Optimizing Spray-Dried Liposomes for Pulmonary Delivery: Impact of Lipids Composition and of Drying Parameters using experimental design

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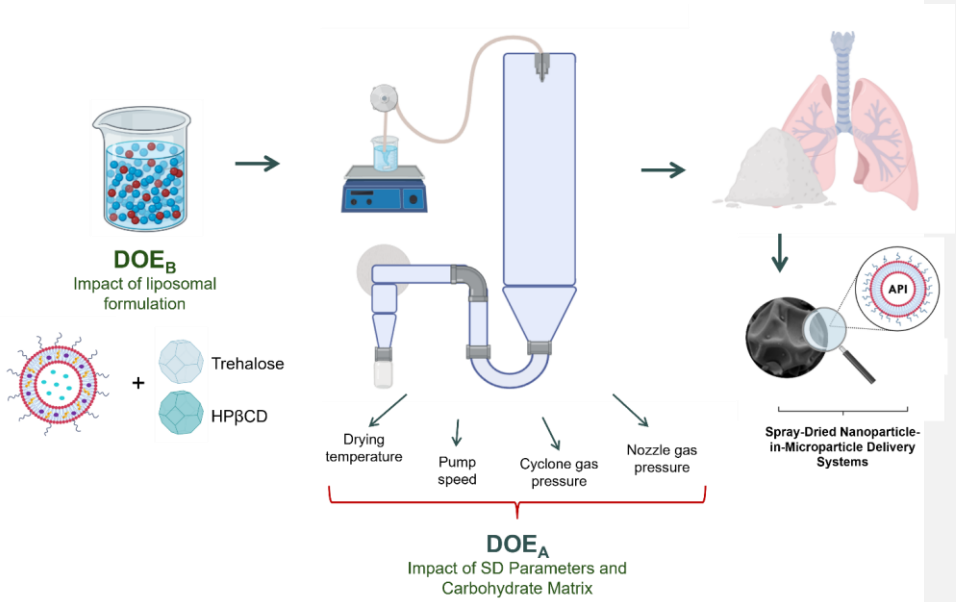
ABSTRACT

Liposomes are promising carriers for pulmonary drug delivery due to their biocompatibility and controlled-release properties. However, their stability in aqueous suspension is limited, leading to aggregation and reduced therapeutic efficiency. Converting liposomal dispersions into dry powders for inhalation (DPIs) is a potential strategy to overcome these limitations. Spray-drying (SD) is particularly attractive for this purpose, offering precise control over particle properties along with speed, cost-efficiency, and scalability. Yet, the thermal and mechanical stresses involved in this process and their effects on liposomal integrity remain poorly understood, limiting its broader application.

In this study, we developed a systematic framework integrating Design of Experiments (DOE) with scale-up-oriented processing, combining supercritical fluid technology (PGSS) for liposome formation with subsequent spray-drying of high solid content suspensions. Two complementary DoE approaches were applied: DOE_A optimized drying parameters and carbohydrate type, testing trehalose and hydroxypropyl-β-cyclodextrin (HPβCD), while DOE_B assessed the effects of active pharmaceutical ingredients (APIs) hydrophobicity, lipid composition (1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-$_{2000}$ ($DSPE\text{-}PEG_{2000}$) percentage), and carbohydrate-to-liposome ratio.

Optimized spray-dried powders exhibited preserved liposome structure (mean diameter <200 nm, narrow polydispersity index (PdI), near-neutral zeta potential (ZP)), favorable aerodynamic properties (~3 µm, extra-fine particle fraction (eFPF) >20% and fine particle fraction (FPF) >60%), low residual moisture (<5%), and high drying yields (>75%). Mechanistic insights revealed that HPβCD significantly protects liposomes during atomization, while drug retention was governed by PEGylated lipid content, lipid-to-carbohydrate ratio, and API hydrophobicity. Co-encapsulation of clinically relevant drug combinations (formoterol/budesonide and ciclesonide/indacaterol) produced uniform powders with efficient aerosolization, highlighting the therapeutic potential of these formulations.

Collectively, these results establish a robust, scalable, and reproducible approach for designing liposomal DPIs, balancing formulation composition, excipient selection, and process parameters. This study demonstrates that strategic integration of scalable technologies can reconcile structural stability, aerodynamic performance, and manufacturability, paving the way for the industrial translation of liposomal dry powders for targeted pulmonary therapies, including asthma and chronic obstructive pulmonary disease (COPD).



KEYWORDS

Dry powder inhalers

Liposome

Spray-drying

Design of Experiments

ABBREVIATION

API, Active Pharmaceutical Ingredient; BUD, Budesonide; Chol, Cholesterol; CIC, Ciclesonide; COPD, Chronic Obstructive Pulmonary Disease; Dae, Aerodynamic Diameter; DepthD, Depth of dimples; DSC, Differential Scanning Calorimetry; DOE, Design of Experiment; DSPE-PEG_{2000}, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-$_{2000}$]; DPI, Dry Powder Inhaler; ED, Emitted Dose; FDA, U.S. Food and Drug Administration; FD, Freeze-Drying; FPD, Fine Particle Dose; FPF, Fine Particle Fraction; eFPF, extra-Fine Particle Fraction; FOR, Formoterol; GSD, Geometric Standard Deviation; glass transition temperature (Tg); HPβCD, Hydroxypropyl-β-Cyclodextrin; HPLC, High Performance Liquid Chromatography; ICS, Inhaled Corticosteroid; ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; ICS, Inhaled Corticosteroid, IND, Indacaterol Maleate; Inlet temperatures (T°in); outlet temperature (T°out); LABA, Long-Acting Beta-2 Agonists, L/C ratio, Lipid-to-carbohydrate ratio; LOD, Limits of detection; LOQ, Limits of quantification; MMAD, Mass Median Aerodynamic Diameter; NGI, Next Generation Impactor; PE, Péclet Number; pMDI, Pressurized Metered-Dose Inhaler; PdI, Polydispersity Index; PSD, Particle Size Distribution; PGSS, Particles from Gas-Saturated Solutions; RD, Recovery Dose; RH, Relative Humidity; RSD, Relative Standard Deviation; SABA, Short-Acting Beta-2 Agonist, SAL, Salbutamol; SD, Spray-Drying; SEM, Scanning Electron Microscopy; SGC, Supercritical CO_2 ($scCO_2$); SMI, Soft-Mist Inhaler; SPC, Soy Phosphatidylcholine; TGA, Thermogravimetric Analysis.

1. Introduction

Liposomes have emerged as highly promising carriers for pulmonary drug delivery, a potential recently highlighted by the U.S. Food and Drug Administration (FDA) approval of Arikayce® in 2018, a liposomal amikacin suspension for inhalation (Bashyal et al., 2025; Pasero et al., 2024). Their biocompatibility, capacity to encapsulate both hydrophilic and lipophilic drugs, membrane-like structure, tunable size, and ability to enhance retention and enable controlled release highlight their strong pharmacological potential (Senjab et al., 2024; Zhang et al., 2025). Additionally, liposomes can penetrate the mucus layer and overcome mucociliary clearance, addressing a major barrier to efficient pulmonary drug delivery and reinforcing their promise as advanced respiratory drug carriers (Lin et al., 2025; Yan & Sha, 2023). When combined with the intrinsic advantages of pulmonary administration, namely targeted lung delivery, rapid onset, limited drug degradation, and minimal systemic side effects, liposomal formulations offer significant opportunities for more effective inhaled therapies (Cipolla et al., 2013).

Despite these beneficial attributes, aqueous liposomal suspensions are prone to aggregation and fusion over time, which can reduce therapeutic efficacy (Xu et al., 2025). To address this limitation, liposomes can be converted into dry formulations, with dry powder inhalers (DPIs) offering a particularly promising approach in the field of inhalation therapy (Yu et al., 2021). Furthermore, unlike conventional inhalation devices such as nebulizers, metered-dose inhalers (pMDIs) and soft-mist inhalers (SMIs), DPIs bypass propellant gases and nebulization, thereby reducing the risk of liposomal disruption during delivery while extending shelf-life (Rudokas et al., 2016; Shahin & Chablani, 2023). Beyond improved stability, DPIs enable precise particle engineering, allowing control over size, surface charge, moisture content, and morphology, which critically influence dispersibility, stability, and deep lung deposition (Chaurasiya & Zhao, 2021; Negi et al., 2023; Scherließ et al., 2022; Spahn et al., 2022).

Liposomal dispersions can be converted into dry powders using well-established techniques such as freeze-drying (FD) and spray-drying (SD) (Negi et al., 2023). Among these, SD is particularly suited for DPIs production, allowing fine control over particle attributes. Indeed, when carefully optimized, this approach yields powders with consistent aerodynamic properties and enhanced pulmonary deposition (Alhajj et al., 2021; Lechanteur & Evrard, 2020), whereas FD typically produces an irregular, lyophilized cake with inadequate flowability (Vishali et al., 2019). Furthermore, from an industrial perspective, SD is also considerably faster, more cost-effective, and highly reproducible (Ziaee et al., 2019). Yet, while FD has been extensively documented for liposomal drying across a wide range of applications (Furst et al., 2016; Kaialy & Nokhodchi, 2013; Nugraheni et al., 2017), the use of SD for liposomal powder formulation remains comparatively less investigated, largely due to the additional challenges of preserving liposomal integrity under this atomizing process. These challenges arise from the structural characteristics that make liposomes attractive carriers, most notably their flexible bilayer and aqueous core, which simultaneously make them highly vulnerable to shear, thermal, and dehydration stresses. Such stresses (high inlet temperatures and atomization pressures) can compromise membrane integrity, leading to drug leakage, vesicle fusion, or even collapse (Dattani et al., 2025; Safaeian Laein et al., 2024). In this context, a suitable carbohydrate matrix is crucial to protect liposomes during drying and ensure efficient aerosolization.

In the pulmonary context, early work by Goldbach et al. (Goldbach et al., 1993) showed that multilamellar soybean phosphatidylcholine (SPC) liposomes spray-dried with 10% lactose retained vesicle size and phospholipid stability upon reconstitution, establishing SD as a viable approach for producing stable pulmonary liposomal powders. However, subsequent studies revealed that SD can induce vesicle shrinkage or transitions from uni- to bi-lamellar structures,

underscoring the need for protective excipients (Wessman et al., 2010). In this regard, cyclodextrins, specifically HPβCD, have been shown to stabilize PEGylated liposomal membranes during SD, preventing bilayer disruption and drug leakage (Van Den Hoven et al., 2012). Trehalose similarly enhances product yield while preserving liposome size and drug encapsulation, and, with or without L-leucine, enables the production of stable, re-dispersible lipid-based powders (Dattani et al., 2025; Khan et al., 2024). Furthermore, Meenach et al. demonstrated that PEGylation facilitates the SD of particles, preserving the bilamellar phospholipid structure post-drying while ensuring favorable aerodynamic performance suitable for pulmonary delivery (Meenach et al., 2013).

Taken together, these studies provide important proof that liposomes can be successfully processed by SD when appropriately formulated and protected. However, most investigations have primarily focused on end-point characterization, offering limited insight into the underlying formulation and process relationships required to derive transferable design principles. As a result, the successful development of liposomal DPIs demands not only confirmation of liposomal integrity, but also a systematic understanding of how formulation and process parameters collectively govern vesicle preservation while enabling inhalation-relevant powder properties. This gap has been explicitly highlighted in the literature, as Ingvarsson et al. (Ingvarsson et al., 2011)noted that liposome integrity following SD is frequently assumed rather than systematically investigated, despite being a critical quality attribute for inhalable liposomal products.

To address this, the present study systematically investigates how SD parameters and formulation strategies influence liposome integrity and DPI quality. A Quality by Design approach based on Design of Experiments (DOE) was applied, integrating formulation development with scale-up-oriented processing. Liposomes were prepared using supercritical fluid technology (PGSS), a solvent-free, one-step process that overcomes key limitations of conventional solvent-based methods, thereby supporting industry-relevant manufacturing (Delma et al., 2023; Penoy et al., 2022), and subsequently dried via SD. An initial DOE evaluated the influence of drying parameters at three distinct levels and compared two carbohydrate matrices, trehalose and HPβCD, for their stabilizing effects on liposomes. Despite the lack of current approval for pulmonary administration, the restricted excipient landscape for inhalation motivates the investigation of well-characterized carbohydrates to broaden the formulation design space and inform regulatory assessment. Subsequently, a second DOE examined the impact of liposomes variables, including active pharmaceutical ingredients (APIs) hydrophobicity, lipid composition (varying the percentage of PEGylated lipid), and carbohydrate-to-liposome ratio. By incorporating APIs with markedly different physicochemical properties, the robustness and transferability of the formulation-process relationships were specifically assessed. Following the identification of optimal drying conditions, structural stability of the liposomes was evaluated across the industrial processes (PGSS and SD), while *in vitro* aerodynamic performance was assessed using a Next Generation Impactor (NGI) to determine their suitability for pulmonary delivery.

Overall, this study aimed to develop optimized DPIs containing PEGylated liposomes capable of delivering multiple APIs. Formulations were designed to preserve liposome integrity while achieving physicochemical properties favorable for inhalation and increased deep lung deposition. This strategy enables the co-encapsulation of agents such as inhaled corticosteroids and β2-agonists, which are recommended for asthma and COPD therapy. To demonstrate the robustness of this approach, clinically used APIs, namely salbutamol (SAL), budesonide (BUD), formoterol (FOR), ciclesonide (CIC), and indacaterol (IND), were deliberately selected to span a broad range of physicochemical properties, particularly in terms of hydrophilicity/lipophilicity and molecular structure, thereby enabling assessment of the

transferability of the formulation–process relationships across diverse APIs. The inclusion of underexplored combinations such as CIC with the ultra-long-acting β2-agonist IND further underscores the translational potential of these liposomal DPIs for future combination therapies.

2. Materials and methods

2.1. Materials

Micronized ciclesonide (CIC, 98.9%) was kindly supplied by NewChem spa (Milan, Italy), and indacaterol maleate (IND, 99.5%) was obtained from LEAP Chem CO (Wan Chai, Hong Kong). Micronized budesonide (BUD, 100.2%) was supplied from Minaken (Dunkerque, France), while salbutamol base (SAL, 99.7%) was purchased from Cambrex (Milan, Italy). Formoterol fumarate dihydrate (FOR, 100.4%) was supplied sourced CHEMO Industriale chimica (Saronno, Italy). All APIs were employed as model compounds in this study.

SPC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), cholesterol (CHOL) from Sigma Aldrich (Brussels, Belgium) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (ammonium salt) (DSPE-PEG_{2000}) from Xiamen Sinopeg Biotech (Xiamen, China).

Tested carbohydrates, including hydroxypropyl-β-cyclodextrin (HPβCD) and Vialose™ trehalose dihydrate, were kindly provided by Roquette (Lestrem, France) and Asland (Schaffhausen, Switzerland), respectively. Saccharose (biochemistry grade) was purchased from Sigma-Aldrich (Darmstadt, Germany).

High-performance liquid chromatography (HPLC)-grade methanol and absolute ethanol were sourced from J.T. Baker (Deventer, The Netherlands) and ThermoFisher Scientific (Geel, Belgium), correspondingly. Ammonium acetate, 25% ammonia solution, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, ≥99.5%), and sodium chloride were all obtained from Sigma-Aldrich (St. Louis, MO, USA, and Brussels, Belgium). Analytical-grade potassium phosphate was sourced from Merck (Darmstadt, Germany), while sodium heptane sulfonate was acquired from VWR Chemicals (Leuven, Belgium). Ultrapure water (resistivity of 18.2 MΩ·cm) was produced using a Milli-Q purification system (Millipore), followed by filtration through 0.22 µm Millipak® 40 disposable filter units (Millipore Corporation, MA, USA). Liquid CO_2 with a purity of 99.7% was purchased from Air Liquide (Zaventem, Belgium).

Size 3 hydroxypropyl methylcellulose capsules suitable for inhalation were supplied by Capsugel® from Lonza) Colmar, France) and used in combination with the Breezhaler® from Novartis Pharma BV) (Basel, Switzerland), a low-resistance inhalation device characterized by a resistance of 0.0177 (kPa)^0.5·(min·L^{-1}), as reported in previous studies (Abadelah et al., 2018; Dal Negro, 2015).

2.2. API quantification

The quantification of CIC, IND, BUD, FOR, and SAL was carried out using an Agilent 1100 Series HPLC system (Santa Clara, USA) equipped with a UV detector. The analytical methods were successfully validated using the accuracy profile approach, with Enoval® software version 3.0 (Arlenda, Liège, Belgium).

2.2.1. Ciclesonide and Indacaterol maleate

For the analysis of CIC and IND, separation was achieved using an XBridge BEH C18 analytical column (3 × 50 mm, 3.5 µm), preceded by a VanGuard Cartridge pre-column. Detection was performed at 243 nm. The mobile phase consisted of an ammonium acetate buffer (pH 10) and methanol, using the following gradient program: 0-1 min: 80/20 (v/v); 1-10

min: linear gradient to 5/95; 10-13 min: isocratic 5/95; 13.5-20 min: return to 80/20. The flow rate was 0.7 mL/min. Column and sampler temperatures were maintained at 30 °C and 10 °C, respectively. Sample preparation involved dilution in methanol (at least 10-fold) to disrupt the liposomal structures and enable accurate quantification of APIs. For this method validation, a 10% risk level (α) and acceptance limits of 10% were defined over the concentration ranges of 1 to 100 µg/mL for CIC and 2 to 100 µg/mL for IND. Accuracy profiles were constructed using weighted linear regression (1/X) for both APIs. The lower limits of detection (LOD) and quantification (LOQ) were 0.4600 µg/mL and 3.205 µg/mL for CIC, and 0.6153 µg/mL and 2.031 µg/mL for IND, respectively.

2.2.2. Budesonide and Formoterol fumarate dihydrate

BUD and FOR quantification employed the same column and detection wavelength (243 nm) as those employed for the quantification of CIC and IND. The mobile phase also consisted of ammonium acetate buffer (pH 10) and methanol, with a different gradient: 0-1 min: 55/45 (v/v); 1-2 min: linear gradient to 35/65; 2-7 min: isocratic 35/65; 8-20 min: return to 55/45. The flow rate, column, and sampler temperatures were identical to the CIC and IND quantification method. Sample preparation involved dilution in methanol (at least 10-fold) to disrupt the liposomal structures and enable accurate quantification of APIs. For method validation, a 10% risk level (α) and acceptance limits were set at ±10% over the concentration ranges of 1 to 100 µg/mL for BUD and 0.1 to 10 µg/mL for FOR. Accuracy profiles for BUD and FOR were constructed using their respective selected calibration models: weighted quadratic regression ($1/x^2$) for BUD and weighted linear regression ($1/x^2$) for FOR. For BUD, LOD and LOQ were determined to be 0.2899 µg/mL and 4.395 µg/mL, respectively. For FOR, the LOD was measured at 0.1161 µg/mL, with a corresponding LOQ of 0.9282 µg/mL (Lechanteur et al., 2022).

2.2.3. Salbutamol

For SAL quantification, separation was performed using a LiChrospher® 100 RP8 column (Hibar® RT 150 × 4.6 mm, 5 µm) under isocratic conditions. The mobile phase comprised of phosphate buffer (pH 3.65) containing sodium heptane sulfonate (as an ion-pairing agent) and acetonitrile (78:22 v/v). The flow rate was 1 mL/min, with the column maintained at 30 °C. Samples (20 µL) were injected at room temperature. Detection was performed at 220 nm. Sample preparation involved a 2-fold dilution in 2.5% Triton X-100 solution, followed by a 5-fold dilution in the mobile phase, to disrupt the liposomal structures and enable accurate quantification of APIs. For the validation of this method, a risk level (α) was set at 5% with acceptance limits at 10%, over the concentration of 0.5 to 20 µg/mL for SAL (Penoy et al., 2022). LOD and LOQ were both determined and found to be equal to 0.039 and 0.13 µg/ml, respectively.

2.3. Quality by design methodology

The first design of experiment (**DOE_A**) is a D-optimal design investigating the main effects, first-order interactions and quadratic effects of the experimental factors. The design incorporated one categorical factor with two levels: carbohydrate type (trehalose vs HPβCD) and four continuous factors: atomization inlet temperature (60-120 °C), pump feed rate (25-150 rpm), nozzle gas pressure (1-4 bar), and cyclone gas pressure (0.05-0.3 bar). The selection of the experimental factors and their corresponding ranges was based on prior scientific knowledge, preliminary experimental investigations, and practical constraints associated with the SD equipment. Overall, the investigated ranges were chosen to remain within physically

meaningful and experimentally feasible limits, while being sufficiently broad to capture relevant effects and interactions among the SD parameters. Experimental work was structured with random blocks of two experiments each plus three central point replicates totaling 24 experiments. Responses included the SD yield, particle size, and residual moisture content, as well as liposome size and polydispersity index (PdI) to monitor liposome integrity. All responses were log-transformed before fitting the model. The initial suspensions contained 5% solid content with a 1:99 lipid-to-carbohydrate ratio and empty liposomes with a SPC/CHOL/DSPE-PEG_{2000} (65/30/5, molar ratio) composition.

Non-significant effects for each response were removed leading to the common mixed effect model for all responses of the following form:

$$\mathbf{Y} = \beta_0 + \beta_1 E + \beta_2 IT + \beta_3 F + \beta_4 NGP + \beta_5 CGP + \beta_6 E * IT + \beta_7 E * F + \beta_8 E * CGP + \beta_9 IT * F + \beta_{10} IT * NGP + \beta_{11} IT * CGP + \beta_{12} F * NGP + \beta_{13} NGP * NGP + \gamma_j + \varepsilon$$

where $\mathbf{Y}$ is the modelled response β_i is the estimate of the fixed effect of the factor *i*, E is the carbohydrate type, IT is the inlet temperature, F is the flowrate, NGP is the nozzle gas pressure, CGP is the cyclone gas pressure, γ_j is the random estimate of the j[th] block and ε is the error term.

The second design of experiment (**DOE_B**) employed a split-plot A-optimal design to investigate the main factors, first-order interactions and quadratic effects of the investigated factors. The split-plot structure was required for practical reasons: the categorical factor API nature (BUD and SAL for hydrophobic and hydrophilic models) and the continuous factor DSPE-PEG_{2000} content (2.5-10%) were selected as hard to change factors. The third factor investigated is the liposome-to-carbohydrate (L/C) ratios (1:99-10:90). These variables were selected based on prior knowledge that DSPE-PEG_{2000} content plays a key role in liposome stability through steric stabilization and membrane protection (Allen & Cullis, 2013), and that the physicochemical nature of the API significantly impacts drug retention in liposomal suspension (Eloy et al., 2014), while also enabling evaluation of formulation feasibility under the selected SD conditions. The evaluated responses included liposome size and PdI to monitor liposome integrity, with particular emphasis on the impact of the tested parameters on the API release after SD. Particle size and residual moisture content were monitored but not modelled during DOE_B. The solid content was held constant at 5% as well, while applying the optimal drying parameters and carbohydrate type established in DOE_A.

The log transformed responses were modelled with the following mixed-effect model:

$$Y = \beta_0 + \beta_1 API + \beta_2 LPEG + \beta_3 LC + \beta_4 API * LPEG + \beta_5 API * LC + \beta_6 LPEG * LC + \beta_7 LPEG * LPEG + \beta_8 LC * LC + \gamma_j + \varepsilon$$

where $\mathbf{Y}$ is the modelled response β_i is the estimate of the fixed effect of the factor *i*, API is the API nature factor, $LPEG$ is the DSPE-PEG_{2000} content, LC is the liposome-to-carbohydrate (L/C) ratio, γ_j is the random estimate of the j[th] whole plot and ε is the error term.

Both DOEs were constructed and analyzed using JMP Pro 18 software.

2.3.1. PGSS-based preparation of liposomal formulations

Liposomes composed of SPC, CHOL, and DSPE-PEG_{2000} in varying molar ratios were produced using the Particles from Gas-Saturated Solutions (PGSS) technique, employing supercritical carbon dioxide ($scCO_2$) as a dispersing agent. The process was carried out under optimized conditions (30min contact with $scCO_2$, 500rpm agitation rate at 80 °C) previously established by Penoy *et al*. (Penoy et al., 2021, 2022), using PGSS equipment (Separex, France), which was also described in detail in their work.

The phospholipid dispersion and APIs were pre-dispersed in HEPES buffer (10 mM, pH 7.4) at 65 °C, followed by stirring at 1200 rpm on a hot plate stirrer for 15 minutes. The resulting dispersion was then introduced into the PGSS system. In **DOE_A**, a pressure of 156 bar was applied to produce 5 mM empty liposomes, while 240 bar was used in **DOE_B** for 45 mM loaded ones.

Liposome dispersions collected after the PGSS process were purified by dialysis using Spectra/Por® cellulose dialysis membranes with a molecular weight cutoff of 20 kDa and a diameter of 10 mm (VWR Chemicals). Dialysis was performed at 4 °C under stirring at 200 rpm for 3 hours, using 20 mL of HEPES buffer per 1 mL of liposome dispersion. The dialysis medium was replaced every hour. Liposomes purified from unencapsulated BUD and SAL were used in **DOE_B** while liposomes purified from free FOR, BUD, CIC and IND were spray-dried in following experiments. This purification also enabled the determination of encapsulation efficiency (EE) after PGSS production. EE (%) was determined as the percentage of the total drug that remained encapsulated within the liposomes after purification, calculated from the difference between the total drug content before purification and the amount of free drug detected after dialysis.

2.3.2. Spray-drying of liposomal formulations

In both DOEs, powders were produced using a Procept 4 M8-Trix Formatrix spray-dryer, equipped with a bi-fluid nozzle. The nozzle diameter was fixed at 0.4 mm. For each batch, 20 mL of solution, in which the components (liposomes and carbohydrate) were dissolved and brought up to volume with Milli-Q water, was used for SD. The process yield for each formulation was calculated using the equation provided in Eq. (1).

$$Drying\ process\ yield\ (\%) = \frac{\text{Amount of powder collected after atomization (g)}}{\text{Total amount of powder implemented in the atomized liquid (g)}} . 100 \qquad (1)$$

Drying conditions were set according to the outcomes of DOE_A and are detailed below.

2.4. Assessment of the glass transition temperature of carbohydrates

Differential Scanning Calorimetry (DSC) measurements were carried out using a Mettler-Toledo instrument (Schwerzenbach, Switzerland) to evaluate the glass transition temperatures (Tg) of the investigated carbohydrates. Samples were analyzed in sealed aluminum pans under a nitrogen atmosphere (20 mL/min). The temperature was increased from 25 to 300 °C at a constant heating rate of 5 °C/min.

2.5. Characterization of liposomes

2.5.1. Particle size, size distribution and zeta potential of liposomes

Liposomes were characterized in terms of Z-Average size (nm), polydispersity index (PdI), and zeta potential (ZP) (mV) after production by PGSS and following SD, which required a rehydration step, using Dynamic Light Scattering (DLS). Measurements were performed at 25 °C and a fixed detection angle of 90° with a Malvern Zetasizer® Nano ZS (Malvern Instruments, UK). Samples produced by the supercritical process and liposomes rehydrated from dry powders in Milli-Q water were diluted in 10 mM HEPES buffer (pH 7.4) to reach an attenuation value of approximately 7, ensuring an optimal particle concentration for reliable measurements. To evaluate potential size changes after drying, a size ratio (Sf/Si) was calculated between liposomes rehydrated from the spray-dried powder (Sf) and freshly prepared liposomes after PGSS production (Si). For both measurements (Si and Sf), freshly prepared and reconstituted liposomes were dispersed in HEPES buffer (pH 7.4) and diluted to achieve an attenuator value of 7 during DLS, ensuring optimal signal quality and reliable size determination. According to Abassi *et al*., a ratio of 1 indicates that the liposome size remained unchanged after drying, reflecting acceptable colloidal stability throughout the drying process

(Abbassi et al., 2025). All measurements were performed in triplicate, and the mean values were reported.

2.5.2. Structural characterization by microscopy analyses

For TEM analysis, nickel grids (300 mesh) coated with a continuous carbon film were glow-discharged (Elmo, Cordouan Technologies) prior to use. A 20 µL drop of sample was applied onto the grid and incubated for 10 min at room temperature. The excess liquid was then blotted with filter paper, and the grids were stained with 2% (w/v) uranyl acetate for 60 s. After staining, the grids were gently washed by sequentially placing them on two drops of ultrapure water, blotted, and air-dried before imaging. Samples were imaged using a Talos transmission electron microscope (ThermoFisher Scientific) operating at 200 kV. Images were acquired using Velox software (ThermoFisher Scientific) and a Ceta camera (ThermoFisher Scientific).

For cryo-TEM analysis, QUANTIFOIL® R1.2/1.3 200 mesh Cu grids were glow-discharged using an ELMO glow discharge system (Cordouan Technologies) at a vacuum of 2.1 x 10^{-1} mbar and a voltage of 1.7 V for 35s. Grids were then transferred to a Vitrobot Mark IV (ThermoFisher Scientific) for a plunge-freezing procedure. A double application of the sample was used: 3 µL of the particle suspension was applied to the grid, followed by blotting (blot force = 2, blot time = 4 s, wait time = 5 s). Immediately after the first blot, a second 3 µL aliquot of the same suspension was applied, followed by a final blot (blot force = 2, blot time = 4 s, wait time = 0 s). Vitrified grids were transferred into a Talos transmission electron microscope (ThermoFisher Scientific) and observed under cryogenic conditions at an accelerating voltage of 200 kV. Images were acquired using EPU software (ThermoFisher Scientific) and a Falcon III EC camera (ThermoFisher Scientific) at a magnification of 73,000x corresponding to a pixel size of 0.14 nm/pixel for an electron dose of 21 $e^{-}/Å^{2}$.

2.6. Characterization of the dried powders

2.6.1. Particle size distribution (PSD)

Particle size distribution (PSD) of the powder samples was evaluated using laser diffraction with a Malvern Mastersizer 3000® (Malvern Instruments, Worcestershire, UK), fitted with the AeroS dry dispersion unit. A small amount of powder, approximately equivalent to a spatula tip, was placed on the micro tray, suitable for low sample volumes, and dispersed using a standard Venturi system. The analysis was conducted at a feed rate of 30% and an air pressure of 4.0 bar, ensuring an obscuration range between 0.5% and 8.0%. Each measurement lasted 10 seconds. Data acquisition and PSD analysis were performed using the dedicated Malvern Mastersizer software. All measurements were conducted in triplicate, and the mean values were reported.

2.6.2. Residual moisture content

The residual moisture content of all spray-dried powders was determined shortly after processing using Thermogravimetric Analysis (TGA; Perkin Elmer, Norwalk, CT). Samples weighing between 5 and 15 mg were placed in platinum pans and heated from 25 °C to 150 °C at a rate of 10 °C/min. This technique measures weight loss as the sample is exposed to temperatures exceeding the evaporation point of water, allowing for accurate quantification of residual water content based on the observed mass decrease. All measurements were conducted in triplicate, and results are expressed as mean values.

2.6.3. Evaluation of dried powders homogeneity

The homogeneity of each powder batch was assessed by collecting five samples from different locations within the powder bed, including both surface and depth. Samples were dissolved in the appropriate mobile phase for API quantification by HPLC (Section 2.2). Blend uniformity was expressed as the coefficient of variation (CV), calculated from the five samples. According to the European Pharmacopoeia, powders with a CV ≤5% for mean API recovery were considered homogeneous.

2.6.4. Particle morphology

After gold metallization, the morphology of the powders obtained by SD was examined using a TESCAN Clara scanning electron microscope equipped with a secondary electron detector.

2.7. Characterization of API leakage from liposomes post drying

After the collection of the lipid-based dry powder, an adequate amount of powder was rehydrated in 3 mL of Milli-Q water. Once fully dissolved, 2 mL of the resulting liquid phase were purified by dialysis using Spectra/Por® cellulose dialysis membranes (20 kDa molecular weight cutoff, 10 mm diameter; VWR Chemicals). Dialysis was carried out at 4 °C under stirring at 200 rpm for 3 hours, using 20 mL of dialysis medium per 1 mL of liposome dispersion. The dialysis medium consisted of a saccharose solution adjusted to match the osmolarity of the sample and was replaced every hour. This purification method was validated in triplicate and enabled the quantification of API released from liposomes following the atomization process. The amount of released API was determined by HPLC (Section 2.2), measuring the API concentration after powder rehydration (total drug) and after dialysis (encapsulated drug), with the released fraction calculated as indicated in Eq. 2 below:

$$Drug\ release\ upon\ drying\ (\%) = \frac{\text{Encapsulated drug (µg/mL)}}{\text{Total drug (µg/mL)}} \cdot 100 \quad (2)$$

2.8. In *vitro* lung deposition evaluation

In *vitro* aerosol performance was evaluated using a Next Generation Impactor (NGI; Apparatus E, Copley, Nottingham, UK) following the guidelines outlined in the European Pharmacopoeia. The NGI, comprising eight stages and a pre-separator, was connected to the inhalation device via an appropriate mouthpiece adapter. Airflow was set at 100 L/min using a flow controller (TPK; Copley, Nottingham, UK) and maintained for 2.4 seconds at each flow rate, or at 60 L/min for 4 s, respectively. The Breezhaler® (Novartis Pharma AG), a low-resistance DPI, was employed for testing, with 20 capsules assessed per run to ensure that the resulting concentrations fell within the calibration curve. Powder deposits collected from all stages of the NGI, including the mouthpiece, capsules, and device, were rinsed with the appropriate mobile phase and subjected to sonication to ensure complete solubilization of APIs, followed by HPLC analysis.

The emitted dose (ED), representing the fraction of drug released from the inhaler, was calculated as the sum of API masses from the induction port to the last NGI stage. The fine particle dose (FPD) was defined as the portion of the dose consisting of particles with an aerodynamic diameter below 5 µm. The fine particle fraction (FPF) was calculated relative to the ED using the following equation (Eq.3):

$$FPF\ (\%) = \frac{\text{Mass of particles<5µm}}{\text{Emitted dose (ED)}} \cdot 100 \quad (3)$$

In addition, the extra-fine particle fraction (eFPF), defined as the fraction of particles with an aerodynamic diameter below 2 µm, was calculated relative to the ED using the following equation (Eq. 4):

$$eFPF\ (\%) = \frac{\text{Mass of particles<2µm}}{\text{Emitted dose (ED)}} \cdot 100 \quad (4)$$

The mass median aerodynamic diameter (MMAD) was determined from the cumulative mass distribution of the aerosol, corresponding to the diameter at which 50% of the total aerosol mass is contained. The geometric standard deviation (GSD) was calculated as the ratio of the diameters at the 84th (D84) and 50th (D50) percentiles.

2.9. Statistical analysis

Data analysis was performed using GraphPad Prism software (version 8.4.3, La Jolla, CA, USA). Results are presented as mean values accompanied by their standard deviations (SD). Statistical differences among groups were assessed via analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison test. Statistical significance was defined as p-values less than 0.05 (*), 0.01 (**), or 0.001 (***).

3. Results and discussion

3.1. DOE_A: Impact of drying parameters and excipient nature on liposome integrity and powder characteristics

To better understand the impact of drying parameters and matrix composition on liposome integrity, a first DOE was conducted. Atomization temperature (inlet), feed flow rate (pump speed), atomization nozzle pressure, and cyclone gas flow were each tested at three levels. Two carbohydrate matrices were investigated: trehalose, for its well-established protective effect on liposomes during drying (Lutta et al., 2024; Susa et al., 2021), and HPβCD, for its potential stabilizing effect of liposomes and ability to produce highly aerosolizable, carrier-free powders with favorable morphologies (Dufour et al., 2015; Lechanteur et al., 2022, 2023; Van Den Hoven et al., 2012). Beyond these functional benefits, both carbohydrates, although not approved yet for inhalation, have been reported to exhibit a favorable safety profile for pulmonary administration (Evrard et al., 2004; Iskandar et al., 2021; Zillen et al., 2021). In particular, HPβCD has demonstrated acceptable local tolerability in preclinical inhalation studies and a favorable safety profile in early-phase clinical evaluations (NCT05148312, NCT05000346, NCT04933383). As a starting point, a lipid-to-carbohydrate ratio of 1:99 was chosen.

Commenté [LB1]: Correct comme ça?

This initial DOE allowed a systematic evaluation of the combined effects of SD parameters and matrix composition on liposome integrity, guiding the development of inhalable powders capable of delivering structurally preserved liposomes. Although the influence of drying parameters on general powder properties is already well established, these properties were included in the DOE to ensure characteristics suitable for pulmonary delivery alongside liposome integrity. All 24 experiments are summarized in Supplementary Data A, which include raw results in terms of SD yield, dry powder particle size, moisture content, as well as liposome size variation and PdI. These results were used to construct a DOE general profiler (Figure 1), providing initial insight into how these parameters collectively influence powder performance and liposome stability.

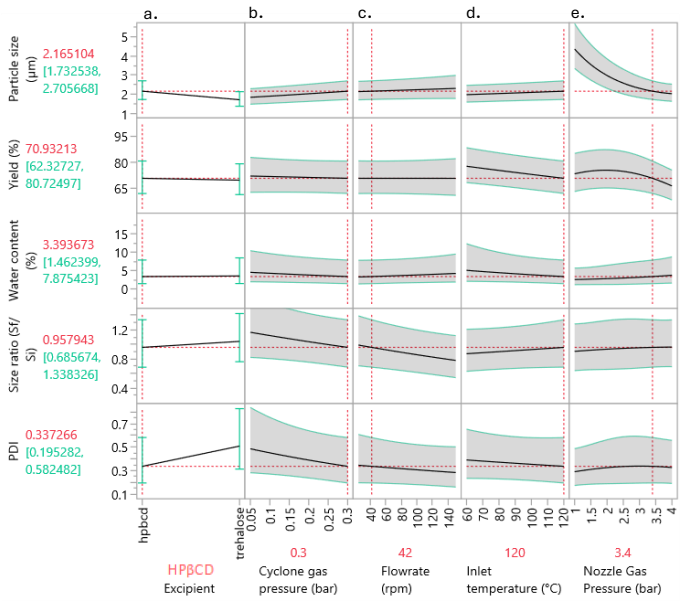


Figure 1: DOE$_A$ prediction profiler: Illustration of the effects of carbohydrate type and spray-drying parameters on key powder characteristics and liposome integrity after drying.

3.1.1. Impact of spray-drying parameters on liposomal properties

Starting with drying parameters, DOE$_A$ analysis revealed a significant combined interaction ($p < 0.05$) between flowrate and drying temperature on liposome integrity, particularly influencing size ratio variation. Specifically, increasing flowrate, directly linked to pump speed, was associated with reduced liposome size and improved size uniformity, as indicated by a lower PdI (Figure 1, column c). Although underexplored in SD, similar trends in microfluidic and extrusion systems, suggested that shear stress and rapid dispersion from higher flow rates can reduce liposome size (Ong et al., 2016; Ota et al., 2023).

Interestingly, increasing the drying temperature from 60 to 120 °C (T°out ~40 and ~75 °C, respectively) appeared to improve liposome integrity, as indicated by a lower PdI and a size ratio approaching 1 (Figure 1, column d). This may result from faster solvent evaporation, which limits lipid rearrangement (Dattani et al., 2025), and from the rapid formation of a protective carbohydrate matrix that stabilizes membranes (Steiner et al., 2022). Importantly, the combined interaction between flowrate and inlet temperature (T°in) suggests a compensatory mechanism rather than a purely synergistic one. At lower flowrates, atomization is generally associated with larger droplets formation and longer drying times. However, increasing the T°in may enhance evaporation kinetics and thereby reduce the duration during which liposomal membranes are exposed to potentially destabilizing conditions. This balance is likely to limit lipid rearrangement and support the preservation of liposomal integrity under conditions combining low flowrate and higher T°in. Though moderate for SD processes, a T°in of 120 °C appears to balance liposome stability and generating powders suitable for effective lung deposition, as supported by findings from recent literature (Khan et al., 2024; Zimmermann et al., 2022).

Furthermore, cyclone and nozzle gas pressure showed no significant effect on liposome integrity, although higher cyclone pressure tended to reduce size ratio and PdI, suggesting improved homogeneity (Figure 1, columns b and e). Additionally, even if increasing nozzle gas pressure showed minimal impact on liposomes integrity, excessive atomization, especially with bi-fluid nozzles, may still disrupt lipid vesicle structure via high shear stress, as reported by recent SD studies on biologics (Poozesh et al., 2025).

Overall, significant effects of process parameters are summarized in Supplementary Data B.

3.1.2. Comparative evaluation of trehalose and HPβCD as protective excipients in liposome spray-drying

Having established the influence of drying parameters, the 24-experiment design then compared the influence of two distinct carbohydrates matrix, HPβCD and trehalose, identifying one optimal condition for each that balanced powder properties with liposomal stability. These conditions, reproduced and validated in triplicate, are summarized in Table 1 below.

Table 1: Optimal drying conditions for liposomes using HPβCd and trehalose, as determined by DOE$_A$.

Optimal condition	HPβCD	Trehalose
Inlet temperature (°C)	120	120
Outlet temperature (°C)	~75	~75
Pump speed (rpm)	42	150
Flowrate (g/min)	~1.56	~5.66
Nozzle gas pressure (bar)	3.4	4.0
Cyclone gas pressure (bar)	0.3	0.05

Although the DOE analysis indicated that the nature of the excipient had no statistically significant impact on PdI values, subsequent validation experiments underscored its practical importance. While both formulations yielded physically acceptable inhalable powders, trehalose was associated with a pronounced increase in liposome size upon reconstitution, exhibiting a size ratio of 1.18 ± 0.04 and a mean PdI of 0.47 ± 0.03 (Figures 2A–B). These findings indicate reduced size homogeneity and potential membrane destabilization, suggesting that the HPβCD-based formulation represents the most favorable condition for preserving liposome integrity.

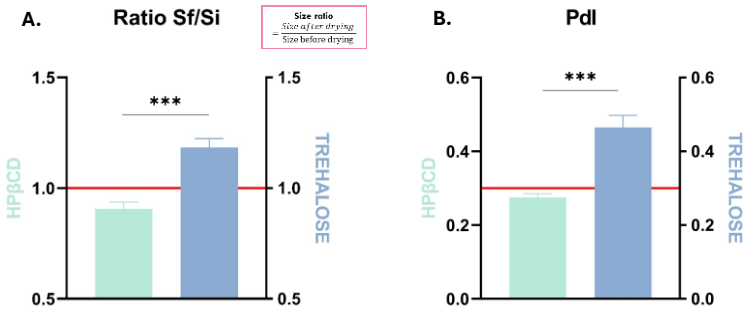


Figure 2: Effect of carbohydrate type on liposome integrity after spray-drying, as determined by DOE statistical analysis. Measured size ratios and PdI values for HPβCD- and trehalose-based formulations under their respective optimal drying conditions, with prediction intervals indicated. Analyses were performed in triplicates (n = 3) and results are presented as mean ± standard deviation.

Differences in performance can be explained by the distinct stabilization mechanisms of both protectants. This may first be attributed to differences in the vitrification behavior of both

carbohydrate matrix. Indeed, during vitrification, an amorphous glassy matrix forms around the liposomes, physically immobilizing them and thereby limiting membrane rearrangements during dehydration (Grasmeijer et al., 2013). The effectiveness of this process largely depends on the excipient's glass transition temperature (Tg): above this temperature, the matrix softens, loses rigidity, and can no longer effectively protect the vesicles, leading to increased lipid mobility and structural disruption (Ingvarsson et al., 2011; Van Den Hoven et al., 2012). In this study, DSC measurements confirmed Tg consistent with literature values, namely approximately 250 °C for HPβCD and approximately 100 °C for trehalose (Roe & Labuza, 2005; Wilhelms et al., 2023; Zheng & Chow, 2009). Consequently, HPβCD remains well above the drying temperatures applied, whereas trehalose operates closer to the outlet temperature (~75 °C), resulting in comparatively weaker stabilization.

Beyond vitrification that plays a central role in liposome stabilization during SD, excipients can also stabilize liposomes by forming hydrogen bonds with membrane surfaces during dehydration, replacing water molecules and stabilizing bilayer structure, similar to freeze-drying mechanisms (Ingvarsson et al., 2011). Van den Hoven ***et al***. notably reported that HPβCD provides enhances protective effects compared to trehalose on PEGylated liposomes, likely due to its higher density of hydrogen bond donors and acceptors (Van Den Hoven et al., 2012). This feature, supported by its greater number of hydroxyl groups (Hammoud et al., 2019), promotes more efficient membrane stabilization and better preservation of liposome characteristics (Kumar et al., 2020; Yergey et al., 2017).

Overall, the protective capacity of HPβCD is reflected in the post-drying liposome characteristics, with preserved size (size ratio = 0.91 ± 0.03) and uniform particle distribution (PdI = 0.27 ± 0.01), as predicted by the DOE profiler (Figure 1, column a). Importantly, the slightly reduced size ratio (0.91) in combination with a relatively low PDI indicates that the minor decrease in liposomal diameter likely reflects subtle bilayer compaction or hydration changes during drying, rather than any loss of vesicle integrity. Under its optimal conditions (Table 1), the formulation achieved high drying efficiency (80.46 ± 4.74%), an inhalable particle size of 2.83 ± 0.24 µm, and low residual moisture (4.60 ± 0.42%), ensuring stability and minimizing degradation. Furthermore, powders produced under the optimal DOE_A conditions using HPβCD exhibited sustained liposomal stability over 1 year of storage, as reflected by unchanged liposomal size and PdI, alongside stable powder water content over time. These long-term stability results are presented in Supplementary C.

These results demonstrate that HPβCD supports the production of inhalable liposomal powders with favorable DPI properties and liposomes structural integrity, confirming its suitability as a drying stabilizer for pulmonary delivery. However, optimal liposome preservation was achieved only within a narrow experimental window, highlighting the critical role of a multifactorial DOE approach in identifying precise formulation conditions, as represented by the experimental design space (Supplementary Data D).

3.1.3. Application of optimized Spray-Drying parameters to budesonide-encapsulated liposomes

The optimal condition identified and validated in DOE_A using HPβCD as a protective carbohydrate matrix was applied to liposomes encapsulating BUD, a hydrophobic inhaled corticosteroid (ICS) commonly used in asthma and COPD, to further assess post-drying integrity and drug retention. The transfer study first confirmed the preservation of liposomal size, with pre-drying and post-drying diameters of 125.95 ± 6.15 and 118.20 ± 8.3 nm, respectively, yielding a size ratio of 0.95 ± 0.04. The produced liposomes exhibited a particle size below 200 nm and a PdI of 0.229 ± 0.01, indicating physicochemical properties appropriate for a pulmonary liposomal formulation and the uniformity required for the clinical

application of lipid-based nanocarriers (Danaei et al., 2018; Zhang et al., 2025). Furthermore, the zeta potential was measured at −4.46 ± 1.30 mV, indicating a near-neutral surface charge that supports effective mucus penetration and cellular uptake, while minimizing the cytotoxicity typically associated with positively charged liposomes (Lechanteur et al., 2016; Zhang et al., 2025; Zhao et al., 2022).

However, despite the preservation of physicochemical properties, post-rehydration dialysis, used to remove free, non-encapsulated drug, revealed that 33.41 ± 3.64% of BUD was released after drying. This partial loss suggests potential leakage or destabilization of the liposomal membrane during atomization, underscoring the need for further optimization (DOE_B) to improve drug retention.

3.2. DOEB: Impact of liposome composition and liposome-to-carbohydrate ratios on liposome integrity and powder characteristics

Following DOE_A, a second experimental plan evaluated the impact of liposome composition (excipients and API) and liposome-to-carbohydrate ratios on liposome integrity and powder characteristics. Specifically, the effects of the nature of the encapsulated API and the liposome-to-carbohydrate (L/C) ratio were investigated to assess drug retention and the protective role of the carbohydrate matrix while increasing the total liposomal content in the resulting powders. BUD and SAL, a short-acting beta-2 agonist (SABA), were selected as models for hydrophobic and hydrophilic drugs, respectively, along with their prominent use in combination therapies for asthma (LaForce et al., 2025). In addition, the influence of liposomal composition was examined by varying the proportion of DSPE-PEG_{2000}, for its potential steric stabilization of liposomes during SD. Although PEGylated lipids are more extensively characterized in parenteral liposomal products, as exemplified by their use in Doxil®, they have also been investigated for pulmonary delivery, with preclinical inhalation studies indicating good tolerability and no major toxicological or pharmacokinetic alterations following repeated administration (Bai & Ahsan, 2010; Muralidharan et al., 2014).
In all experiments conducted within this design, particle size and residual moisture content were systematically monitored but were not incorporated into the modeling process within the DOE_B framework. The experimental design, along with the corresponding raw data, is provided in Supplementary Data E.

DOE_B experiments performed with BUD and SAL loaded liposomes revealed that the DPI properties and liposome integrity (size ratio and PdI) consistently remained within acceptable limits, regardless of API type, lipid composition and quantity. Specifically, all produced powders exhibited a particle size of 2.75 ± 0.50 µm and a residual moisture content of 3.58 ± 1.28%, values well within the range suitable for inhalation. Moreover, a liposomal size ratio of 0.93 ± 0.03 and an average PdI of 0.22 ± 0.02 further confirmed the preservation of liposome integrity after drying. Collectively, these findings highlight that DPI performance and liposome integrity are predominantly governed by SD parameters and carbohydrate matrix type, as identified in DOE_A, whereas the variables examined in DOE_B, although exerting minor effects, still resulted in outcomes meeting the desired specifications. The effects of the tested parameters on liposome integrity and API release are illustrated in the DOE_B profiler (Figure 3), along with a summary of the parameters significantly affecting drug release after drying is presented in Supplementary Data F.

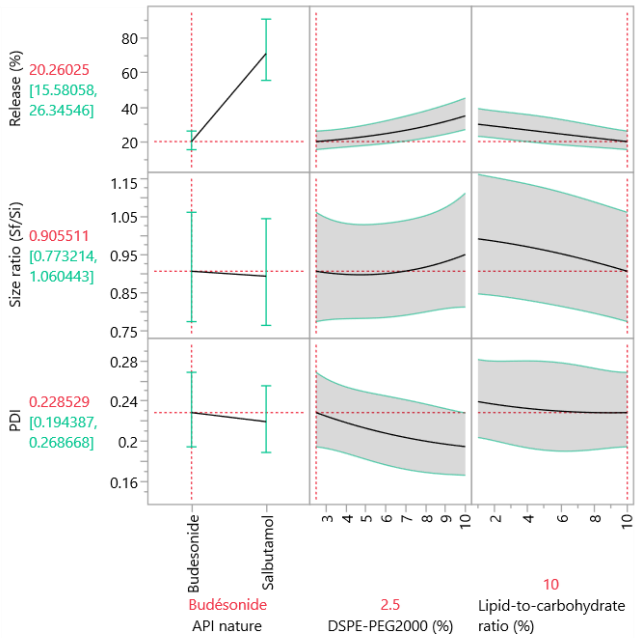


Figure 3: DOE_B prediction profiler illustrating the influence of API type, DSPE-PEG_{2000} content, and L/C ratio on key powder properties and liposome integrity post-drying, with particular emphasis on drug release after drying. (B) Predicted optimal drying conditions for BUD-loaded liposomes, highlighting the effects of DSPE-PEG_{2000} content and L/C ratio on BUD release. (C) Predicted optimal drying conditions for SAL-loaded liposomes, highlighting the effects of DSPE-PEG_{2000} content and L/C ratio on SAL release.

When examining the extent of API release from liposomes during the drying process, the nature of the encapsulated compound appears to play a critical role. Liposomes loaded with BUD could exhibit limited release when optimized, stabilizing around 20%, whereas those encapsulating SAL show significantly higher release levels, reaching approximately 70% (Figure 3). This imbalance is likely due to structural reorganization of the liposomes during drying, which promotes leakage from the aqueous core. Given SAL's high water solubility, it diffuses more readily out of the aqueous heart of the liposome, while BUD, with a stronger affinity for the lipid bilayer, remains largely retained. Another possible explanation highlighted by Wessman *et al.* (Wessman et al., 2010), is that cyclodextrin concentration increases during solvent evaporation, creating an osmotic gradient that may drive the efflux of the internal aqueous compartment, leading to the selective release of the hydrophilic drug, consistent with classical osmotic theory. This hypothesis was experimentally excluded (see Figure 6).

Importantly, the key finding of DOE_B reveals that an increased percentage of DSPE-PEG_{2000} in liposomes encapsulating hydrophobic drugs markedly enhances their release during the drying process, as illustrated by the zoomed section of the profiler corresponding to BUD optimal condition (Figure 4A). This effect is likely attributable to the combined influence of two factors: the increased hydrophilicity conferred by the PEGylated lipids at the liposome surface and the elevated drying temperatures, which together could compromise the structural integrity of the lipid membrane and increase bilayer fluidity, thereby facilitating the release of the

entrapped hydrophobic drug (Giakoumatos et al., 2022; Khan et al., 2024). Nevertheless, a minimal proportion of DSPE-PEG_{2000} remains essential to prevent vesicle aggregation during atomization by providing a steric hydrophilic barrier (Li et al., 2015; Tunsirikongkon et al., 2019). This effect is reflected in the profiler, where an increase in the PEGylated fraction correlates with a lower PdI (Figure 3). Additionally, PEGylation offers benefits in the pulmonary context by enhancing mucus penetration, thereby reducing mucociliary clearance and extending residence time (Li et al., 2021; Osman et al., 2018). A slight percentage of PEGylated lipid thus remains essential. In contrast, the release of hydrophilic drugs, which are confined to the aqueous core of the liposomes, is minimally impacted by variations in DSPE-PEG_{2000} content (Figure 4B), highlighting the selective influence of PEGylation on drug retention in the membrane.

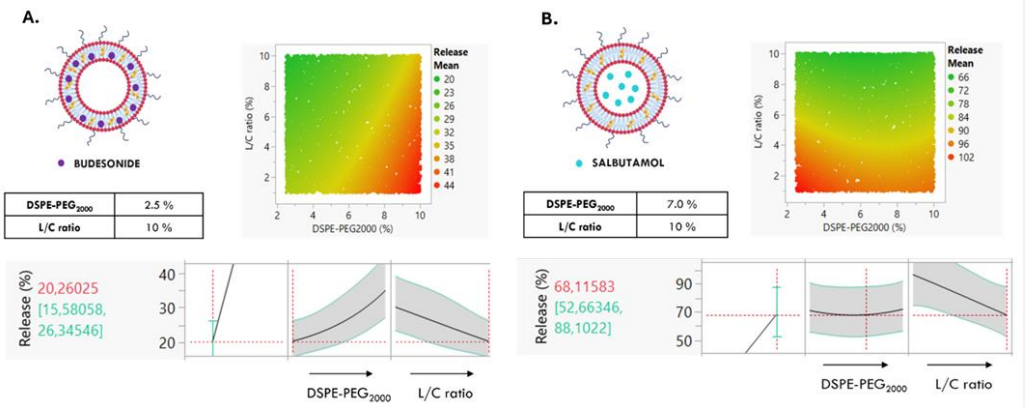


Figure 4: (A) Predicted optimal drying conditions for BUD-loaded liposomes, illustrating the influence of DSPE-PEG_{2000} content and lipid-to-carbohydrate (L/C) ratio on BUD release during SD. An increase in DSPE-PEG_{2000} content is associated with enhanced BUD leakage, whereas increasing the L/C ratio results in reduced BUD release, leading to an optimal formulation containing 2.5% DSPE-PEG_{2000} and an L/C ratio of 10% to minimize BUD loss during drying. (B) Predicted optimal drying conditions for SAL-loaded liposomes, illustrating the influence of DSPE-PEG_{2000} content and L/C ratio on SAL release during SD. While variations in DSPE-PEG_{2000} content have no significant impact on SAL release, increasing the L/C ratio reduces SAL leakage, resulting in an optimal formulation containing 7% DSPE-PEG_{2000} and an L/C ratio of 10% to limit SAL loss during drying.

Moreover, and notably, despite the influence of PEGylated lipid content, a consistent and significant ($p < 0.0001$) reduction in drug release is observed for both API-loaded liposome formulations as the L/C ratio increases (Figures 4A-B). This trend may be attributed to a higher concentration of liposomes within the atomized liquid, resulting in a greater number of liposomes within each droplet. Such a configuration likely promotes mutual shielding among liposomes, particularly those located at the core of the droplet, thereby reducing their direct exposure to drying stresses. This protective effect contributes to better preservation of liposomal membrane integrity and limits premature API leakage. Additionally, increasing the lipid content raises the viscosity of the droplets, potentially slowing API diffusion from destabilized liposomes during the rapid drying phase. Although still highly hypothetical, these two combined mechanisms could be responsible for the decreased API release observed after drying with increasing L/C ratio.

Furthermore, Figure 5 shows the correlation between observed and predicted release percentages. The data closely follows the line of identity (red), indicating a strong agreement between predicted and measured values. The high R^2 (0.99081), and highly significant p-value (<0.0001) confirm the excellent predictive performance of the model.

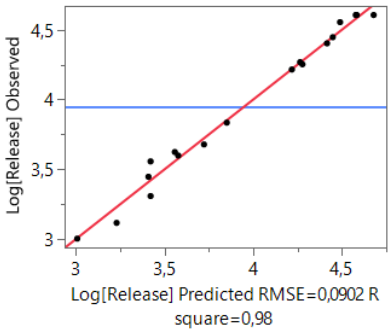


Figure 5: Observed (measured) and predicted (via linear regression: solid red line) drug release values for all considered powders.

Ultimately, based on the comprehensive analysis of all measured parameters, optimal formulations were identified for both BUD (Figure 4A) and SAL-loaded liposomes (Figure 4B) to minimize drug release during the drying while preserving liposome structural integrity. For both drugs, the total lipid content was maintained at 10%, with the optimal DSPE-PEG_{2000} content being 2.5% for BUD and 7% for SAL. These conditions effectively balance drug retention and liposome stability during drying, thereby ensuring the best preservation of liposomal properties. Both conditions were validated in triplicate. Furthermore, given the minimal effect of PEGylated lipid on SAL release and considering the therapeutic interest of combining an ICS with a SABA in the treatment of asthma, co-encapsulation of both APIs was subsequently evaluated using the BUD-optimal formulation. All results are presented in Table 4 and fall within the validation ranges predicted by the DOE analysis, confirming their suitability for inhalation based on particle characteristics, as well as liposome properties and integrity after drying.

Table 1: Overall results of the validation of the BUD-optimal condition, the SAL-optimal condition, and the BUD-optimal condition applied to liposomes co-encapsulating both APIs. Analyses were performed in triplicates (n = 3) and results are presented as mean ± standard deviation.

		BUD	SAL	Co-encapsulation
DPI properties	**Drying Yield (%)**	75.85 ± 6.77	74.41 ± 2.70	76.03 ± 0.08
	Particle size (µm)	2.79 ± 0.05	2.55 ± 0.03	2.64 ± 0.03
	Water content (%)	4.26 ± 0.77	2.71 ± 0.48	3.50 ± 0.70
Liposomes properties	**Liposomes size after powders rehydration (nm)**	127.1 ± 6.79	127.43 ± 1.07	128.63 ± 4.21
	Size ratio (Sf/Si)	0.92 ± 0.05	0.96 ± 0.01	0.92 ± 0.01
	PdI	0.21 ± 0.03	0.21 ± 0.00	0.22 ± 0.01
	ZP (mV)	-2.73 ± 0.39	-2.31 ± 0.25	-2.92 ± 0.08

Regarding the percentage of drug that leaked upon drying, Figure 6 demonstrates that, under their respective optimal conditions, BUD exhibited drug release value ranging from 11.42 and 30.06%, with a mean drug release of 23.57 ± 4.38%, while SAL ranged from 62 and 76.03%, with a mean release of 75.38 ± 4.21%, respectively. Moreover, when applying the BUD-optimal conditions to liposomes co-encapsulating both drugs, the results remained within the validated ranges, with release values of 26.35 ± 2.24% for BUD and 64.50 ± 4.71% for SAL. These values fall within the validation limits for both optimal conditions, thereby confirming the statistical robustness and compliance with this second design.

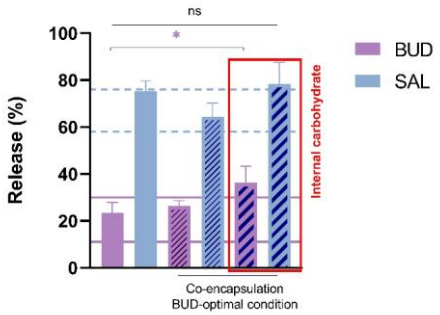


Figure 6: Drug release values of BUD- and SAL-loaded liposomes under their respective optimal spray-drying conditions, and for co-encapsulated liposomes processed under BUD-optimal conditions with or without internal carbohydrate incorporation. Analyses were performed in triplicates (n = 3) and results are presented as mean ± standard deviation.

Finally, dry liposomal formulations are well known to preserve liposome stability over time (Changsan et al., 2009; Dattani et al., 2025), particularly by maintaining encapsulation efficiency during storage. Initial stability studies on the final formulation, shown in Supplementary Data G, support this concept.

3.3. Application of the optimized spray-drying process to additional APIs

3.3.1. Evaluation of the influence of API hydrophobicity on drug release upon drying

To evaluate the transferability and robustness of the combined findings from both DOE studies, the optimized drying parameters and carbohydrate matrix selection identified in DOE_A, together with insights from DOE_B, resumed in Table 5, were applied to additional APIs displaying varying degrees of hydrophobicity. These included formoterol fumarate (FOR), indacaterol maleate (IND), and ciclesonide (CIC). The hydrophobicity of the molecules was determined by their logP values, which reflect the lipophilic character of a drug by measuring its affinity for the octanol phase (Kujawski et al., 2012). The logP values are 0.44, 1.91, 2.42, 3.31, and 4.08 for SAL, FOR, BUD, IND, and CIC, respectively (DrugBank, 2025). Beyond their distinct hydrophobicity profiles, these compounds were chosen due to their therapeutic relevance in the management of pulmonary diseases such as asthma and COPD. Additionally, their selection reflects their importance in innovative combination therapies, exemplified by the co-administration of FOR and BUD in the marketed formulation Symbicort®, as well as emerging dual-molecule strategies involving the co-encapsulation of IND and CIC (Bya et al., 2025). Accordingly, and for therapeutic relevance, liposomes co-encapsulating FOR and BUD, as well as IND and CIC, were prepared, and their performance was compared with that of liposomes co-encapsulating SAL and BUD.

Table 5: Overview of the optimized spray-drying (SD) conditions for PEGylated liposomes, combining the SD parameters and carbohydrate selection identified in DOEA with the lipid composition and lipid-to-carbohydrate ratio determined in DOEB.

FINAL OPTIMAL CONDITION FOR PEGYLATED DRYING LIPOSOMES

Cyclodextrin matrix	**HPβCD**
Inlet temperature (°C)	120
Outlet temperature (°C)	~75

SPRAY-DRYING PROCESS OPTIMAL PARAMETERS (DOE_A)	Pump speed (rpm)	42
	Flowrate (g/min)	~1.56
	Nozzle gas pressure (bar)	3.4
	Cyclone gas pressure (bar)	0.3
LIPOSOMAL OPTIMAL COMPOSITION (DOE_B)	$DSPE\text{-}PEG_{2000}$ content (%)	2.5
	Lipid-to-carbohydrate ratio (%)	1:9

Building on this experimental framework, the additional results revealed a notable decrease in drug release after drying, with measured values of 26.31 ± 8.13% for FOR, 14.47 ± 1.07% for CIC, and 13.47 ± 2.27% for IND, compared to previous results obtained for BUD and SAL. When related to their logP values, a clear trend emerges showing that drug release decreases as lipophilicity increases, supported by a strong exponential correlation (R^2 = 0.98) between API hydrophobicity and release behavior during SD, as initially observed in DOE_B (Figure 7).

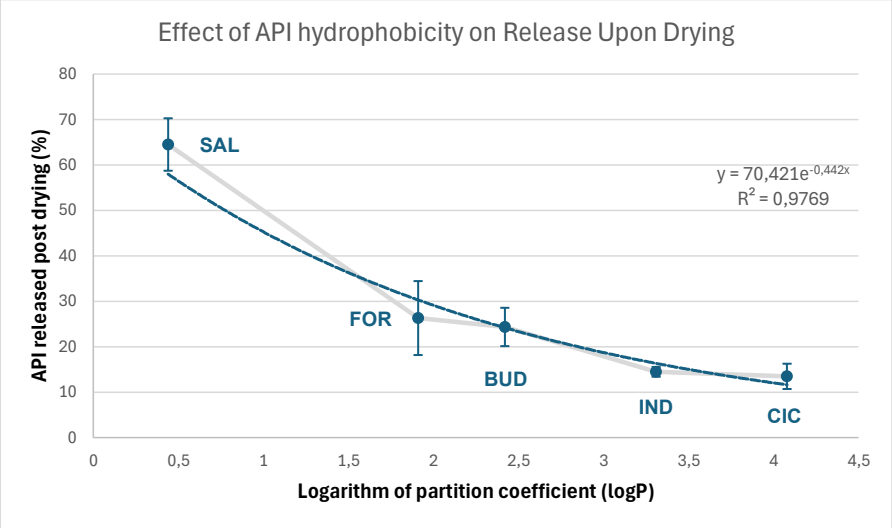


Figure 7: Influence of API hydrophobicity (log P) on drug release during the spray-drying process under optimized conditions, showing a strong correlation (R^2 = 0.98). Analyses were performed in triplicates (n = 3) and results are presented as mean.

These findings reinforced those firstly observed in DOE_B, where hydrophobic APIs are better retained during drying due to their higher affinity for the lipid bilayer, which protects them despite membrane reorganization. For highly hydrophobic APIs, higher $DSPE\text{-}PEG_{2000}$ contents could be envisaged, as their strong affinity for the lipid bilayer may allow increased PEGylation without compromising drug retention and potentially improving mucopenetration (Piñol-Cancer et al., 2025). In contrast, compounds with lower logP exhibited increased leakage during drying. While the optimized SD process effectively preserved liposomal integrity, the limited retention of hydrophilic APIs represents a key limitation of this study. Several formulation strategies reported in the literature may be explored to address this limitation (Yu et al., 2021), including the use of hydrophobic amino acids (Chen et al., 2012), polymer coating of liposomes (Altin et al., 2018; Sarabandi & Jafari, 2020), or modulation of bilayer rigidity through cholesterol enrichment or the use of saturated phospholipids (Anderson & Omri, 2004; Charnvanich et al., 2010).

Accordingly, only liposomes encapsulating relatively hydrophobic APIs were selected for further characterization in the subsequent stages of the project.

3.3.2. Characterization of formulated powders and assessment of aerosolization performance

Finally, to further characterize the powders obtained under conditions combining both DOEs, additional analyses were conducted to assess API distribution, the morphology of liposomes and dry powder particles, and the lung deposition profile, complementing the previously evaluated powder properties and liposome integrity. These investigations focused on the powders containing liposomes co-encapsulating BUD-FOR and CIC-IND. Powders properties (particle size and water content) and liposomes integrity (size and PdI) remained consistent with predicted values by DOE_A under optimal conditions, as summarized in Table 5 below.

Table 2: Summary of powder and liposome properties for BUD–FOR and CIC–IND co-encapsulated formulations produced under combined DOE-optimized conditions. Analyses were performed in triplicates (n = 3) and results are presented as mean ± standard deviation.

		BUD-FOR	CIC-IND
DPI properties	**Drying Yield (%)**	72.87 ± 5.50	76.07 ± 0.01
	Particle size (µm)	2.80 ± 0.30	2.79 ± 0.31
	Water content (%)	3.31 ± 0.54	4.84 ± 0.35
Liposomes properties	**Liposomes size after powders rehydration (nm)**	141.8 ± 7.47	151.83 ± 3.52
	Size ratio (Sf/Si)	0.98 ± 0.03	0.88 ± 0.08
	PdI	0.23 ± 0.01	0.24 ± 0.02
	ZP (mV)	-2.82 ± 0.14	-2.92 ± 0.32

To confirm the integrity of the final liposomal formulation under optimal drying conditions, complementary microscopy were performed using both TEM and cryo-TEM. Before atomization, the liposomes exhibited smooth, spherical vesicles with well-defined bilayers (Figures 8A and 9A), a morphology that was preserved after drying, as evidenced by the rehydrated powders displaying structures comparable to the pre-drying liposomes (Figures 8B and 9B). Although the presence of a high carbohydrate content slightly altered bilayer definition, the post-drying liposomes retained their lamellarity and overall morphology, consistent with size estimation. Importantly, TEM and cryo-TEM analyses yielded consistent structural and size observations, providing complementary evidence for the preservation of liposomal integrity after SD. Together, these findings confirm the protective role of HPβCD and highlight the suitability of the optimized drying parameters and liposomal composition for preserving structural integrity.

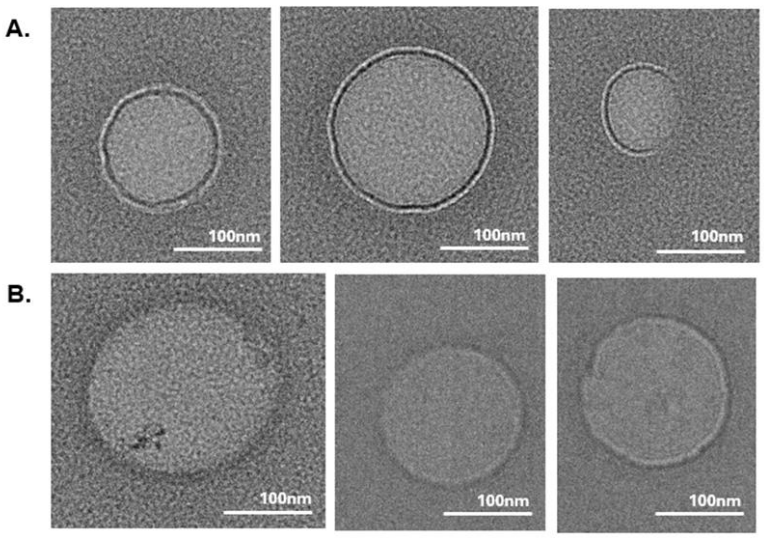


Figure 8: (A) TEM images of liposomes before SD and (B) rehydrated liposomes after SD, within the rehydrated carbohydrate matrix.

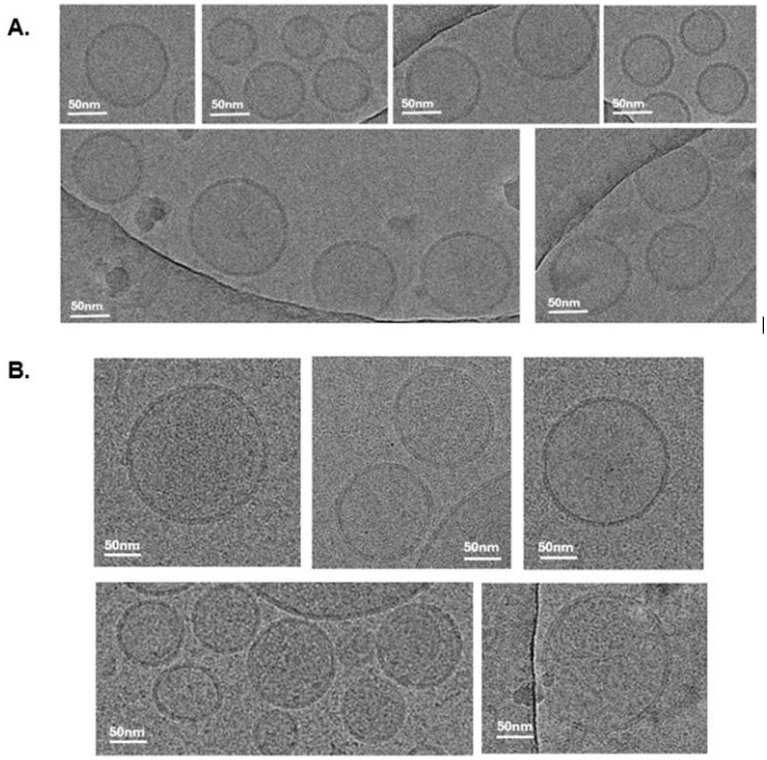


Figure 9: (A) Cryo-TEM images of liposomes before SD and (B) rehydrated liposomes after SD, within the rehydrated carbohydrate matrix.

In the development of DPIs, the therapeutic agent generally constitutes only a minor proportion of the overall powder mass. This proportion becomes even smaller when the API is encapsulated in liposomes, as such an approach aims to lower the required dose and thereby minimize potential adverse effects (Willis et al., 2012). Consequently, a homogeneous distribution of the API within the resulting powders is essential to ensure consistent therapeutic performance, with each delivered dose containing the same amount of drug (Marianni et al., 2021). In this study, powder homogeneity was evaluated by calculating the CV of API content after drying. As reported by Nguyen *et al*., API distribution within the powder is generally deemed acceptable when the CV is below 5% (Nguyen et al., 2015). Analysis of different samples from the produced powders containing co-encapsulated drug combinations showed CV values below 5% for all APIs: 1.22 ± 0.42 for FOR, 1.88 ± 1.00 for BUD, 2.17 ± 1.99 for IND, and 2.69 ± 1.55% for CIC (Figure 10A). These results confirm a homogeneous distribution of APIs within the produced powders, reflecting uniform liposome distribution in the droplets formed during atomization. Moreover, together with the unchanged size and PdI values, they indicate that no liposome fusion occurred during the process. Indeed, such fusion could have resulted in a heterogeneous API distribution in the final powder.

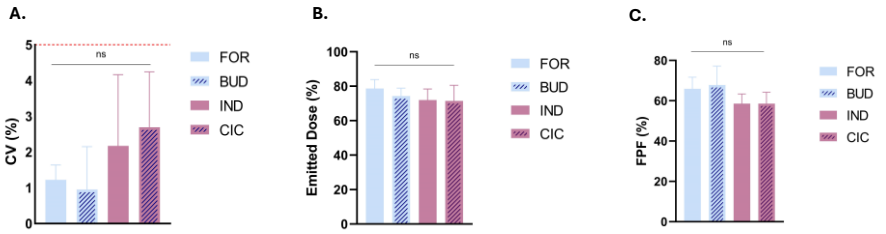


Figure 10: (A) Coefficient of variation of APIs calculated across multiple powder samples, reflecting the uniformity of API distribution within the powders. (B) Emitted Dose (ED) and (C) Fine Particle Fraction (FPF) measured using a Next Generation Impactor (NGI) on powders containing liposomes co-encapsulating FOR–BUD and IND–CIC, at 100L/min. Analyses were performed in triplicates (n = 3) and results are presented as mean ± standard deviation.

Furthermore, the *in vitro* aerodynamic performance of both powders was evaluated using the NGI, with one formulation co-encapsulating FOR and BUD and the other CIC and IND. The BUD-FOR and CIC-IND powders exhibited mass median aerodynamic diameters (MMAD) of 4.19 ± 1.41 µm and 4.60 ± 0.47 µm, respectively, with geometric standard deviations (GSD) of 1.82 ± 0.05 and 1.83 ± 0.04, indicating relatively narrow particle size distributions. The SD lipid-based powders exhibited aerodynamic diameters slightly above 3 µm, which falls within the size range generally considered optimal for deposition in the lower respiratory tract and relevant for treating a wide variety of pulmonary disorders, including asthma and COPD (Ou et al., 2020). Importantly, the particle sizes remained within the recommended 1-5 µm range, allowing efficient lung deposition while minimizing the risks of exhalation or oropharyngeal impaction (Magramane et al., 2023; Zillen et al., 2021). Regarding particle size distribution, although a perfectly monodisperse aerosol would have a GSD close to 1, inhaler-generated powders typically exhibit GSD values around 1.8 (Louey et al., 2004; Mitchell et al., 2003).

Among the parameters evaluated, the ED, defined as the fraction of the drug exiting the device, ranged from 75 to 85% for both powders (Figure 10B), considered as high-efficiency DPIs (Behara et al., 2014). Additionally, when calculating the fraction of drug reaching the deepest regions of the lung based on the ED, as characterized by the FPF, all formulations demonstrated efficient pulmonary deposition, with FPF values of 58.54 ± 4.82% for IND, 58.58 ± 5.67% for CIC, 63.44 ± 12.22% for BUD, and 65.87 ± 5.85% for FOR (Figure 10C). Detailed stage-by-stage deposition data were also generated, showing that all APIs exhibited highly comparable deposition profiles across the NGI stages, thereby indicating consistent aerodynamic behavior throughout the impactor (Supplementary Data H).

Furthermore, as COPD predominantly affects the peripheral airways and the lung parenchyma, evaluation of the eFPF represents a more clinically relevant parameter for assessing deep lung delivery (Braido et al., 2016; Usmani & Barnes, 2012). The eFPF corresponds to the fraction of particles with an aerodynamic diameter below 2 µm, which are more likely to penetrate and deposit in the small airways and alveolar regions (Hillyer et al., 2018; Usmani et al., 2020). For the tested powders, eFPF values exceeded 20% for all formulations, reaching 23.74 ± 3.63%, 25.35 ± 0.96%, 25.28 ± 3.23%, and 22.68 ± 3.64% for FOR, BUD, CIC, and IND, respectively. These results are particularly encouraging, as eFPF values above 5% have been widely reported in the literature as indicative of an effective potential for deep lung deposition (Tse et al., 2021).

In addition, previous studies indicate that patients using the Breezhaler® typically achieve an inspiratory flow rate of approximately 60 L/min (Abadelah et al., 2018). Therefore, to evaluate aerodynamic performance under clinically relevant conditions, lung deposition experiments

were conducted at 60 L/min using the powder encapsulating liposomes co-delivering CIC and IND.

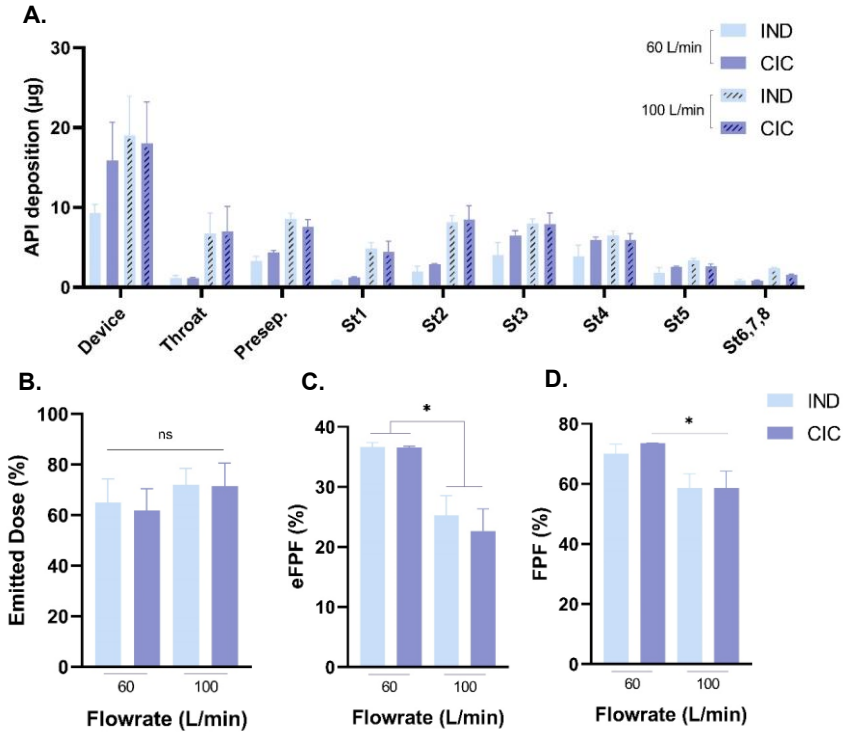


Figure 11: (A) Stage-by-stage deposition profiles, (B) Emitted Dose (ED), (C) extra-Fine Particle Fraction (eFPF) and Fine Particle Fraction (FPF) obtained from NGI lung deposition studies of the optimized powder formulation containing liposomes co-encapsulating CIC and IND at a clinically relevant flow rate of 60 L/min. Results are compared with those obtained at 100 L/min. Analyses were performed in triplicates (n = 3) and results are presented as mean ± standard deviation.

Compared with 100 L/min, powder retention within the inhaler was similar at both flow rates, as confirmed by the stage-by-stage mass recovery profiles (Figure 11A) and comparable ED values at 60 L/min with 61.83 ± 8.66% for CIC and 64.98 ± 9.41% for IND (Figure 11B). Although total deposition across the NGI stages was higher at 100 L/min, increased impaction in the throat and pre-separator was observed at this flow rate, likely due to enhanced inertial effects. In contrast, deposition in the respirable fraction remained high at both flow rates, with eFPF values of 36.58 ± 0.21% and 36.61 ± 0.79% (Figure 11C), and FPF values of 73.55 ± 0.12% and 70.12 ± 0.79% for CIC and IND, respectively (Figure 11D). Notably, both FPF and eFPF were slightly higher at 60 L/min, suggesting more efficient deep lung delivery under lower-flow conditions, confirming that the optimized powder formulation retains robust and clinically relevant aerodynamic performance across physiologically realistic inhalation flow rates.

Overall, these pulmonary deposition results are especially noteworthy given the fixed L/C ratio in the powders and the SD parameters optimized to preserve liposome integrity, especially since higher lipid content has been shown to be associated with a decrease in FPF (Almurshedi et al., 2021). Notably, previous studies on powders composed solely of HPβCD, without liposomes, reported similar FPF values when ultra-fine particles were generated under optimized drying conditions (Bya et al., 2025; Gresse et al., 2024; Santos Gomes et al., 2025).

Finally, the morphology of powders obtained under optimal conditions was examined. Despite the presence of a non-negligible fraction of liposomes and drying parameters optimized for liposomal drying, HPβCD-based powders maintained their characteristic deflated structure (Figure 12), which has been previously associated with improved aerodynamic properties and enhanced pulmonary deposition (Dufour et al., 2015; Gresse et al., 2024; Lechanteur et al., 2023).

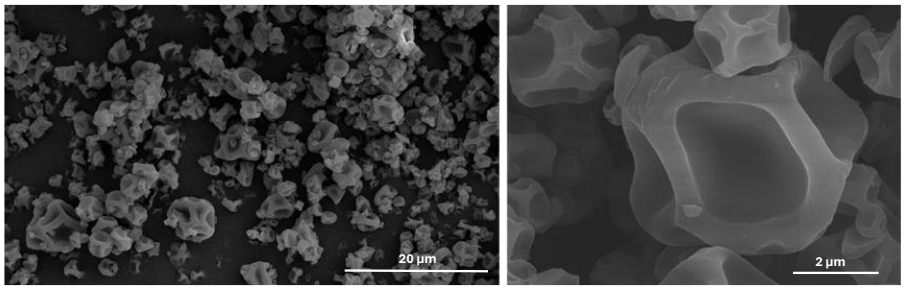


Figure 12: Scanning electron micrographs of spray-dried powders produced under the combined optimal conditions.

4. Conclusion

This study demonstrates that SD of liposomes, when carefully optimized, can produce inhalable powders that maintain liposome structure while exhibiting favorable aerodynamic performance. By combining two scalable, one-step processes, PGSS liposome formation and

subsequent SD, robust formulations were achieved even at a high solid content of 5%, highlighting the feasibility of industrial-scale production. Importantly, the use of a systematic DOE approach allowed this work to move beyond formulation-specific outcomes and establish clear relationships between process parameters, formulation attributes, and critical quality attributes relevant to inhalable liposomal products. This integrated, scale-up-oriented strategy provides practical insight into how liposome integrity and DPI performance can be jointly engineered under industrially relevant drying conditions.

Notably, coupled DOE results demonstrated that HPβCD plays a critical role in protecting liposomes during atomization, while drug retention was primarily influenced by PEGylated lipid content, lipid-to-carbohydrate ratio and API hydrophobicity, with highly hydrophilic compounds exhibiting significant release. Under the optimized conditions, carrier-free powders with a suitable aerodynamic size (~3 µm), low residual moisture (<5%), and high drying yield (>75%) were obtained. Liposomes retained their structural integrity throughout processing, as confirmed by minimal size variation (ratio 0.9–1.0), mean diameters below 200 nm, narrow size distribution (PdI ~0.2), near-neutral zeta potential, and preservation of their morphology as observed by microscopy. These attributes collectively support efficient pulmonary delivery.

Optimized formulations co-encapsulating clinically relevant drug combinations (formoterol/budesonide and ciclesonide/indacaterol maleate) further exhibited uniform drug distribution (CV <5%), characteristic deflated morphology, and efficient aerosolization (eFPF >20% and FPF >60%), underscoring their potential for inhalation therapy. Moreover, the selection of formulation components such as HPβCD, cholesterol, and PEGylated lipids, supported by encouraging preclinical and early clinical experience, reinforces the translational relevance of the proposed approach, while acknowledging that regulatory acceptance for pulmonary delivery must ultimately be addressed in the context of the final product.

Taken together, these findings establish a clear framework for designing liposomal dry powders for deep-lung delivery, balancing formulation composition, excipient choice, and process parameters. Ultimately, this work paves the way for the industrial-scale translation of inhalable liposomal therapeutics, demonstrating that a strategic combination of scalable manufacturing methods can reconcile formulation performance with manufacturability.

Future investigations, including pharmacokinetic and toxicological evaluation of the final dry powder formulation in an asthmatic rat model using the PreciseInhale® system, will be critical to further substantiate the translational potential of combining liposomal encapsulation with carrier-free powder technologies for enhanced pulmonary drug delivery.

CRediT authorship contribution statetement

Laure-Anne Bya: Writing – original draft, Writing – review & editing, Investigation, Data curation, Conceptualization. **T. Nghia Dinh**: Data curation, Review. **Noémie Penoy**: Conceptualization, Supervision, review. **Pierre-Yves Sacré**: Conceptualization, Investigation, Review. **Didier Cataldo:** Supervision. **Erika Hendrickx:** Data curation. **Louise Conrard:** Data curation. **Brigitte Evrard**: Supervision. **Anna Lechanteur**: Supervision, Writing – review & editing.

Declaration of 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.

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SUPPLEMENTARY DATA

Supplementary Data A: Summary of DOE_A tested parameters and corresponding responses.

Tested SD parameters and carbohydrate matrix	Responses

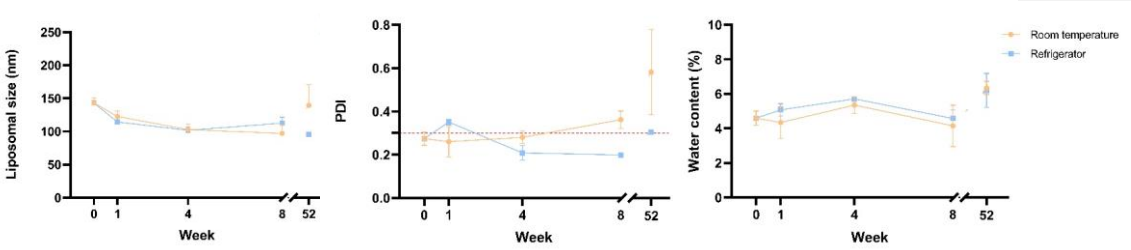
Random Block	Excipient	Inlet temperature (°C)	Flowrate (rpm)	Nozzle Gas Pressure (bar)	Cyclone gas pressure (bar)	Particle size (µm)	SD yield (%)	Water content (%)	Sf/Si size ratio	PdI upon SD
1	trehalose	120	25	1	0.05	3.099	72.915	2.229	0.946	0.393
1	HPβCD	120	25	1	0.3	3.962	75.707	2.293	0.923	0.291
2	trehalose	90	88	2.5	0.18	2.127	72.873	4.806	1.045	0.631
2	trehalose	60	150	1	0.05	4.26	63.769	5.422	0.995	0.465
3	trehalose	120	25	4	0.3	1.389	63.798	3.435	1.207	0.532
3	HPβCD	60	25	4	0.3	1.644	75.807	5.308	0.938	0.373
4	HPβCD	120	150	1	0.05	4.699	75.808	4.339	0.87	0.402
4	trehalose	90	88	2.5	0.18	2.341	72.912	3.874	0.998	0.471
5	trehalose	90	150	1	0.3	5.07	72.922	5.594	0.817	0.409
5	HPβCD	60	25	1	0.18	3.378	85.199	6.264	0.691	0.39
6	trehalose	120	150	2.5	0.3	2.63	81.996	3.654	0.891	0.472
6	HPβCD	120	150	4	0.3	2.055	66.273	5.719	0.75	0.261
7	HPβCD	60	88	1	0.05	3.134	75.657	8.813	0.759	0.332
7	trehalose	120	150	4	0.05	1.847	72.972	5.447	0.966	0.395
8	trehalose	60	150	4	0.3	/	9.119	9.111	0.958	0.358
8	HPβCD	90	88	2.5	0.18	2.235	75.657	6.759	0.904	0.327
9	trehalose	60	25	4	0.05	1.68	72.94	5.323	0.85	0.428
9	HPβCD	60	150	4	0.05	1.596	75.731	6.733	0.921	0.428
10	trehalose	120	88	1	0.3	4.721	72.814	4.151	0.968	0.441
10	HPβCD	90	25	1	0.05	3.787	75.741	5.072	1.111	0.454
11	HPβCD	120	25	4	0.05	1.715	66.264	5.04	1.104	0.477
11	trehalose	60	25	1	0.3	3.456	59.226	4.8	0.996	0.464
12	trehalose	120	150	1	0.18	5.416	72.982	4.066	0.889	0.474
12	HPβCD	60	150	1	0.3	5.301	56.786	5.484	0.918	0.401

Supplementary Data B: Summary of drying parameters showing significant effects on response variables (DPI properties and liposomes integrity) identified through statistical analysis in $DOE_{A.}$

	Response variable	***Significant drying parameters***
DPI properties	**Particle size**	• Cyclone gas pressure ($p < 0.0001$) • Excipient*Cyclone gas pressure ($p < 0.05$) • Excipient*Flowrate ($p < 0.0001$) • Excipient*Inlet temperature ($p < 0.0001$) • Flowrate ($p < 0.0001$) • Flowrate*Nozzle gas pressure ($p < 0.0001$) • Inlet temperature ($p < 0.0001$) • Inlet temperature*Cyclone gas pressure ($p < 0.05$) • Nozzle gas pressure ($p < 0.0001$)
	Drying Yield	• Excipient ($p < 0.05$) • Cyclone gas pressure ($p < 0.05$) • Excipient*Flowrate ($p < 0.05$)

		• Inlet temperature*Nozzle Gas pressure ($p < 0.05$) • Nozzle gas pressure*Nozzle gas pressure ($p < 0.05$)
	Moisture content	• Inlet temperature ($p < 0.05$)
Liposomes integrity	**Size ratio (Sf/Si)**	• Inlet temperature*Flowrate ($p < 0.05$)
	PdI	/

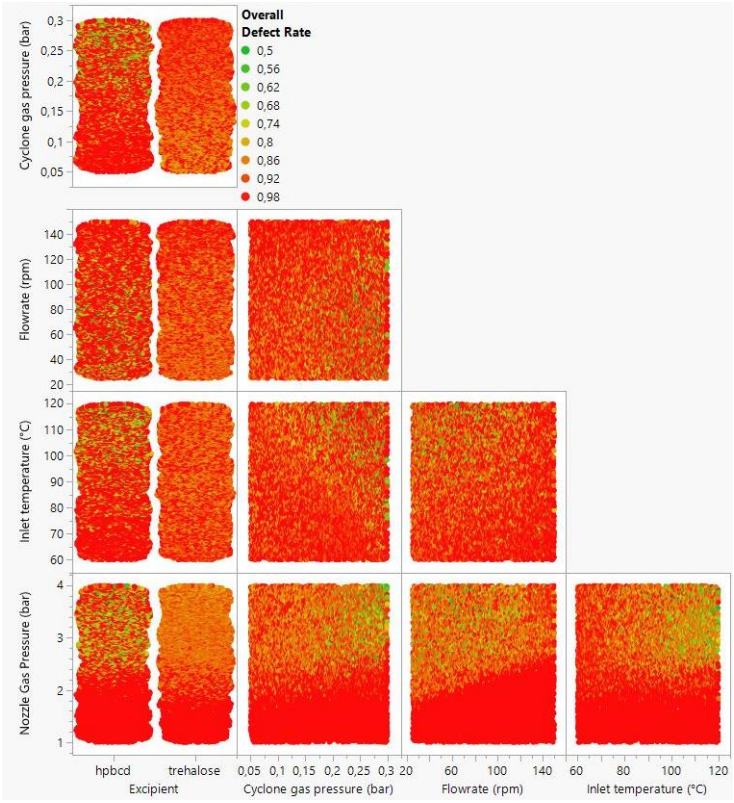
Supplementary Data C: Long-term stability of DOE_A processed empty liposomal powders



Long-term stability was evaluated for empty liposomal powders produced by DOE_A under optimal processing conditions using HPβCD, as trehalose-based formulations were not retained after drying. Liposomal powders were stored at 5 °C and at room temperature, and physicochemical properties were monitored over a 12-month period.

Throughout the storage period, liposomal integrity was largely preserved under both conditions, as evidenced by stable mean particle size values. A slight reduction in mean liposomal diameter (from approximately 140 to 120 nm) was observed over time but remained within the expected variability of DLS measurements and did not indicate structural destabilization. PdI values remained stable during refrigerated storage, whereas a gradual increase in PdI was detected at room temperature, suggesting the onset of increased size heterogeneity. In parallel, powder water content remained stable at approximately 5% throughout the study, which is within acceptable limits for inhalable dried formulations.

While this short-term stability assessment is encouraging, further studies conducted under ICH-recommended conditions and using final packaging configurations will be required to confirm long-term stability and ensure reproducibility in clinically relevant delivery systems.**Supplementary Data D**: DOEA's design space. The design space analysis revealed that only a narrow range of spray-drying parameters and carbohydrate matrix compositions achieved the desired conditions (DPI parameters suitable for inhalation, while preserving liposome integrity), as shown by the green dots, highlighting the complexity and critical importance of this experimental design.



Supplementary Data E: Summary of DOE_B tested parameters and corresponding responses.

	Tested parameters				Responses			
Whole Plots	API nature	DSPE-PEG2000 content (%)	L/C ratio (%)	Water content (%)	Particle size (µm)	Release upon SD (%)	Sf/Si ratio	PdI
1	SAL	10	5.5	3.79	2.66	85.31	0.94	0.219
1	SAL	10	1	1.82	2.59	100	0.94	0.251
1	SAL	10	10	3	2.48	70.27	0.91	0.215
2	SAL	6.25	1	4.06	2.72	100	0.91	0.257
2	SAL	6.25	5.5	3.32	2.57	81.63	0.88	0.216
2	SAL	6.25	10	1.96	2.58	67.7	0.87	0.214
3	SAL	2.5	10	3.4	2.69	71.36	0.89	0.216
3	SAL	2.5	5.5	2.7	2.7	95.01	0.98	0.231
3	SAL	2.5	1	1.74	2.48	100	1.04	0.269
4	BUD	10	5.5	6.22	2.44	39.6	0.93	0.205
4	BUD	10	10	4.16	2.52	37.5	0.94	0.186
4	BUD	10	1	4.29	2.71	46.29	0.93	0.215
5	BUD	6.25	10	5.43	2.537	22.59	0.91	0.192

5	BUD	6.25	5.5	5.2	3.567	35.08	0.96	0.227
6	BUD	2.5	1	3.49	4.489	31.42	0.97	0.238
6	BUD	2.5	10	3	2.74	20.18	0.9	0.235
7	BUD	6.25	1	4.68	2.549	36.58	0.94	0.208
7	BUD	6.25	5.5	2.18	2.463	27.37	0.93	0.213

Supplementary Data F: Summary of tested parameters (API nature, L/C ratio and PEGylated lipid content) showing significant effects on response variables (liposomes integrity) identified through statistical analysis in DOE_B.

	Response variable	***Significant drying parameters***
Liposomes integrity	**API release**	• API nature ($p < 0.05$) • L/C ratio ($p < 0.0001$)
	Size ratio (Sf/Si)	• API nature ($p < 0.05$) • L/C ratio ($p < 0.05$)
	PdI	• Lipid content ($p < 0.05$)

Supplementary Data G: Short-term stability comparison of liquid and spray-dried liposomal formulations

An initial stability study was conducted comparing the stability of the liposome suspension obtained after the PGSS process with that of the dried liposomes under BUD-optimized condition. Both the powders and the liposome suspensions were stored at +4 °C. This study was inspired by and supplemented with previously published results from Penoy *et al*. on the same liposome composition and production (Penoy et al., 2022). When examining the results, Figure below shows that, for non-dried liposomes, after three weeks, the release reached 93 ± 0.20 for SAL and 19.74 ± 1.77% for BUD. In contrast, in the spray-dried powders, encapsulation remained stable between time zero and three weeks. In addition to its drying protective effect, HPβCD also serves as an effective stabilizing agent after drying. Unlike other carbohydrates matrix such as trehalose or sucrose, HPβCD possesses a high Tg above 200 °C and exhibits low hygroscopicity, and exhibits low hygroscopicity, which helps preserve the amorphous glassy state during storage. This unique combination of properties ensures superior long-term stability of dried liposomal formulations (Chang et al., 2020; Haeuser et al., 2019; Hammoud et al., 2019; Milani et al., 2020).

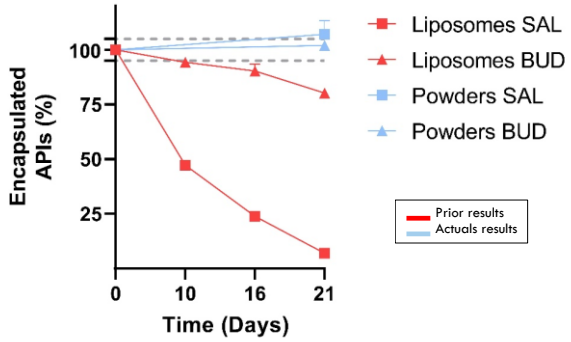


Figure: Stability of liposomal formulations over time, showing the percentage of encapsulated API for PGSS-processed suspensions versus spray-dried powders under BUD-optimized conditions. Values are normalized to 100% at time zero. Analyses were performed in triplicates (n = 3) and results are presented as mean ± standard deviation.

Supplementary Data H: Stage-by-stage NGI deposition profiles of the different APIs at 100 L/min.