Sou et al., 2013), and this effect may have ramifications for aerosolisation performance as the interparticulate cohesive forces intensify, inducing agglomeration, and the particles encounter more resistive airflow, which impacts powder fluidisation (Cui et al., 2018).

Another possible explanation for the improved aerosolisation by the addition of leucine into the formulation could be its ability to regulate water content in the powder by anti-hygroscopic effect. Even with the inclusion of a protein up to 50% w/w in the formulation, the residual moisture content of the assessed powders was no greater than 3% by weight. Such aridity is enough to inhibit crystallisation of the amorphous components and confer excellent powder flowability (Mah et al., 2019). Bevacizumab was co-spray-dried with trehalose and leucine (at 40%, 40%, 20% w/w, respectively) for pulmonary delivery in a study and the measured moisture content was between 3% and 4%, while the FPF (cutoff $d_{ae} < 5$ μm) was 82% (Shepard et al., 2021). Notwithstanding the somewhat different formulation compositions and experimental conditions, these results were comparable to those obtained here. There are two projected moisture-protective mechanisms, the crystallinity of leucine and its enrichment on the particle surface. With a sufficient concentration of leucine in the feed solution, e.g., greater than 10% w/w, leucine should reach supersaturation early in the spray-drying process and crystallise, considering that it is sparingly soluble in water (~22 mg/mL at room temperature) (Yalkowsky et al., 2010) and migrates to the droplet surface (Mangal et al., 2015; Vehring, 2008). The moisture uptake by crystalline leucine on SD particle surface is limited (Li et al., 2016). The accumulation of leucine on the surface of SD particles quantified by X-ray photoelectron spectroscopy has previously been reported (Mah et al., 2019; Mangal et al., 2015; Xu et al., 2022). The chemical structure of a leucine molecule comprises distinct hydrophilic (amino and carboxylic acid groups) and lipophilic (aliphatic isobutyl side chain) moieties (Chow et al., 2017). This amphiphilicity of leucine predisposes its accretion on the particle surface (Weissbuch et al., 1990). Protein molecules too display the tendency to amass at air-solution interfaces due to their surface-active nature, however, their much larger molecular weight retards diffusion towards the core (Adler et al., 2000; Grasmeijer et al., 2016). Hence, it was hypothesised that leucine would compete with the antibody for accumulation on the surface, decreasing the relative amount of proteins there. This not only enhances protein stability, since adsorption at interfaces promotes unfolding and aggregation of the protein (Wang et al., 2010), but also increases the level of powder dryness.

In the context of dehydrated biopharmaceuticals, a higher level of dryness is usually preferred, however, this is not always the case, as established in studies involving lyophilised excipient-free tissue-type plasminogen activator (Hsu et al., 1992) and plasma-derived IgG with sucrose (Duralliu et al., 2020). Beyond a certain threshold, denaturation resumes, therefore, it can be interpreted that an optimum residual moisture content may exist for each protein. Over-drying (e.g., < 1% water content) can transpire even in the presence of stabilising sugar excipients, especially when large rigid polysaccharides, for instance, some cyclodextrins, are used. This is perhaps due to inefficient hydrogen bonding, which makes local mobility more facile (Mensink et al., 2017). Moreover, electrostatic charges on particle surfaces may build up in extreme dryness (Elajnaf et al., 2006), facilitating agglomeration and could seriously hinder powder fluidisation (Kaialy, 2016). Nevertheless, based on the physical stability observed in the gel electrophoretic and filtration analyses, the SD antibody formulations here did not appear to be overdried.

The binding ability of the antibody to IL-4Rα was investigated using ELISA and found to be tantamount to that of the unformulated antibody. Likewise, in the antiproliferation assays, the cytokine rescue of the starved TF-1 cells that were bereft of essential growth factors was inhibited in a concentration-dependent manner to similar extents by the

formulated and unprocessed antibodies. These results were construed as adequate stabilisation of the SD antibody. The smaller IC_{50} values observed in the rhIL-13 assays compared to those in the rhIL-4 could be explained by the existence of an additional signalling pathway, independent of STAT6 activation, for the IL-4/IL-4Rα complex to produce growth factors that stimulate TF-1 proliferation. Indeed, this complex recruits the common γc subunit to activate gene transcription through a downstream signalling adaptor molecule, insulin receptor substrate-2 (McCormick and Heller, 2015). At the single cytokine concentration (8 ng/mL) added to the cells, the stimulatory effect was saturated for IL-4, but not for IL-13, which mediated roughly 60% maximal effect. This could presumably be rationalised by the different binding affinities and proliferative responses reported for IL-4 and IL-13. IL-4 binds to IL-4Rα with a much higher affinity than IL-13 to IL-13Rα1 (LaPorte et al., 2008). The stimulation index of IL-4 is also higher than that of IL-13 (Drexler et al., 1997). In addition, the number of binding sites on TF-1 cells for IL-4 is double that for IL-13 (Lefort et al., 1995). Consequentially, the EC_{50} is higher for IL-13 compared with IL-4.

The active ingredients presented in this study included not only a full-length mAb, but also the Fab. In local inhaled therapy, larger biomacromolecules may be preferable since unintended absorption from the lung (Patton, 1996) and degradation by lung proteases (Fröhlich and Salar-Behzadi, 2021) are both inversely related to molecular weight. However, utilising just the Fab without the crystallisable fragment (Fc) region that is present in the full-length mAb is logical given that the antibody mechanism of action relies on the Fab engaging with IL-4Rα for therapeutic efficacy in asthma. Furthermore, the role of the Fc domain is less pertinent in inhaled therapy where systemic absorption and extended serum half-life is not sought (Chow et al., 2023). Future work might be pursued in two directions. Firstly, the *in vivo* biological activity of the SD antibody in animal models should be explored, in conjunction with pulmonary toxicity assessment of the excipients in the respiratory tract. Secondly, more comprehensive characterisation of the powder formulations, for example, in relation to the protein-excipient interactions (Chen et al., 2021), antibody-antigen binding kinetics (Yang et al., 2017), and surface area-porosity analysis (Ji et al., 2016), could be undertaken. In spite of their medical utility and applications in pharmaceutical research, certain relevant physicochemical properties of cyclodextrins, in particular, hygroscopicity and density, remain ambiguous (Day et al., 2020), thus a better understanding of these properties would also be useful.

5. Conclusions

This study co-formulated 2HPβCD with leucine to produce a dual-excipient platform by spray drying for pulmonary delivery of solid-state biomacromolecules. The incorporation of leucine into the formulation boosted the processing yield and aerosol performance, with a 1:1 weight ratio of both excipients exhibiting the most favourable outcomes among the tested powders. The improvement in powder aerosolisation was imputed to particle surface and morphology modifications as well as water repellent by leucine. Spray-dried powders of an anti-IL-4Rα mAb and its Fab founded upon this excipient ratio emulated the physicochemical and aerodynamic characteristics of their formulation platform predecessor. The moisture content of the powder antibody formulations was within an acceptable range of between 1% and 3% w/w, while only minor changes in antibody monomer content over a period of 10 months at ambient storage conditions were detected. The *in vitro* antigen-binding capability and cellular inhibitory potency of the dehydrated antibody were comparatively well-preserved. Collectively, the results here validate the feasibility of a successful inhaled biologic product for the local treatment of severe asthma.

CRediT authorship contribution statement

Harry W. Pan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Jinlin Guo:** Investigation. **Lingqiao Zhu:** Writing – review & editing. **Susan W.S. Leung:** Writing – review & editing. **Chenghai Zhang:** Supervision. **Jenny K.W. Lam:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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