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DEVELOPMENT AND OPTIMIZATION OF POWDERS CONTAINING LIPOSOMES FOR PULMONARY ADMINISTRATION

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SUMMARY

English

Pulmonary drug delivery is a promising strategy for treating chronic respiratory diseases, as it enables high local drug concentrations while minimizing systemic side effects. However, its effectiveness is often limited by physiological barriers that hinder drug deposition and therapeutic efficiency. To address this, liposomes have emerged as attractive carriers, enabling the encapsulation of active pharmaceutical ingredients, their protection from degradation and improved bioavailability, although their limited aqueous stability remains a major challenge.

In this context, formulating liposomes as dry powders for inhalation represents a relevant approach to enable efficient pulmonary delivery and improve stability. Spray-drying, a widely used technique to produce respirable particles, allows control over powder properties but may also compromise liposome integrity and induce premature drug leakage. This thesis therefore aimed to evaluate the feasibility of developing optimized inhalable liposome-based dry powder formulations.

A Quality by Design approach supported by Design of Experiments was implemented to assess the feasibility of spray-drying liposomes and to identify critical formulation and process parameters. This work provided fundamental insights into the impact of drying conditions and liquid feed composition on liposome integrity and powder properties. Liposome stability during spray-drying was shown to depend on a complex interplay of factors, where active ingredient properties influence susceptibility to leakage, while lipid composition, excipient environment and process conditions govern membrane response to stress and powder properties. These findings highlight the need to consider liposomal systems as integrated formulations. Additionally, optimized spray-drying conditions were identified and validated, enabling the production of inhalable powders with preserved liposome integrity, limited drug leakage and suitable aerodynamic properties, resulting in high *in vitro* lung deposition (FPF ~70%).

Subsequently, optimized inhalable dry powder formulations were developed, incorporating an innovative combination of ciclesonide and indacaterol for asthma treatment, which is not currently available on the pharmaceutical market in this form. The spray-drying conditions identified were successfully applied to liposomes designed to co-encapsulate this combination for pulmonary delivery, yielding inhalable powders with preserved liposome integrity, enhanced lung deposition and satisfactory stability under refrigerated conditions. In parallel, the same drug combination was formulated into non-liposomal inhalable dry powders, including a clinically relevant optimized formulation and a reference formulation matched in composition and drug content to the optimized liposome-based DPI, enabling assessment of the added value of liposomal incorporation.

Overall, this work demonstrates the feasibility of developing liposome-based inhalable dry powders, highlights the challenges associated with their formulation and provides a better understanding of the parameters governing their performance. These findings establish a solid foundation for the design of next-generation inhalable formulations and open new perspectives for the development of more efficient and targeted therapeutic strategies for the treatment of respiratory diseases.

French

L'administration pulmonaire constitue une stratégie prometteuse pour traiter les maladies respiratoires chroniques, car elle permet d'atteindre des concentrations locales élevées de principes actifs tout en limitant les effets systémiques. Cependant, son efficacité reste limitée par des barrières physiologiques qui entravent le dépôt et réduisent l'efficacité thérapeutique. Pour y remédier, les liposomes apparaissent comme des vecteurs attractifs, capables d'encapsuler des principes actifs, de les protéger de la dégradation et d'en améliorer la biodisponibilité, bien que leur stabilité en milieu aqueux reste limitée.

Dans ce contexte, leur formulation sous forme de poudres sèches inhalables constitue une approche pertinente pour améliorer leur stabilité et permettre une administration pulmonaire efficace. Le séchage par atomisation, largement utilisé pour produire des particules respirables, permet de contrôler les propriétés des poudres mais peut altérer l'intégrité des liposomes et induire une libération prématurée de leur contenu. Cette thèse vise donc à évaluer la faisabilité du développement de poudres sèches inhalables optimisées à base de liposomes.

Une approche « Quality by Design » couplée à des plans d'expériences a permis d'évaluer le séchage par atomisation des liposomes et d'identifier les paramètres critiques. Ce travail a permis de mieux comprendre l'impact des conditions de séchage et de la composition de la solution d'alimentation sur l'intégrité des liposomes et les propriétés des poudres. Les résultats montrent que la stabilité des liposomes dépend d'une interaction complexe entre les propriétés des molécules actives, la composition lipidique, les excipients et les conditions de séchage, influençant l'intégrité de la bicouche lipidique et le relargage des substances actives au cours du procédé. Ils soulignent que les systèmes liposomaux ne peuvent être considérés comme de simples vecteurs, leurs performances dépendant de l'ensemble de la formulation et du procédé. Des conditions optimisées ont ainsi été identifiées, permettant d'obtenir des poudres inhalables contenant des liposomes intègres avec un dépôt pulmonaire élevé (FPF ~70%), mesuré *in vitro*.

Ensuite, des formulations inhalables optimisées ont été développées à partir d'une combinaison innovante de ciclesonide et d'indacatérol, non disponible sur le marché pharmaceutique sous forme de poudre sèche pour inhalation. Les conditions de séchage identifiées ont été appliquées à des liposomes co-encapsulant ces molécules et optimisés pour l'administration pulmonaire, conduisant à des poudres adaptées au traitement de l'asthme avec un dépôt pulmonaire amélioré. En parallèle, cette combinaison non encapsulée dans des liposomes a également été formulée en poudre sèche, incluant une formulation optimisée cliniquement pertinente et une formulation de référence ajustée à la formulation liposomale, afin d'évaluer l'impact des liposomes.

Dans l'ensemble, ces travaux démontrent la faisabilité du développement de poudres sèches inhalables à base de liposomes, mettent en évidence les défis associés à leur formulation et apportent une meilleure compréhension des paramètres qui régissent leur performance. Ces résultats constituent une base solide pour la conception de formulations inhalables de nouvelle génération et ouvrent des perspectives pour le développement de stratégies thérapeutiques plus efficaces et ciblées dans le traitement des maladies respiratoires.

ABBREVIATIONS

ACI, Andersen Cascade Impactor
ASD, Amorphous Solid Dispersion
API, Active Pharmaceutical Ingredient
BUD, Budesonide
CGP, Cyclone Gas Pressure
CHOL, Cholesterol
CI, Cascade Impactor
CIC, Ciclesonide
CO₂, Carbon Dioxide
COPD, Chronic Obstructive Pulmonary Disease
CV, Coefficient of Variation
d_{ae}, Aerodynamic Diameter
DepthD, Depth of Dimples
Des-CIC, Desisobutyl-ciclesonide
DLPC, Dilinoleoyl-sn-glycero-3-phosphocholine
DLS, Dynamic Light Scattering
DMPA, Dimyristoyl-sn-glycero-3-phosphate
DoE, Design of Experiment
DOTAP, Dioleoyl-3-trimethylammonium-propane
DPPC, Dipalmitoylphosphatidylcholine
DPI, Dry Powder Inhaler
DSC, Differential Scanning Calorimetry
DSPE-PEG₂₀₀₀, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-₂₀₀₀]
DSPC, Distearoylphosphatidylcholine
ED, Emitted Dose
eFPF, Extra-Fine Particle Fraction
EMA, European Medicines Agency
FDA, U.S. Food and Drug Administration
FD, Freeze-Drying
FDKP, Fumaryl Diketopiperazine
FIB, Focused Ion Beam
FOR, Formoterol
FPD, Fine Particle Dose
FPF, Fine Particle Fraction
GINA, Global Initiative for Asthma
GOLD, Global Initiative for Chronic Obstructive Lung Disease
GMP, Good Manufacturing Practices

GRAS, Generally Recognized as Safe
GSD, Geometric Standard Deviation
GUV, Giant Unilamellar Vesicle
HFA, Hydrofluoroalkane
HP β CD, Hydroxypropyl- β -cyclodextrin
HP γ CD, Hydroxypropyl- γ -cyclodextrin
HPLC, High Performance Liquid Chromatography
HR, High resistance
iBCS, inhalation-based Biopharmaceutics Classification System
ICH, International Council for Harmonisation
ICS, Inhaled Corticosteroid
IND, Indacaterol Maleate
LABA, Long-Acting β_2 -Agonists
LAMA, Long-Acting Muscarinic Antagonists
L/C ratio, Lipid-to-Carbohydrate Ratio
LNP, Lipid Nanoparticle
LOD, Limit of Detection
LOQ, Limit of Quantification
LR, Low resistance
LUV, Large Unilamellar Vesicle
MeOH, Methanol
MLV, Multilamellar Vesicle
MMAD, Mass Median Aerodynamic Diameter
MOC, Micro-Orifice Collector
mRNA, Messenger RNA
MSLI, Multistage Liquid Impinger
MVV, Multivesicular vesicle
Nd, Number of Dimples
NGI, Next Generation Impactor
NGP, Nozzle Gas Pressure
NP, Nanoparticle
NTA, Nanoparticle Tracking Analysis
O₂, Oxygen
OLV, Oligolamellar Vesicle
P_c, Critical Pressure
Pdl, Polydispersity Index
Pe, Péclet Number
PEG, Polyethylene Glycol
Ph.Eur., European Pharmacopoeia
PIF, Peak Inspiratory Flow

PK, Pharmacokinetic
PLA, Polylactic Acid
PLGA, Poly(lactic-co-glycolic acid)
pMDI, Pressurized Metered-Dose Inhaler
PGSS, Particles from Gas Saturated Solutions
PSD, Particle Size Distribution
QbD, Quality by Design
RD, Recovery Dose
RH, Relative Humidity
RRT, Relative Retention Time
RSD, Relative Standard Deviation
R-HSI, Raman Hyperspectral Imaging
SABA, Short-Acting β_2 -Agonists
SAL, Salbutamol
SAMA, Short-Acting Muscarinic Antagonists
SC, Solid Content
SCFs, Supercritical Fluids
scCO₂, Supercritical Carbon Dioxide
SD, Spray-Drying
SEM, Scanning Electron Microscopy
SFD, Spray-Freeze Drying
SLN, Solid Nanoparticle
SMI, Soft-Mist Inhaler
SPC, Soy Phosphatidylcholine
SUV, Small Unilamellar Vesicle
T_c, Critical Temperature
TFH, Thin Film Hydration
T_g, Glass Transition Temperature
TGA, Thermogravimetric Analysis
T_{in}, Inlet Temperature
T_m, Phase Transition Temperature
T_{out}, Outlet Temperature
ULV, Unilamellar Vesicle
XRPD, X-Ray Powder Diffraction
ZP, Zeta Potential

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INTRODUCTION

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I. Pulmonary drug delivery

Pulmonary drug administration ranks among the oldest therapeutic approaches. Evidence from ancient Egypt includes a medical papyrus describing the inhalation of vapors from heated plant materials to alleviate respiratory distress [1]. Similar practices appeared across multiple civilizations and persisted in the Middle Ages, with physicians such as Hippocrates documenting the therapeutic use of inhaled substances including atropine, opium, arsenic or ephedrine [2].

Progress in pulmonary drug administration remained limited until the early modern period (19th century), when inhalation therapy began to adopt more structured delivery strategies (Figure 1). Notable advances include an early dry powder inhaler (DPI) developed by Ira Warren in 1852 and a portable atomizer introduced by Jean Sales-Girons in 1858, both regarded as key precursors of modern inhalation devices [2,3]. At the same time, inhalation salons and medicinal smoke cigarettes were widely used for asthma treatment, collectively laying the groundwork for contemporary inhalation therapy [1].

However, major technological advances did not occur until 1956, when the first pressurized metered-dose inhaler (pMDI) emerged from an unexpectedly simple question asked by a young asthma patient wondering why her medication could not be delivered like a hairspray [2]. This question led to the adaptation of aerosol valve technology for pharmaceutical applications, which ultimately led to the development and approval of the Medihaler®, the first portable, standardized device capable of delivering precise and reproducible doses directly to the lungs. This marked the beginning of the modern era of inhalation therapy [4]. The major milestones in the development of inhalation devices and formulation technologies are summarized in Figure 1.

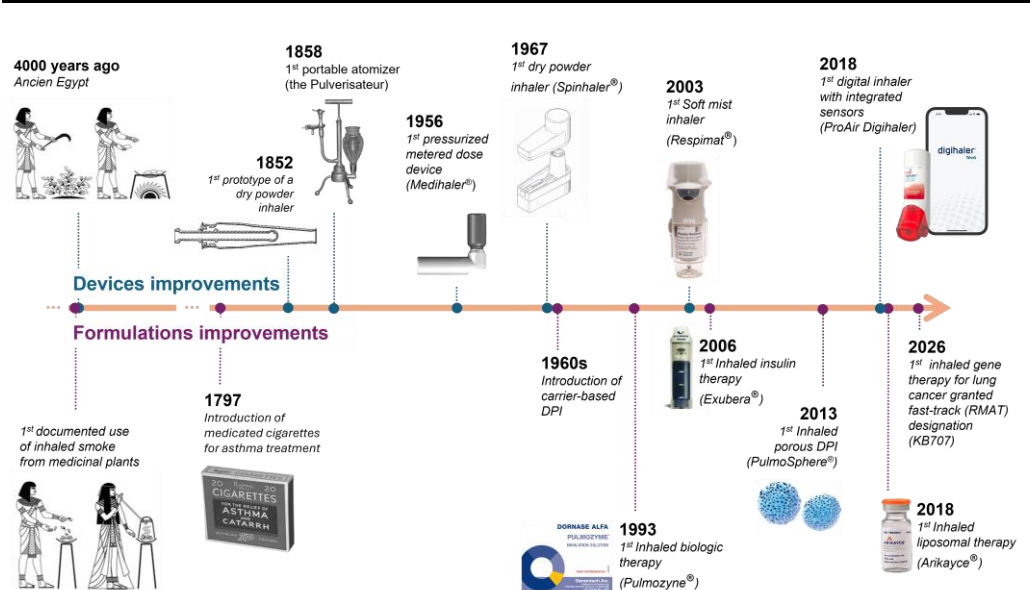


Figure 1: Overview of the historical development of inhalation devices and formulation technologies for pulmonary drug delivery. The items shown are intended solely as representative examples and should not be regarded as exhaustive.

Over the subsequent decades, technological advances have led to the development of new inhalation devices, such as hydrofluoroalkane (HFA)-based pMDIs and soft-mist inhalers (SMIs), alongside continuous improvements in existing systems, including DPIs and nebulizers, ultimately culminating in modern digitally assisted devices [5]. In parallel, significant advancements in drug formulations have transformed pulmonary therapy. The late 1960s saw the emergence of carrier-based DPI systems and a major milestone was achieved in the 1990s with the first inhaled biologic therapy, namely dornase alfa (Pulmozyme®) [2,6]. The 2000s saw considerable advances in complex inhalation formulations, including inhaled insulin (Exubera® and later Afrezza®), engineered porous particles (PulmoSphere™) and nanomedicine approaches such as liposomal formulations (Arikayce®), with gene therapy emerging more recently [7–9].

This substantial expansion over recent decades has led to several hundred medicinal products now approved for pulmonary administration worldwide, reflecting both ongoing scientific innovation and increasing clinical interest [10]. Yet, the urgent need for more effective treatments is highlighted by the persistent global burden of pulmonary diseases, which remain a major cause of mortality and affect hundreds of millions of

people [11]. This burden is expected to grow substantially, with tracheal, bronchial and lung cancer incidence and mortality expected to rise by ~90% by 2050 [12].

Meeting this pressing need, however, faces significant formulation and patient-related challenges, as well as regulatory hurdles [13,14]. Together, these challenges underscore that, despite the maturation of pulmonary drug delivery, ongoing innovation remains crucial to enhance treatment effectiveness and address the global impact of pulmonary diseases.

II. Human respiratory tract: from structure to function

II.1. The human respiratory tract: anatomy, histology and physiology

The lungs are essential organs of the respiratory system responsible for enabling gas exchange between the external environment and the bloodstream. Through this process, oxygen (O_2) is delivered to the body while carbon dioxide (CO_2) is removed, thereby ensuring the proper functioning of cellular metabolism.

The human pulmonary airways are functionally divided into two distinct regions (Figure 2). The upper respiratory tract includes the conducting airways responsible for directing air to the lungs, namely the nasal and oral cavity, pharynx and larynx, whereas the lower respiratory tract contains both the conducting airways and the respiratory region and includes the trachea, bronchi, bronchioles and alveoli. Within this system, the trachea, bronchi and most bronchioles constitute the conducting airways that transport air, while the respiratory region begins at the respiratory bronchioles and extends to the alveoli, where gas exchange with the blood takes place [10]. The cellular composition of the conducting airways varies along the bronchial tree but generally includes ciliated epithelial cells, mucus-secreting goblet cells and basal cells that function as epithelial progenitors. Additional specialized cells, including club cells in the bronchioles, contribute to detoxification processes and epithelial maintenance [15].

Within the lower respiratory tract, the bronchial tree undergoes approximately 23 dichotomous divisions, representing the progressive branching of the bronchi into bronchioles, which culminates with the alveolar regions [16]. At the alveolar level, this architecture forms a vast gas-exchange surface ($\sim 100\text{ m}^2$), supported by a dense and highly permeable capillary network that facilitates efficient gas exchange [15,17].

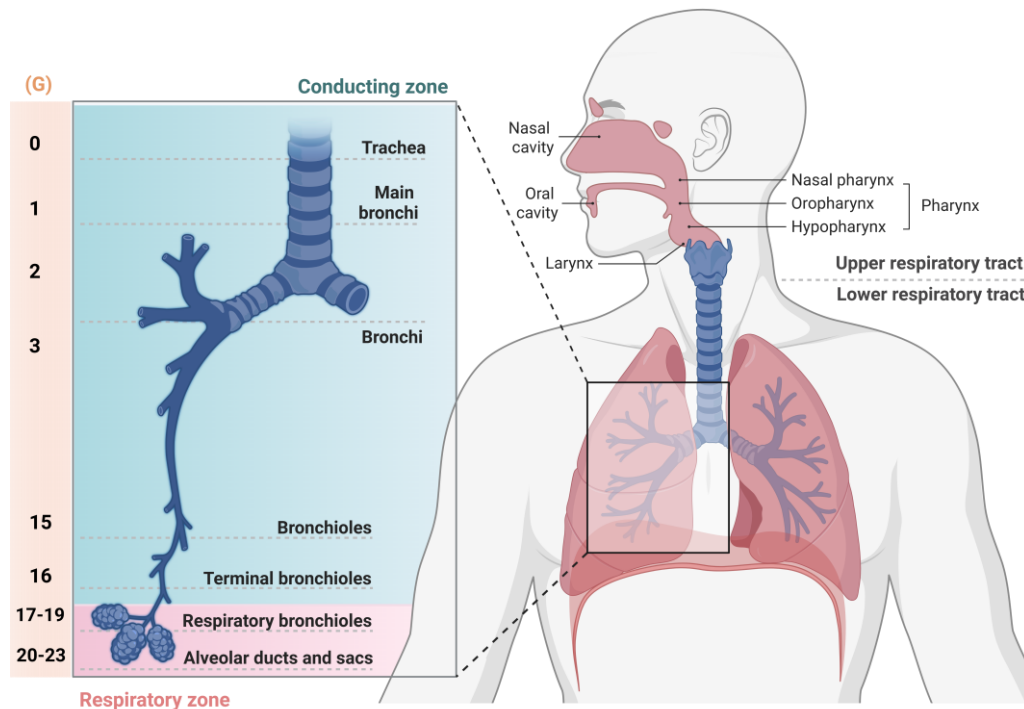


Figure 2: Schematic representation of the anatomy of the human respiratory system. Created with BioRender.

Gas exchange occurs across the alveolar-capillary barrier, which represents the cornerstone of pulmonary function (Figure 3). This barrier is primarily formed by type I pneumocytes, which cover more than 95% of the alveolar surface and facilitate efficient diffusion of O_2 and CO_2 through their close association with the capillary endothelium [18]. Type II pneumocytes complement this function by secreting pulmonary surfactant, a surface-active substance that reduces alveolar surface tension and prevents alveolar collapse during expiration [19,20]. In addition, type II pneumocytes act as progenitor cells, contributing to the regeneration of damaged type I pneumocytes and thus maintaining alveolar integrity [21].

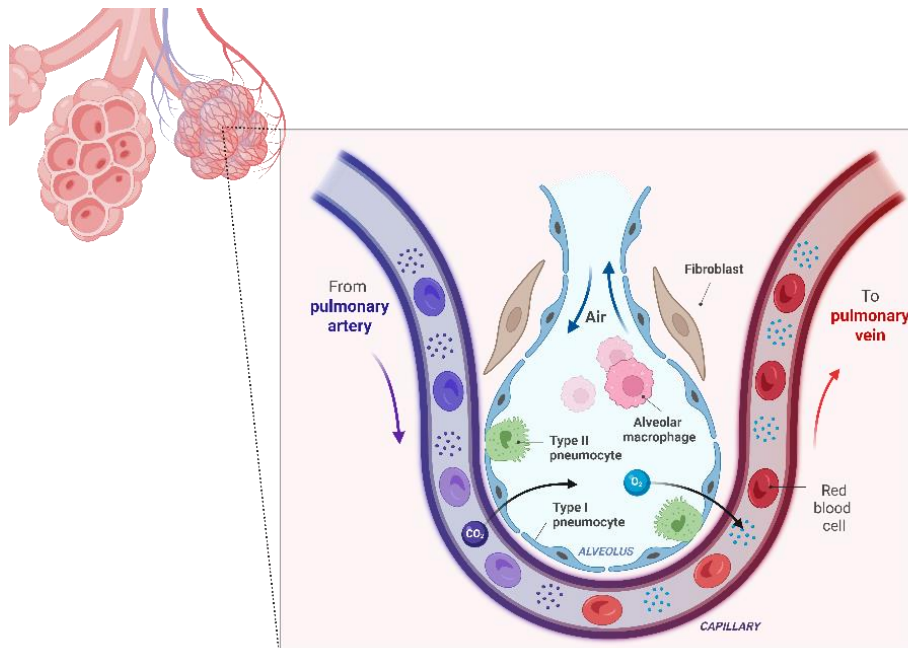


Figure 3: Schematic representation of the lung respiratory region. Created with BioRender.

Beyond its primary role in gas exchange, the lung contributes to homeostasis through multiple functions, including metabolism of various substances, regulation of pulmonary blood flow, maintenance of acid-base balance, thermoregulation and protection against inhaled particles and pathogens [22]. In particular, this protective function is supported by several physiological barriers that limit particle penetration and retention within the respiratory tract. Among these defenses, the mucociliary clearance system represents one of the most important protective mechanisms in both upper and lower airways [17,23]. This mechanism relies on the coordinated action of mucus-secreting goblet cells and ciliated epithelial cells, forming a dynamic mucociliary layer that traps inhaled particles and microorganisms and transports them toward the upper airways, where they are eliminated by swallowing or coughing.

In the distal regions of the lung, where mucociliary clearance is absent, alveolar macrophages represent the predominant immune cell population and provide an additional line of defense. These phagocytic cells eliminate debris and pathogens that reach the alveoli, although their clearance efficiency strongly depends on particle size and occurs at a slower rate than mucociliary transport [17].

Together, the highly specialized cellular composition, the extensive branching of the airways and the complementary defense mechanisms illustrate how the lung functions as a remarkably complex and multifunctional organ, capable of efficient gas exchange while maintaining protection against inhaled particles and pathogens.

However, despite the efficiency of these structural and physiological defense mechanisms, the lungs remain inherently vulnerable due to their open architecture and constant exposure to the external environment. Continuous contact with inhaled pathogens, environmental particles, allergens and pollutants challenges pulmonary homeostasis and can overwhelm or impair these protective systems.

As a result, this persistent exposure makes the lungs particularly susceptible to viral and bacterial infections, as well as to the development of various pulmonary disorders. Moreover, dysregulated or excessive activation of these defense mechanisms may itself contribute to disease pathogenesis, as observed in conditions such as lung cancer or more commonly, chronic inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) [24,25].

In this context, the lungs not only represent a major site of disease but also constitute an attractive target for therapeutic intervention. Consequently, the pulmonary route has attracted considerable interest for the development of effective therapeutic approaches.

II.2. Therapeutic potential of pulmonary administration

The fundamental objective of drug delivery is to achieve an effective therapeutic concentration at the appropriate site [26]. While multiple routes of administration are available, each presents inherent limitations. Oral delivery often suffers from low and variable bioavailability, intravenous administration is invasive and subject to rigorous sterility requirements and other routes are constrained by physiological barriers such as limited skin permeability, rapid ocular clearance or mucociliary and enzymatic defenses in the nasal cavity [17]. These limitations underscore the need for alternative strategies, among which pulmonary administration offers distinct advantages when clinically relevant.

Therefore, pulmonary administration has emerged as a versatile route of drug delivery for both local and systemic therapeutic applications. For systemic delivery, the unique

physiological features of the lung, including a large alveolar surface area, a dense capillary network and a thin alveolar-capillary barrier, provide an efficient interface for rapid drug absorption into the bloodstream [27]. These characteristics have stimulated research into inhalable formulations for systemically acting therapeutics, including proteins such as insulin, as well as vaccines [28,29]. Pulmonary administration also offers several advantages over conventional routes by bypassing first-pass hepatic metabolism and reducing enzymatic degradation, thereby improving bioavailability. In addition, it represents a non-invasive alternative to parenteral administration that may enhance patient compliance [30,31]. However, despite these advantages, only a limited number of inhaled products intended for systemic delivery have been approved to date. Consequently, pulmonary administration remains predominantly used for local therapeutic applications, which constitute the primary focus of this thesis.

In this setting, pulmonary delivery is particularly well suited for the treatment of respiratory diseases such as asthma and COPD, as well as other pulmonary conditions including cystic fibrosis, pulmonary infections and pulmonary arterial hypertension [17,32]. By enabling direct drug deposition at the site of action, this route allows rapid therapeutic onset and the achievement of high local drug concentrations while limiting systemic exposure when the formulation is appropriately optimized [30]. As a result, lower doses can be administered, reducing the risk of adverse effects. Furthermore, the ease of inhalation-based delivery supports patient autonomy and contributes to improved adherence to treatment [33].

Despite these notable advantages, pulmonary administration remains subject to several limitations (Figure 4), including some inherent to the physicochemical properties of the therapeutic substance, which are shared with other administration routes. Indeed, the performance of inhaled therapies is strongly dependent on the physicochemical properties of the active pharmaceutical ingredient (API) and its formulation. For locally acting therapies, inhaled APIs must first dissolve in the pulmonary lining fluid and subsequently diffuse across or interact with the airway epithelium to exert their pharmacological effect [34]. In this context, both drug dissolution and epithelial permeability play a critical role in therapeutic efficacy. Limited aqueous solubility or slow dissolution can restrict drug availability at the site of action, while epithelial permeability influences drug retention within the lungs. For local pulmonary delivery, insufficient

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dissolution reduces local availability, whereas excessive permeability promotes rapid systemic absorption, thereby decreasing pulmonary residence time [35,36]. Overall, these limitations can be rationalized using an inhalation-based Biopharmaceutics Classification System (iBCS), a proposed framework in which drug performance is governed by the interplay between solubility and epithelial permeability [37].

Beyond API-related constraints, pulmonary administration is also influenced by patient-specific pulmonary factors, including inspiratory capacity, inhalation technique and airway geometry [38]. From an anatomical perspective, the highly branched and progressively narrowing architecture of the respiratory tract makes the aerodynamic diameter (d_{ae}) of inhaled aerosols a critical determinant of their ability to reach and be retained in the distal regions of the lung.

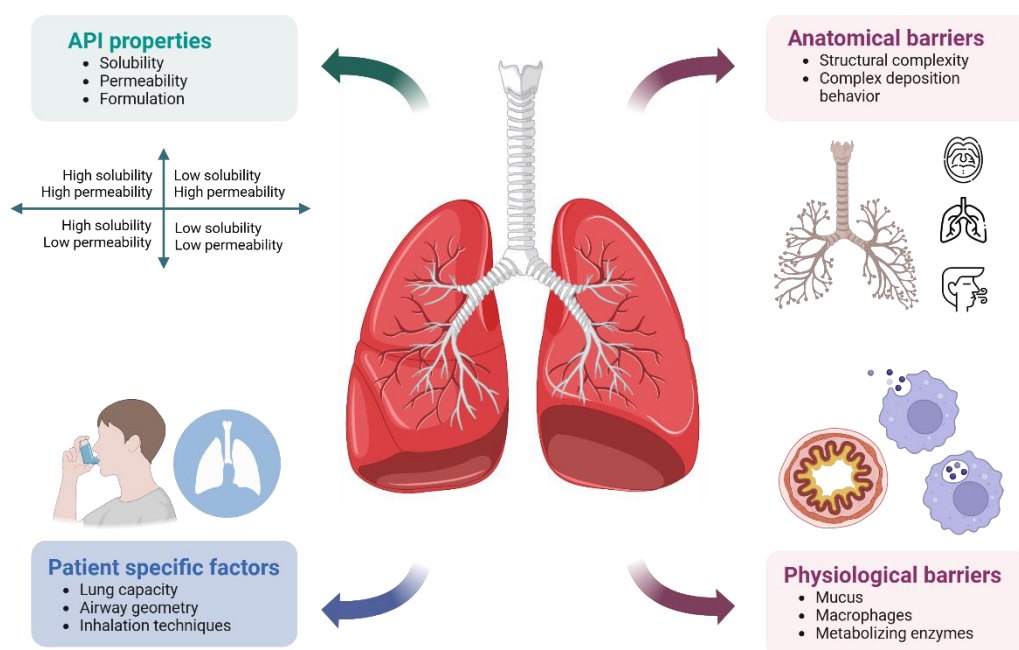


Figure 4: Overview of the main limitations of pulmonary administration. Created with BioRender.

Pulmonary deposition is governed by three primary mechanisms, each depending on the d_{ae} of the aerosol particles: impaction, sedimentation and Brownian diffusion (Figure 5). Impaction mainly affects particles larger than $5\ \mu\text{m}$, which tend to collide with airway

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walls during changes in airflow direction, leading to deposition primarily in the upper respiratory tract and potentially leading to off-target adverse effects. In contrast, Brownian diffusion dominates the behavior of very fine particles below $0.5\ \mu\text{m}$, which exhibit random motion and are frequently exhaled rather than retained. For effective therapeutic action in the deep lung, particles with a d_{ae} between 1 and $5\ \mu\text{m}$ are generally considered optimal, as gravitational forces promote sedimentation and enable deposition within the small airways, particularly the bronchioles, which are key targets in local pulmonary therapy [39–41].

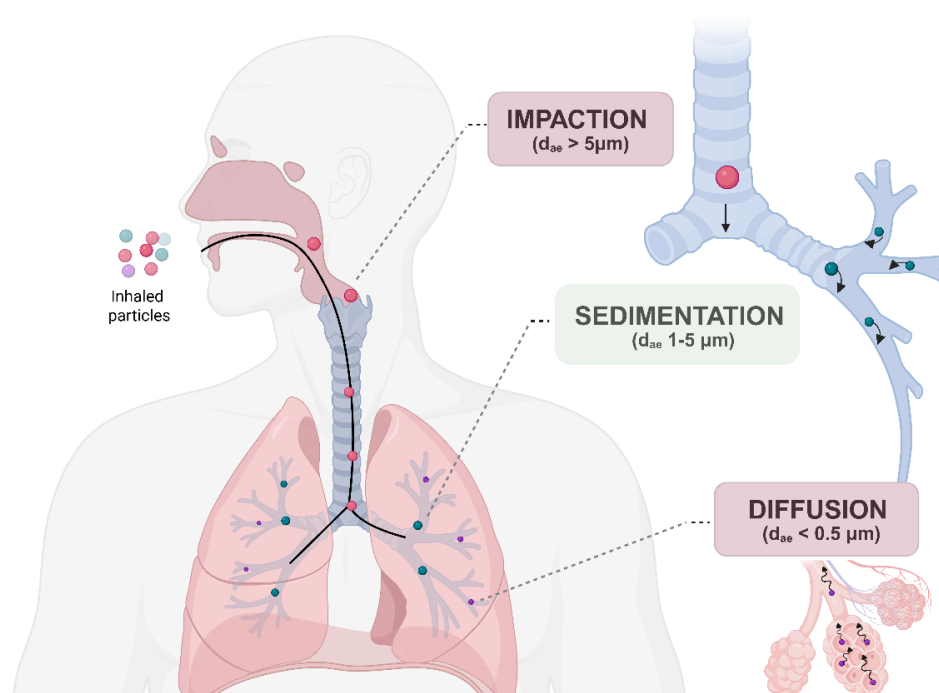


*Figure 5: Physical mechanisms governing inhaled particle deposition in the respiratory tract.
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Beyond mechanical constraints, inhaled drug carriers must overcome the chemical and physiological defense mechanisms of the lungs [42]. Following deposition on the respiratory epithelium, drugs interact with components of the pulmonary surfactant, including metabolizing enzymes, which may affect their stability and local availability. However, enzymatic activity in the lungs remains lower than in the gastrointestinal tract or liver [30]. In parallel, key pulmonary clearance mechanisms, notably the mucociliary escalator and alveolar macrophages, while being essential for pulmonary defense, can substantially reduce drug residence time and therapeutic efficacy by removing inhaled

particles [43]. Although a d_{ae} between 1 and 5 μm is critical for deep lung deposition, it is equally important for evading clearance by the mucociliary escalator, as studies indicate that approximately 90% of coarse particles above 6 μm are removed within 24 hours [17]. Nevertheless, even within the optimized d_{ae} range, particles remain vulnerable to phagocytosis by alveolar macrophages, which preferentially target particles between 0.5 and 5 μm [35].

Consequently, most inhaled therapies exhibit a short pulmonary half-life, necessitating multiple daily administrations, which can negatively affect patient compliance and overall treatment efficacy [17]. To primarily address these physiological barriers and improve pulmonary drug retention, nanocarrier-based drug delivery platforms represent a promising strategy [44].

III. Pulmonary delivery of liposomes

Among the various nanocarrier-based strategies developed to overcome pulmonary physiological barriers and improve drug retention, liposomes have emerged as particularly attractive candidates owing to their unique structural and physicochemical properties [44,45]. Nevertheless, numerous other nanoparticle (NP) platforms have been investigated for pulmonary delivery and are briefly outlined here to situate liposomes within the broader landscape of inhalable nanocarriers.

These nanocarriers are generally classified into inorganic and organic NPs. Inorganic NPs, such as gold, iron oxide or silica-based carriers, provide high physicochemical stability and tunable properties. However, their use in pulmonary delivery, as in other routes of administration, is limited by concerns related to toxicity, low biodegradability and potential accumulation in lung tissues [46–48].

By contrast, organic NPs are more widely investigated for inhalation due to their superior biocompatibility and formulation flexibility. In particular, polymeric systems enable efficient drug encapsulation and controlled release. For instance, chitosan-based NPs offer advantages such as biodegradability and mucoadhesion, although limitations such as cytotoxicity and variable clearance remain [44,47,49].

Lipid-based NPs represent another major class of organic carriers for pulmonary delivery, including micelles, solid lipid nanoparticles (SLNs), liposomes and lipid

nanoparticles (LNPs) [47,49]. Micelles enhance the solubilization of poorly water-soluble drugs, while SLNs provide drug protection and controlled release through a solid lipid matrix [49,50]. In parallel, LNPs, composed of ionizable lipids, are increasingly investigated for pulmonary applications, particularly for nucleic acid delivery [51,52]. Finally, liposomes are well-established and clinically validated drug delivery systems. These vesicular structures, composed of phospholipid bilayers surrounding an aqueous core, have been widely investigated for pulmonary applications [44,49,53].

Accordingly, liposomes constitute the main strategy investigated in this thesis. The following section provides an overview of their fundamental characteristics and principal production methods, which form the basis for understanding their design and broader pharmaceutical applications, while their relevance for pulmonary delivery will be discussed in Section III.2.

III.1. Liposomes as drug delivery system: an overview

Liposomes were first described in the mid-1960s by Bangham as self-assembled phospholipid vesicles, quickly attracting attention as multifunctional drug delivery systems [54]. Over the following decades and continuing to the present day, liposomal formulations have been extensively studied and successfully applied across several therapeutic areas, notably oncology, infectious diseases and vaccinology [55,56]. The clinical approval of multiple liposomal products for intravenous administration, including Doxil® (liposomal doxorubicin) and AmBisome® (liposomal amphotericin B), among others, has firmly established liposomes as a mature, reliable and clinically validated drug delivery platform [57].

Regarding pulmonary applications, a major milestone was reached with the approval of Arikayce® (amikacin liposome suspension), the first inhaled liposomal therapy, by the U.S. Food and Drug Administration (FDA) in 2018 and subsequently by the European Medicines Agency (EMA) in 2020. These approvals significantly strengthened interest in the development of liposomes for pulmonary drug delivery [58].

III.1.1. Definition and composition

Over time, liposomal systems have undergone significant evolution, progressing from conventional vesicles characterized by limited stability and rapid clearance by the

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mononuclear phagocyte system to stealth formulations with prolonged systemic circulation times. Further advancements have led to the development of actively targeted liposomes designed for enhanced cellular specificity, and more recently to multifunctional platforms capable of combining drug delivery with imaging or other auxiliary functions within a single entity [59].

While formulation strategies have significantly evolved, liposomal systems rely on the same fundamental structural principles. Indeed, liposomes are vesicular drug delivery systems predominantly composed of naturally derived or synthesized phospholipids that spontaneously self-assemble into well-defined bilayer structures in aqueous environments (Figure 6). This self-organization arises from the intrinsic amphiphilicity of phospholipids, whereby hydrophilic headgroups, composed of a phosphate group associated with a hydrophilic functional group, orient toward the aqueous medium, while hydrophobic fatty acid chains align inward to form a water-excluded lipid bilayer [60,61]. As a result, liposomes exhibit a distinctive architecture consisting of an internal aqueous compartment enclosed by one or several lipid bilayers, enabling the simultaneous encapsulation of hydrophilic APIs within the aqueous core and lipophilic compounds within the lipid bilayer [59].

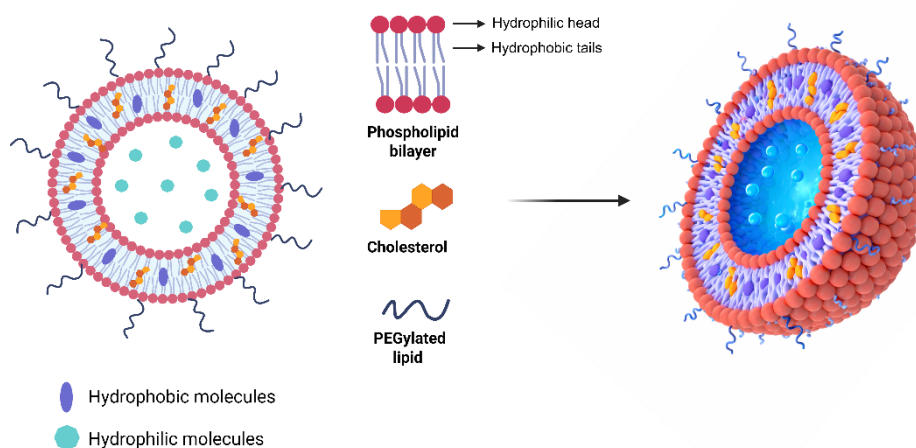


Figure 6: Schematic representation of the principal structural components of a liposome and the spatial distribution of encapsulated molecules. Created with BioRender.

Liposomes can be classified according to their structural characteristics, particularly their lamellarity, which refers to the number of lipid bilayers composing the vesicle.

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Based on this criterion, liposomes are categorized into unilamellar vesicles (ULVs), oligolamellar vesicles (OLVs), multilamellar vesicles (MLVs) and multivesicular vesicles (MVs), which contain multiple internal vesicles within a single outer bilayer (Figure 7) [55,60]. Liposomes are also classified by their hydrodynamic diameter: small unilamellar vesicles (SUVs) measure 20 to 100 nm, large unilamellar vesicles (LUVs) range from approximately 100 nm up to 1 μm and giant unilamellar vesicles (GUVs), which exceed 1 μm , are mainly used as model systems rather than for drug delivery [62,63].

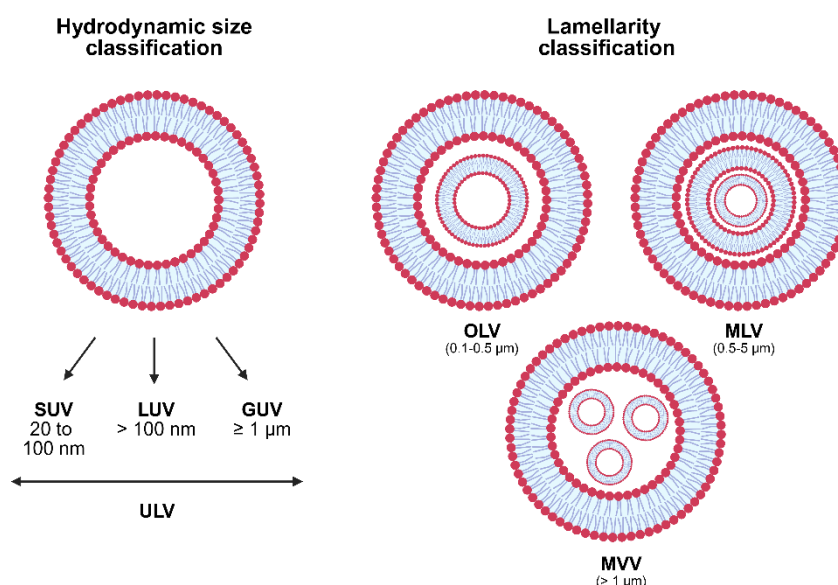


Figure 7: Classification of liposomes according to their size and lamellarity. Created with BioRender and adapted from [56].

These structural features are closely associated with key physicochemical parameters, including hydrodynamic diameter, polydispersity index (Pdl) and zeta potential (ZP), which critically influence liposome stability and performance. The Pdl reflects size distribution homogeneity, with values ≤ 0.3 generally indicating acceptable uniformity for drug delivery applications [56]. Additionally, ZP reflects the overall surface charge and governs colloidal stability: particles with high positive or negative ZP repel each other, preventing aggregation, whereas low ZP can promote flocculation. Surface charge depends on lipid headgroups, composition and environmental factors such as pH and ionic strength [64]. Several analytical techniques are available to characterize these properties, with dynamic light scattering (DLS) being the most widely used technique for determining liposome size and Pdl. Other complementary methods, such

as microscopy-based techniques and nanoparticle tracking analysis (NTA), may also be employed [65].

In addition to classification based on lamellarity and size, liposomes can also be categorized according to the charge of their constituent phospholipids. Phospholipids can be classified as zwitterionic (neutral), cationic or anionic based on their polar headgroups, which allows modulation of liposome surface charge, influencing both the stability and complexation of the encapsulated cargo, as well as interactions with biological environments. While neutral phospholipids are commonly employed in drug delivery, such as phosphatidylcholine derivatives including soy phosphatidylcholine (SPC), charged or ionizable lipids may be incorporated to confer specific functionalities and enhance therapeutic performance. In this context, cationic liposomes formulated with lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) are particularly effective for nucleic acid delivery through electrostatic interactions with negatively charged genetic material [66]. Conversely, anionic liposomes incorporating phospholipids such as 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA) are more frequently explored for transdermal drug delivery, where their negative surface charge has been associated with enhanced skin permeation [67].

Beyond structural and colloidal characteristics, the intrinsic physicochemical properties of phospholipids, particularly the phase transition temperature (T_m), play a crucial role in determining bilayer behavior. The T_m depends on the length and degree of saturation of fatty acid chains, as longer chains promote tighter lipid packing and increase T_m , whereas a higher degree of unsaturation introduces bends that prevent tight lipid packing, thereby lowering T_m and increasing membrane fluidity [68]. When the temperature exceeds the T_m , the bilayer transitions from an ordered gel phase to a more disordered liquid-crystalline phase, enhancing molecular mobility [59,69]. This behavior is particularly relevant during liposome production and post-production, where thermal and mechanical stresses can impact bilayer integrity, vesicle size and drug retention [70].

Building upon these intrinsic properties, liposomal formulations are often further optimized through the incorporation of additional components. Among these, cholesterol (CHOL) is commonly incorporated, where it modulates bilayer fluidity, reduces permeability and improves mechanical robustness. Adjusting its content allows

fine control over these properties, thereby enhancing liposomal integrity under physiological conditions and promoting prolonged drug retention [71,72]. Additionally, the modification of the liposomal surface through incorporation of hydrophilic polymer-conjugated lipids, most commonly polyethylene glycol (PEG) in a process known as PEGylation, is frequently employed in liposome development. This strategy not only reduces aggregation and helps maintain liposome size during production and storage, but also minimizes nonspecific interactions with biological components, thereby enhancing colloidal stability and *in vivo* performance [73,74].

Overall, the structural characteristics and physicochemical properties of liposomes are key determinants of their colloidal stability, biological interactions, drug encapsulation and release behavior, and ultimately their therapeutic performance and reproducibility [59]. These parameters are particularly critical in the context of pulmonary delivery, where precise control over liposome properties is required to ensure efficient deposition, retention and drug release within the respiratory tract.

III.1.2. Production methods

Liposome production relies on a variety of preparation methods that can be broadly classified into conventional laboratory-scale techniques and more recent approaches developed for industrial application.

Among conventional methods, the thin film hydration (TFH) method, known as the Bangham method, represents the earliest and most widely used approach for liposome preparation [62]. This technique involves dissolving lipidic components in an organic solvent, followed by solvent evaporation to form a thin, homogeneous lipid film on the vessel wall. Subsequent hydration of the dried film with an aqueous phase, typically under mechanical agitation, induces spontaneous self-assembly into vesicular structures, leading predominantly to the formation of multilamellar vesicles (Figure 8) [75].

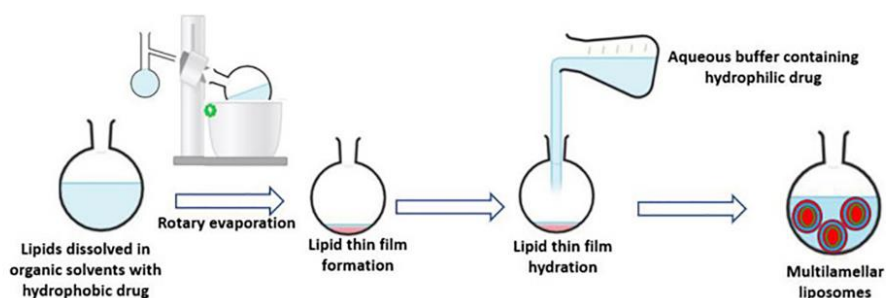


Figure 8: Illustration of the key stages in liposome formation via the thin film hydration (TFH) method [76].

Despite its simplicity and widespread use, TFH presents several limitations, including broad and heterogeneous size distributions that require post-processing steps such as extrusion or sonication [77]. Furthermore, its multistep nature, limited batch volumes and reliance on organic solvents with challenging removal may complicate large-scale implementation [78]. Other well-established conventional techniques, including detergent removal, reverse-phase evaporation and ethanol injection, have also been described for liposome preparation. While some of these methods, notably ethanol injection, have been successfully translated into the manufacturing of clinically approved liposomal products, conventional approaches may still present challenges related to process complexity, scalability and solvent removal requirements [79]. Consequently, increasing attention has been directed toward alternative manufacturing strategies, such as supercritical fluid (SCF)-based methods such as the Particle from Gas Saturated Solution (PGSS) technique, an industrial-scale approach [62,80].

SCFs have attracted increasing attention in pharmaceutical manufacturing and analysis due to their relatively environmentally friendly profile, low chemical reactivity, cost-effectiveness and their potential to replace conventional organic solvents [81,82]. A fluid reaches the supercritical state, characterized by an intermediate region between the liquid and gas phases, when its temperature and pressure exceed the critical point, defined by a critical temperature (T_c) and a critical pressure (P_c). SCFs exhibit liquid-like densities, which confer solvent power, along with low, gas-like viscosities that promote high diffusivity [80]. By adjusting temperature and pressure, as well as selecting fluids with different initial densities, both solvent strength and viscosity can be finely tuned, allowing control over particle diffusivity and enabling adaptation to a wide range of applications [83,84].

Among the SCFs investigated for pharmaceutical applications, supercritical carbon dioxide (scCO₂) has emerged as a key industrial solvent due to its low toxicity, low cost, chemical stability and inertness, along with a favorable environmental profile, as it is largely recycled from existing industrial processes [81]. Furthermore, the relatively low T_c and P_c of CO₂, 31.1 °C and 73.8 bar (Figure 9), respectively, make scCO₂ particularly suitable for the processing of thermolabile pharmaceutical drugs, while being recognized as safe for pharmaceutical applications [62]. Consequently, scCO₂ is widely used for extraction, SCFs chromatography, sterilization and liposome formation [85].

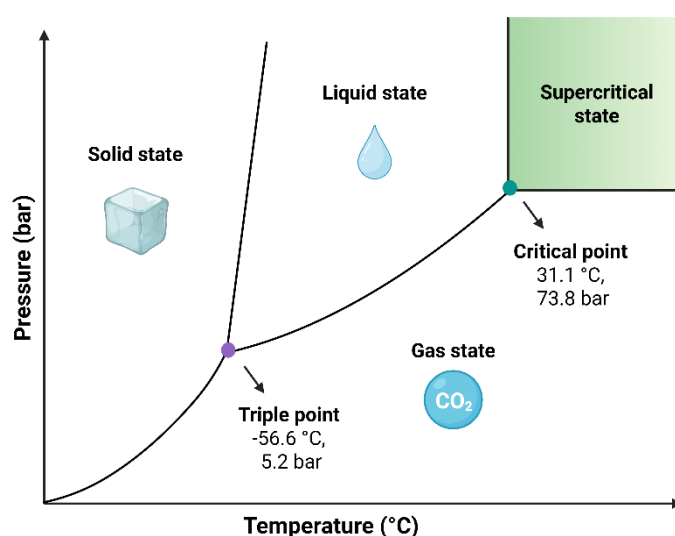


Figure 9: Phase diagram of carbon dioxide (CO₂) (reproduced from [80]), illustrating the critical point defined by the critical temperature (T_c) and critical pressure (P_c).

Regarding liposome production, the PGSS technique, which specifically exploits scCO₂ as a dispersing agent, has attracted increasing interest as a potential industrially scalable strategy. In this approach, lipids are first dispersed in an aqueous medium, where they naturally self-assemble into bilayer structures and are then exposed to scCO₂ under high pressure, leading to the formation of a macroemulsion [86]. Under pressurized conditions, scCO₂ diffuses into the hydrophobic region of the lipid bilayer, causing a temporary swelling and plasticization of the membrane and increasing its fluidity. Upon depressurization, CO₂ is rapidly released from the lipid phase, reducing the solvent capacity of the system and destabilizing the swollen bilayer structure. Liposome formation then results from two complementary mechanisms: the release of

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CO₂ generates shear and collision forces that disperse lipid assemblies, while hydrophobic interactions and interfacial tension drive the spontaneous reorganization of phospholipids into stable spherical bilayers [80,87,88]. The different steps of the overall process are illustrated in Figure 10, while the experimental configuration of the implemented PGSS setup is provided in Appendix I.

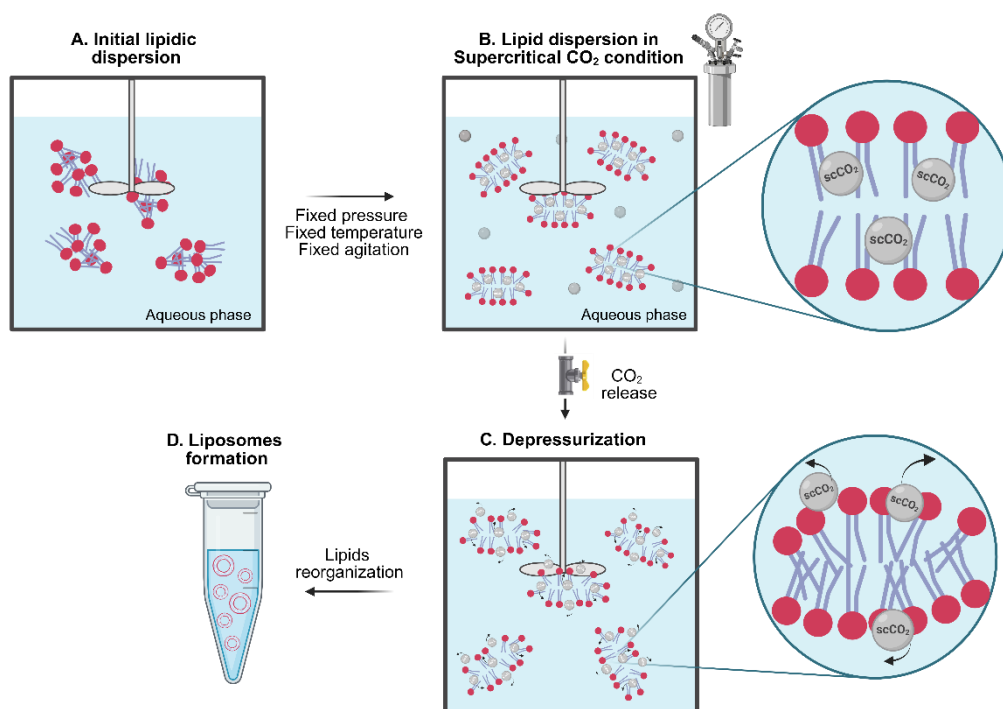


Figure 10: Schematic representation of the mechanism of liposome formation using the PGSS method: (A) phospholipid dispersion in the aqueous medium (B) formation of phospholipid bilayers after pressurization and equilibration with CO₂ (C) generation of a fine phospholipid dispersion during depressurization (D) liposome formation driven by hydrophobic interactions following depressurization. Created with BioRender.

Under optimized conditions, this process enables the one-step, solvent-free production of liposomes with high reproducibility, satisfactory API encapsulation efficiency and well-controlled particle size and polydispersity, while offering scalability and the potential for simultaneous sterilization [85,89].

III.2. Rationale for liposomal delivery to the lung

Combined with the advantages of pulmonary administration for localized drug delivery, namely targeted lung delivery, rapid onset of action, high local bioavailability and non-

invasive self-administration, liposomal pulmonary delivery offers additional distinct advantages (Figure 11).

Regarding their safety profile, liposomes are generally considered as biocompatible and biodegradable carriers with a low toxicity profile, primarily due to their structural similarity to biological membranes and pulmonary surfactant [61,90]. They closely mimic the composition of biological membranes, consisting primarily of phospholipids and CHOL. Indeed, CHOL is a key structural component that typically accounts for approximately 30 to 50% of total cell membrane lipids in the body [91]. Similarly, pulmonary surfactants mainly consist of phospholipids, predominantly dipalmitoylphosphatidylcholine (DPPC), along with neutral lipids and specific surfactant proteins [84,92]. This compositional similarity promotes good compatibility with lung tissue, limits inflammatory responses and contributes to the overall lower toxicity of liposomes compared to other NP-based delivery systems [55,93]. Although liposomes are generally regarded as biocompatible and biodegradable carriers, the composition of individual formulations should be carefully considered, as the inclusion of non-endogenous components, such as certain synthetic lipids or PEG, may alter their biological interactions and require further evaluation of their pulmonary safety.

Beyond these favorable safety attributes, a key advantage of liposomes for pulmonary administration lies in their ability to overcome major pulmonary clearance mechanisms, notably alveolar macrophage uptake and the mucus barrier, thereby enhancing drug retention at the target site. This property is particularly valuable in respiratory diseases characterized by excessive and altered mucus production, such as asthma, COPD and cystic fibrosis, where the presence of a thicker and more heterogeneous mucus layer substantially hinders drug diffusion and access to the underlying epithelial cells [48]. Therefore, formulation parameters are critical, as liposome size, lamellarity, surface charge and membrane rigidity strongly influence these clearance-related mechanisms [56]. Small vesicles, particularly those smaller than 200 nm, are generally preferred for pulmonary delivery, as they are less susceptible to phagocytosis and mucus entrapment, resulting in delayed lung clearance compared to larger particles [61,94]. In this context, Romero *et al.* demonstrated that ~100 nm liposomes exhibit enhanced colloidal stability, reduced cytotoxicity and improved diffusion through mucus compared to larger vesicles, while Chono *et al.* showed that macrophage uptake of liposomes

increases with particle size, highlighting the advantage of smaller particles for pulmonary delivery [95,96]. Likewise, neutral liposomes (ZP around 0 mV) are favored to minimize electrostatic interactions with negatively charged mucins, facilitating mucus penetration while avoiding the toxicity often associated with cationic liposomes, as highlighted by Zhang *et al.* [94,97]. Furthermore, multilamellar liposomes formulated with high T_m phospholipids, such as DPPC or distearoylphosphatidylcholine (DSPC), in combination with CHOL, provide enhanced thermodynamic and mechanical stability in pulmonary fluid [98]. Additionally, surface modification through PEGylation has been shown to further improve mucus penetration, reduce pulmonary clearance and extend residence time in the lung [99]. These effects are primarily attributed to the hydrophilic steric barrier provided by PEG, which minimizes hydrophobic and electrostatic interactions with mucus constituents, thereby facilitating diffusion through the mucus layer [100]. This is further supported by Martina *et al.*, who demonstrated that PEGylation reduces macrophage uptake by preventing the initial recognition of liposomes [101].

On the other hand, liposomes can be deliberately engineered to promote uptake by alveolar macrophages or, conversely, to be retained within the mucus layer, highlighting the versatility and tunability of liposomal systems for pulmonary drug delivery. These design strategies depend on the therapeutic objective, whether to modulate excessive inflammatory responses, to achieve prolonged local activity within the airway mucus, or to target intracellular pathogens persisting in macrophages, for example those involved in non-tuberculous mycobacterial lung infections [102,103]. For the latter, this approach is exemplified by Arikayce[®], currently the only inhaled liposomal formulation approved by both the FDA and the EMA, in which liposomes are designed for uptake by alveolar macrophages to deliver amikacin intracellularly, where pathogens persist [104].

In addition to their ability to modulate pulmonary clearance mechanisms, liposomes provide important formulation-related advantages, particularly in terms of controlled drug release and protection of encapsulated compounds. Building on these properties, liposomes can sustain and finely regulate drug release, thereby prolonging lung residence time and reducing dosing frequency [105,106]. For example, Cipolla *et al.* showed that inhaled liposomal ciprofloxacin achieved sustained lung retention with prolonged half-life and high local drug concentrations, while maintaining low systemic

exposure, thereby supporting once-daily administration and favorable tolerability [107]. In addition, liposomes protect encapsulated APIs from chemical degradation or instability in pulmonary fluids, which helps to minimize both local and systemic toxicity and to prevent local irritation of lung tissue associated with poorly soluble or undissolved drugs [52,108]. Regarding degradation, this has been demonstrated by Stark *et al.*, who showed that liposomal encapsulation protects vasoactive intestinal peptide, a peptide with bronchodilatory and anti-inflammatory properties, from enzymatic degradation in bronchoalveolar lavage fluid [109]. Furthermore, liposomes enable the co-encapsulation of both hydrophilic and lipophilic compounds, as illustrated by Penoy *et al.*, who reported the liposomal co-encapsulation of salbutamol (SAL) and budesonide (BUD), which may support less frequent administration and improve patient compliance in inhalation therapies [89]. Finally, at the terminal stage of pulmonary residence, liposomal membranes can interact with surfactant and cell membranes, potentially leading to gradual lipid exchange and eventual degradation, which contributes to controlled drug release and enhanced biocompatibility, as suggested by Mehta *et al.* [110]. Ferguson *et al.* also demonstrated that liposomes remain structurally intact and stable in pulmonary surfactant for extended periods, without immediate disruption or integration into surfactant or cellular membranes and are instead gradually cleared following cellular uptake and slow degradation [111].

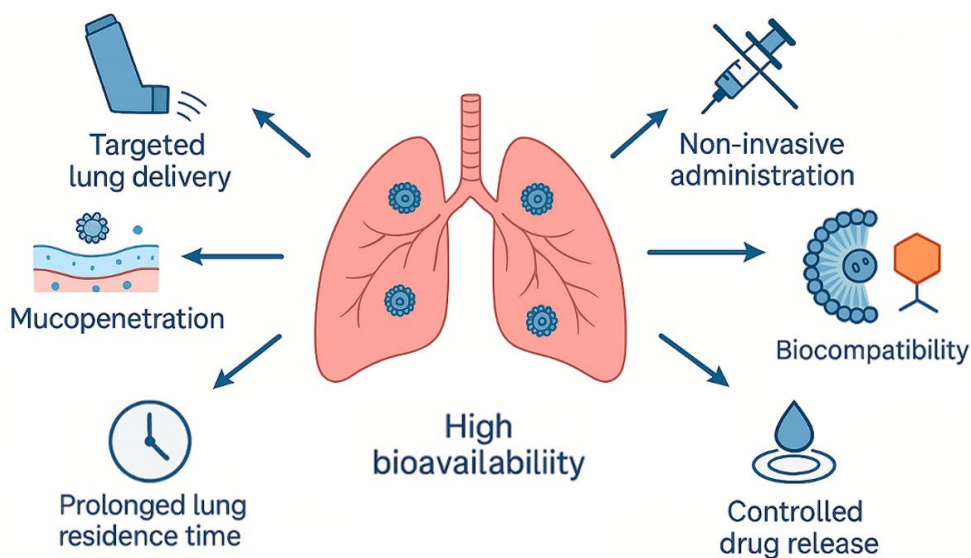


Figure 11: Advantages and interest of pulmonary administration of liposomes. Refined using Copilot.

Taken together, these properties position pulmonary delivery as a particularly attractive extension of liposomal drug delivery strategies, fully leveraging the unique physiological and therapeutic advantages of the lung. Accordingly, numerous liposomal formulations have been investigated for pulmonary administration in both preclinical and clinical settings, including antimicrobial agents such as ciprofloxacin, amikacin, tobramycin and amphotericin B, anti-inflammatory drugs such as BUD, as well as chemotherapeutics including camptothecin derivatives, cisplatin and doxorubicin [53,92]. These efforts culminated in the approval of Arikayce® by the FDA in 2018 and by the EMA in 2020, marking a key milestone in the clinical validation of liposomes for pulmonary delivery. More recently, several formulations have demonstrated promising clinical outcomes, including inhaled liposomal amphotericin B for pulmonary infections and liposomal cyclosporine A in transplant-related complications, further supporting the translational potential of inhaled liposomal systems [112,113].

Nevertheless, Arikayce® remains the only liposomal formulation currently approved for pulmonary delivery, and only a limited number of lipid excipients have been authorized for inhalation to date [61]. This regulatory landscape underscores both the early stage of the field and the substantial opportunities for innovation in the development of safe and effective inhaled liposomal therapies.

III.3. Devices for inhalation therapy

Pulmonary administration of liposomes, as well as other pulmonary therapies, relies on inhalation devices capable of generating aerosols that ensure efficient transport and deposition within the respiratory tract. Owing to their nanometric size, liposomes alone exhibit an excessively low d_{ae} and would therefore be largely exhaled with the respiratory airflow [114]. Effective deposition in the bronchiolar and alveolar regions generally requires aerosols with a d_{ae} between 1 and 5 μm [115]. Inhalation devices thus play a pivotal role by transforming liposome-based formulations into respirable aerosols, either as liquid droplets or solid microparticles, enabling their delivery to the distal regions of the lung.

Beyond drug formulation, the choice of the inhalation device is a key determinant of therapeutic efficacy, a consideration that is particularly critical for liposomal delivery. Indeed, selecting an appropriate formulation-device combination remains challenging,

as liposomes are highly sensitive to their physical state and to mechanical stresses during administration. The inhalation device must therefore preserve liposome integrity while generating aerosols with suitable aerodynamic properties for effective lung deposition.

In addition, the inhalation device must not only be appropriately matched to the formulation but also suited to the patient's inspiratory capacity and ability to use the device correctly. Incorrect handling or poor coordination between actuation and inhalation remains a major cause of suboptimal outcomes. Conversely, when appropriately matched to both the formulation and the patient, the device can improve drug stability, optimize deposition at the target site and ensure dose consistency, thereby maximizing clinical effectiveness.

To date, four main categories of inhalation devices have been developed for pulmonary drug delivery, differing in design and operating mechanisms: nebulizers, pMDIs, SMIs, which deliver drugs as liquid formulations and DPIs, designed for the administration of drugs as dry powders. In the following section, these devices will be discussed according to their relevance and feasibility for the administration of liposomes.

III.3.1. Pressurized metered-dose inhalers

In pMDIs, a liquid medicinal formulation, a propellant and, when applicable, co-solvents or excipients, are maintained under pressure within a sealed canister. Upon actuation, a metering valve opens, releasing a precisely measured dose, which is transformed into an inhalable aerosol as the sudden pressure drop causes rapid propellant evaporation and fragmentation of the liquid into fine droplets (Figure 12) [116]. Since their introduction in 1950, pMDIs have played a pivotal role in the evolution of inhaler technology, offering enduring advantages, such as portability, cost-effectiveness and the reliable delivery of drug particles to the distal airways [2].

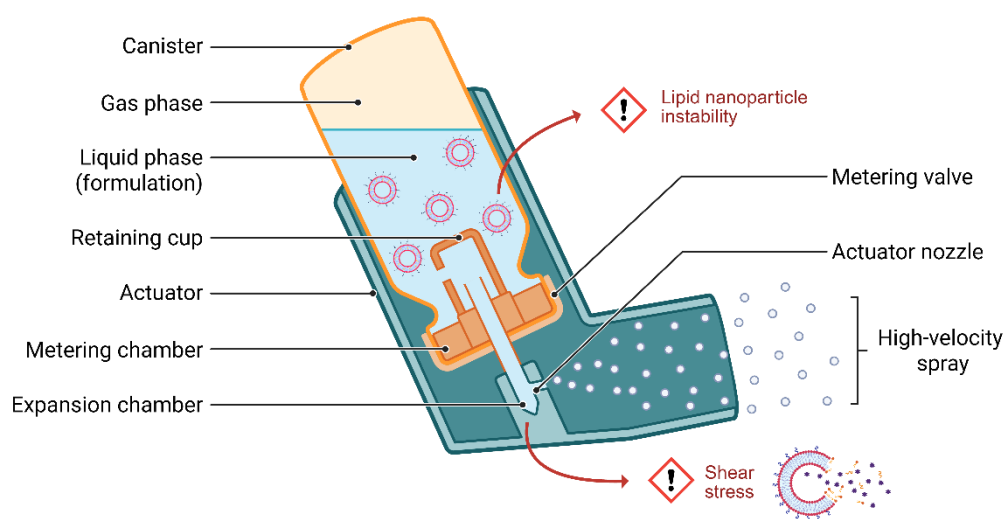


Figure 12: Schematic representation of a pressurized metered-dose inhaler (pMDI) and potential risk for liposomal administration. Created on BioRender.

Nevertheless, longstanding concerns remain regarding both user-related handling challenges and the intrinsic dependence of pMDIs on propellant gases. Regarding the former issue, pMDIs require precise coordination between actuation and inhalation, adequate inhalation depth and breath-holding [117]. Even when used correctly, only about half of the administered medication reaches the distal airways, with the remainder depositing in the upper respiratory tract [118]. To this matter, continuous advancements in pMDI technology have led to the development of enhanced devices, including breath-actuated MDIs, such as the Redihaler®, as well as pMDIs designed for use with accessory devices like spacers or valve-holding chambers [4,119]. By eliminating the need for coordination or stabilizing the aerosol prior to inhalation, these approaches aim to simplify device handling and improve usability. However, while these technological refinements have improved usability and patient handling, they do not resolve a growing concern associated with pMDIs: their environmental impact linked to propellant gases.

Following the adoption of the 1987 Montreal Protocol, ozone-depleting chlorofluorocarbon propellants used in early pMDIs were replaced by HFAs [2,120]. Although HFAs do not contribute to ozone depletion, their climatic footprint remains non-negligible. This impact is illustrated by Wilkinson *et al.*, who showed that replacing just 10% of pMDI prescriptions in the United Kingdom with equivalent non-propellant

devices (such as DPIs) could save over 68.6 kilotonnes of CO₂-equivalent emissions annually, an amount comparable to the emissions from approximately 43 000 round-trip transatlantic flights between London and New York [121]. Accordingly, contemporary research on pMDIs largely concentrates on propellant transition strategies, aiming to replace HFAs with alternatives that maintain aerosol performance while substantially reducing environmental impact [122].

Despite their notable environmental impact and challenges related to proper use, pMDIs remain widely used worldwide and account for a substantial proportion of inhaler use in routine clinical practice [5].

Beyond environmental and use considerations, additional challenges arise from a liposomal formulation perspective. The apolar nature and limited solubilization capacity of HFA propellants, combined with rapid propellant evaporation, high shear forces during actuation and the frequent use of co-solvents such as ethanol, can destabilize sensitive lipid nanostructures and adversely affect their structural integrity and drug retention [58,123,124]. Accordingly, liposomal pMDI formulations remain underexplored, despite stabilization strategies proposed by Huang *et al.*, such as biomineralization-inspired shell coating to enhance liposome robustness [125].

III.3.2. Soft Mist Inhalers

SMLs are multidose, pocket-sized, propellant-free inhalers that use mechanical energy from the actuation of a spring to transform a liquid drug formulation into an aerosol mist via a micro-nozzle system (Figure 13) [126]. The key advantage of this technology lies in the slow and prolonged generation of the aerosol, which minimizes oropharyngeal deposition caused by inertial impaction, a limitation commonly observed with high velocity pMDIs [3,126,127]. This feature allows SMLs to achieve high pulmonary drug deposition, enhancing therapeutic efficacy while reducing local side effects [5].

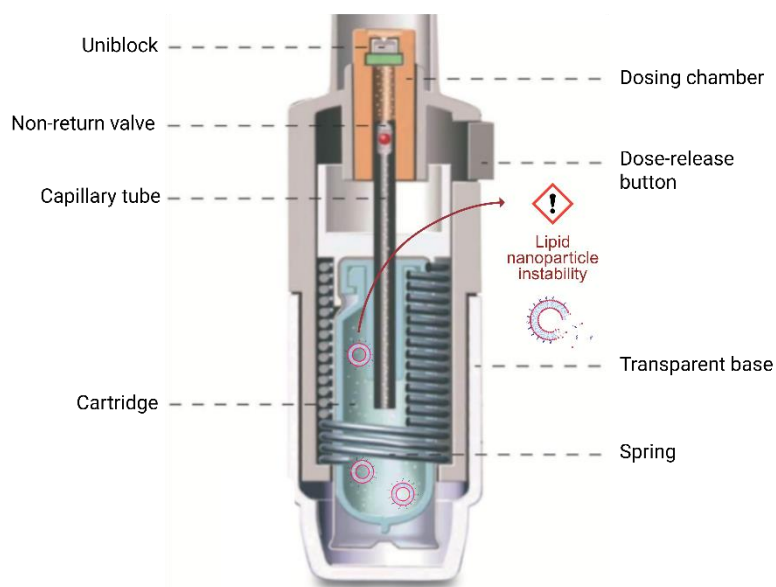


Figure 13: Schematic representation of a soft mist inhaler (SMI) and potential risk for liposomal administration. Modified from [126].

SMIs also provide consistent dosing through the incorporation of a metering chamber and are patient-friendly, as the mechanically generated mist is independent of the patient's inspiratory effort and facilitates coordination between actuation and inhalation [128]. These characteristics improve adherence and make SMIs suitable for patients of all ages [5].

Despite their clinical benefits, their wider implementation is limited by higher development and manufacturing costs arising from complex engineering [128], as well as by stringent formulation requirements. In particular, achieving consistent soft mist aerosol characteristics necessitates low-viscosity aqueous solutions and precise control of fluid properties [129]. These factors likely contribute to the observation that, to date, Respimat®, which integrates the low-velocity aerosol generation and the micro-nozzle (Uniblock) system, remains the only SMI commercially available [130].

Nonetheless, for liposomal formulation development, these formulation requirements introduce additional challenges. The necessity for low-viscosity formulations to ensure proper atomization through the micro-nozzle may limit compatibility with viscous or highly structured lipid dispersions, while the aqueous environment required for SMI operation can exacerbate long-term stability issues, such as aggregation, hydrolysis, or

drug leakage (Figure 13) [129]. Despite these limitations, Biesel *et al.* recently demonstrated that SMI-based aerosolization of liposomal messenger RNA (mRNA) formulations preserves particle size, colloidal stability and mRNA integrity while enabling efficient pulmonary deposition, thereby highlighting its potential as a viable delivery platform.

Nonetheless, despite their favorable aerodynamic performance, the use of SMIs for complex lipid-based nanocarriers, particularly liposomes, remains underexplored, similarly to pMDIs, largely due to rheological limitations and stability challenges.

III.3.3. Nebulizers

Nebulizers are among the earliest modern devices for producing aerosols, converting liquid formulations into fine droplets suitable for pulmonary delivery. Over time, they have evolved into three main types, namely pneumatic, ultrasonic and mesh nebulizers, each distinguished by its specific mechanism of droplet generation (Figure 14).

Pneumatic or jet nebulizers generate aerosols by directing compressed air through a narrow nozzle, where it accelerates and interacts with the liquid formulation according to the Venturi principle. The resulting high-velocity airflow creates shear forces at the air-liquid interface, leading to the fragmentation of the liquid into respirable droplets that are subsequently entrained and transported by the air stream [119,131]. Ultrasonic nebulizers generate aerosols by applying high-frequency oscillations produced by a piezoelectric element to the liquid formulation. These vibrations transmit energy through the liquid, forming surface waves at the air-liquid interface that destabilize the fluid and release fine droplets into the surrounding air [131,132]. Finally, mesh nebulizers, which may be static or vibrating, generate aerosols by forcing the liquid through micro-orifices and, compared with jet nebulizers, offer faster drug delivery, lower residual volumes and more compact device designs [132,133].

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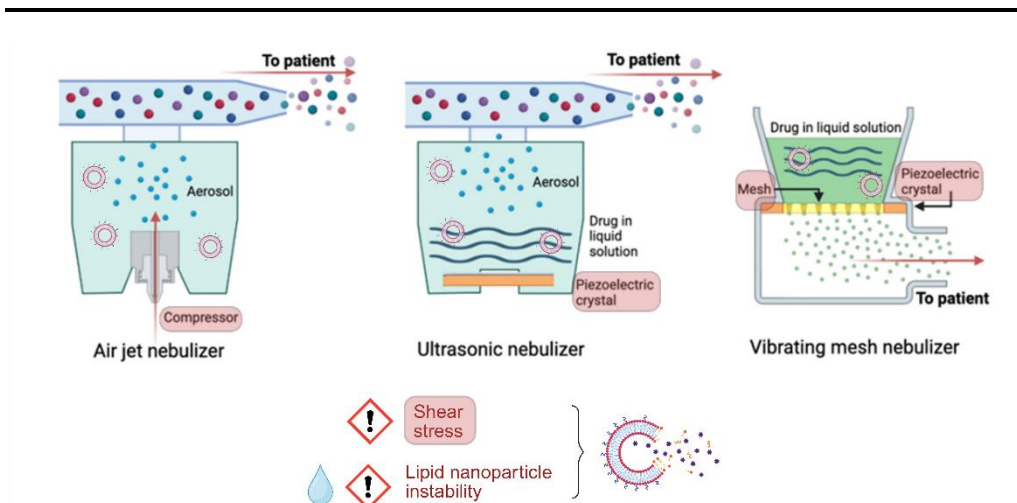


Figure 14: Schematic representations illustrating the operating principles of predominant nebulizer types: air-jet nebulizer (left), ultrasonic nebulizer (center) and vibrating mesh nebulizer (right). Modified from [5].

More recently, a fourth category of nebulizers, called smart or adaptive devices, has emerged, integrating sensors and microprocessor-controlled aerosol delivery to optimize dosing and monitor patient inhalation patterns [5].

Beyond their technological diversity, nebulizers continue to hold an important place in clinical practice owing to their ease of use and versatility [3]. Drug delivery does not require precise coordination between inhalation and actuation, making them particularly suitable for elderly and pediatric patients, as well as for those who are ventilated or unconscious [5,131]. Moreover, nebulizers not only allow the administration of relatively large doses but also provide the flexibility to combine compatible liquid formulations for simultaneous API delivery, which can be advantageous in certain clinical situations [4].

Despite their advantages, nebulizers present several limitations, including variable and often poorly reproducible dosing due to environmental drug losses, prolonged administration times and bulky designs that limit portability and routine daily use [119,134]. Aerosol losses can be substantial, reaching up to 50% with jet nebulizers, which not only reduces the delivered dose but may also result in environmental contamination, particularly when cytotoxic drugs are administered [135]. In addition, their higher cost and potential for drug degradation, together with challenges in nebulizing viscous or suspension formulations, further constrain their practicality [133].

Consequently, nebulizers are typically reserved for acute exacerbations, particularly in patients who are unable to generate sufficient inspiratory flow [134].

Furthermore, in the context of pulmonary delivery of liposomal formulations, nebulization can induce significant structural stress, including shear forces, thermal effects and mechanical deformation, leading to vesicle disruption and drug leakage [92]. Experimental studies support these limitations, with Elhissi *et al.* reporting liposome destabilization following air-jet nebulization [136], while Valle *et al.* observed a progressive decrease in encapsulation efficiency during ultrasonic nebulization [137]. In contrast, vibrating-mesh nebulizers, although often considered the most suitable devices for inhalable nanoformulations due to their improved aerosolization efficiency and reduced formulation stress, as highlighted by Peng *et al.* [58], may still induce liposome instability. Indeed, Klein *et al.* showed that vibrating-mesh nebulization can lead to leakage of encapsulated content and liposome aggregation, indicating that careful consideration remains necessary [138].

Formulation strategies can partially mitigate these effects, as studies have shown that increasing CHOL content or incorporating high T_m phospholipids can mitigate membrane alterations during nebulization [92,139]. This approach is exemplified by Arikayce®, a CHOL-enriched DPPC liposomal formulation delivered via a vibrating-mesh nebulizer (Lamira®), specifically designed to limit mechanical stress [92,140]. While this product demonstrates the clinical feasibility of liposomal pulmonary delivery, it also illustrates formulation and device-specific limitations. In particular, partial drug leakage may still occur during aerosolization, with approximately 30% of the encapsulated amikacin being released during nebulization, highlighting the persistence of mechanical stress even in optimized systems [141]. In addition, the treatment regimen is associated with practical constraints such as pre-use preparation, relatively long administration times (15-20 minutes) and the need for dedicated powered devices with limited portability [142]. Altogether, these factors may reduce patient convenience and adherence, particularly in the context of chronic treatments requiring repeated long-term administration.

Consequently, these limitations have contributed to a growing interest in alternative delivery strategies, particularly DPIs, which may offer improved stability, portability, reproducibility and patient adherence [119,134].

III.3.4. Dry Powder Inhalers

DPIs are distinguished from other inhaler devices by their ability to deliver medications to the lungs in a solid dry powder form. In DPIs, APIs are typically combined with one or more excipients, most commonly carbohydrates, which play a crucial role in optimizing the powder's physical properties, thereby ensuring efficient aerosolization and pulmonary deposition.

III.3.4.1. Classification

In most DPIs, the powder is converted into an inhalable aerosol through a process primarily driven by the patient's inspiratory effort and supported by the device's internal airflow resistance [13]. This breath-actuated mechanism classifies DPIs as passive devices (Figure 15). While this system eliminates the need for external energy or coordination between actuation and inhalation, it also imposes a key limitation: inspiratory capacity varies between patients due to factors such as age, lung function and disease severity, which can lead to inconsistent powder dispersion and reduced dose uniformity [119].

To address this limitation and improve dose consistency, DPIs incorporating active aerosolization mechanisms have been extensively investigated. These devices aim to assist powder dispersion using an internal energy source, such as a spring, battery, or compressed air during inhalation [35]. For instance, Zhang *et al.* developed an innovative active DPI utilizing compressed air to disperse very small quantities of extra-fine powder, achieving 50 to 70% lung deposition *in vitro*, along with consistent dose delivery and minimal residual drug in the device [143]. While such active DPIs show considerable promise, only passive DPIs are currently available on the market, owing to their lower cost, greater portability and reduced technical complexity [13].

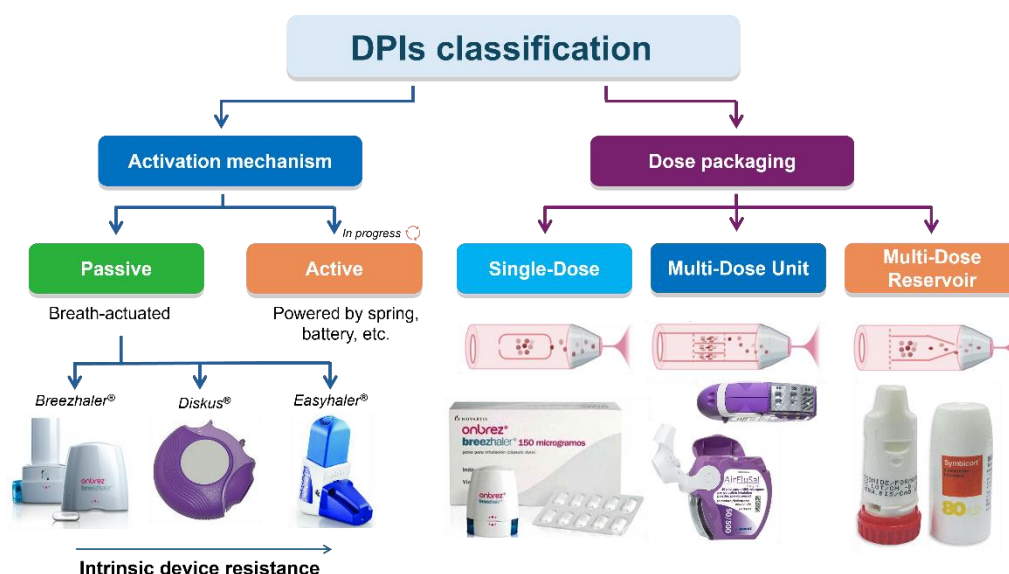


Figure 15: Dry powder inhalers (DPIs) classification depending on their activation mechanism and dose packaging.

Beyond their classification by activation mechanism, DPIs can also be categorized according to drug packaging, specifically the number of doses each device contains. Single-dose DPIs hold medication in individual capsules, often in foil blisters, strips or removable disks, which are placed in the device for each use and discarded once inhaled. In contrast, multi-dose DPIs may either store multiple pre-loaded unit doses or incorporate a built-in reservoir that dispenses a single dose per actuation, allowing repeated use without handling individual capsules [35]. While multi-dose DPIs are convenient for patients because they eliminate the need to handle individual capsules, single-dose devices are often considered more reliable in terms of dose consistency and stability. By isolating each dose, these devices protect the powder from moisture and other environmental factors, thereby enhancing long-term storage stability and ensuring accurate delivery [119].

In addition to the conventional classification of DPIs based on their activation mechanism or dose packaging, pulmonary deposition is also strongly influenced by device-dependent internal flowrate resistance [144]. As previously mentioned, in passive breath-actuated DPIs, aerosol generation primarily relies on the patient's inspiratory effort. However, the device's internal airflow resistance, defined as the opposition to the patient's inspiratory flow, plays a key role in converting inhalation

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energy into deagglomeration forces, such as turbulence, shear stress and inertial interactions, required to break down powder agglomerates and aerosolize the powder within the respirable size range [145]. This aspect is particularly critical for carrier-based formulations, where efficient detachment of the micronized API from the carrier is necessary to enable deposition in the deepest regions of the lung [146]. On this basis, DPIs are commonly classified as low, medium or high resistance devices based on design features such as swirl chambers, grids, orifices and mouthpiece dimensions [147,148]. The details of each inhaler resistance are listed in the table below (Table 1).

Table 1: Classification of dry powder inhalers (DPIs) according to airflow resistance and powder deagglomeration mechanism.

RESISTANCE TYPE	INTERNAL AIRFLOW FEATURES	FLOW REQUIREMENT	MECHANISM OF POWDER DEAGGLOMERATION	EXAMPLE DEVICES	REF
LOW	Wide air intakes, minimal opposition	High (~90-100 L/min)	Relies on patient-generated flow to generate turbulence and shear	Aerolizer®, Breezhaler®	[144,149]
MEDIUM	Intermediate air inlets and geometries	Moderate (~60-70 L/min)	Combination of patient flow and device-induced turbulence	Diskus®	[144,150]
HIGH	Narrow air inlets, complex internal design	Low (~30-60 L/min)	Device converts flow into turbulence, shear and particle collisions → less dependent on patient flow	Turbuhaler® (double-helix swirl), Easyhaler® (narrow air inlet)	[13,148,151]

In low-resistance (LR) DPIs, air flows easily through the device due to wide air intake openings and minimal opposition to airflow, resulting in limited internal turbulence. Consequently, powder deagglomeration in these devices is largely dependent on the magnitude of the patient-generated inspiratory flow, which generally requires higher inspiratory flow rates to achieve efficient powder dispersion and generate particles within the respirable size range [144].

In contrast, high-resistance (HR) DPIs impose greater opposition to airflow through narrower air inlets and more complex internal geometries. This increased resistance promotes the conversion of inspiratory flow into localized turbulence, shear forces and particle-particle or particle-wall collisions, which may facilitate powder deagglomeration at lower inspiratory flow rates. Aerosolization performance may therefore be less

sensitive to variations in peak inspiratory flow (PIF), although it remains dependent on the interplay between device characteristics, formulation properties and patient inspiratory capacity [13,145].

III.3.4.2. Strategic relevance of DPI in modern pulmonary drug delivery

In current inhaled drug development, DPIs offer considerable advantages. Although they are breath-actuated and therefore dependent on the patient's inspiratory capacity, this feature eliminates the need for precise coordination between actuation and inhalation, thereby enhancing pulmonary drug deposition. Combined with their portability, this simplifies device use and ensures suitability across patient populations, from children to the elderly [119,152]. Furthermore, DPIs do not rely on propellant gases, offering a clear advantage in terms of environmental sustainability. Jeswani *et al.* reported that substituting pMDIs with DPIs could reduce the climate impact associated with inhaler use by up to 96% [121,122].

As implied by their name, DPIs deliver the drug in a solid dry state, which confers a key and non-negligible advantage in terms of active substances stability [153]. As inhaled drug development increasingly explores advanced modalities such as biologics and NP-based systems to enhance bioavailability and therapeutic efficacy, formulation stability has become as critical as device selection [154]. In this context, the dry environment provided by DPIs is especially advantageous for preserving the long-term stability of these innovative formulations [155].

Taken together, the limitations of alternative inhalation devices, combined with the inherent advantages of DPIs, underscore their strategic relevance as a preferred platform for pulmonary drug delivery, particularly for advanced formulations with stringent stability requirements.

In addition to their stability advantages, the development of inhalable pharmaceutical formulations remains challenging, as achieving sufficient aerosolization and efficient deep lung deposition requires careful optimization. DPI performance depends not only on the device design and the patient's inspiratory capacity, but also critically on the physicochemical properties of the powder formulation. Importantly, a major strength of DPI systems lies in the wide range of available production methods, encompassing diverse particle engineering strategies that enable precise control of aerodynamic

behavior and the tailoring of formulations to specific therapeutic and patient-related needs [13,153].

The following section examines the key physicochemical properties governing the performance of inhalation powders, with a particular focus on their impact on pulmonary deposition. Building on this foundation, formulation strategies, excipient roles and particle engineering approaches for DPIs are then discussed, with special emphasis on liposomal formulations.

IV. Formulation and engineering of DPI

IV.1. Key particle properties governing pulmonary deposition

The performance of inhalable powders depends on a set of interdependent physicochemical properties that must be finely tuned to achieve efficient aerosolization, consistent dose delivery and optimal pulmonary deposition. In liposome-based DPIs, this challenge is amplified by the need to simultaneously preserve microparticle aerodynamic properties and liposomal integrity.

From a formulation and particle engineering perspective, this work is based on “the nano-in-micro” strategy, a widely established approach in DPIs, in which liposomes (nanoscale) are incorporated into inhalable microparticles [156]. This strategy requires careful control of microparticle characteristics to ensure appropriate aerodynamic behavior for inhalation.

This section outlines the key physicochemical parameters governing pulmonary deposition and discusses their optimal ranges in the context of liposome-based powder formulations.

IV.1.1. Particle size

Particle size is a key determinant of the fate of inhaled powders, directly controlling both aerosolization efficiency and deposition within the respiratory tract. As discussed in Section II.2, particles must typically fall within the 1-5 μm range to reach the deep lung, while avoiding impaction in the upper airways or exhalation. The size of powder particles can be assessed in different ways.

The geometric diameter of a powder is commonly evaluated using laser diffraction, a technique that analyzes the distribution of light scattered by particles suspended in a medium. The D50, representing the median particle size, indicates that 50% of the particle volume is smaller and 50% larger than this value [115]. Laser diffraction also provides complementary metrics such as D10 and D90, corresponding to the particle diameters below which 10% and 90% of the cumulative particle volume are found, respectively, thereby providing information on the width of the particle size distribution. This variability can be further described by the span, calculated as (D90 – D10)/D50, with higher values indicating greater heterogeneity [157].

Although the geometric diameter provides a useful estimation of the particle size range within a powder, it corresponds to the diameter of an idealized spherical particle and therefore does not account for particle morphology. Consequently, the d_{ae} is regarded as the most relevant parameter for inhalation formulations, as it integrates both particle geometry and density [35,158].

The d_{ae} of a particle is defined as the diameter of a hypothetical spherical particle with a unit density of 1 g/cm³ that settles at the same terminal velocity as the particle of interest in air and is calculated using Equation 1.

$$d_{ae} = d \times \sqrt{\frac{\rho}{\rho_0 \chi}}$$

Equation 1: Aerodynamic diameter (d_{ae}).

As highlighted in Equation 1, the d_{ae} is governed by multiple particle attributes, including geometric size (d), density (ρ) and morphology, the latter being accounted for through the dynamic shape factor (χ). The parameter ρ_0 represents the unit reference density (1 g/cm³) [39]. By incorporating variations in particle shape and density, the d_{ae} provides a robust and physiologically relevant metric for predicting particle behavior in an airstream and their likely deposition within the respiratory tract.

To this end, *in vitro* cascade impaction techniques described in the European Pharmacopoeia (Ph. Eur) 11.0 (general chapter 2.9.18), including the Multi-Stage Liquid Impinger (MSLI) (Apparatus C), the Andersen Cascade Impactor (ACI) (Apparatus D) and the Next Generation Impactor (NGI) (Apparatus E), are commonly employed. These methods fractionate aerosolized particles within a controlled airflow according to their

d_{ae} , enabling determination of key performance indicators such as emitted dose (ED), fine particle dose (FPD), fine particle fraction (FPF), mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD). ED reflects the amount of API released from the device, while FPF represents the proportion of particles within the respirable range ($< 5 \mu\text{m}$). The MMAD corresponds to the d_{ae} at which 50% of the aerosol mass lies above and 50% below this value and the GSD characterizes the extent of the aerodynamic size distribution [158]. Collectively, these parameters provide a comprehensive evaluation of aerosol quality and expected deposition performance.

Among the available standard instruments, the NGI is the most widely used and remains the reference for detailed aerodynamic profiling of DPI formulations. It consists of seven impaction stages followed by a terminal micro-orifice collector (MOC). The cut-off diameters of these stages are logarithmically spaced, and the device operates at flow rates between 30 and 100 L/min, corresponding to particle sizes of approximately 0.24-11.7 μm . Overall, the NGI simulates different regions of the respiratory tract, including the mouth, throat and upper airways, via its induction port and optional pre-separator, which can contain liquid to better replicate respiratory tract humidity. A schematic representation of the impactor and the correspondence between its stages and the respiratory tract regions is shown in Figure 16. Powders collected from each stage of the NGI can subsequently be rehydrated and quantified, allowing precise assessment of dose distribution across the device [159].

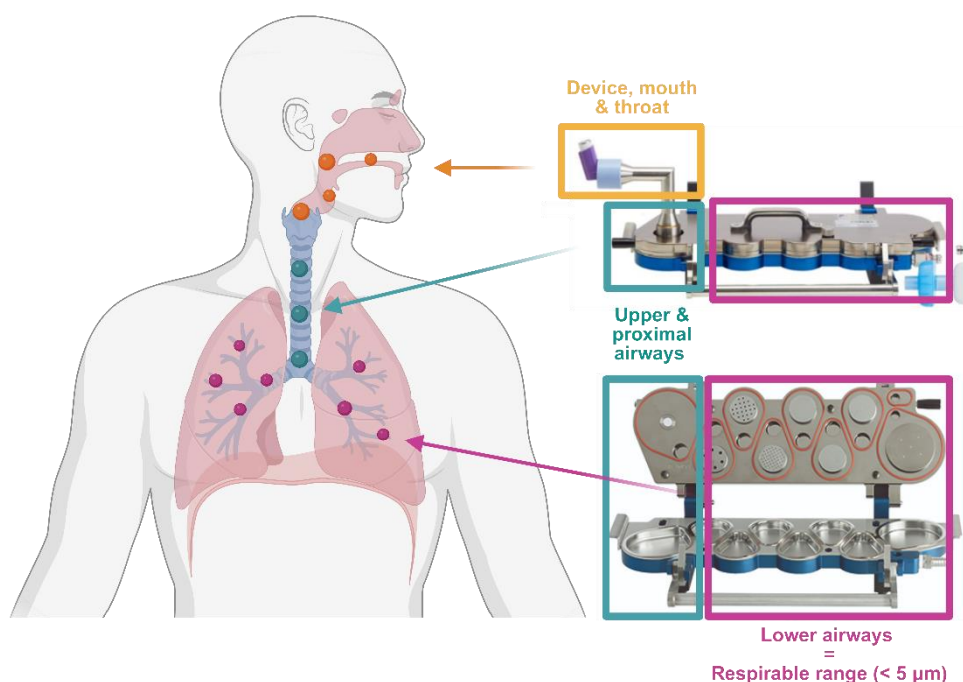


Figure 16: Representation of the Next Generation Impactor (NGI) illustrating the relationship between impactor stages and deposition sites within the respiratory system.

Overall, in the context of DPI development, evaluating aerosolization efficiency and pulmonary deposition represents a critical step. Insight into how particles disperse upon device actuation and how they distribute according to their aerodynamic properties is essential for predicting deposition patterns in the respiratory tract [13].

IV.1.2. Structural organization and crystallinity

The thermodynamic state of a powder dictates its molecular organization. Crystalline materials display a regular, ordered arrangement of molecules, whereas amorphous solids exhibit a disordered organization. Although some substances are inherently amorphous, crystalline materials may undergo amorphization during manufacturing processes [160].

For DPI formulations, physical state plays a critical role in aerosol performance and pulmonary bioavailability by influencing particle cohesion, solubility and dissolution behavior in the lungs. Amorphous powders, often produced under high-temperature or rapid drying conditions, generally exhibit smaller and more homogeneous particle sizes,

leading to improved dispersion and deeper lung deposition [158,161]. Moreover, amorphous APIs generally exhibit higher solubility and faster dissolution in pulmonary fluids than their crystalline counterparts, thereby enhancing pulmonary absorption and systemic bioavailability [162]. Amorphous matrices also have been reported to be advantageous for formulations containing lipid-based nanocarriers, as they enable improved redispersion of lipid NPs from dried formulations compared with crystalline matrices [163].

Despite their advantages, amorphous powders are thermodynamically unstable and typically highly hygroscopic. Moisture uptake can increase molecular mobility and promote recrystallization over time, potentially compromising the stability of the API, the carrier matrix and liposomal formulations [164].

In contrast, crystalline powders are generally less hygroscopic but tend to exhibit greater cohesiveness, which may promote agglomeration and heterogeneous lung deposition. Their lower apparent solubility and slower dissolution rate in pulmonary fluids can also limit drug absorption and bioavailability, ultimately reducing therapeutic efficacy [158].

Therefore, monitoring the physical state during storage is essential. Differential scanning calorimetry (DSC) and X-ray powder diffraction (XRPD) are commonly used to detect recrystallization and assess formulation stability [40].

IV.1.3. Residual moisture content

Moisture content is a critical factor affecting the stability and performance of dry powder formulations for pulmonary delivery. It is influenced by the composition of the powder, storage conditions and manufacturing parameters [115]. Due to their high specific surface area, DPIs are particularly sensitive to humidity, especially when hygroscopic components are present [165].

Excess moisture promotes the formation of liquid bridges between particles, increasing cohesive forces and leading to agglomeration. This alters particle size distribution and reduces aerosolization efficiency, particularly the fraction of particles depositing in the deep lung [166,167]. Moreover, changes in moisture content can also affect bulk density and flowability, thereby influencing dose uniformity and aerosol consistency [158].

From a stability perspective, moisture can induce recrystallization of amorphous components and accelerate chemical degradation, leading to reduced efficacy or altered bioavailability [168]. Excessive residual water should be minimized, as it not only increases the risk of microbial growth [169], but also enhances molecular mobility within the matrix. In lipid-based nanocarrier formulations, increased water content may also destabilize lipid bilayers, promoting structural changes and nanocarrier degradation [170,171].

Moisture content is typically measured using Karl Fischer titration or thermogravimetric analysis (TGA). To limit moisture uptake during storage, strategies such as desiccant incorporation, moisture-protective blister packaging and unit-dose systems are commonly employed [84]. Overall, although no strict regulatory threshold has been universally established, residual moisture is generally recommended to remain below 5% to ensure physicochemical stability and optimal aerosol performance [172].

IV.1.4. Shape and surface morphology

Particle morphology and surface characteristics are major determinants of aerosol performance and pulmonary deposition in DPI formulations, as they directly influence particle density, d_{ae} and interparticle interactions [115].

Surface roughness plays a major role in powder dispersion. Irregular or corrugated particles reduce contact area and cohesion, facilitating deagglomeration, whereas smooth particles promote stronger interactions and agglomeration. However, excessive roughness may lead to mechanical interlocking and hinder dispersion, highlighting the need for an optimal balance [173,174]. Surface texture also affects electrostatic behavior, with irregular surfaces promoting triboelectric charging due to increased interparticle collisions [175,176].

Beyond surface texture, overall particle morphology significantly influences aerodynamic performance and deposition behavior. Irregularly shaped particles tend to reduce interparticle contact and aggregation, whereas elongated or non-spherical particles can modify airflow interactions and aerodynamic drag, thereby affecting deposition patterns within the respiratory tract [35]. Morphology also affects particle and bulk density, as porous or corrugated particles typically exhibit lower density, which reduces their d_{ae} and can enhance lung deposition [177,178], although these particles

may be more challenging to handle and may compromise capsule-filling accuracy. In contrast, denser particles generally exhibit improved handling characteristics, although tighter control of their geometric size is required to achieve a d_{ae} suitable for efficient lung deposition [39].

Overall, precise control of particle morphology and surface is crucial for optimizing dispersion, aerodynamic behavior and lung deposition. These characteristics are commonly assessed using techniques such as scanning electron microscopy (SEM) or focused ion beam (FIB) imaging [179].

IV.1.5. Flowability

The flowability of inhalation powders is critical at multiple stages, from industrial manufacturing, such as capsule filling, to aerosolization during inhalation [180,181]. Powder flow behavior is strongly influenced by key physicochemical properties, including particle size, shape, surface morphology and moisture content, which can also be affected over time by excipient choice and environmental factors such as relative humidity (RH) and temperature [115].

As inhalable powders are typically highly cohesive due to their low micronized d_{ae} , they often exhibit poor and challenging flowability and carrier-based strategies remain the primary approach to improving powder flow in DPI development [84,182]. This challenge is thus further exacerbated in carrier-free formulations.

Powder flow can be assessed using several standardized methods. The Ph.Eur. 11.0 (monograph 2.9.36) recommends techniques such as the repose angle and Hausner ratio. Additional insights into powder flow can be obtained using specialized instruments, such as the Granupack™ (Granutools™, Awans, Belgium) for bulk and tapped density measurements and the Granudrum™ (Granutools™, Awans, Belgium) for assessing dynamic cohesion [183,184]. Together, these tools provide a more complete characterization of flow properties relevant to DPI performance.

IV.2. Carrier-based and carrier-free powders

As previously mentioned, effective pulmonary deposition depends on the d_{ae} , which must fall within the micron-sized range for deep lung delivery. However, particle size reduction introduces significant formulation challenges. As size decreases, the specific

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surface area increases, intensifying cohesive forces, particularly van der Waals interactions, that dominate at the micron scale [185]. As a result, micronized powders exhibit poor flowability and a strong tendency to agglomerate, compromising filling processes and aerosolization performance [173,181].

To overcome these limitations, the incorporation of carrier particles acting as diluents was introduced early in the development of DPI formulations (Figure 17) [186]. This approach typically involves blending the micronized API with a coarse carrier, most commonly lactose monohydrate, valued for its well-established safety profile, with particle sizes considerably larger than the API, usually ranging from 50 to 200 μm [84,185]. Carrier-based formulations remain the predominant approach for most commercially available DPIs [187], exemplified by widely prescribed products in the Belgian market, including Inuvair®, Novolizer®, Symbicort® and Airbufo® [142].

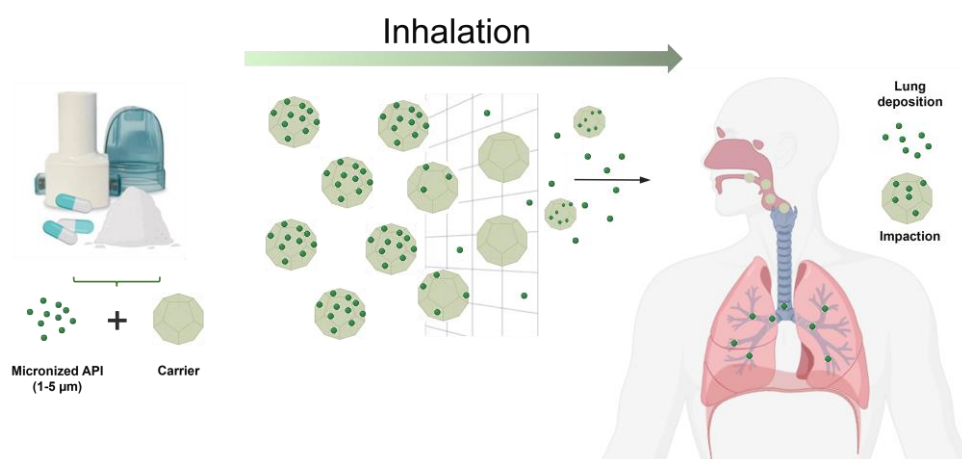


Figure 17: Schematic representation of a carrier-based dry powder inhaler (DPI) formulation, where micronized drug particles adhere to the surface of carrier particles and are released upon carrier impaction in the device and upper respiratory tract. Created with BioRender.

Their widespread use reflects the ability of carrier particles to facilitate the delivery of low API doses while improving bulk powder properties. Because the carrier constitutes the majority of the formulation, it enhances flowability, handling and dose reproducibility by reducing interparticulate cohesion and promoting a more homogeneous API distribution [188,189]. During inhalation, carriers promote dispersion of the micronized drug and facilitate API detachment under mechanical forces generated by the inhaler, such as turbulence, collision, shear and friction, while their large d_{ae} leads to impaction

either within the device grid or in the upper respiratory tract [84]. Efficient drug delivery depends on a balanced carrier-drug interaction: strong enough to maintain blend stability during handling, yet weak enough to allow effective release, an equilibrium that is inherently difficult to achieve [190].

Despite these advantages, carrier-API interactions and coarse particle impaction limit lung deposition, with typically only 20 to 30% of the dose reaching the peripheral lung [189,191]. Although optimization of carrier properties, including particle size adjustment, surface modification or the addition of fine carriers, can provide incremental improvements, these approaches remain inherently limited in enhancing deep lung delivery [192].

Building on these limitations, the development of carrier-free powders has been extensively explored to improve aerosolization efficiency and increase the fraction of API reaching the peripheral lung through advanced particle engineering techniques [40,182]. This strategy is particularly advantageous for sensitive systems such as liposomes, which are typically produced as aqueous dispersions and therefore require a dedicated drying step to obtain stable powders suitable for DPI administration.

As a result, particle engineering strategies not only enable the production of carrier-free systems but also provide precise control over particle characteristics. This versatility is reflected in the wide range of morphologies that can be achieved, particularly in carrier-free formulations where particles are directly formed from the API and excipients. By modulating formulation composition and process conditions, it is possible to generate structures such as hollow, porous, corrugated, or collapsed particles (Figure 18). These morphological features play a critical role in determining key functional attributes, including aerodynamic performance, dispersibility and payload stability. The different production methods employed in particle engineering are described in the following section (Section IV.4).

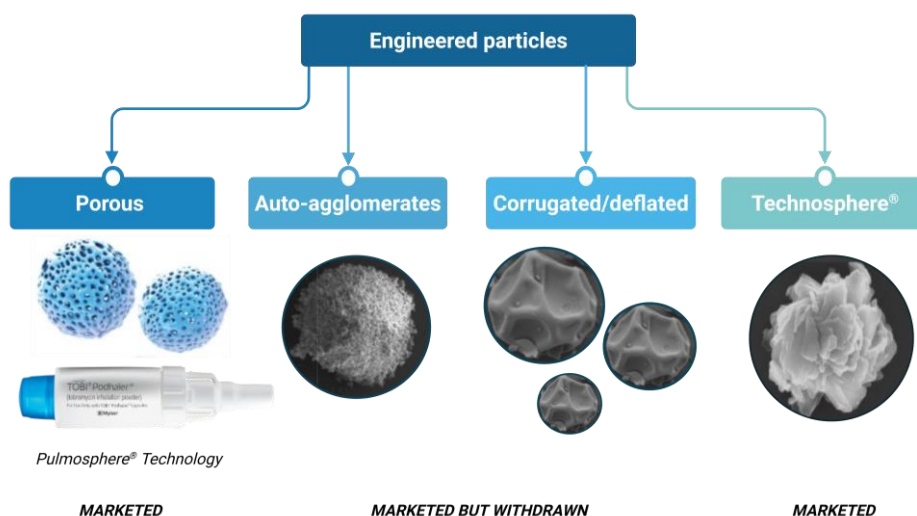


Figure 18: Representative particle morphologies obtained through particle engineering.

Porous particles

Porous particle formulations are of particular interest for DPIs because they combine a relatively large geometric size with low density, resulting in a favorable d_{ae} for deep lung deposition. This architecture reduces interparticle cohesion and agglomeration, improves aerosolization performance and may also decrease uptake by alveolar macrophages, thereby prolonging pulmonary residence time [35]. Porosity can be generated during spray-drying (SD) through the incorporation of porogenic agents, such as ammonium carbonate salts that decompose to create internal voids, or by using lipid-based excipients, especially phospholipids, capable of structuring low-density matrices [179,193].

PulmoSphere™ represents a well-known example of this strategy. These hollow, sponge-like particles are typically produced via emulsion followed by SD, where a dispersed oil phase acts as a blowing agent to form internal pores [84,182,194]. Phospholipids such as DSPC, a major component of endogenous lung surfactant with established safety for inhalation, are incorporated to stabilize the emulsion system, contribute to particle formation and enhance biocompatibility [61]. The resulting particles exhibit tunable size and density, low cohesion and high respirable fractions, making them effective carriers for pulmonary drug delivery. This technological approach has

been successfully translated into clinical practice, notably in the marketed TOBI® Podhaler®, which consists of porous tobramycin PulmoSphere™ particles [194,195].

Additionally, building on these porous carrier systems, the concept of Trojan particles has emerged as another promising strategy for the pulmonary delivery of lipid nanocarriers, including liposomes, representing a typical nano-in-micro approach. In this strategy, inhalable porous microparticles encapsulate NPs and release them after deposition in the lungs. By combining favorable aerodynamic behavior, low interparticle cohesion, rapid disintegration and efficient deep-lung deposition, Trojan particles enable NPs to reach their target site while overcoming limitations associated with their intrinsic nanoscale size, which would otherwise promote exhalation and poor pulmonary retention, as discussed previously [196–198].

Agglomerates or Pellets

Another carrier-free strategy involves formulating powders as spheroidized agglomerates or softpellets obtained by the self-agglomeration of the API, either alone or in combination with excipients. These loosely bound agglomerates disperse efficiently under the turbulent airflow of inhalation devices and break apart easily during inhalation, ensuring effective pulmonary delivery [199,200]. Pulmicort® Turbuhaler®, containing only BUD, is a classic example of this approach. Despite their advantages, softpellets remain relatively underexplored and their fragility contributed to the withdrawal of this product from the Belgian market [175].

Carrier-free self-agglomerating powders have been investigated using various manufacturing methods to control agglomerate formation while preserving effective deagglomeration during inhalation. These methods include vibration, agitation, powder rolling, air-jet mixing, resonant acoustic mixing and spheronization in tilted rotating containers [201]. Recently, self-inclined vibrating systems developed by Gresse *et al.* have shown the ability to generate flowable yet easily deagglomerating agglomerates for inhalation purposes [175].

Corrugated or deflated particles

Corrugated, wrinkled or deflated particles are spherical structures with surface folds that offer notable advantages for inhalation therapy. Unlike hollow porous particles, deflated particles do not exhibit an internal void but rather present a collapsed structure with a

corrugated exterior, which reduces interparticle cohesion, lowers density and promotes deeper lung deposition [40,178]. Their formation arises from formulation and process conditions that favor solvent evaporation over solute diffusion during drying. In particular, the selection of appropriate excipients, combined with parameters such as elevated drying temperatures, low feed flow rates, or the use of surface-active components capable of forming a hydrophobic shell, promotes early shell formation followed by particle collapse and the development of corrugated morphologies [40,202,203].

Amino acids such as L-leucine or trileucine are well known to induce surface wrinkling by precipitating at the droplet interface during drying, thereby forming a hydrophobic barrier that limits water diffusion and promotes particle folding [204]. Deflated structures can also be obtained using carbohydrates, particularly cyclodextrins. Dufour *et al.* reported that hydroxypropyl- β -cyclodextrin (HP β CD) has been employed to complex hydrophobic drugs prior to SD, notably corticosteroids such as BUD, resulting in improved aerosol performance [205]. By promoting corrugated morphologies, HP β CD can increase the respirable fraction of the active compound, illustrating the potential of such excipients to modulate particle architecture and optimize pulmonary delivery [177,181,202].

Pulmosol™ technology represents one of the earliest commercial applications of wrinkled particles for inhalation. In products such as Exubera®, the first FDA-approved inhaled insulin, formulations containing excipients including mannitol, glycine and sodium citrate were spray-dried under controlled conditions to produce highly soluble particles with wrinkled surfaces suitable for alveolar deposition [186,206]. Although this product was withdrawn from the market for commercial reasons, the underlying technology remains highly promising for the future development of carrier-free inhalable powders [207].

Technosphere®

In contrast to the previously described particle engineering strategies, which are mainly based on SD processes, freeze-drying (FD) can also be employed to design inhalable engineered particles with tailored properties. One notable example is the Technosphere® platform, a technology based on the formation of porous microparticles

composed of fumaryl diketopiperazine (FDKP), which self-assemble into crystalline structures ($\sim 2\ \mu\text{m}$) with a high specific surface area under acidic conditions. The drug-containing suspension is subsequently subjected to rapid freezing followed by FD to obtain a dry powder suitable for inhalation. Afrezza®, an FDA-approved inhaled insulin, is a clinically relevant example of this technology, in which insulin is adsorbed onto these microparticles. Upon administration, the particles rapidly dissolve at physiological pH, as FDKP becomes highly soluble above pH 6, enabling fast systemic absorption of insulin [208].

IV.3. Functional Excipients in DPI

In both carrier-based and carrier-free DPI formulations, excipient selection is critical, as excipients can represent a substantial fraction of the powder mass and strongly influence overall product performance [209]. Depending on their nature, excipients can modify powder properties or be selected deliberately to achieve specific aerosol, stability or delivery characteristics. They may enhance API stability and solubility, modulate release and pulmonary targeting, reduce mucociliary clearance and improve flowability, thereby supporting dose reproducibility and therapeutic performance [152].

Depending on their nature and properties, excipients can be classified into different functional categories (Figure 19).

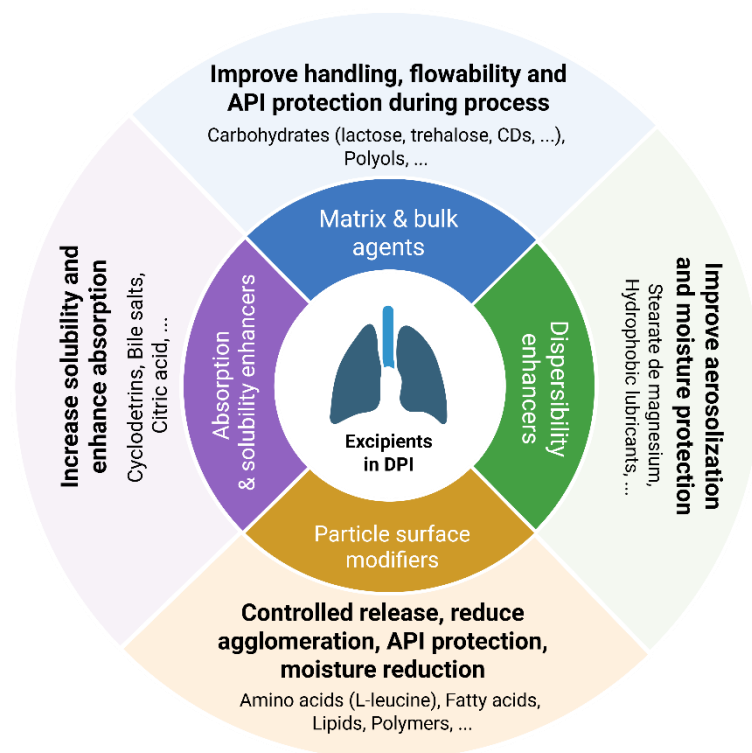


Figure 19: Overview of excipient roles in dry powder inhalers (DPIs) with selected examples.
Created with BioRender.

IV.3.1. Dispersibility and aerosolization enhancers

Dispersibility enhancers are incorporated into DPI formulations to reduce interparticle cohesion and improve aerosolization. By decreasing contact between micronized particles and modulating surface interactions, they enhance powder flow and facilitate drug detachment during inhalation, thereby increasing aerodynamic efficiency. These excipients are typically combined with the therapeutic agent and diluent through blending processes.

Common examples include magnesium stearate, a fatty acid salt and well-established force-control agent in DPI formulations, used in marketed products such as Relvar® Ellipta and Ultibro® Breezhaler [192,210]. It reduces particle adhesion, acts as a moisture barrier and improves flow and dose reproducibility, particularly under humid conditions. Other dispersibility enhancers include sodium stearate, hydrophobic lubricants (sucrose tristearate) or fine lactose particles [211].

IV.3.2. Particle surface modifiers and coating agents

Surface modifiers and coating agents are widely used in DPI formulations to enhance powder dispersibility, reduce moisture uptake and improve aerodynamic performance. Hydrophobic amino acids, particularly L-leucine, are the most employed surface modifiers [212,213]. Due to its low solubility and surface-active properties, L-leucine tends to migrate to the particle surface during the SD process, forming a protective hydrophobic layer that limits cohesion, stabilizes amorphous powders and protects them from moisture-induced agglomeration [214,215].

Other coating agents, including fatty acids, lipids (e.g., DPPC, DSPC, CHOL) and biodegradable polymers (natural like alginates, chitosan and synthetic such as polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA)), can similarly modulate interfacial properties, reduce agglomeration, protect sensitive or hygroscopic drugs and enable controlled-release strategies [84,156,216].

IV.3.3. Absorption and solubility enhancers

Absorption and solubility enhancers are used to improve pulmonary epithelial uptake of poorly water-soluble drugs, such as many corticosteroids employed in asthma and COPD. Cyclodextrins, cyclic oligosaccharides with a hydrophobic cavity, form inclusion complexes with hydrophobic APIs, enhancing aqueous solubility, dissolution rate and pulmonary residence time [216]. Cyclodextrins, such as HP β CD and hydroxypropyl- γ -cyclodextrin (HP γ CD), are considered the safest options, enabling effective delivery at reduced doses while sometimes exerting beneficial effects on lung inflammation and hyperresponsiveness [84,205]. Other absorption enhancers, including bile salts, unsaturated fatty acids, citric acid and chitosan, can increase epithelial permeability but must be used at low concentrations or in carefully controlled formulations to limit local toxicity and inflammation [217].

IV.3.4. Carbohydrate matrix formers, stabilizers and carriers

Cyclodextrins, along with other carbohydrates such as lactose, trehalose and polyols like mannitol, are also widely used in DPI formulations as bulk agents and protective matrices [40,153]. They provide structural support, facilitate handling and ensure accurate and reproducible dosing, which is critical for drugs administered at low doses

(μg range), such as in asthma and COPD [218,219]. Additionally, the use of sugars as bulk agents can enhance patient compliance, providing a recognizable taste or sensation upon inhalation [152].

As stabilizing excipients, these carbohydrates protect sensitive APIs, including amorphous drugs and biologics, during processing and storage. Non-reducing sugars, such as trehalose, are generally preferred for labile molecules as they limit chemical interactions, whereas reducing sugars like lactose must be carefully evaluated due to their potential to undergo Maillard reactions with amine groups present in certain APIs or commonly found in biopharmaceuticals [40,220]. The choice of bulk agent also influences the final powder's physical properties, including amorphous versus crystalline structure, density, porosity, surface roughness and overall stability over time.

IV.3.5. Regulatory aspects of excipients for pulmonary delivery

Despite the wide range of excipients investigated for inhalation purposes, their use remains strongly constrained and is further complicated by the lack of full international harmonization [84,156]. Candidates are largely confined to biocompatible compounds that are endogenous to the lung or readily metabolized and cleared and generally recognized as safe (GRAS) [221]. However, only a small number of these compounds have been authorized for inhalation by the EMA and FDA [14,84]. Regulatory complexity is further increased by dosage-form specificity, since excipients acceptable for liquid inhalation systems are often unsuitable for DPIs due to their impact on powder processing and stability.

Among commonly investigated excipients, only a limited number have been approved for inhalation and have established use in marketed DPI products, including carbohydrates such as lactose and mannitol, as well as dispersibility enhancers such as magnesium stearate [14,222]. For instance, trehalose and cyclodextrins such as HP β CD have demonstrated favorable pulmonary tolerability [156,223,224], and amino acids such as leucine are considered low risk, although most are not yet formally approved for inhalation use [84].

The constraints are even more pronounced for lipid-based pulmonary systems, such as liposome-based DPI considered in this work. Although many lipids are approved for parenteral use, only a limited number, mainly endogenous phospholipids such as DPPC

and DSPC along with CHOL, have recognized inhalation status due to their similarity to lung surfactant [61,225]. Their use is exemplified by liposomal formulations such as Arikayce®, composed of DPPC and CHOL, while DSPC is also employed in the PulmoSphere™ technologies [226,227]. In contrast, other lipid components, including PEGylated lipids, such as distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀), remain unapproved for inhalation despite favorable preclinical safety data and their use in parenteral products [228].

Overall, the limited number of excipients approved for pulmonary use, together with regulatory uncertainties, remains a key challenge in formulation development, while also driving ongoing research and innovation in the field.

IV.4. Production methods for DPIs

Beyond excipient selection, the manufacturing process also plays a critical role in defining the physicochemical properties and performance of inhalable powders. Particle engineering techniques can be broadly classified into top-down and bottom-up approaches. Top-down methods involve the size reduction of bulk materials through mechanical processes, such as ball milling and jet milling. In contrast, bottom-up approaches rely on the controlled formation of particles from liquid systems, enabling precise tuning of particle properties during the transition to dry powders [158]. In the context of carrier-free inhalable systems, bottom-up techniques are particularly advantageous, as they allow optimization of aerodynamic performance while preserving the stability of sensitive systems, such as liposomes. Key methods include SD, spray-freeze drying (SFD), FD and SCF technologies, which are increasingly investigated for the development of liposome-based DPIs.

IV.4.1. *Supercritical fluid technology*

The theory regarding the use of SCFs, as previously described in Section III.1.2 for liposome production, can also be applied to powder production, with generation of inhalable size particles.

As for liposome production, SCF technologies, most commonly using scCO₂, can play different roles depending on the process, acting as a solvent, dispersing agent, co-solvent or anti-solvent. For example, as a dispersing agent, the SCF is mixed with the

formulation and then rapidly expanded, leading to particle precipitation or dispersion, such as in the PGSS process. In anti-solvent approaches, the active material is first dissolved in an organic solvent and the SCF induces precipitation because of its poor solubilizing power for the solute [229,230].

These techniques are particularly attractive for pulmonary drug delivery because they allow fine control of particle size and morphology, improving aerosol performance [35]. For example, scCO₂ has been used to produce respirable terbutaline sulfate particles with improved dispersion and FPF [231], along with development of a carrier-free BUD DPI showing comparable results to the Pulmicort® drug product [232]. Interestingly, for liposome-based inhalation systems, Costa *et al.* demonstrated that SCF-assisted SD enables the production of liposome-based powders with preserved structural integrity and suitable aerosol performance [233]. Although SCF technologies have attracted interest, they remain less widely applied to DPI manufacturing than conventional approaches, with methods such as FD still being more commonly used for liposomal formulations intended for inhalation.

IV.4.2. *Freeze-drying*

FD, also known as lyophilization, is a low-temperature, low-pressure solvent-removal process that generates a dry matrix through three sequential stages: freezing, primary drying and secondary drying.

During freezing, the formulation is cooled from ambient to subzero temperatures (typically -20 to -80 °C), promoting solidification and separation of solvent from solutes. Primary drying is the longest and most energy-demanding stage, during which most of the frozen water is removed. In this step, under reduced pressure (approximately 0.03 to 1 mbar, below the triple point of water), ice undergoes sublimation, with gentle heat applied to provide the energy required for this phase change. Secondary drying removes residual water by desorption, with shelf temperature controlling the rate, until low moisture levels (typically 0.5-1%) are achieved for long-term stability [234,235]. The cycle concludes with the chamber being returned to atmospheric pressure and ambient temperature, after which the dried samples are collected, typically forming a porous matrix that facilitates reconstitution while preserving the product's physicochemical stability [236]. The FD process is represented in Figure 20.

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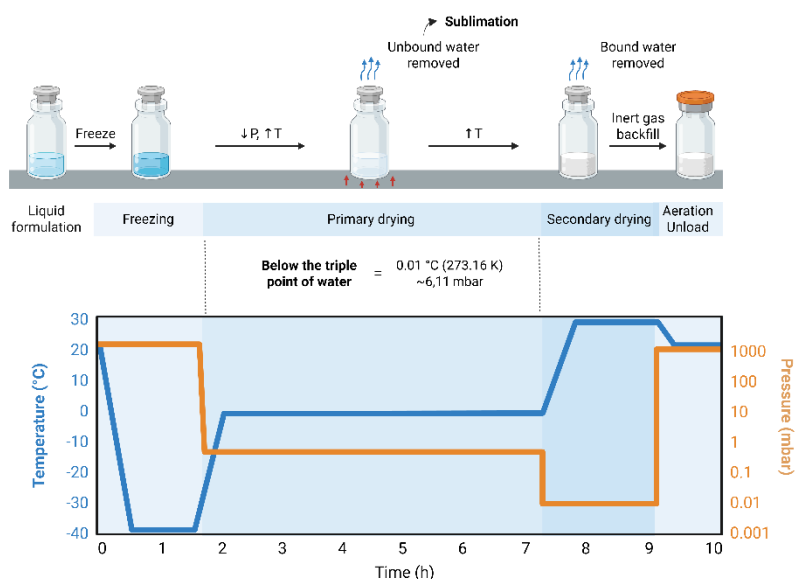


Figure 20: Schematic overview of the freeze-drying (lyophilization) process. Created with BioRender.

However, the stability of the final product, particularly for liposomes or other sensitive actives, can be compromised at each stage of the process due to specific stresses. Freezing can form ice crystals that disrupt lipid membranes or cause aggregation, while incomplete primary drying may leave residual moisture and excessive heat during secondary drying can result in structural collapse of the dried matrix [50,237]. To mitigate these effects, cryoprotectants, commonly sucrose or trehalose, are added to stabilize the formulation and support redispersion [238]. Along with careful excipient selection, control of process parameters such as chamber pressure, shelf temperature, freezing rate, and heat transfer positions FD as a particle engineering strategy [239].

FD is widely used to stabilize pharmaceuticals, biopharmaceuticals and nanomedicines that are unstable in liquid form, including biologics such as monoclonal antibodies and vaccines [35,234]. In some cases, FD is also used to enhance the solubility and bioavailability of poorly soluble drugs through amorphous solid dispersions (ASDs), as exemplified by the BCS Class II drug itraconazole [240]. Moreover, a clinically relevant illustration, as previously exemplified, is Afrezza®, an FDA-approved inhaled insulin based on the Technosphere® technology [208].

Despite several advantages, such as being well suited for heat-sensitive products and extensively documented for drying lipid nanocarriers, including liposomes, conventional FD is generally not ideal for optimized DPI production. FD typically yields irregular lyophilized cakes with poor flowability [241] and offers limited control over particle size compared with other particle engineering techniques, compromising aerosolization performance [242]. Furthermore, FD is a time and energy-intensive process, often requiring drying cycles of several days [243], which complicates large-scale manufacturing, while the resulting products commonly require refrigerated storage [244]. Consequently, its application for direct DPI production remains limited. Nevertheless, recent studies by Valle *et al.* and Vanza *et al.* demonstrated the feasibility of producing DPIs from lyophilized liposomes, including sildenafil and afatinib-loaded formulations, with satisfactory aerosolization performance and preserved liposomal integrity [137,245].

However, conventional FD still presents important limitations for the development of optimized inhalable formulations, which has driven the growing interest in alternative approaches such as SFD or SD.

IV.4.3. *Spray-freeze drying*

Combining features of both FD and SD, SFD enables powder production through three successive steps: spray atomization, rapid cryogenic freezing and sublimation drying [216]. In this process, a liquid formulation containing the therapeutic and excipients is atomized into fine droplets using hydraulic, pneumatic, ultrasonic or piezoelectric nozzles. These droplets undergo ultra-fast freezing upon contact with a cryogenic medium, either by direct immersion or deposition onto a cold surface, which promotes amorphous embedding of the API, limits component segregation during solidification and preserves thermolabile compounds. The frozen droplets are subsequently lyophilized under vacuum, during which solvent sublimation generates highly porous, low-density particles with large surface area [246,247]. The overall process is schematically illustrated in Figure 21.

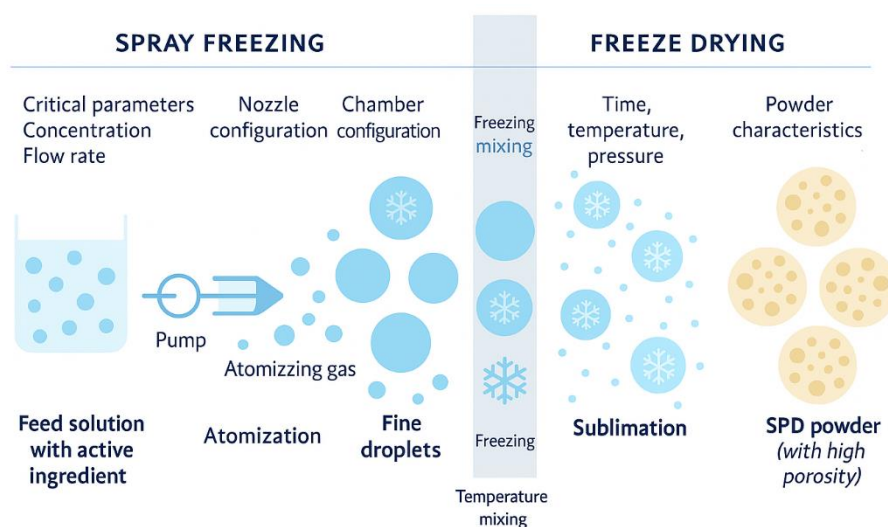


Figure 21: Illustration of the different steps involved in the spray-freeze-drying (SFD) process. Adapted from [241], with assistance from Copilot.

Overall, SFD integrates the advantages of SD, such as size control, with those of FD, including thermal protection and improved preservation of labile substances, while potentially reducing drying time and energy consumption compared with conventional bulk lyophilization [216]. The resulting powders have been shown to enhance the apparent solubility of poorly water-soluble drugs, stabilize biologics and vaccines and provide favorable aerodynamic properties (high porosity, low density and high FPF), making them well suited for pulmonary delivery [241,248]. However, in the context of inhalable liposome-based systems, only a few studies to date have investigated the application of SFD to produce liposome-based DPIs. Among these, Ye *et al.* reported that clarithromycin-loaded liposome-based powders obtained by SFD exhibited high drug recovery, efficient liposome reconstitution, good aerosolization performance and stability under ambient conditions [249].

Although SFD has produced encouraging results, its application, both for inhalable liposome-based formulations and for other DPI systems, remains largely restricted to early-stage research. This is mainly due to process complexity, limited industrial maturity and persistent scalability challenges [250]. Consequently, SD remains a particularly attractive and industrially relevant particle engineering method for producing inhalable liposome-based powders.

IV.4.4. Spray-drying

SD is one of the most widely used techniques for producing dry powders. Initially established in the food industry, with markedly expanded use during World War II for milk powder production, the technology was subsequently expanded in the pharmaceutical field [251]. Its application to pulmonary delivery emerged in the 1980s with the development of fine respirable particles for inhalation [252]. Beyond converting liquid formulations into particles, it enables the preparation of ASDs to enhance the solubility of poorly water-soluble drugs and the encapsulation of actives within biodegradable matrices for controlled release [253,254]. SD is widely regarded as a rapid, continuous, cost-effective, scalable and reproducible manufacturing technique that also enables precise particle engineering [255,256]. Furthermore, its energy requirements have been reported to be substantially lower than those of FD, with an estimated total energy consumption of approximately 1.88 kWh compared with 22.38 kWh for FD under comparable conditions [257]. These advantages make SD a versatile platform for developing inhalable formulations.

The rapid solvent evaporation and short thermal exposure contribute to the preservation of heat-sensitive compounds, while high encapsulation efficiencies allow the production of pure drug particles or drug-loaded nano and microparticles with narrow and controllable size distributions [258,259]. When appropriately optimized, the resulting powders often exhibit favorable flow properties and enhanced dissolution performance, particularly in the case of amorphous systems [260,261]. Importantly, the process can be readily translated from laboratory to industrial scale with high material recovery, further supporting its widespread use in pharmaceutical manufacturing [255].

The process involves the transformation of a liquid feed, such as a solution, suspension, or emulsion containing the API and excipients, into a dry powder within a continuous operation. This transformation occurs through three main stages: atomization and droplet formation, rapid solvent evaporation during drying and subsequent powder recovery. A typical SD system therefore comprises several key components, including a feed pump, an atomization unit, a drying chamber, a gas-particle separation device (commonly a cyclone) and a powder collector [252,262]. A schematic representation of the spray-dryer setup and the different processing stages is shown in Figure 22.

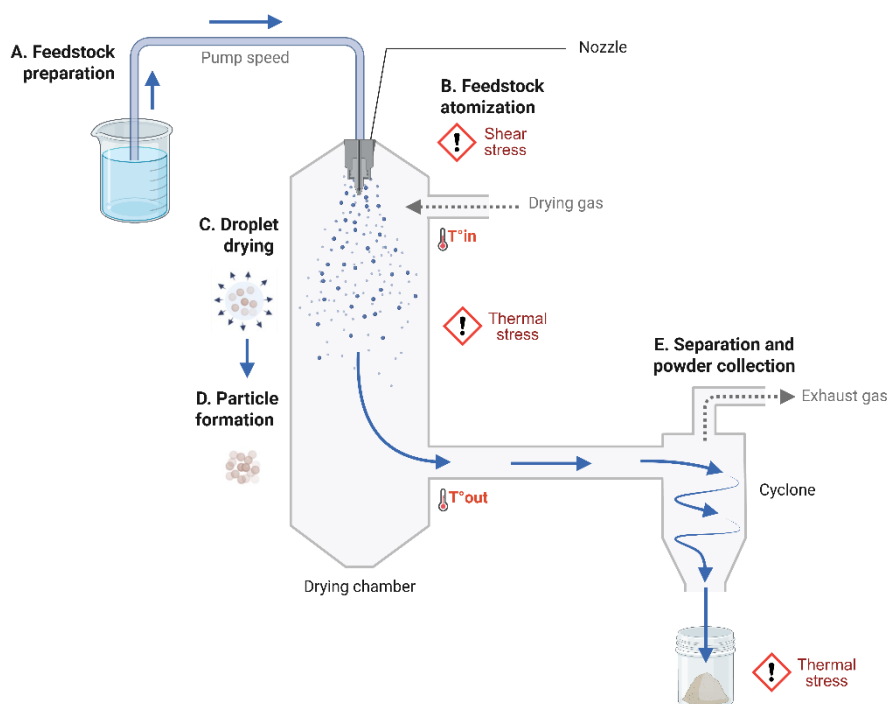


Figure 22: Schematic representation of the spray-drying process: (A) feedstock preparation and transfer to the atomization nozzle, (B) feedstock atomization, (C) droplet drying, (D) particle formation and (E) particle separation and powder collection. Created by BioRender.

IV.4.4.1. Atomization and droplet formation

Atomization constitutes the initial and critical step of the SD process, during which the liquid feed is converted into a fine mist of droplets. The formulation is continuously delivered to the atomizer at a controlled flow rate and dispersed through kinetic, centrifugal, pressure, or acoustic forces, depending on the nozzle design [263]. This step largely determines droplet size distribution and, by generating droplets with a high surface-to-volume ratio, promotes efficient heat and mass transfer during subsequent drying.

The mechanisms governing droplet formation vary according to the atomization technology employed. Rotary atomizers rely on centrifugal forces generated by a high-speed rotating disk or wheel, producing relatively narrow droplet size distributions that can be adjusted through rotational speed and geometry. The single-fluid nozzle, or pressure nozzle, forces the solution through a small orifice under high pressure, thereby

converting pressure energy into kinetic energy to promote droplet formation. Ultrasonic atomizers employ piezoelectric vibrations to generate capillary waves on a liquid film, enabling droplet formation and are therefore recommended primarily for non-thermally sensitive systems because of potential heat generation. Finally, in bi-fluid (air-assisted) nozzles, a high-velocity compressed gas stream shears the liquid feed, generating interfacial instabilities that promote droplet breakup, with smaller droplets obtained at higher gas-to-liquid mass ratios [179,258,264]. Among the available atomization technologies, these nozzles are the most widely used in the pharmaceutical field, particularly at laboratory scale, owing to their versatility. Their tolerance to varying viscosities, independent control of liquid and gas flow rates and suitability for producing fine inhalable droplets in small-batch conditions make them particularly advantageous for pharmaceutical applications [251]. Typically, these atomization systems generate droplets in the range of 10-100 μm , which generally results in dried particle sizes of approximately 0.5-50 μm , depending on formulation composition and drying conditions [179].

IV.4.4.2. Droplet drying kinetics

Once generated, the droplets enter the drying chamber, where they come into contact with a heated drying gas, typically air or nitrogen [265]. The relative direction of the droplets and the drying gas defines the flow configuration, which may be co-current, counter-current or mixed-flow (Figure 23).

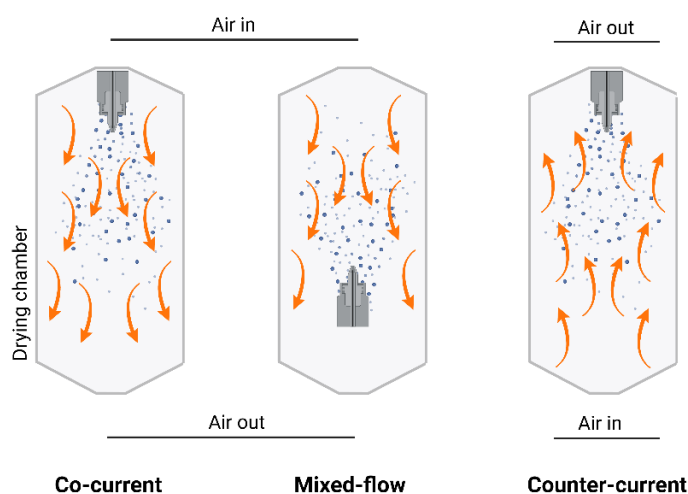


Figure 23: Schematic representation of different drying chamber configurations: co-current, mixed-flow and counter-current. Created with BioRender and inspired by [179].

In co-current systems, both droplets and drying gas flow in the same direction, from the top to the bottom of the chamber. Droplets encounter the hot gas immediately upon entry, promoting rapid solvent evaporation while minimizing thermal stress, which is particularly advantageous for heat-sensitive materials such as biomolecules. In contrast, counter-current systems feature droplets descending against an upward flow of gas, resulting in more efficient heat transfer but exposing dried particles to higher temperatures and increasing the risk of thermal degradation. Mixed-flow systems combine elements of both configurations: the gas flows from top to bottom, as in co-current systems, but the atomization nozzle is positioned in the middle of the chamber, producing a hybrid flow pattern [179,262,266].

A temperature gradient is established along the height of the drying chamber, characterized by the inlet temperature (T_{in}) at the top and the outlet temperature (T_{out}) at the bottom, as represented in Figure 22. T_{in} corresponds to the temperature of the drying gas upon entry and primarily determines the thermal driving force for solvent evaporation immediately after atomization, whereas T_{out} reflects the overall thermal exposure experienced by the droplets during drying [267,268]. Beyond thermal conditions, the chamber geometry also plays an important role: its dimensions, particularly height, determine droplet residence time. Longer drying chambers enhance solvent evaporation by increasing residence time but also alter flow dynamics and

droplet trajectories, potentially increasing wall deposition and reducing yield, thus making chamber dimensions a parameter to optimize [251].

Following the characterization of the drying chamber configuration and temperature conditions, attention can be focused on the drying kinetics of individual droplets, which are governed by coupled heat and mass transfer phenomena and proceed through successive stages (Figure 24). After atomization, droplets encounter the hot gas stream, initiating rapid heat transfer and solvent evaporation. The initial phase corresponds to the constant-rate drying period, during which evaporation occurs predominantly at the droplet surface. This process induces droplet shrinkage while liquid from the interior migrates outward to replenish the evaporated solvent. Because the droplet remains largely liquid at this stage, most of the supplied thermal energy is consumed by evaporation rather than by increasing droplet temperature. Consequently, the droplet temperature remains relatively stable, which helps preserve heat-sensitive components such as biologics or lipid-based nanocarriers [258,266,269].

As drying progresses, solvent loss leads to an increase in solute concentration and viscosity, eventually causing the formation of a solid or semi-solid shell at the droplet surface. This transition marks the onset of the falling-rate drying period, in which evaporation becomes limited by internal mass transfer. Moisture must then diffuse through the increasingly rigid particle matrix, causing the drying rate to decrease while the particle temperature gradually approaches that of the surrounding gas. During this stage, the particle typically consists of a porous outer shell surrounding a progressively shrinking wet core until complete solidification is achieved [266,269].

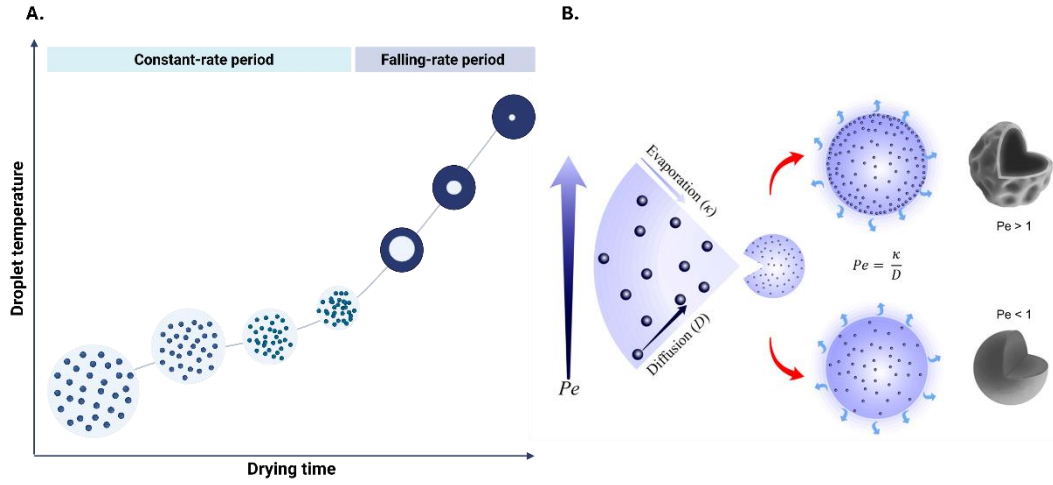


Figure 24: (A) Drying kinetics of droplets during spray-drying. Inspired by [40]. (B) Illustration of the Péclet number concept: as the Péclet number increases, the radial contraction of spray droplets occurs more rapidly compared with the inward diffusion of solute molecules, promoting solute accumulation at the droplet surface. Modified from [270].

The evolution of solute concentration during drying generates concentration gradients between the surface and the droplet interior, which play a critical role in determining the final particle structure. The duration and relative importance of each drying stage depend on both formulation properties, such as viscosity, solid content (SC) and solvent volatility and process parameters, including T_{in} , gas velocity and initial droplet size. The competition between evaporation kinetics and internal solute diffusion is commonly represented by the dimensionless Péclet number (Pe), given by the following equation (Equation 2):

$$Pe = \frac{\kappa}{D} = \frac{\text{Evaporation rate}}{\text{Diffusion rate}}$$

Equation 2: Péclet number.

When solvent evaporation proceeds more rapidly than solute diffusion, the resulting high Pe ($Pe > 1$) promotes solute accumulation at the droplet surface, leading to the formation of hollow, porous, or corrugated particles. Conversely, lower Pe ($Pe < 1$), associated with faster internal diffusion relative to evaporation, generally produce more homogeneous and dense particle structures. Overall, the Pe number is influenced by both formulation composition and processing conditions [40,251,269].

Beyond this diffusion-evaporation balance, the mechanical properties of the shell formed during drying further influence particle morphology. Rigid shells may fracture or develop internal voids as a result of vapor pressure buildup during continued solvent removal, whereas more flexible shells tend to collapse inward, yielding denser particles. Consequently, the final particle density does not depend solely on the Pe itself but also on post-formation shell deformation phenomena, such as bending or folding [271].

IV.4.4.3. Powder collection

After drying, the resulting particles are transported by the gas stream to a separation unit, most commonly a cyclone separator. Within the cyclone, centrifugal forces generated by the swirling gas flow separate the solid particles from the gas phase, allowing the powder to be collected in a dedicated container while the exhaust gas exits the system [272,273]. The collected powder can then undergo further processing or characterization as required.

In addition to other components of the spray dryer, the design of the cyclone separator, regarding its dimensions, can be optimized to enhance particle recovery and increase the overall yield of the drying process, with narrower cyclones shown to improve the efficient collection of micrometer-sized powders [273,274].

IV.4.4.4. Particle engineering of spray-dried particles

SD is a versatile technique for engineering DPI particles, enabling precise control of critical attributes at industrial scale. Powder properties arise from the combined effects of dryer configuration, as described in the previous section, together with feed composition and process parameters (Figure 25), making their understanding essential for the rational design of inhalable drug delivery systems [46].

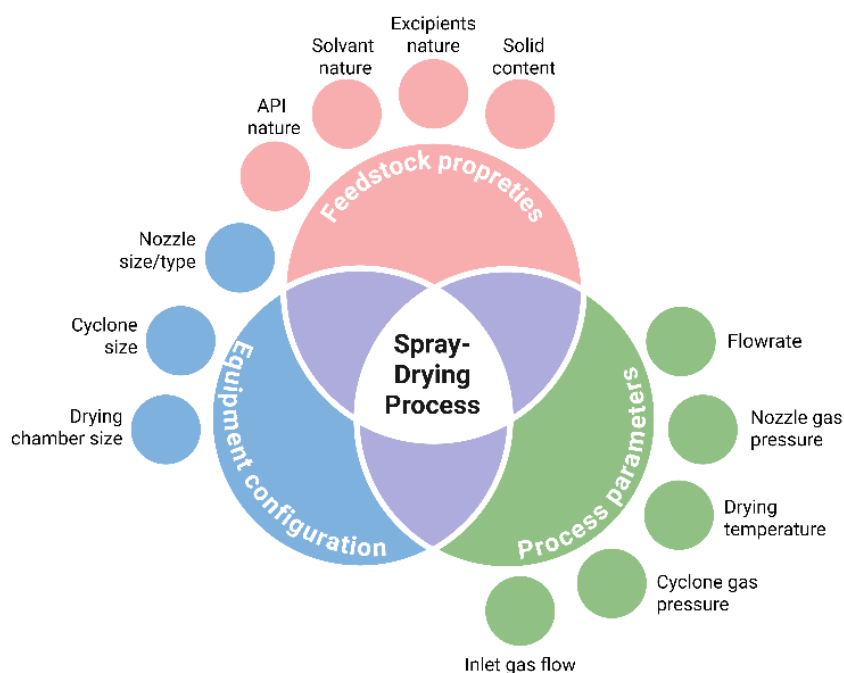


Figure 25: Overview of the parameters affecting powder properties after spray-drying. Created with BioRender.

Influence of the atomized liquid composition:

The SC of the atomized feed, defined as the mass fraction of dissolved or suspended solids, together with the physical state of the feed (solution, suspension, or emulsion), strongly influences powder properties. These factors modulate feed viscosity, which in turn affect droplet formation, drying behavior, particle size and residual moisture [275]. Excessive viscosity may also reduce atomization efficiency and increase the risk of nozzle blockage, especially when small nozzle orifices are used, highlighting the importance of careful feed preparation [251,276].

In addition, excipient properties further shape powder behavior and stability, particularly in carrier-free DPI formulations. Key factors include the glass transition temperature (T_g), defined as the temperature at which an amorphous material transitions from a brittle glassy state to a rubbery state, as well as molecular weight and hygroscopicity [40,168]. These physicochemical properties also influence solute diffusion during drying through their effect on the Pe , which governs particle morphology [177,202].

In the context of liposomal formulations, excipients are particularly critical, as they contribute to matrix vitrification, control residual moisture, and help preserve liposomal integrity during drying and storage [46,170].

As detailed in Section IV.3, a wide range of excipients can be selected based on their physicochemical properties to modulate particle morphology, cohesion and aerosol performance.

Influence of the SD parameters:

Regarding the drying process, key controllable SD parameters include feed flow rate, nozzle gas pressure (NGP), nozzle orifice diameter, drying temperature and cyclone gas pressure (CGP).

Feed flow rate governs the speed at which liquid reaches the nozzle, impacting atomization efficiency, droplet size and residence time in the drying chamber and overall drying performance [277]. It must therefore be optimized to balance efficient drying while maintaining industrial throughput [278,279].

NGP governs atomization shear forces and primarily controls droplet size, thereby determining particle size distribution and aerosol performance. Higher NGP is generally associated with the formation of smaller, more uniform droplets and faster drying, although excessively high NGP may induce mechanical and shear stresses that compromise lipid bilayer integrity and therefore require careful consideration [70,280]. The nozzle orifice diameter also influences droplet formation, as part of the overall nozzle design governing atomization efficiency and mechanical stress [84].

Another critical parameter for liposomal stability during drying is temperature. T_{in} governs evaporation kinetics, while T_{out} reflects droplet thermal exposure. Increasing T_{in} may improve drying efficiency but also raises T_{out} , which can affect liposome stability. Precise control of the temperature window is therefore required to balance drying performance and liposome integrity [279,280].

Finally, CGP also contributes to drying performance by modulating particle separation and influencing powder collection and overall yield [273].

Overall, SD parameters are highly interdependent, and their combined effects govern particle properties and liposome stability. Understanding these interactions is essential for the rational design of stable, inhalable powders with preserved liposomal integrity.

IV.4.4.5. Spray-drying of liposomes for pulmonary delivery

Liposomal formulations have been processed into dry powders using several particle engineering approaches (Section IV.4). Among these, SD has emerged as a promising technique to produce inhalable liposomal systems, owing to its ability to generate respirable particles with controlled physicochemical properties and favorable deposition profiles [40,50,179]. From a technological standpoint, the use of SD for liposomal systems is particularly challenging, as the lipid bilayer structure is highly sensitive to the shear, thermal and dehydration stresses encountered during atomization and drying. These stresses can compromise membrane integrity and reduce encapsulation efficiency [170], inducing vesicle fusion, aggregation or structural disruption and ultimately leading to cargo leakage. In addition, elevated temperatures may further promote lipid degradation processes, including phospholipid oxidation and hydrolysis [280–282].

In this context, the application of SD to liposomes remains at an early stage of development and has only been investigated in a limited number of studies. The existing literature is predominantly based on proof-of-concept approaches, often relying on simplified or model liposomal systems in the absence of encapsulated APIs. As a result, the generalizability of these findings to clinically relevant, drug-loaded formulations remains uncertain, and systematic investigations addressing the impact of SD on API stability, encapsulation retention and therapeutic performance are still lacking.

Nevertheless, although limited in scope, available studies demonstrate that liposome-based DPIs can be successfully produced by SD when appropriate formulation strategies are implemented. Early work by Goldbach *et al.* showed that multilamellar SPC liposomes spray-dried with lactose retained vesicle size and phospholipid integrity upon reconstitution [283], whereas subsequent studies by Wessman *et al.* highlighted the risk of vesicle shrinkage or lamellar transitions in the absence of adequate stabilization [284].

To address these challenges, stabilizing excipients have been employed. Carbohydrates such as lactose, sucrose, mannitol and trehalose have been shown to protect lipid bilayers during drying and preserve key physicochemical properties after redispersion, including particle size, encapsulation efficiency and cargo stability [272,285]. Additionally, cyclodextrins such as HP β CD have been shown to protect PEGylated liposomal membranes, preventing bilayer disruption and cargo leakage [286]. Similarly, trehalose, alone or combined with L-leucine, has been shown to improve product yield and enable the production of stable, redispersible liposomal powders while preserving vesicle integrity and aerodynamic properties suitable for deep lung deposition [170,287]. Khatib *et al.* also demonstrated that optimized SD conditions can help maintain liposome performance, resulting in high drug entrapment efficiency and controlled release profiles suitable for inhalation powder formulations [288].

In addition to formulation strategies, intrinsic lipid properties also contribute to the final powder characteristics. Pilcer *et al.* reported that variations in phospholipid type and Tg affect particle morphology and d_{ae} , with higher Tg generally associated with lower MMAD values and improved stability [84]. Furthermore, liposomal PEGylation has been shown to facilitate SD by maintaining bilayer membrane structure while supporting favorable aerosol performance [114].

Overall, although less extensively investigated than FD, available studies indicate that SD holds significant potential for the production of inhalable liposome-based powders. However, the field remains at an early stage of development, with existing studies primarily focused on formulation feasibility rather than on clinically relevant, drug-loaded systems. Consequently, a comprehensive mechanistic understanding of liposome behavior during drying is still lacking, highlighting the need for further systematic investigations on clinically relevant formulations to support the rational development of liposome-based DPIs.

V. Clinical context and therapeutic relevance of liposome-based DPIs in asthma and COPD

Although liposomal drug delivery systems have been increasingly investigated for a broad range of pulmonary diseases, including bacterial and fungal infections, lung cancer and inflammatory or immune-related disorders, their design and therapeutic

performance can be tailored according to the targeted pathology, as previously illustrated (Section III.2). In the present work, particular emphasis is placed on the relevance of liposomes, and in particular liposome-based DPI formulations, for the treatment of asthma and COPD.

Asthma and COPD represent the two most prevalent chronic inflammatory airway disorders and account for the majority of patients requiring long-term inhalation therapy [289]. Understanding their pathophysiology, current pharmacological management and the limitations of existing inhaled therapies provide a strong rationale for the development of advanced lipid-based DPI systems capable of improving drug deposition, stability, and therapeutic efficacy. The following section therefore outlines the key features of these diseases in order to clarify the clinical motivation behind the formulation strategies developed in this thesis.

V.1. Pathophysiology of the lung

As mentioned earlier, despite their defense mechanisms, the lungs have an open architecture and are in continuous contact with inhaled pathogens, environmental particles, allergens and pollutants, making them particularly susceptible to viral or bacterial infections and other lung disorders. Furthermore, dysregulated or excessive activation of these defense systems may contribute to lung pathology, as observed in lung cancer and, most commonly, in chronic inflammatory diseases [24]. Among these, asthma and COPD represent the most prevalent and well-characterized conditions [25].

V.1.1. *Asthma*

Asthma is a chronic, heterogeneous respiratory disorder marked by chronic airway inflammation, bronchial hyper-responsiveness and variable airflow obstruction that produces symptoms such as wheezing, cough, chest tightness and dyspnoea [290]. Asthma development and exacerbation are influenced by a combination of environmental exposures, including allergens as well as non-allergic factors such as air pollutants, respiratory infections and tobacco smoke, in addition to genetic predisposition involving multiple susceptibility genes [291,292]. Disease severity and control are further modulated by comorbidities such as obesity, gastro-oesophageal reflux disease and allergic rhinitis, while demographic factors including age and sex contribute to differences in asthma phenotypes and prevalence [293].

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Asthma can also be classified according to its underlying inflammatory endotypes, most commonly eosinophilic and neutrophilic asthma. Eosinophilic asthma represents the most prevalent endotype and is typically associated with type 2 immune responses involving Th2 lymphocytes and eosinophil recruitment to the airways (Figure 26A). This inflammatory profile contributes to inflammation, mucus overproduction and bronchial hyperresponsiveness, which underlie the characteristic symptoms of asthma (Figure 26B) [27,294].

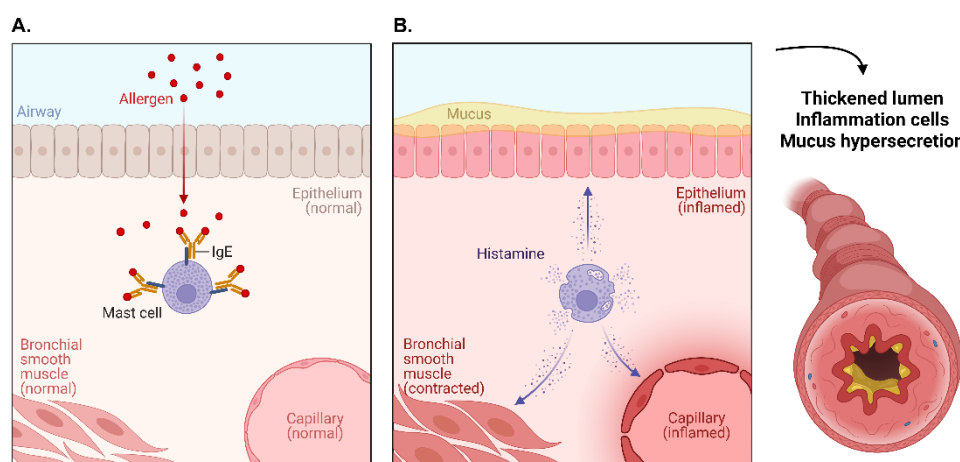


Figure 26: Simplified overview of the asthma response: (A) Allergens enter via inhalation and bind to IgE on mast cells. (B) Mast cells are activated, leading to increased mucus production, inflammation and contraction of smooth bronchial muscle. Created with BioRender.

In contrast, neutrophilic asthma is less common and is often associated with non-allergic triggers. It is characterized by increased neutrophil infiltration in the airways and is thought to involve alternative immune pathways, particularly Th1 and Th17 responses, which promote neutrophil recruitment and contribute to persistent airway inflammation [27,295].

V.1.2. Chronic obstructive pulmonary disease

COPD shares several pathophysiological features with asthma, including airway inflammation, mucus hypersecretion and narrowing of the airways while exhibiting a higher prevalence of chronic cough. However, these two diseases differ primarily in their triggering factors, the involvement of the lung tissue and the reversibility of airflow limitation. Indeed, COPD is mainly caused by tobacco smoking and biomass exposure

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and is characterized by irreversible and progressive airflow obstruction resulting from a combination of emphysema and chronic bronchitis. Furthermore, unlike asthma, which affects all populations from childhood, COPD primarily develops in mid-to-late adulthood and is associated with an increased risk of comorbidities [296,297].

Regarding its pathophysiology, COPD is characterized by a predominantly neutrophilic inflammatory response involving neutrophils, macrophages and CD8⁺ T lymphocytes. These cells release pro-inflammatory cytokines and chemokines that sustain chronic airway inflammation and drive structural changes in the small airways. Increased oxidative stress further impairs anti-inflammatory mechanisms, contributing to the reduced responsiveness to corticosteroids [298]. Beyond inflammation, COPD involves airway and lung parenchymal remodeling, including small airway fibrosis, mucus hypersecretion and alveolar destruction leading to emphysema (Figure 27).

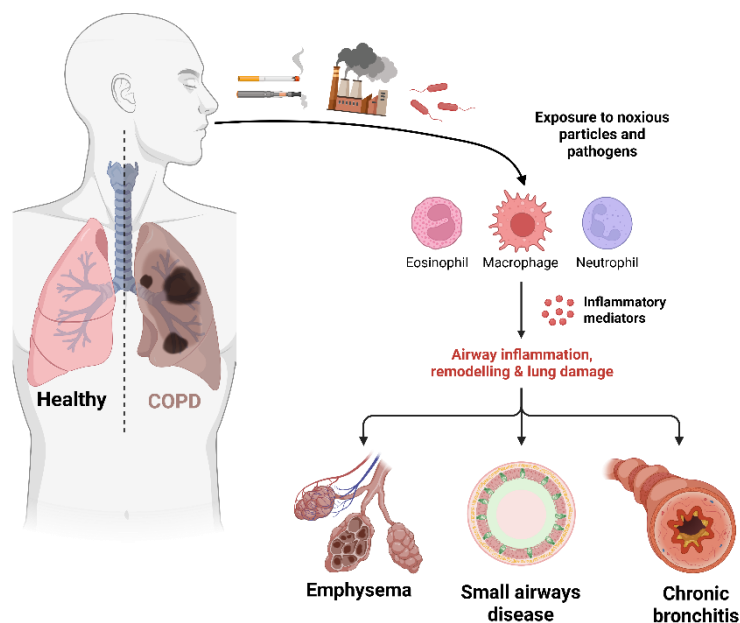


Figure 27: Overview of COPD pathophysiology. Created with BioRender.

Together, these processes result in persistent and largely irreversible airflow limitation and systemic manifestations of the disease, often requiring alternative therapeutic strategies.

V.1.3. *Treatment strategies for asthma and COPD*

The management of asthma and COPD relies on pharmacological and non-pharmacological strategies aimed at controlling symptoms and preventing exacerbations. Overall, these approaches remain symptomatic rather than curative and differ according to the distinct underlying pathophysiological mechanisms of the two diseases.

In asthma, acute symptoms are now primarily managed using as-needed low-dose inhaled corticosteroids (ICS)-formoterol combinations, which provide both rapid bronchodilation and anti-inflammatory effects. Short-acting β_2 -agonists (SABAs) (e.g., salbutamol) may still be used as reliever therapy in specific cases but should not be administered as monotherapy. Short-acting muscarinic antagonists (SAMAs, e.g., ipratropium) may be used as adjunct therapy in more severe episodes due to their complementary bronchodilatory effect [299].

Long-term asthma control focuses on suppressing chronic airway inflammation, with ICS (e.g., ciclesonide, budesonide) forming the cornerstone of maintenance therapy, generally effective in eosinophilic, IgE-driven asthma but less so in neutrophilic [300]. Long-acting β_2 -agonists (LABAs, e.g., salmeterol, formoterol, indacaterol) are frequently prescribed in combination with ICS, as current Global Initiative for Asthma (GINA) recommendations indicate that β_2 -agonists should not be used as monotherapy in asthma management. Additional therapeutic options include leukotriene receptor antagonists (e.g., montelukast) or anti-IgE monoclonal antibodies (e.g., omalizumab), which may be considered in selected patients [294,299].

Regarding COPD management, smoking cessation and regular physical activity remain fundamental components of care. Bronchodilators are the mainstay of pharmacological treatment: short-acting bronchodilators (SABA and/or SAMA) are used as needed for symptom relief, while long-acting bronchodilators (LABAs and long-acting muscarinic antagonists (LAMAs, e.g., tiotropium)) are preferred for maintenance therapy. Dual LABA/LAMA therapy is recommended for many symptomatic patients and improves lung function and symptoms, while reducing exacerbations. ICS are not routinely used in COPD and are mainly indicated in patients with persistent exacerbations despite optimal bronchodilator therapy, particularly when blood eosinophil counts are elevated.

In such cases, escalation to triple therapy (LABA/LAMA/ICS) may be considered [296,301].

Despite the wide range of existing treatments and APIs, as well as personalized, stepwise management strategies, asthma and COPD continue to affect millions worldwide, imposing substantial socioeconomic costs, with prevalence steadily rising and COPD remaining a leading cause of mortality [11]. This persistent burden underscores the need for continued research and therapeutic innovation to improve disease management and patient outcomes.

V.2. Potential of liposome-based DPIs for asthma and COPD

Taken together, the pathophysiological features of asthma and COPD, including mucus hypersecretion, chronic airway inflammation and increased pulmonary clearance mechanisms such as mucociliary transport and alveolar macrophage uptake, represent major barriers to effective drug delivery and retention in the lungs [48]. In this context, liposomal formulations offer several key advantages as their physicochemical properties can be tailored to enhance mucus penetration, reduce premature clearance by macrophages and improve drug stability within the pulmonary environment [114].

Furthermore, liposomes enable the controlled release of encapsulated drugs and can accommodate both hydrophilic and lipophilic compounds, making them particularly suitable for combination therapies, such as ICS and LABA co-delivery in asthma and COPD [114,302]. When formulated as DPIs, improved lung deposition and enhanced stability can be achieved, potentially leading to improved therapeutic efficacy and disease control [84]. Liposome-based DPI systems therefore represent a promising strategy to optimize pulmonary drug delivery, enhance therapeutic efficacy and improve patient adherence through more efficient and sustained drug action.

In this context, the development of liposome-based DPIs emerges as a rational and promising approach to address current limitations in inhalation therapy and improve the management of chronic respiratory diseases such as asthma and COPD, while representing a fertile area for future scientific and clinical innovation.

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CONTEXT AND OBJECTIVES

I. Context

This doctoral thesis is carried out within the **LN Pulmo project**, funded by the Walloon Region (Win2Wal program, 2022-2026, agreement No. 2210043).

The LN Pulmo project, initiated in September 2022, aims to develop an innovative dry powder inhaler platform based on optimized liposome-loaded particles for the treatment of asthma and COPD. The project builds on a novel therapeutic association between ciclesonide (CIC), an ICS requiring intracellular activation, and indacaterol maleate (IND), a LABA providing sustained bronchodilation, and combines this strategy with scalable manufacturing processes based on scCO₂ liposome production and spray-drying (SD). This approach focuses on engineering muco-penetrating and macrophage-avoiding liposomes to enhance pulmonary bioavailability. Their formulation as an inhalable dry powder is further intended to improve lung deposition and long-term stability while ensuring industrial scalability and enabling translational *ex vivo* and *in vivo* evaluation of pulmonary pharmacokinetics, pharmacodynamics and safety to demonstrate enhanced bioavailability of the final formulation.

This project is conducted within a collaborative framework involving several academic and one industrial partner. A **first academic partner** (LTPB, Prof. Géraldine Piel and PhD Student Tuan Nghia Dinh) is responsible for developing the optimal liposomal composition co-encapsulating CIC and IND, as well as evaluating its performance through dedicated *in vitro*, *ex vivo* and *in vivo* studies (in collaboration with the third partner). In addition, this partner is involved in the transfer of liposome production to the PGSS pilot scale. The **second academic partner**, serving as the project promotor (LTPB, Prof. Brigitte Evrard and Dr. Anna Lechanteur, PhD Student Laure-Anne Bya), focuses on the development and optimization of the liposome-based DPI formulations. This part of the whole project constitutes the core contribution of this doctoral thesis. Finally, the **third academic partner** (LBTD, Prof. Didier Cataldo, PhD Student Benedetta Bottero) oversees the biological investigations aiming to demonstrate the therapeutic relevance and bioavailability of the liposome-containing DPI formulations. The project also involves an **industrial collaborator**: Aquilon Pharma (CEO: Paul

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Maes), a spin-off company of the University of Liège founded in 2013, specializing in the enhancement of the efficacy of inhaled medicines.

The schematic representation of the final deliverable, as well as the roles of the different partners involved, are presented in Figure 28.

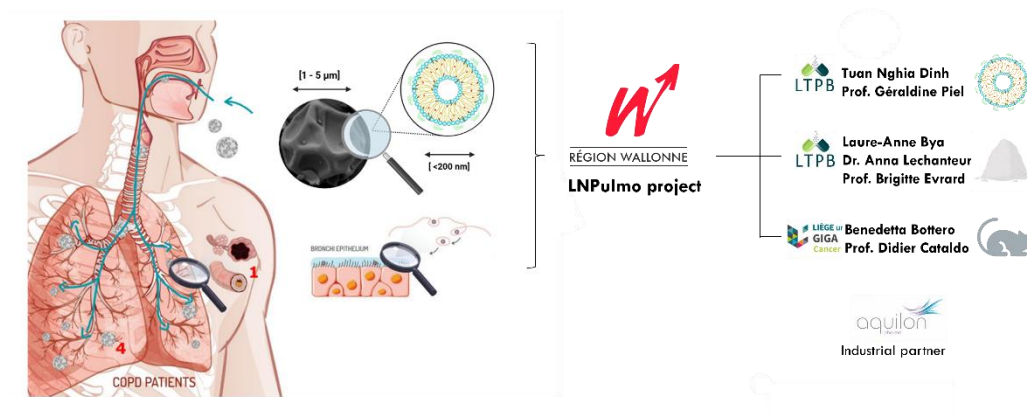


Figure 28: LNPulmo project goal and partners.

II. Objectives of the thesis

Pulmonary drug delivery has emerged as a promising approach for the local treatment of respiratory diseases, enabling high drug concentrations at the site of action while minimizing systemic side effects. Among inhalation technologies, DPIs can offer notable advantages, including improved drug stability and the absence of propellant gases. Their performance can be achieved through particle engineering strategies that optimize powder properties such as aerodynamic behavior and dispersibility. In this context, spray-drying (SD) technology is widely employed to produce engineered inhalable particles, allowing precise control over both formulation and process parameters.

In parallel, liposomal drug delivery systems have gained increasing attention for pulmonary administration due to their ability to co-encapsulate both hydrophilic and lipophilic APIs, protect them from degradation and improve bioavailability through controlled release. Following inhalation, PEGylated liposomes can enhance mucus penetration and reduce alveolar macrophage uptake, thereby prolonging pulmonary residence time and increasing drug exposure at the airway epithelium. When incorporated into DPI formulations, these advantages are further strengthened through

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improved lung deposition and stability, making liposome-based DPIs a promising strategy for optimizing therapeutic outcomes in chronic respiratory diseases such as asthma and COPD.

While liposome-based DPIs represent a promising approach by combining the advantages of liposomal nanocarriers and inhalable solid formulations, the SD process is inherently associated with elevated temperatures and mechanical stresses that can compromise liposome structural integrity and promote premature drug leakage. At the same time, the resulting powders must meet stringent aerodynamic, physicochemical and stability requirements to ensure efficient pulmonary delivery. In this context, the **general objective of this thesis** is to assess the feasibility of developing **optimized inhalable dry powder formulations incorporating liposomes with properties suitable for effective pulmonary administration.**

In line with this general objective, this thesis focuses on production methods with strong potential for future transfer to the pharmaceutical industry. Indeed, the work employs two scalable particle engineering technologies: liposome production by PGSS followed by SD to produce the final powder. This integrated approach aims to enable the reproducible manufacture of liposome-based DPIs.

To achieve this general objective, the thesis is structured into two main sub-objectives, each corresponding to a chapter (Figure 29).

1. The first sub-objective aims to generate fundamental scientific insights about the SD of liposomal systems. This is achieved by systematically investigating how formulation variables and SD process parameters jointly influence powder physicochemical properties, as well as liposome structural integrity and API retention after drying. A Quality by Design (QbD) approach is employed through the implementation of Design of Experiments (DoEs). To ensure broad relevance and transposability, model liposomes encapsulating APIs with different lipophilicity profiles were studied, including the co-encapsulation of clinically relevant combinations for asthma and COPD therapy. This integrated approach enables the identification of key formulation-process drivers required to obtain reproducible and stable dry powders with high pulmonary deposition while preserving liposome functionality, thereby establishing an interesting framework for the development of liposome-based DPIs.

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2. The second sub-objective aims to compare the properties and aerosolization performance of dry powder formulations with and without liposomes. To this end, a therapeutic combination of two APIs of interest for asthma treatment, ciclesonide (CIC) and indacaterol maleate (IND), was selected as a common pharmacological basis. The liposome-based DPI was developed by applying the optimized SD framework identified in Chapter 1. In parallel, a non-liposome-based DPI containing the same API combination was specifically developed to serve as a reference system. This comparative approach enables the systematic evaluation of the impact of liposome incorporation on powder properties and aerosol performance.

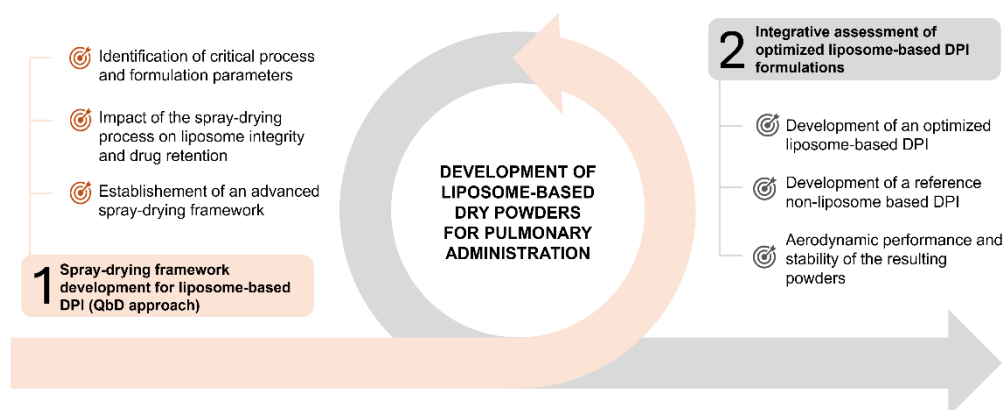


Figure 29: Schematic overview of the main objective of the thesis and its associated sub-objectives.

EXPERIMENTAL PART

CHAPTER 1

INVESTIGATION OF THE SPRAY-DRYING PROCESS GOVERNING THE PRODUCTION OF OPTIMIZED LIPOSOME-BASED INHALABLE DRY POWDERS

EXPERIMENTAL PART

Chapter 1. Investigation of the spray-drying process governing the production of optimized liposome-based inhalable dry powders

I. Introduction

While spray-drying (SD) is widely recognized as an effective technique for producing inhalable dry powders with controlled aerodynamic properties, its application to formulations containing lipid-based nanocarriers, particularly liposomes, remains insufficiently understood. In particular, the combined impact of SD process parameters and lipid composition on liposome integrity, API retention and resulting powder properties has not yet been fully elucidated. This reflects the relatively emerging nature of liposome-based systems for inhalation, as no liposome-based DPI has yet been approved for clinical use. Consequently, a deeper understanding of the interplay between formulation variables and drying parameters is essential for the rational design of inhalable liposome-based delivery systems.

In this context, the objective of the first chapter is to build a mechanistic understanding of the SD of liposomal systems by elucidating how process parameters and formulation variables govern powder properties, liposome structural integrity and API retention after drying. This approach aims to establish a robust formulation-process framework that supports the identification of SD conditions suitable for the production of reproducible inhalable powders while preserving liposome functionality.

To achieve this objective, preliminary screening experiments were conducted to determine factor ranges to assess the feasibility of drying liposomal suspensions over a range of process parameters, based on existing literature. These results enabled the set-up of a DoE, which served as the foundation for this chapter. All experiments were performed using well characterized and reproducible model liposomes produced by the PGSS process developed by Penoy *et al.*, based on a simple and well-defined lipid composition (SPC/CHOL/DSPE-PEG₂₀₀₀, 67.5/30/2.5, molar ratio), slightly adapted from the original formulation [89]. Notably, the PGSS-based liposome formation process had been previously optimized using a DoE approach at the LTPB (Prof. Géraldine Piel), ensuring high reproducibility and robustness of the resulting formulation. While not fully

characterized for inhalation-related functionalities such as mucus penetration or avoidance of alveolar macrophage uptake, these liposomes are nevertheless suitable for pulmonary delivery, as supported by their low toxicity on A549 lung epithelial cells [89]. They therefore represent relevant and reliable candidates for investigating the impact of SD on liposomal integrity and powder properties relevant to pulmonary administration.

The results of this chapter are reported in the research article entitled “*Optimizing spray-dried liposomes for pulmonary delivery: impact of lipid composition and drying parameters using experimental design*” (European Journal of Pharmaceutics and Biopharmaceutics, 2025), provided in the following Section II.2.

II. Published results

II.1. Summary of results

A DoE-based approach was used to investigate the impact of SD and formulation variables on key responses, providing the basis for defining suitable SD conditions for liposomal formulations. In this context, two complementary experimental designs were implemented to systematically investigate the influence of formulation and process parameters. The first experimental design (DoE_A) was performed using empty model liposomes produced by PGSS to investigate the impact of SD process parameters and carbohydrate matrix selection on liposome structural integrity and powder physicochemical properties. Trehalose and HP β CD were compared as matrix-forming excipients, while key SD parameters, namely inlet temperature (T_{in}), feed rate, nozzle gas pressure (NGP) and cyclone gas pressure (CGP), were systematically evaluated.

Results demonstrated that HP β CD significantly improved liposome preservation during drying, maintaining vesicle size and low Pdl after rehydration, whereas trehalose led to pronounced structural disruption. An optimal set of SD conditions was identified and consisted of a T_{in} of 120 °C (T_{out} of 75 °C), a feed rate of 1.6 g/min, a NGP of 3.4 bar and a CGP of 0.3 bar. These conditions yielded inhalable powders with suitable particle size (~2.8 μ m), low residual moisture (<5%) and a characteristic deflated particle morphology, which has been reported to enhance powder dispersibility and pulmonary deposition.

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The second design (DoE_B) investigated the influence of liposome-related formulation parameters and API properties on liposome behavior during drying. To this end, previously optimized API-loaded liposomes developed in the laboratory were employed, using budesonide (BUD) and salbutamol (SAL) as representative hydrophobic and hydrophilic model drugs, respectively, to evaluate the influence of API physicochemical properties on drug leakage during drying. In parallel, the influence of lipid-related formulation parameters was evaluated by varying the DSPE-PEG₂₀₀₀ content (2.5-10%) and the lipid-to-carbohydrate ratio (1-10%). A lipid-to-carbohydrate ratio of 10% and a DSPE-PEG₂₀₀₀ content of 2.5% were identified as optimal.

Beyond elucidating the impact of SD parameters on liposome integrity and DPI properties, which led to the identification of optimized formulation conditions, this experimental design also revealed that drug retention during drying is primarily governed by API lipophilicity, with more lipophilic compounds exhibiting markedly lower leakage upon SD. In addition, increasing the proportion of PEGylated lipids was associated with enhanced leakage of lipophilic APIs, likely due to increased membrane fluidity, while having minimal impact on hydrophilic compounds. A strong correlation between API lipophilicity and post-drying retention was observed ($R^2 = 0.98$), emphasizing the critical role of drug-liposome interactions and highlighting a limitation of the SD process for liposomes encapsulating hydrophilic APIs.

Subsequently, the optimized conditions (DoE_A + DoE_B) were combined and applied to liposomal formulations co-encapsulating APIs of interest for asthma and COPD therapy. The investigated liposomes contained the following co-encapsulated combinations: SAL with BUD, formoterol fumarate (FOR) with BUD and IND with CIC. These pairs correspond to clinically relevant therapeutic associations of β_2 -agonists and ICS. Results show that, along with suitable inhalation properties and preserved liposomal integrity, as confirmed by microscopy after rehydration, the powders exhibited favorable aerodynamic performance, with FPF above 58%, homogeneous drug distribution and deflated morphology.

Overall, this work demonstrates that SD can effectively produce liposome-based DPIs when formulation and process parameters are jointly optimized. It highlights the critical role of excipient selection, lipid-related formulation parameters and API properties in preserving liposomal integrity and ensuring efficient pulmonary delivery.

II.2. Publication

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

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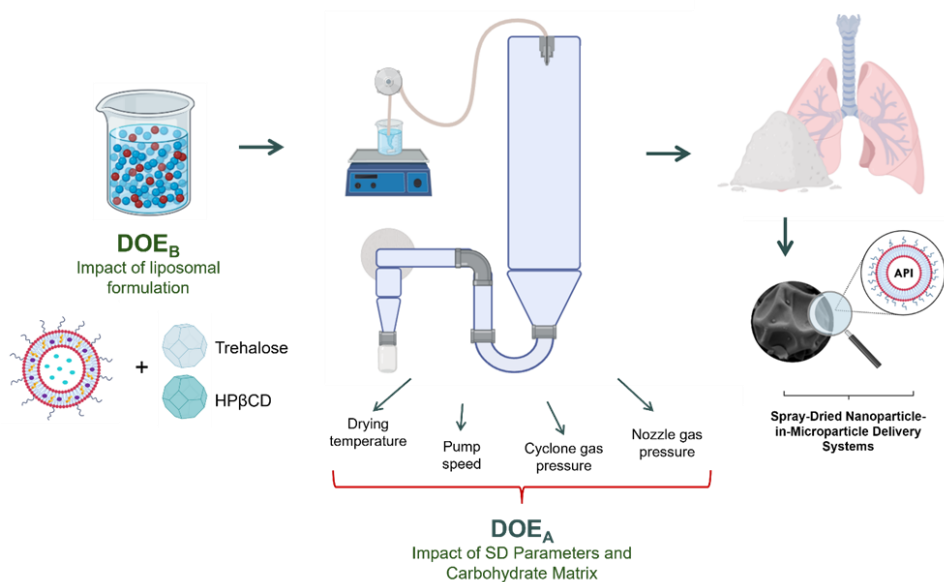
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ABSTRACT

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 (HP β CD), 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-PEG₂₀₀₀) percentage) and carbohydrate-to-liposome ratio.

Optimized spray-dried powders exhibited preserved liposome structure (mean diameter <200 nm, narrow polydispersity index (Pdl), near-neutral zeta potential (ZP)), favorable aerodynamic properties ($\sim 3\ \mu\text{m}$, 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 HP β CD 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).

II.2.1. 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 [46,303]. 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 [55,94]. 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 [103,304]. 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 [90].

Despite these beneficial attributes, aqueous liposomal suspensions are prone to aggregation and fusion over time, which can reduce therapeutic efficacy [63]. 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 [70]. 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 [35,92]. 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 [115,158,305,306].

Liposomal dispersions can be converted into dry powders using well-established techniques such as freeze-drying (FD) and spray-drying (SD) [158]. 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 [40,179], whereas FD typically produces an irregular, lyophilized cake with inadequate flowability [241]. Furthermore, from an industrial perspective, SD is also considerably faster, more cost-effective and highly reproducible [264]. Yet, while FD has been extensively documented for liposomal drying across a wide range of applications [307–309], 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 [170,310]. 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.* [283] 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 bilamellar structures, underscoring the need for protective excipients [284]. In this regard, cyclodextrins, specifically HP β CD, have been shown to stabilize PEGylated liposomal membranes during SD, preventing bilayer disruption and drug leakage [286]. 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 [170,287]. 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 [114].

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.* [280] 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 [85,89] 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 HP β CD, 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 β 2-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 β 2-agonist IND further underscores the translational potential of these liposomal DPIs for future combination therapies.

II.2.2. Materials and methods

II.2.2.1. 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-PEG₂₀₀₀) from Xiamen Sinopeg Biotech (Xiamen, China).

Tested carbohydrates, including hydroxypropyl- β -cyclodextrin (HP β CD) 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, $\geq 99.5\%$) 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).

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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 CO₂ 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 [144,311].

II.2.2.2. 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).

II.2.2.2.a. 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.

The development and validation of this analytical method, developed at the beginning of this thesis, can be found in Appendix II.

II.2.2.2.b. 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 $\pm 10\%$ over the concentration ranges of 1 to 100 $\mu\text{g/mL}$ for BUD and 0.1 to 10 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$ and 4.395 $\mu\text{g/mL}$, respectively. For FOR, the LOD was measured at 0.1161 $\mu\text{g/mL}$, with a corresponding LOQ of 0.9282 $\mu\text{g/mL}$ [202].

II.2.2.2.c. Salbutamol

For SAL quantification, separation was performed using a LiChrospher® 100 RP8 column (Hibar® RT 150 $\times$ 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 $\mu\text{g/mL}$ for SAL [89]. LOD and LOQ were both determined and found to be equal to 0.039 and 0.13 $\mu\text{g/mL}$, respectively.

II.2.2.3. 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 HP β CD) 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 ranges investigated 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 (Pdl) 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-PEG₂₀₀₀ (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:

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

where 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-PEG₂₀₀₀ 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-PEG₂₀₀₀ content plays a key role in liposome stability through steric stabilization and membrane protection [302] and that the physicochemical nature of the API significantly

impacts drug retention in liposomal suspension [312], while also enabling evaluation of formulation feasibility under the selected SD conditions. The evaluated responses included liposome size and Pdl to monitor liposome integrity, with particular emphasis on the impact of the tested parameters on the API leakage 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 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-PEG₂₀₀₀ 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.

II.2.2.3.a. PGSS-based preparation of liposomal formulations

Liposomes composed of SPC, CHOL and DSPE-PEG₂₀₀₀ in varying molar ratios were produced using the Particles from Gas-Saturated Solutions (PGSS) technique, employing supercritical carbon dioxide (scCO₂) as a dispersing agent. The process was carried out under optimized conditions (30 min contact with scCO₂, 500 rpm agitation rate at 80 °C) previously established by Penoy *et al.* [79,89], 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

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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.

II.2.2.3.b. 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 Equation 3.

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

Equation 3: Drying process yield calculation.

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

II.2.2.4. 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 (T_g) 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.

II.2.2.5. Characterization of liposomes

II.2.2.5.a. Particle size, size distribution and zeta potential of liposomes

Liposomes were characterized in terms of Z-Average size (nm), polydispersity index (Pdl) 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 [313]. All measurements were performed in triplicate and the mean values were reported.

II.2.2.5.b. 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 2.1×10^{-1} mbar and a voltage of 1.7 V for 35 s. 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⁻/Å².

II.2.2.6. Characterization of the dried powders

II.2.2.6.a. Particle size distribution

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.

II.2.2.6.b. 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.

II.2.2.6.c. 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 II.2.2.2). Blend uniformity was expressed as the coefficient of variation (CV), calculated

from the five samples. Powders with a CV ≤5% for mean API recovery were considered to exhibit satisfactory content uniformity.

II.2.2.6.d. 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.

II.2.2.7. 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 osmolality 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 retained API was determined by HPLC (Section II.2.2.2), measuring the API concentration after powder rehydration (total drug) and after dialysis (encapsulated drug). Since Equation 4 provides the percentage of retained drug after drying, the drug release upon drying was obtained by subtracting this value from 100.

$$\text{Drug retention upon drying (\%)} = \frac{\text{Encapsulated drug (\mu g/mL)}}{\text{Total drug (\mu g/mL)}} \cdot 100$$

Equation 4: Drug retention upon drying calculation.

II.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 either 100 L/min for 2.4 s or 60 L/min for 4 s using a flow controller (TPK; Copley, Nottingham, UK). The Breezhaler® (Novartis Pharma AG), a low-resistance DPI, was employed for

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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 (Equation 5):

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

Equation 5: Fine Particle Fraction (FPF) calculated based on the Emitted dose (ED).

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 (Equation 6):

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

Equation 6: Extra Fine Particle Fraction (eFPF) calculated based on the Emitted dose (ED).

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.

II.2.2.9. Statistical analysis

Data were analyzed using GraphPad Prism software (version 8.4.3, La Jolla, CA, USA) and are presented as mean $\pm$ standard deviation. Statistical comparisons between two groups were performed using unpaired t-tests, whereas multiple-group comparisons were assessed by one-way ANOVA followed by Tukey's post hoc test. Two-way ANOVA with Sidak's post hoc test was used when two experimental factors were analyzed simultaneously. Statistical significance was set at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

II.2.3. Results and discussion

II.2.3.1. 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 [314,315] and HP β CD, for its potential stabilizing effect on liposomes and ability to produce highly aerosolizable, carrier-free powders with favorable morphologies [177,202,205,286]. Beyond these functional benefits, both carbohydrates, although not approved yet for inhalation, have been reported to exhibit a favorable safety profile for pulmonary administration [210,316,317]. In particular, HP β CD 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.

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 (Appendix III), which include raw results in terms of SD yield, dry powder particle size, moisture content, as well as liposome size variation and Pdl. These results were used to construct a DoE general profiler (Figure 30), providing initial insight into how these parameters collectively influence powder performance and liposome stability.

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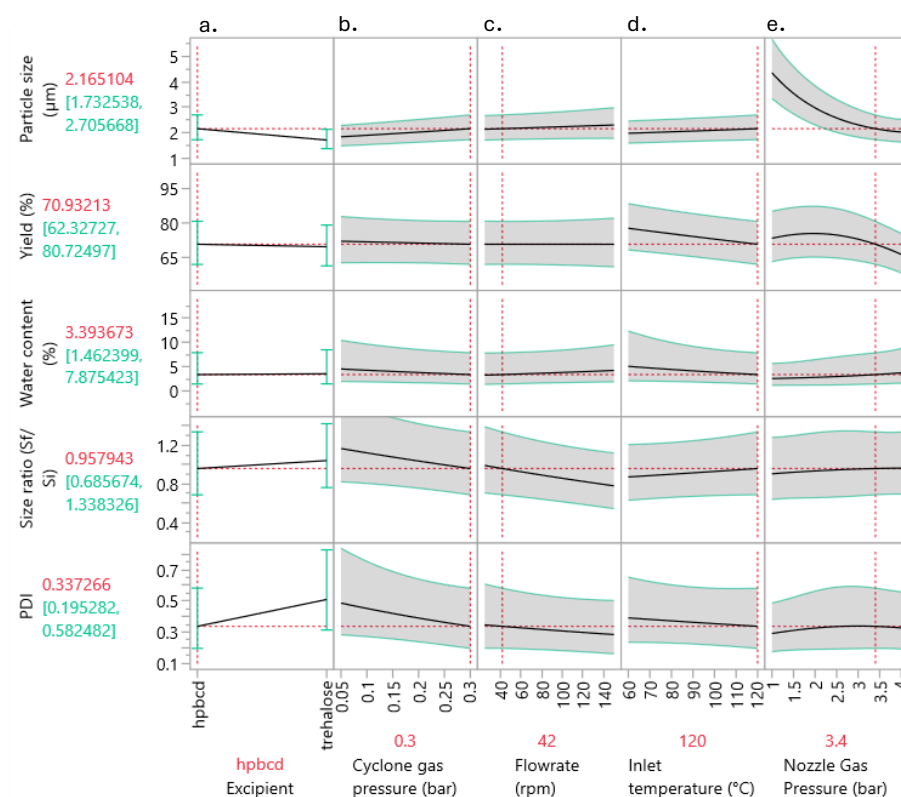


Figure 30: DoEA prediction profiler: Illustration of the effects of carbohydrate type and spray-drying parameters on key powder characteristics and liposome integrity after drying.

II.2.3.1.a. Impact of spray-drying parameters on liposomal properties

Starting with drying parameters, DoEA 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 30, 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 [318,319].

Interestingly, increasing the drying temperature from 60 to 120 °C ($T_{out} \sim 40$ and ~ 75 °C, respectively) appeared to improve liposome integrity, as indicated by a lower PDI and a size ratio approaching 1 (Figure 30, column d). This may result from faster solvent evaporation, which limits lipid rearrangement [170] and from the rapid formation of a

protective carbohydrate matrix that stabilizes membranes [163]. 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 [171,287].

Furthermore, cyclone and nozzle gas pressure showed no significant effect on liposome integrity, although higher cyclone pressure tended to reduce size ratio and Pdl, suggesting improved homogeneity (Figure 30, 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 [320].

Overall, significant effects of process parameters are summarized in Supplementary Data B (Appendix III).

II.2.3.1.b. Comparative evaluation of trehalose and HP β CD 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, HP β CD 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 2 below.

Table 2: Optimal drying conditions for liposomes using HP β CD and trehalose, as determined by DoE_A.

Optimal condition	HP β CD	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)	0.30	0.05

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Although the DoE analysis indicated that the nature of the excipient had no statistically significant impact on Pdl 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 Pdl of 0.47 ± 0.03 (Figure 31A-B). These findings indicate reduced size homogeneity and potential membrane destabilization, suggesting that the HP β CD-based formulation represents the most favorable condition for preserving liposome integrity.

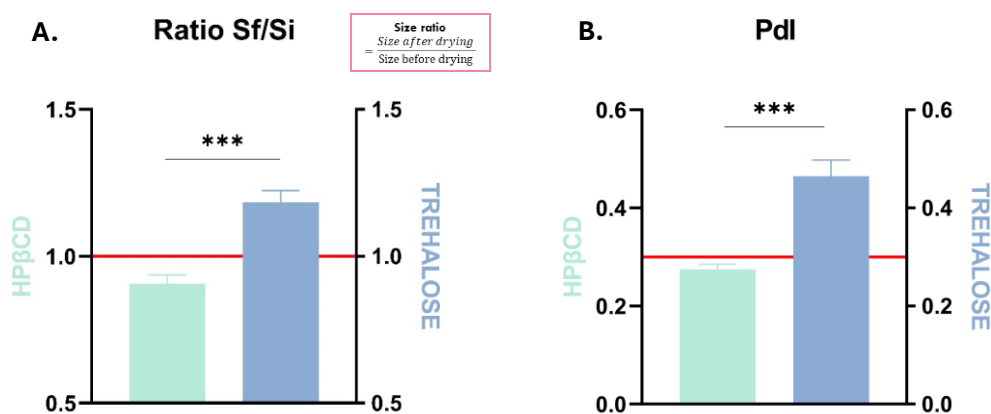


Figure 31: Effect of carbohydrate type on liposome integrity after spray-drying, as determined by DoE statistical analysis. Measured size ratios (A) and Pdl values (B) for HP β CD- and trehalose-based formulations under their respective optimal drying conditions, with prediction intervals indicated. Analyses were performed in triplicates ($n=3$) and results are presented as mean $\pm$ 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 matrixes. Indeed, during vitrification, an amorphous glassy matrix forms around the liposomes, physically immobilizing them and thereby limiting membrane rearrangements during dehydration [321]. The effectiveness of this process largely depends on the excipient's glass transition temperature (T_g): above this temperature, the matrix softens, loses rigidity and can no longer effectively protect the vesicles, leading to increased lipid mobility and structural disruption [280,286]. In this study, DSC measurements confirmed T_g consistent with literature values, namely approximately 250 °C for HP β CD and approximately 100 °C for trehalose [322–324]. Consequently, HP β CD remains well above the drying temperatures applied, whereas

trehalose operates closer to the T_{out} (~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 [280]. Van den Hoven *et al.* notably reported that HP β CD provides enhances protective effects compared to trehalose on PEGylated liposomes, likely due to its higher density of hydrogen bond donors and acceptors [286]. This feature, supported by its greater number of hydroxyl groups [325], promotes more efficient membrane stabilization and better preservation of liposome characteristics [326,327].

Overall, the protective capacity of HP β CD is reflected in the post-drying liposome characteristics, with preserved size (size ratio = 0.91 ± 0.03) and uniform particle distribution ($PdI = 0.27 \pm 0.01$), as predicted by the DoE profiler (Figure 30, 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 2), the formulation achieved high drying efficiency ($80.46 \pm 4.74\%$), an inhalable particle size of $2.83 \pm 0.24 \mu m$ and low residual moisture ($4.60 \pm 0.42\%$), ensuring stability and minimizing degradation. Furthermore, powders produced under the optimal DoE_A conditions using HP β CD 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 Data C (Appendix III).

These results demonstrate that HP β CD 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, Appendix III).

II.2.3.1.c. Application of optimized Spray-drying parameters to budesonide-encapsulated liposomes

The optimal condition identified and validated in DoE_A using HP β CD 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 ± 8.30 nm, respectively, yielding a size ratio of 0.95 ± 0.04 . The produced liposomes exhibited a particle size below 200 nm and a Pdl of 0.23 ± 0.01 , indicating physicochemical properties appropriate for a pulmonary liposomal formulation and the uniformity required for the clinical application of lipid-based nanocarriers [94,328]. 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 [94,329,330].

However, despite the preservation of physicochemical properties, post-rehydration dialysis, used to remove free, non-encapsulated drug, revealed that $33.41 \pm 3.64\%$ 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.

II.2.3.2. DoE_B: 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 [331]. In addition, the influence of liposomal composition was examined by varying the proportion of DSPE-PEG₂₀₀₀, 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 [228,332].

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 (Appendix III).

DoE_B experiments performed with BUD and SAL loaded liposomes revealed that the DPI properties and liposome integrity (size ratio and Pdl) consistently remained within acceptable limits, regardless of API type, lipid composition and quantity. Specifically, all produced powders exhibited a particle size of $2.75 \pm 0.50 \mu\text{m}$ and a residual moisture content of $3.58 \pm 1.28\%$, values well within the range suitable for inhalation. Moreover, a liposomal size ratio of 0.93 ± 0.03 and an average Pdl 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 32), along with a summary of the parameters significantly affecting drug release after drying is presented in Supplementary Data F (Appendix III).

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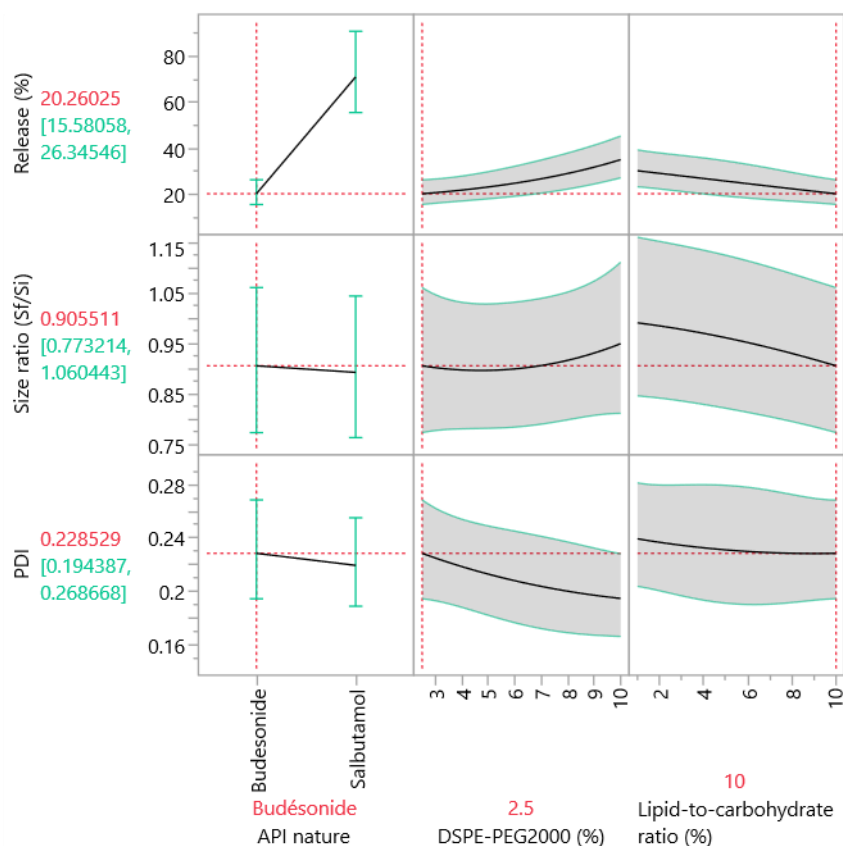


Figure 32: DoE_B prediction profiler illustrates the influence of API type, DSPE-PEG₂₀₀₀ content and L/C ratio on key powder properties and liposome integrity post-drying, with particular emphasis on drug release after drying.

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 32). 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.* [284], 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 35).

Importantly, the key finding of DoE_B reveals that an increased percentage of DSPE-PEG₂₀₀₀ 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 33A). 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 [287,333]. Nevertheless, a minimal proportion of DSPE-PEG₂₀₀₀ remains essential to prevent vesicle aggregation during atomization by providing a steric hydrophilic barrier [334,335]. This effect is reflected in the profiler, where an increase in the PEGylated fraction correlates with a lower Pdl (Figure 32). Additionally, PEGylation offers benefits in the pulmonary context by enhancing mucus penetration, thereby reducing mucociliary clearance and extending residence time [336,337]. 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-PEG₂₀₀₀ content (Figure 33B), highlighting the selective influence of PEGylation on drug retention in the membrane.

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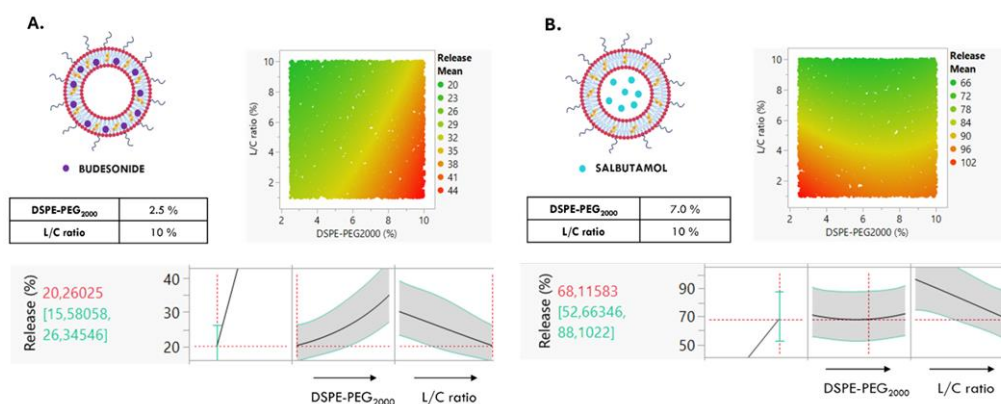


Figure 33: (A) Predicted optimal drying conditions for BUD-loaded liposomes, illustrating the influence of DSPE-PEG₂₀₀₀ content and lipid-to-carbohydrate (L/C) ratio on BUD release during SD. An increase in DSPE-PEG₂₀₀₀ content is associated with enhanced BUD leakage, whereas increasing the L/C ratio results in reduced BUD release, leading to an optimal formulation containing 2.5% DSPE-PEG₂₀₀₀ 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-PEG₂₀₀₀ content and L/C ratio on SAL release during SD. While variations in DSPE-PEG₂₀₀₀ content have no significant impact on SAL release, increasing the L/C ratio reduces SAL leakage, resulting in an optimal formulation containing 7% DSPE-PEG₂₀₀₀ 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 (Figure 33A-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 34 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 (0.99) and highly significant p -value (< 0.0001) confirm the excellent predictive performance of the model.

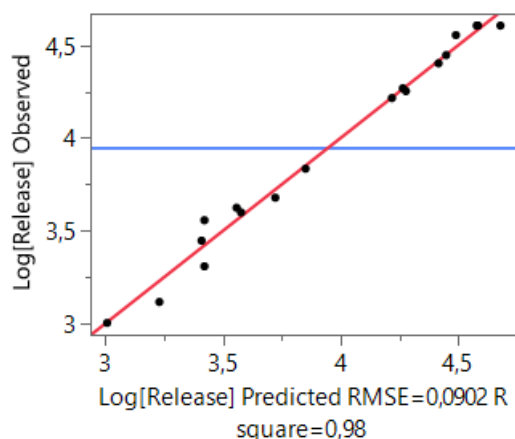


Figure 34: Observed (measured) and predicted (via linear regression: solid red line) drug release values for all considered powders.

Ultimately, based on the comprehensive analysis of all measured parameters, optimal formulations were identified for both BUD (Figure 33A) and SAL-loaded liposomes (Figure 33B) 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-PEG₂₀₀₀ 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 BUD-optimal formulation. All results are presented in Table 3 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.

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Table 3: 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 $\pm$ standard deviation.

		BUD	SAL	Co-encapsulation
DPI properties	Drying Yield (%)	75.85 $\pm$ 6.77	74.41 $\pm$ 2.70	76.03 $\pm$ 0.08
	Particle size (μ m)	2.79 $\pm$ 0.05	2.55 $\pm$ 0.03	2.64 $\pm$ 0.03
	Water content (%)	4.26 $\pm$ 0.77	2.71 $\pm$ 0.48	3.50 $\pm$ 0.70
Liposomes properties	Liposomes size after powders rehydration (nm)	127.10 $\pm$ 6.79	127.43 $\pm$ 1.07	128.63 $\pm$ 4.21
	Size ratio (Sf/Si)	0.92 $\pm$ 0.05	0.96 $\pm$ 0.01	0.92 $\pm$ 0.01
	Pdl	0.21 $\pm$ 0.03	0.21 $\pm$ 0.00	0.22 $\pm$ 0.01
	ZP (mV)	-2.73 $\pm$ 0.39	-2.31 $\pm$ 0.25	-2.92 $\pm$ 0.08

Regarding the percentage of drug that leaked upon drying, Figure 35 demonstrates that, under their respective optimal conditions, BUD exhibited drug release value ranging from 18.62 and 29.80%, with a mean drug release of $23.57 \pm 4.38\%$, while SAL ranged from 72.67 and 80.23%, with a mean release of $75.38 \pm 4.21\%$, 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 $26.35 \pm 2.24\%$ for BUD and $64.50 \pm 4.71\%$ for SAL. These values fall within the validation limits for both optimal conditions, thereby confirming the statistical robustness and compliance with this second design.

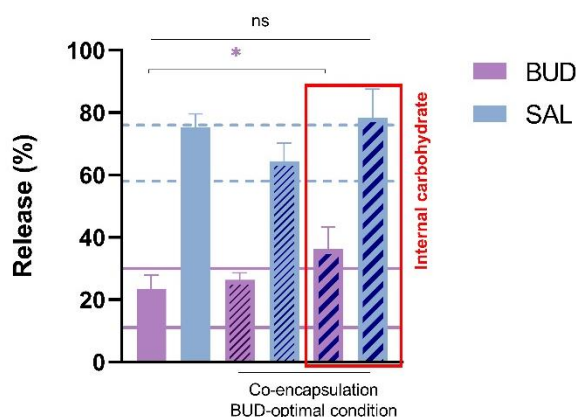


Figure 35: Drug release values of BUD- and SAL-loaded liposomes under their respective optimal spray-drying conditions and for co-encapsulated liposomes processed under BUD-optimal conditions with or without internal carbohydrate incorporation. Analyses were performed in triplicates (n=3) and results are presented as mean $\pm$ standard deviation.

Finally, dry liposomal formulations are well known to preserve liposome stability over time [170,338], particularly by maintaining encapsulation efficiency during storage. Initial stability studies on the final formulation, shown in Supplementary Data G (Appendix III), support this concept.

II.2.3.3. Application of the optimized spray-drying process to additional APIs

II.2.3.3.a. 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, summarized in Table 4, 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 [339]. The logP values are 0.44, 1.91, 2.42, 3.31 and 4.08 for SAL, FOR, BUD, IND and CIC, respectively [340]. 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 [341]. 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.

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Table 4: Overview of the optimized spray-drying (SD) conditions for PEGylated liposomes, combining the SD parameters and carbohydrate selection identified in DoE_A with the lipid composition and lipid-to-carbohydrate ratio determined in DoE_B.

Final optimal condition for PEGylated drying liposomes		
Spray-drying process optimal parameters (DoE _A)	Cyclodextrin matrix	HP β CD
	Inlet temperature (°C)	120
	Outlet temperature (°C)	~75
	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-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 $26.31 \pm 8.13\%$ for FOR, $14.47 \pm 1.07\%$ for CIC and $13.47 \pm 2.27\%$ 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 36).

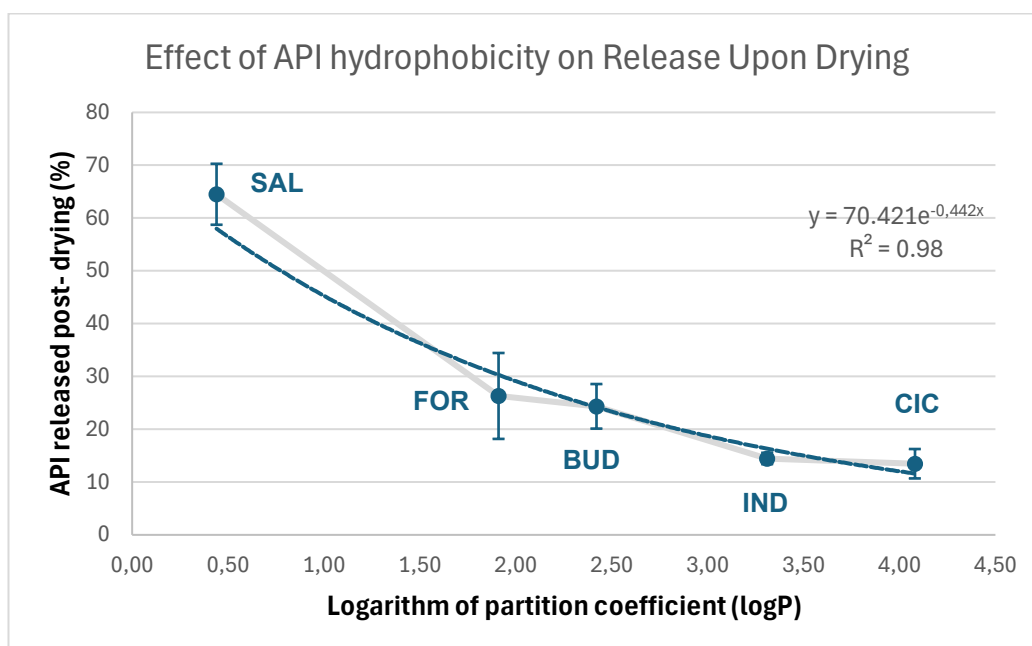


Figure 36: Influence of API hydrophobicity ($\log P$) on drug release during the spray-drying process under optimized conditions, showing a strong correlation ($R^2 = 0.98$). Analyses were performed in triplicates ($n=3$) and results are presented as mean.

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-PEG₂₀₀₀ contents could be envisaged, as their strong affinity for the lipid bilayer may allow increased PEGylation without compromising drug retention and potentially improving mucopenetration [342]. In contrast, compounds with lower $\log P$ 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 [70], including the use of hydrophobic amino acids [343], polymer coating of liposomes [344,345], or modulation of bilayer rigidity through cholesterol enrichment or the use of saturated phospholipids [346,347].

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

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II.2.3.3.b. 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 Pdl) remained consistent with predicted values by DoE_A under optimal conditions, as summarized in Table 5 below.

Table 5: 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
Liposome properties	Liposomes size after powders rehydration (nm)	141.80 ± 7.47	151.83 ± 3.52
	Size ratio (Sf/Si)	0.98 ± 0.03	0.88 ± 0.08
	Pdl	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 was performed using both TEM and cryo-TEM. Before atomization, the liposomes exhibited smooth, spherical vesicles with well-defined bilayers (Figure 37A-B), a morphology that was preserved after drying, as evidenced by the rehydrated powders displaying structures comparable to the pre-drying liposomes (Figure 38A-B). 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.

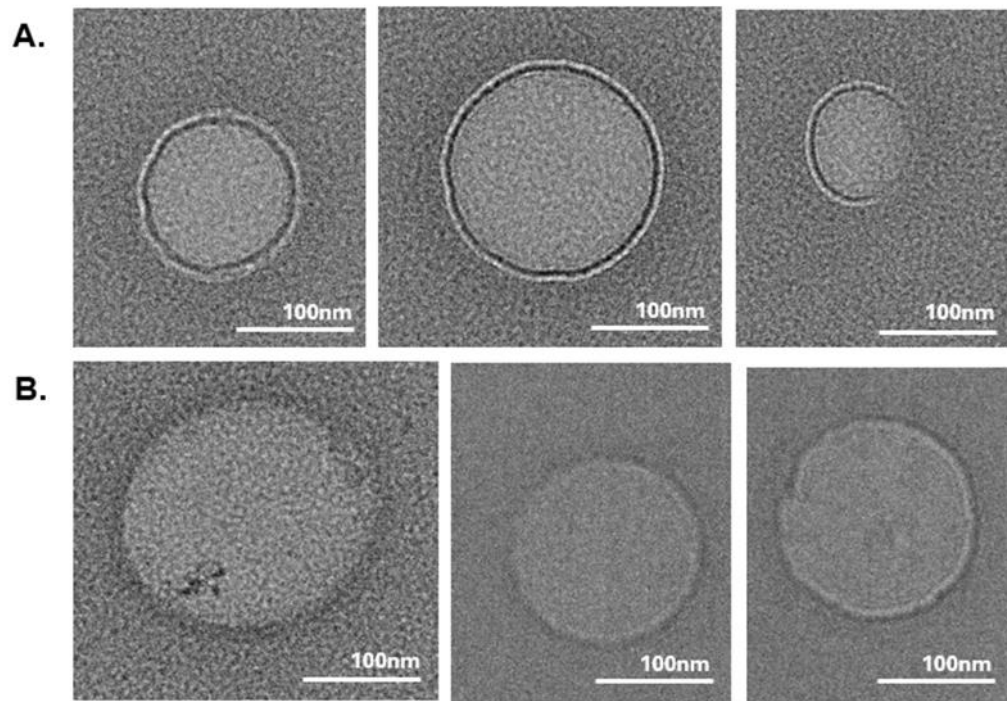


Figure 37: (A) TEM images of liposomes before SD and (B) rehydrated liposomes after SD, within the rehydrated carbohydrate matrix.

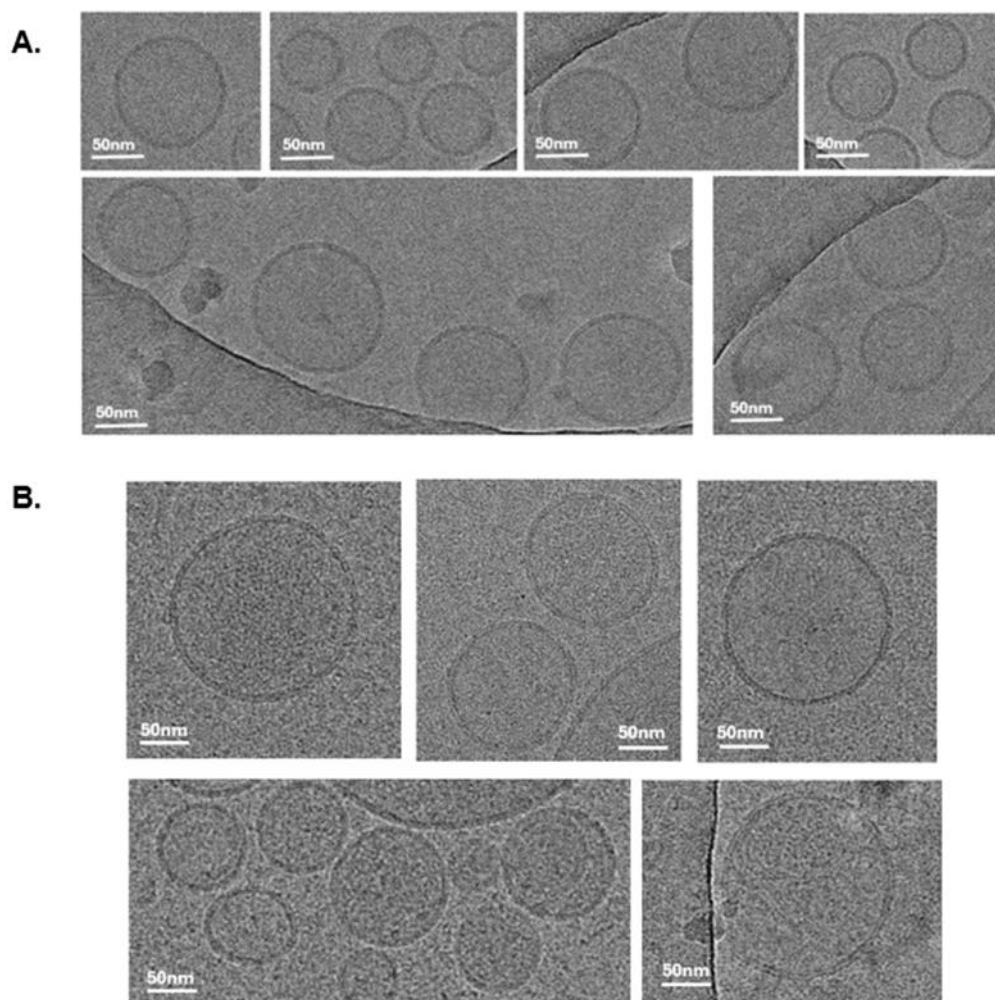


Figure 38: (A) Cryo-TEM images of liposomes before SD and (B) rehydrated liposomes after SD, within the rehydrated carbohydrate matrix.

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 [348]. 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 [349]. 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% [350]. Analysis of different samples from the produced powders containing co-encapsulated drug combinations

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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 $2.69 \pm 1.55\%$ for CIC (Figure 39A). 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 Pdl 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.

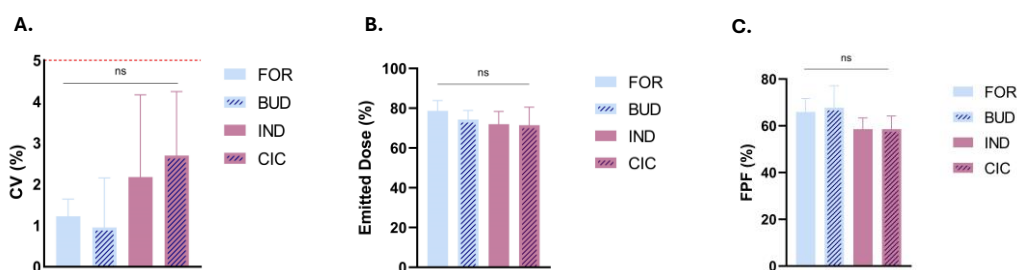


Figure 39: (A) Coefficient of variation of APIs calculated across multiple powder samples, reflecting the uniformity of API distribution within the powders. (B) Emitted Dose (ED) and (C) Fine Particle Fraction (FPF) measured using a Next Generation Impactor (NGI) on powders containing liposomes co-encapsulating FOR-BUD and IND-CIC, at 100L/min. Analyses were performed in triplicates ($n=3$) and results are presented as mean $\pm$ 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 $4.19 \pm 1.41 \mu\text{m}$ and $4.60 \pm 0.47 \mu\text{m}$, 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 $3 \mu\text{m}$, 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 [351]. Importantly, the particle sizes remained within the recommended $1\text{--}5 \mu\text{m}$ range, allowing efficient lung deposition while minimizing the risks of exhalation or oropharyngeal impaction [152,210]. 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 [352,353].

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 39B), considered as high-efficiency DPIs [354]. 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 $58.54 \pm 4.82\%$ for IND, $58.58 \pm 5.67\%$ for CIC, $63.44 \pm 12.22\%$ for BUD and $65.87 \pm 5.85\%$ for FOR (Figure 39C). 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, Appendix III).

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 [355,356]. The eFPF corresponds to the fraction of particles with a d_{ae} below $2\ \mu\text{m}$, providing an estimate of the proportion of the inhaled dose likely to deposit in the deeper regions of the lungs, including the distal conducting airways and the respiratory bronchioles [357,358]. For the tested powders, eFPF values exceeded 20% for all formulations, reaching $23.74 \pm 3.63\%$, $25.35 \pm 0.96\%$, $25.28 \pm 3.23\%$ and $22.68 \pm 3.64\%$ 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 [359].

In addition, previous studies indicate that patients using the Breezhaler® typically achieve an inspiratory flow rate of approximately 60 L/min [311]. 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.

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 40A) and comparable ED values at 60 L/min with $61.83 \pm 8.66\%$ for CIC and $64.98 \pm 9.41\%$ for IND (Figure 40B). 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 $36.58 \pm 0.21\%$ and $36.61 \pm 0.79\%$ (Figure 40C) and FPF values of $73.55 \pm 0.12\%$ and $70.12 \pm 0.79\%$ for CIC and

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IND, respectively (Figure 40D). 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.

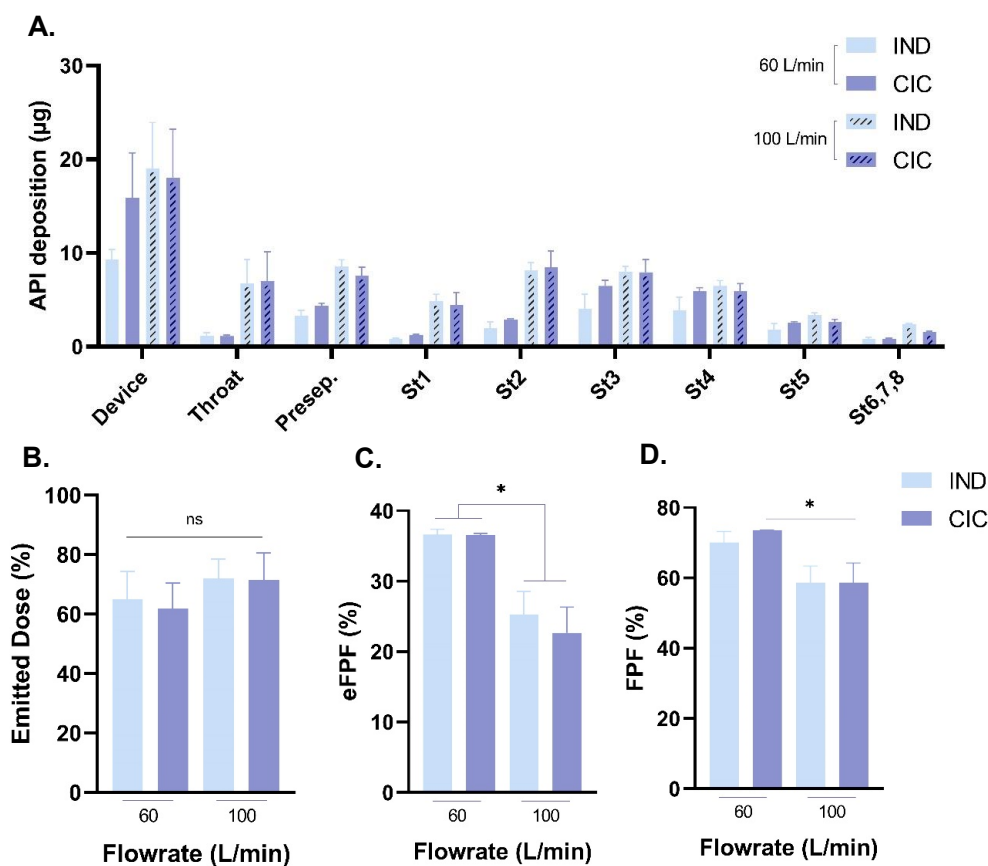


Figure 40: (A) Stage-by-stage deposition profiles, (B) Emitted Dose (ED), (C) extra-Fine Particle Fraction (eFPF) and Fine Particle Fraction (FPF) obtained from NGI lung deposition studies of the optimized powder formulation containing liposomes co-encapsulating CIC and IND at a clinically relevant flow 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 $\pm$ standard deviation.

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 [360]. Notably, previous studies on powders composed solely of

HP β CD, without liposomes, reported similar FPF values when ultra-fine particles were generated under optimized drying conditions [181,341,361].

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, HP β CD-based powders maintained their characteristic deflated structure (Figure 41), which has been previously associated with improved aerodynamic properties and enhanced pulmonary deposition [177,181,205].

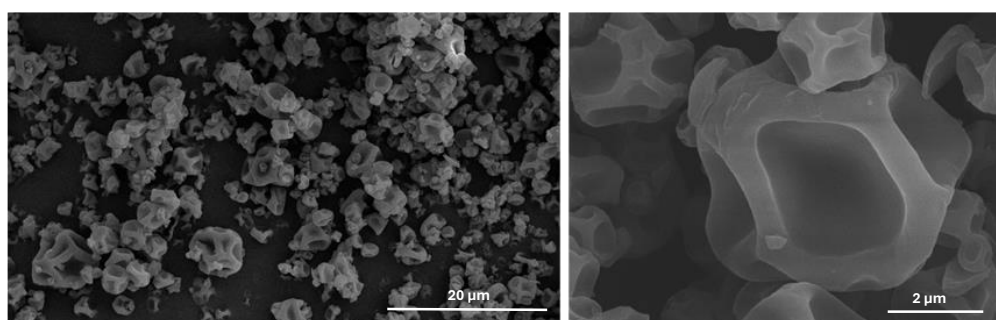


Figure 41: Scanning electron micrographs of spray-dried powders produced under the combined optimal conditions.

II.2.4. 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 HP β CD 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 ($\sim 3\ \mu\text{m}$), 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 (Pdl ~ 0.20), 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 (FOR/BUD and CIC/IND) 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 HP β CD, 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.

III. Conclusion

The first chapter of this thesis successfully established an interesting strategy for converting liposomal suspensions into inhalable dry powders through the combined use of PGSS liposome production and SD. Together, the two complementary DoEs fulfilled the primary objective of the study by elucidating how formulation parameters and SD conditions interact to govern vesicle stability, drug retention and final particle

characteristics. Based on this comprehensive understanding of the SD process, an optimal SD condition was subsequently identified, enabling the production of powders suitable for deep lung delivery while preserving liposomal integrity.

The optimized process, based on the use of HP β CD as a stabilizing matrix, controlled SD parameters and adjusted lipid-related formulation variables, successfully yielded liposome-based DPIs with properties suitable for pulmonary administration. These systems exhibited high structural stability, uniform API distribution, and excellent aerodynamic performance across different inhalation flow rates. Importantly, this chapter identified the key determinants of drug retention in liposomes during drying, highlighting the critical roles of PEGylation level, lipid-to-carbohydrate ratio and most importantly, API lipophilicity.

Collectively, these findings confirm the feasibility of producing a dry inhalable liposome-based formulation capable of co-delivering clinically relevant drug combinations for asthma and COPD. By providing mechanistic insight and actionable design principles, this first chapter establishes the scientific foundation for the development of optimized liposomal DPIs. The knowledge gained in this chapter will directly support the drying of an optimized liposomal formulation specifically designed for asthma treatment, which forms the basis of the second chapter of this thesis.

CHAPTER 2

COMPARATIVE EVALUATION OF
OPTIMIZED LIPOSOME-BASED AND NON-
LIPOSOME-BASED INHALABLE DRY
POWDER CONTAINING A NOVEL ASTHMA
API COMBINATION

Chapter 2. Comparative evaluation of optimized liposome-based and non-liposome-based inhalable dry powder containing a novel asthma API combination

I. Introduction

This chapter focuses on the comparison of inhalable dry powder formulations with and without liposomal carriers in terms of physicochemical and aerodynamic properties. To this end, liposome-based and non-liposome-based DPI formulations co-containing a novel asthma API combination, ciclesonide (CIC) and indacaterol maleate (IND) (Figure 42), were developed and optimized.

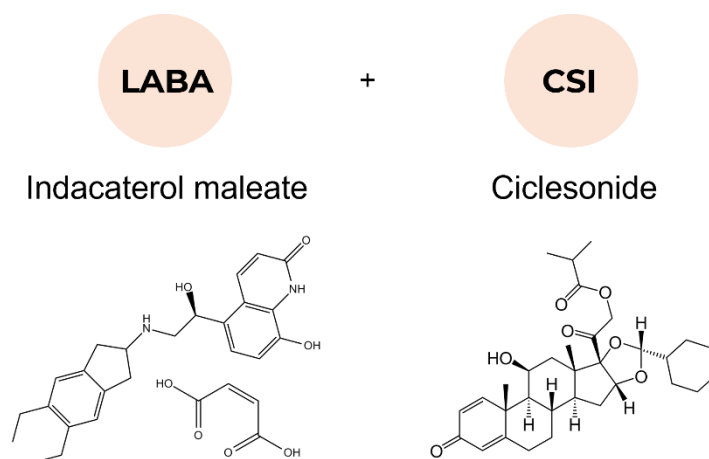


Figure 42: Therapeutic combination of interest: indacaterol maleate (a long-acting β_2 -agonist, LABA) and ciclesonide (an inhaled corticosteroid, ICS).

This combination is supported by a strong pharmacological and therapeutic rationale. More specifically, IND is an ultra-LABA characterized by a rapid onset of action and a 24-hour bronchodilatory effect, enabling once-daily administration, which may contribute to improved treatment adherence [362]. In contrast, CIC is an ICS administered as a prodrug, which undergoes intracellular activation in bronchial epithelial cells to its pharmacologically active metabolite, desisobutyryl-ciclesonide (des-CIC) [363,364]. This prodrug approach has been shown to reduce oropharyngeal side effects and systemic exposure by promoting localized activation in the lungs [365]. Taken together, the complementary bronchodilatory and anti-inflammatory actions of IND and CIC align with the therapeutic principles underlying ICS-LABA regimens

recommended by current GINA and Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines for the management of asthma, as well as COPD [296,299].

Despite this strong therapeutic relevance, this combination does not currently exist on the market. Furthermore, although IND is commercialized as a DPI, either alone (Onbrez®), in combination with mometasone furoate (Ateectura®) or in triple therapy with glycopyrronium (Enerzair®) [142], CIC is only marketed as a pMDI under the pharmaceutical product Alvesco®, which is available in France but not in Belgium [5,366]. For this reason, investigating this association, particularly in a DPI format, could improve lung deposition through particle engineering while simultaneously leveraging the therapeutic complementarity of both APIs, ultimately enhancing clinical efficacy [306].

Beyond this pharmacological rationale, the incorporation of liposomes offers additional advantages for pulmonary delivery. Inhaled drugs are often rapidly cleared from the lungs by mucociliary transport and alveolar macrophages, which can significantly limit their residence time and therapeutic efficacy. Liposomal systems can help overcome these barriers by enhancing mucus penetration, reducing macrophage uptake and acting as local drug reservoirs, thereby prolonging pulmonary retention and enabling sustained drug release [42,94,367]. This approach is particularly advantageous for CIC, whose pharmacological activity depends on intracellular uptake and therefore benefits from prolonged residence within the lung environment. Moreover, even for APIs such as IND, which primarily exert their effect at the cell surface, improved mucus penetration and reduced clearance may enhance drug availability at the site of action [365,367,368]. In addition, liposomal delivery systems may enable dose reduction by improving local drug retention and targeting, thereby potentially minimizing systemic exposure and reducing the risk of side effects. The sustained release profile may also decrease dosing frequency, which could contribute to improved disease control and enhanced patient adherence to treatment [226,369].

To address these objectives, this chapter is structured into three complementary parts:

Part I – Development and optimization of a liposome-based DPI formulation containing CIC and IND

Building on the formulation-process framework established in Chapter 1, this part focuses on the development and optimization of a liposome-based DPI specifically

engineered for pulmonary administration and co-encapsulating the CIC-IND combination. While the model liposomes (SPC/CHOL/DSPE-PEG₂₀₀₀, 67.5/30/2.5 molar ratio) previously investigated were suitable for elucidating the effects of SD on liposomal integrity and powder properties, they were not fully characterized for inhalation-specific applications. Accordingly, the optimized SD conditions identified in Chapter 1 are applied to liposomes tailored for pulmonary delivery in the context of asthma treatment (optimized by collaborators). The aim is thus to develop a liposome-based DPI combining appropriate liposomal attributes with robust physicochemical and aerodynamic performance required for inhalation.

Part II – Development of optimized non-liposome-based DPI formulations containing CIC and IND

This part addresses the development of optimized non-liposome-based DPI formulations co-containing IND and CIC. The initial formulation aims to enable the development of this novel API combination as a DPI with improved lung deposition and clinically relevant dosing. This powder represents a reference for comparison with the liposomal formulation, using matched composition and dose levels based on the optimized liposome-based DPI developed in Part I.

Part III – Comparative evaluation of liposome-based and non-liposome-based DPI formulations containing CIC and IND

The final part is dedicated to a head-to-head comparison of the two optimized DPI systems developed in this work, namely the liposome-based formulation from Part I and the reference non-liposomal formulation established in Part II. This evaluation aims to assess the impact of liposome incorporation into DPI formulations by comparing physicochemical properties (e.g., particle size and residual water content), aerosol performance, pulmonary deposition and physicochemical stability. In addition, this comparative analysis provides optimized formulations that constitute a solid basis for future studies investigating the potential benefits of liposomal encapsulation in terms of bioavailability and safety.

II. Part I. Development and optimization of a liposome-based DPI formulation containing CIC and IND

II.1. Context

II.1.1. *Scientific basis for the optimized liposomal formulation for asthma treatment*

In the context of the **LNPulmo project**, a systematic optimization of a liposomal formulation for the co-delivery of CIC and IND was undertaken, particularly through the contributions of **PhD students Tuan Nghia Dinh (Promotor: Prof. Géraldine Piel, LTPB)** and **Benedetta Bottero (Promotor: Prof. Didier Cataldo, LBTB)**.

The initial step of this work consisted in identifying the most suitable PEGylated lipid for the formulation. Among several PEGylated lipids, DSPE-PEG₂₀₀₀ was identified as the most suitable candidate. Indeed, its terminal amine function enhances IND loading through amine-phosphate interactions, while its C18 acyl chain confers superior macrophage compatibility relative to shorter-chain analogs. Incorporation of DSPE-PEG₂₀₀₀ at 15% enabled efficient mucopenetration and reduced macrophage uptake while leading to a marked improvement in the *ex vivo* bronchodilatory effect of IND compared with the free-drug solution and formulations containing different PEGylation percentages.

Following PEGylated lipid selection, several phospholipids were systematically assessed. Among them, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC) emerged as the optimal phospholipid matrix, as its high degree of unsaturation supported the co-encapsulation of hydrophobic agents and facilitated epithelial cell uptake. Moreover, *ex vivo* studies performed on isolated rat tracheal rings demonstrated that DLPC-based liposomes induced a significantly greater relaxation of airway smooth muscle than free IND, as reflected by an increased maximal relaxant effect (E_{max}) and a substantially reduced EC₅₀. This improved bronchodilatory response was not attributable to an intrinsic effect of the liposomes themselves and was observed independently of the presence of CIC, indicating that DLPC specifically promotes IND availability and action at the target tissue level.

Beyond its enhanced *ex vivo* bronchodilatory activity, the optimized liposomal formulation also demonstrated marked therapeutic benefits *in vivo*. In an ovalbumin (OVA)-induced murine model of asthma, the formulation significantly reduced airway

hyperresponsiveness and attenuated both upstream epithelial alarmin release and downstream type 2 inflammatory cytokine production, as well as inflammatory cell recruitment. Importantly, these effects were achieved at reduced doses of both CIC and IND, suggesting a potential dose-sparing effect and underscoring the promise of this liposome-based formulation as a more efficient pulmonary delivery strategy.

Taken together, these findings highlight the **DLPC/CHOL/DSPE-PEG₂₀₀₀ (55/30/15, molar ratio)** formulation as an optimized liposomal system co-encapsulating CIC and IND, and as a promising candidate for improved asthma therapy. The complete set of results generated in this work has been submitted for publication to *International Journal of Pharmaceutics* on May 13th 2026, under the title “*Ciclesonide/indacaterol-encapsulated liposomes: optimizing corticosteroid-bronchodilator therapy for a more effective approach to asthma management*”. The manuscript was co-written by Tuan Nghia Dinh (LTPB, CIRM) and Benedetta Bottero (LBTD, GIGA-Cancer). The co-authors are Fabienne Perin (GIGA-Cancer and CHU of Liège), Laure-Anne Bya, Anna Lechanteur, Brigitte Evrard and Géraldine Piel (LTPB, CIRM, University of Liège), and Didier Cataldo (GIGA-Cancer and CHU of Liège).

II.1.2. Objectives of this part

The selection of the optimal liposomal formulation, **DLPC/CHOL/DSPE-PEG₂₀₀₀ (55/30/15, molar ratio)**, provides the scientific basis for its further development and in-depth investigation, particularly with respect to drying. Overall, this first part of Chapter 2 aims to evaluate the feasibility of transferring the optimized SD conditions established in Chapter 1 (T_{in} 120 °C, flowrate 1.56 g/min, NGP 3.4 bar and CGP 0.3 bar, with a 10% lipid-to-carbohydrate ratio and using HPBCD as the protectant carbohydrate) to the drying of this formulation. This approach is intended to support the development of an optimized liposome-based DPI for asthma management based on the innovative combination of CIC and IND.

It is worth noting that DoE_B conducted in Chapter 1 identified 2.5% DSPE-PEG₂₀₀₀ as the optimal level to limit drug leakage during drying, as higher PEGylated lipid contents were shown to increase membrane fluidity and promote lipophilic drug leakage under thermal stress. However, from this point onward, the final liposomal formulation contains a substantially higher proportion of PEGylated lipid (15%) to enhance mucus

penetration. The impact of this increased PEG content on liposomal integrity and stability during drying will therefore be specifically evaluated.

Prior to this, this part of the work addressed the continuity between liposome production methods across different scales. To ensure methodological consistency with Chapter 1 and enable the production of larger volumes, the feasibility of transitioning optimized liposomes production from TFH (total lipid concentration: 5 mM) to the PGSS method at higher total lipid concentration (45 mM) was evaluated. These preliminary experiments, conducted by PhD student Tuan Nghia Dinh, relied on a PGSS process for liposome production previously optimized at the LTPB and also applied in Chapter 1, building on earlier work by Delma *et al.* and Penoy *et al.* under the supervision of Prof. Géraldine Piel [85,89]. However, this concentration (45 mM total lipids) did not yield satisfactory results under the selected conditions, particularly in terms of API encapsulation efficiency and reproducibility (Table 6, Results section), leading to the selection of 27.5 mM total lipids for subsequent experiments.

Spray-dried powders were subsequently prepared from liposomes produced by both TFH and PGSS, each at a total lipid concentration of 27.5 mM, allowing evaluation of the applicability and robustness of the optimized SD conditions across liposomes generated using two distinct production methods.

Ultimately, this section aims to **evaluate the feasibility of developing an optimized liposome-based DPI for asthma therapy** by integrating two industrially relevant production steps, PGSS for liposome manufacture and SD for powder formation, into a coherent and scalable process chain.

II.2. Materials and methods

The materials and methods are largely consistent with those described in Section II.2.2 (Chapter 1) of the publication “Optimizing Spray-Dried Liposomes for Pulmonary Delivery: Impact of Lipids Composition and of Drying Parameters using experimental design”. Additional materials and methods are therefore detailed in the present section.

II.2.1. Materials

In addition to the lipids previously described in Section II.2.2.1 (Chapter 1), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC) was added in this part of the thesis. DLPC was purchased from Avanti Polar Lipids, Inc. (AL, USA).

II.2.2. Preparation of the optimized liposomal formulation

Liposome dispersions obtained after TFH and PGSS processes were purified by dialysis for 4 hours according to the protocol described in Section II.2.2.3.a (Chapter 1). The purified liposomal suspensions were then stored at 4 °C until further characterization.

II.2.2.1. Thin film hydration

SPC, cholesterol, DSPE-PEG₂₀₀₀ (55:30:15 molar ratio) and CIC and IND (5% of total lipids) were dissolved in ethanol in a round-bottom flask. The organic solvent was removed at 30 °C using a rotary evaporator (BÜCHI Labortechnik GmbH, The Netherlands), resulting in the formation of a thin lipid film. This film was subsequently hydrated with HEPES buffer (10 mM, pH 7.4) to obtain a liposomal dispersion. The suspension was then extruded three times successively through polycarbonate membranes with pore sizes of 400 nm followed by 200 nm (Whatman, UK) in order to control vesicle size. The theoretical lipid concentration in the final liposome suspension was 27.5 mM.

II.2.2.2. Particles from gas saturated solutions

Liposomes composed of SPC or DLPC, CHOL and DSPE-PEG₂₀₀₀ (55:30:15 molar ratio) and CIC and IND (5% of total lipids) were produced using the PGSS technique, employing scCO₂ as a dispersing agent. The process was conducted under optimized conditions (30 min contact with scCO₂, agitation at 500 rpm, 80 °C and 240 bar), following the procedure described in Section II.2.2.3.a (Chapter 1). The theoretical lipid concentration in the final liposome suspension was 27.5 mM.

II.2.3. Oxidation study

Lipid oxidation was evaluated by monitoring the formation of conjugated dienes using UV-visible spectrophotometry, based on the method reported by Delma *et al.* [85]. Absorbance measurements were performed at 234 nm with a UV/VIS

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spectrophotometer (U-3000, Hitachi, Ltd., Tokyo, Japan), using absolute ethanol as the reference solvent.

Lipid dispersions prior to PGSS processing, liposomal suspensions obtained after PGSS processing and powders rehydrated after SD were dissolved in absolute ethanol to obtain a final lipid concentration of 1 mg/mL. The extent of lipid oxidation was determined from the absorbance of conjugated dienes measured at 234 nm.

The concentration of peroxidized lipids (mol/L) was calculated from the difference in absorbance between samples measured before and after PGSS processing, considering the molar extinction coefficient and the optical path length. The percentage of lipid oxidation was subsequently determined by relating the concentration of oxidized lipids to the total lipid concentration in the sample. The equations used to calculate the concentration of peroxidized lipids and the corresponding oxidation percentage were applied according to the recommendations provided by Avanti Polar Lipids [370], and are given below (Equation 7 and Equation 8).

$$C_{\text{peroxidized lipids}} \left(\frac{\text{mol}}{\text{L}} \right) = \frac{A}{B \times C}$$

Equation 7: Calculation of the concentration of peroxidized lipids.

where A = Absorbance reading at 234 nm, B = Extinction coefficient in (OD*mL)/(mmol*cm) (27,000 for peroxidized lipid) and C = Path length in cm.

$$\text{Percent oxidation (\%)} = \frac{D}{E} \times 100$$

Equation 8: Calculation of the corresponding oxidation percentage.

where D = Concentration of peroxidized lipid in mg/mL E = Concentration of lipid sample = 1.0 mg/mL.

II.3. Results and discussion

II.3.1. *Pre-drying optimization and assessment of SD robustness across liposomes produced by TFH and PGSS*

As previously outlined, the optimized liposomal formulation, initially produced by TFH at a total lipid concentration of 5mM, was subsequently transposed to the PGSS process at 45 mM (total lipid concentration). However, this approach showed limited robustness, as reflected by poor reproducibility of API encapsulation and high variability in the results (Table 6). This reduced formulation quality was attributed to the combined effects of the modified lipid composition (DLPC), the elevated PEGylated lipid content (15%) [333,371], and the high lipid concentration, which likely altered membrane organization and increased heterogeneity [372].

Rather than modifying the previously optimized PGSS parameters or liposome composition, the total lipid concentration was reduced to 27.5 mM, resulting in improved formulation homogeneity and reproducibility, as reflected by a reduced Pdl (0.26 ± 0.05) and more consistent encapsulation efficiencies (Table 6).

Table 6 : Characterization of optimal liposomal formulations produced by TFH and PGSS, prepared at two total lipid concentrations (5 and 27.5 mM). Results at 45 mM (PGSS) were obtained from preliminary experiments conducted by PhD student Tuan Nghia Dinh. Analyses were performed in triplicate (n=3), and results are expressed as mean $\pm$ standard deviation.

	Formulations			
	5 mM - TFH	27.5 mM - TFH	27.5 mM - PGSS	45 mM - PGSS
Size (nm)	141.32 $\pm$ 3.13	140.57 $\pm$ 5.44	126.06 $\pm$ 4.03	126.03 $\pm$ 9.82
Pdl	0.09 $\pm$ 0.03	0.10 $\pm$ 0.02	0.26 $\pm$ 0.05	0.34 $\pm$ 0.04
ZP (mV)	-2.72 $\pm$ 0.56	-3.33 $\pm$ 0.36	-2.63 $\pm$ 0.51	-6.63 $\pm$ 1.19
CIC EE (%)	97.28 $\pm$ 0.16	99.26 $\pm$ 3.91	96.19 $\pm$ 2.19	96.04 $\pm$ 10.46
IND EE (%)	94.62 $\pm$ 0.88	95.99 $\pm$ 3.52	90.20 $\pm$ 2.76	98.16 $\pm$ 9.19

Although the Pdl of liposomes produced at 27.5 mM by PGSS remained higher than that of TFH-produced liposomes, it remained below 0.3 and therefore within an acceptable range for pulmonary delivery and subsequent SD. Liposomes produced at a total lipid concentration of 27.5mM exhibited a suitable size for inhalation (126.06 ± 4.03 nm), a near-neutral ZP (-2.63 ± 0.51 mV), and high encapsulation efficiencies for both APIs ($96.19 \pm 2.19\%$ for CIC and $90.20 \pm 2.76\%$ for IND). Consequently, the 27.5 mM optimal liposomal formulation was selected for subsequent

drying and liposome-based DPI production, and the optimized SD conditions from Chapter 1 were applied to liposomes produced by both TFH and PGSS to assess their suitability for liposomes generated using two distinct production methods.

Process performance during SD was comparable for liposomal formulations produced by both methods, with yields of $75.96 \pm 0.07\%$ for TFH-derived liposomes and $79.89 \pm 5.19\%$ for PGSS-derived liposomes. The resulting powders also exhibited similar particle size characteristics, with mean diameters of $2.80 \pm 0.39 \mu\text{m}$ and $2.65 \pm 0.09 \mu\text{m}$ and span values of 1.85 ± 0.38 and 1.51 ± 0.01 , for TFH and PGSS produced powders, respectively, which are within the suitable range for inhalation [373]. Residual moisture contents were likewise comparable, reaching $4.56 \pm 0.70\%$ for TFH and $4.30 \pm 0.59\%$ for PGSS, both remaining below the recommended 5% threshold for dry powder formulations [172]. Concerning API content, both powders exhibited similar drug loading, with IND contents of $0.26 \pm 0.01\%$ and $0.24 \pm 0.01\%$ and CIC contents of $0.27 \pm 0.01\%$ and $0.23 \pm 0.02\%$ for powders obtained from TFH and PGSS-produced liposomes, respectively. API distribution within the powders remained homogeneous, as indicated by low CV, ranging from $1.49 \pm 0.74\%$ to $2.48 \pm 0.68\%$ for IND and from $2.56 \pm 0.62\%$ to $3.32 \pm 2.17\%$ for CIC. Taken together, these results indicate that the physicochemical properties of the spray-dried powders were largely independent of the upstream liposome production method, provided that liposomal composition, L/C ratio and drying conditions were maintained constant.

Liposomal integrity following SD was also evaluated after powder rehydration. In both cases, the optimized drying conditions preserved liposomal characteristics, as evidenced by size ratios of 0.92 ± 0.02 for TFH-derived liposomes and 0.82 ± 0.03 for PGSS-derived liposomes, together with Pdl values of 0.12 ± 0.01 and 0.18 ± 0.01 , respectively. In both cases liposomes exhibited sizes of $130.68 \pm 4.44 \text{ nm}$ and $107.72 \pm 3.62 \text{ nm}$ for TFH and PGSS-produced liposomes after SD (Figure 43A), respectively, with size ratios close to unity, Pdl values below 0.30 (Figure 43B), and near-neutral ZP (Figure 43C), indicating well-preserved liposomal characteristics suitable for inhalation.

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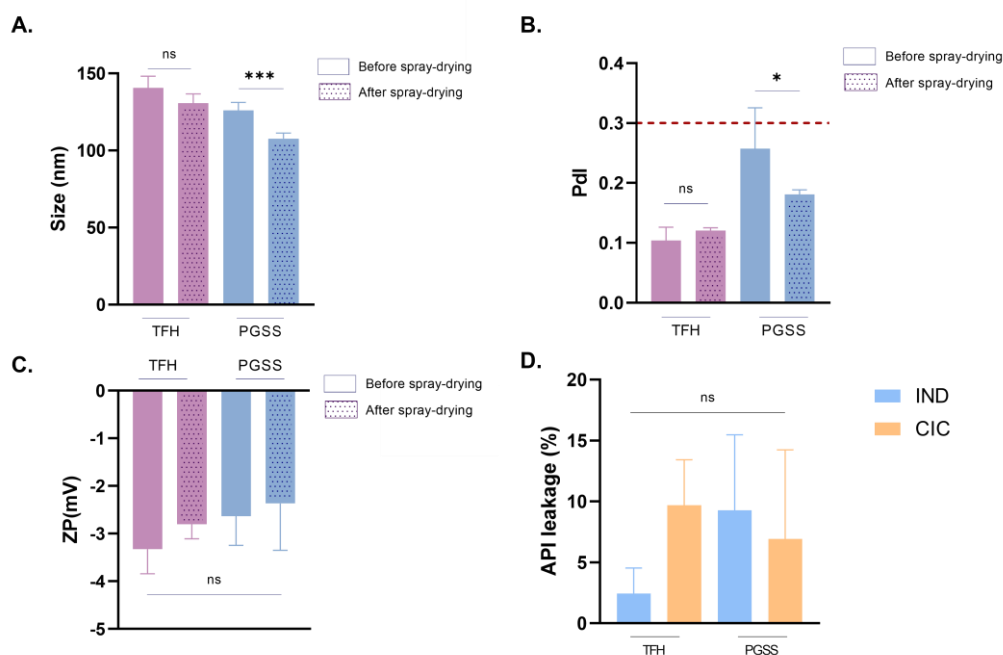


Figure 43: Comparison of liposomal integrity before and after spray-drying for optimized formulations prepared by thin-film hydration (TFH) and PGSS. (A) Mean liposome size, (B) polydispersity index (Pdl), and (C) zeta potential (ZP) of liposomes before spray-drying and after rehydration of the spray-dried powders, allowing assessment of the impact of the drying process on liposomal integrity for both production methods. (D) Percentage of API release from liposomes after spray-drying for the investigated formulations. Analyses were performed in triplicate ($n = 3$), and results are presented as mean $\pm$ standard deviation.

Furthermore, API release after SD remained limited, reaching $2.44 \pm 1.50\%$ and $8.16 \pm 7.08\%$ for IND and $9.69 \pm 2.76\%$ and $9.23 \pm 5.68\%$ for CIC in liposomes produced by TFH and PGSS, respectively (Figure 43D). This high retention is consistent with the pronounced lipophilicity of both molecules ($\log P = 3.31$ for IND and 4.08 for CIC), which favors their partitioning within the lipid bilayer. Importantly, although results from Chapter 1 (DoE_B) indicated that increasing DSPE-PEG₂₀₀₀ content could promote API release, particularly for BUD, this effect was negligible for the highly lipophilic compounds investigated here. Their strong affinity for the lipid bilayer likely outweighed any destabilizing effects induced by higher PEGylated lipid content during atomization, thereby ensuring drug retention. This observation is particularly encouraging, as a high proportion of PEGylated lipid is required to ensure effective mucus penetration in the optimized formulation. Overall, while PEGylated lipid content may affect the retention of moderately lipophilic APIs, its impact remains limited for highly lipophilic molecules, whose membrane affinity governs their stability.

Taken together, increasing the lipid concentration from 5 to 27.5 mM was successfully validated using the TFH method, with no significant impact on liposome physicochemical properties. In addition, the optimal liposomal formulation was successfully spray-dried under the optimized SD conditions developed in Chapter 1, irrespective of the liposome production method, thereby highlighting the interest and broad applicability of the drying process.

In this section, DLPC was temporarily replaced by the structurally similar and more cost-effective SPC in order to assess the scalability and robustness of the production and drying processes under industrially relevant conditions. Once these process-related aspects were validated using SPC, the next step was to confirm that the optimized SD conditions could be successfully transferred to the final formulation based on DLPC at 27.5 mM, produced with PGSS. Section II.3.2 therefore focuses on evaluating this transferability and further assessing liposomal integrity after drying.

II.3.2. Spray-drying of an optimized liposomal formulation for inhalation: validation and further evaluation

The optimized liposomal formulation, with DLPC as the main phospholipid, was produced using the PGSS process, yielding liposomes with a mean size of 108.79 ± 4.35 nm, a Pdl of 0.26 ± 0.04 , a near-neutral ZP of -4.16 ± 2.62 mV while assuring high encapsulation efficiency of 95.53% and 95.85% for CIC and IND, respectively. The optimized SD condition (Chapter 1) was subsequently applied to these liposomes to assess the feasibility of coupling scalable liposome production with SD for the development of an inhalable DPI, while ensuring the preservation of liposomal integrity and appropriate powder properties. The results confirm that optimized SD conditions yield inhalable powders from PGSS-produced liposomes while preserving liposomal integrity. A summary of the resulting powder properties and post-drying liposome characteristics is provided in the table below (Table 7).

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Table 7: Physicochemical properties of the optimized liposomal dry powder and liposomal integrity after spray-drying. Analyses were performed in triplicate (n=3), and results are expressed as mean $\pm$ standard deviation.

	PROPERTIES	VALUE (MEAN $\pm$ SD)
DRY POWDER	Drying yield (%)	77.92 $\pm$ 4.48
	Particle size (D50) (μ m)	2.65 $\pm$ 0.25
	Moisture content (%)	3.66 $\pm$ 0.41
	CIC content (%)	0.27 $\pm$ 0.03
	IND content (%)	0.27 $\pm$ 0.02
LIPOSOMAL INTEGRITY	Particle size post-drying (nm)	108.79 $\pm$ 4.35
	Size ratio (Sf/Si)	1.02 $\pm$ 0.04
	PdI	0.22 $\pm$ 0.01
	ZP (mV)	-3.45 $\pm$ 2.52
	IND release (%)	5.05 $\pm$ 5.41
	CIC release (%)	5.38 $\pm$ 7.19

Regarding powder properties, the drying yield reached 77.92 $\pm$ 4.48%, and the resulting DPI powder displayed a suitable median particle size of 2.65 $\pm$ 0.25 μ m with a low residual moisture content of 3.66 $\pm$ 0.41%. In addition, the API content in the powder was determined and reached 0.27 $\pm$ 0.03% for CIC and 0.27 $\pm$ 0.02% for IND. Importantly, API recovery after SD reached 101.51 $\pm$ 2.64% for IND and 101.29 $\pm$ 4.20% for CIC, indicating that no significant degradation occurred during the SD process and that both APIs were quantitatively retained in the final powder.

After redispersion, liposomes maintained a particle size of 108.79 $\pm$ 4.35 nm and a size ratio of 1.02 $\pm$ 0.04, confirming that the drying process induced minimal change in vesicle size. This was further supported by a low PdI value of 0.22 $\pm$ 0.01, indicative of a narrow size distribution and a near-neutral ZP of -3.45 $\pm$ 2.52 mV. In addition, API leakage remained limited after drying, reaching 5.05 $\pm$ 5.41% for IND and 5.38 $\pm$ 7.19% for CIC, suggesting that the lipid bilayer retained its barrier properties and that both compounds remained largely associated with the liposomes throughout the process. Overall, these results confirm that the optimized SD conditions are well suited for producing inhalable liposome-based DPI while preserving the essential physicochemical properties of the vesicles.

To further validate the robustness of the drying conditions and assess lipid bilayer integrity, complementary microscopy analyses were performed. Although multilamellar liposomes were observed post SD compared to after PGSS production (Figure 44), this

phenomenon is well documented in the literature and is generally attributed to lipid bilayer rearrangements occurring during dehydration and subsequent rehydration. Such rearrangements reflect a reorganization of the lipid membranes rather than degradation of the vesicular structure. Indeed, liposomes can undergo dehydration and reconstitution without major alterations in vesicle size or drug retention when the formulation is properly stabilized [280,374]. Consistent with these observations, microscopy revealed intact lipid bilayer structures before and after drying, together with preservation of liposome size, confirming that the overall integrity of the liposome-based system was maintained.

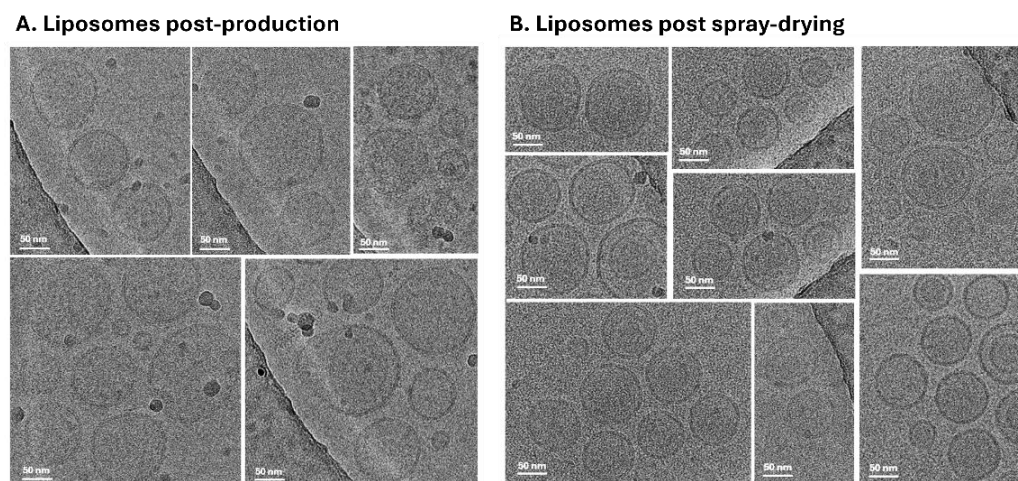


Figure 44: (A) Cryo-TEM images of liposomes before SD and (B) rehydrated liposomes after SD, within the rehydrated carbohydrate matrix.

Moreover, powder homogeneity was assessed and revealed low CV of $2.14 \pm 1.64\%$ and $2.44 \pm 1.23\%$ for CIC and IND, respectively. Such low CV values indicate excellent content uniformity [350], confirming that API distribution remained homogeneous despite liposomal rearrangements. This is a critical parameter for ensuring dose reproducibility, consistent aerosol performance, and ultimately reliable therapeutic efficacy [84].

In addition to structural integrity, the chemical stability of the lipid bilayer was also considered. Indeed, liposomes are known to be susceptible to hydrolytic and oxidative degradation. In particular, phospholipids containing unsaturated acyl chains, such as DLPC used in this study, are more prone to oxidative degradation due to the presence of double bonds (Figure 45B) [89,375]. Therefore, lipid oxidation was evaluated by

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measuring the absorbance at 234 nm, enabling the detection of conjugated dienes, which are widely recognized as early markers of lipid peroxidation resulting from the oxidation of double bonds in phospholipid acyl chains [85,89]. The increase in lipid oxidation was monitored at each production step by comparing absorbance values measured before and after liposome production, accounting for the initial oxidation level present in the phospholipids, and before and after SD. As shown in Figure 45A, although a slight increase in absorbance was observed after SD, this corresponded to an oxidation increase of only $0.04 \pm 0.01\%$. This very low level of oxidation was not associated with any measurable alteration of liposomal physicochemical properties, indicating that liposomal integrity was maintained under the optimized SD conditions. In addition to oxidation, phospholipids may undergo hydrolytic degradation through ester bond cleavage, generating lysophospholipids and free fatty acids [85]. Since this process can be promoted by moisture and elevated temperatures, liposomal lipid hydrolysis was also investigated. However, interference from the carbohydrate matrix prevented reliable assessment following SD, precluding definitive conclusions regarding hydrolytic degradation under the optimized processing conditions. Nevertheless, the preservation of liposomal physicochemical properties after rehydration suggests that any hydrolysis was limited and did not significantly compromise liposomal integrity.

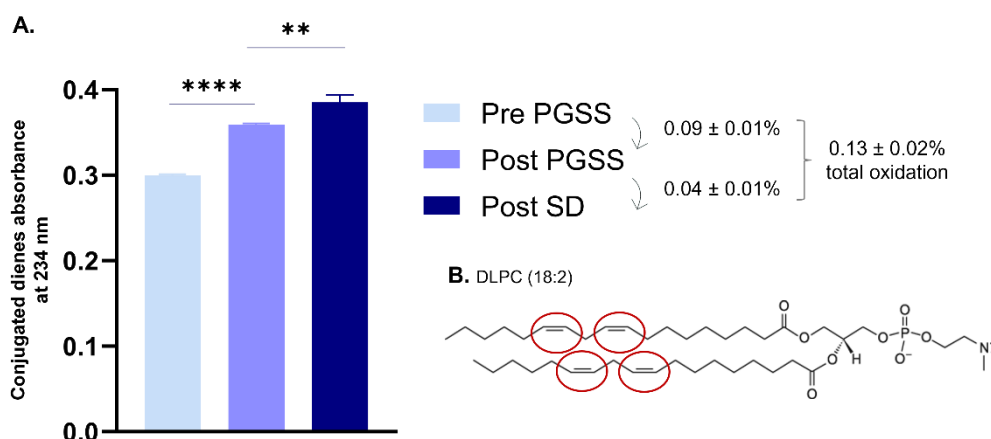


Figure 45: (A) Conjugated diene absorbance (indicator of lipid oxidation) measured at each stage of the production process. (B) Chemical structure of the main phospholipid used in the formulation, 1,3-bis(sn-3'-phosphatidyl)-sn-glycero-3-phosphocholine (DLPC). Analyses were performed in triplicate ($n=3$), and results are presented as mean $\pm$ standard deviation.

Overall, the application of the optimized SD conditions enabled the successful transformation of the liquid liposomal formulation developed for asthma management

into an inhalable dry powder, while maintaining its structural and physicochemical integrity. The preservation of vesicle size, drug retention and lipid stability confirm the robustness of the drying process and support its suitability for the production of liposome-based DPI formulations. This optimized dried liposomal powder will be further characterized in the following section.

II.4. Conclusion

This first part of this Chapter 2 aimed to evaluate the feasibility of applying the optimized SD conditions identified in Chapter 1 to a liposomal formulation specifically developed for asthma therapy and co-encapsulating CIC and IND.

The results presented in this section demonstrate that this objective was successfully achieved. The use of these optimized conditions on inhalation-tailored liposomes resulted in inhalable dry powders exhibiting suitable aerodynamic properties, low residual moisture content and homogeneous API distribution. Importantly, these drying conditions preserved the structural and physicochemical integrity of the optimized liposome formulation after drying, as evidenced by stable vesicle size, low Pdl, near neutral ZP, limited CIC and IND release, while showing negligible lipid oxidation. This preservation of liposomal integrity suggests that the formulation retains key features likely required to support the therapeutic benefits previously demonstrated *in vivo* by collaborators using the corresponding non-dried liposomal system.

In addition, to challenge the robustness of the optimized SD process, liposomes produced by different manufacturing methods (TFH and PGSS) were subjected to identical drying conditions. Irrespective of the upstream production method, the optimized SD conditions consistently yielded powders with comparable properties and well-preserved liposomal characteristics. This demonstrates that the drying process itself is robust and largely insensitive to moderate variations in liposome production method, assuming that formulation composition and key process parameters are controlled.

Overall, these findings confirm the feasibility of developing an optimized liposome-based DPI for asthma therapy by applying the SD conditions established in Chapter 1 to a clinically relevant, highly PEGylated liposomal formulation encapsulating CIC and IND. They further support the relevance of this drying strategy for the continued

development of liposome-based DPI formulations within a scalable and coherent process framework.

III. Part II. Development of optimized non-liposome-based DPI formulation containing CIC and IND

III.1. Context

While IND is available as a DPI, CIC is only marketed as a pMDI and no DPI combining both APIs currently exists. This highlights the need for a formulation co-delivering CIC and IND within a single inhalation system to combine bronchodilator and anti-inflammatory effects. As a reminder, this combination is particularly attractive, as IND provides rapid and ultra-long-acting bronchodilation, while CIC, as a prodrug, ensures targeted pulmonary activation and an improved safety profile.

In this context, non-liposome-based DPI systems incorporating the same API combination were developed. An initial formulation was designed to assess the feasibility of delivering the CIC-IND association as a DPI, with appropriate lung deposition and clinically relevant dosing. This formulation therefore represents a novel therapeutic approach by enabling the delivery of this API combination in a DPI format. The results obtained for the development of this formulation are specifically reported in the published article entitled *“Formulation of a Dry Powder for Inhalation Combining Ciclesonide and Indacaterol Maleate Using Spray-Drying”*, published in the *International Journal of Pharmaceutics* and presented in the following Section III.2.

Building on the insights gained from this first system, an optimal non-liposomal formulation was subsequently developed as a reference for direct comparison, with composition and dose levels matched to those of the liposomal DPI described in Part I. This strategy supports a key objective of the LNPulmo project (partner 3), which is to evaluate the added value of liposomal encapsulation in terms of pulmonary bioavailability, drug retention and potential for sustained release, as well as its impact on pulmonary deposition and powder stability. The development of a non-liposomal reference formulation is therefore essential to ensure that any observed differences in performance can be specifically attributed to the presence of liposomes. The results associated with the development of this reference formulation are further detailed in this section.

This second part therefore **aims to evaluate the feasibility of developing optimized DPI co-delivering CIC and IND**, with **suitable aerosol performance, enhanced pulmonary deposition and adequate stability over time**.

III.2. Published results

III.2.1. *Summary of results*

This study aimed to develop a carrier-free DPI combining CIC and IND, representing a novel therapeutic approach for asthma, using SD technology to produce an optimized inhalable formulation. Due to the high hydrophobicity of CIC, the feasibility of atomizing either a solution or a suspension was investigated. The results clearly demonstrate that the solubilization state of CIC prior to atomization plays a critical role in determining the final physicochemical and aerodynamic properties of the powders.

Phase-solubility studies of cyclodextrin-CIC complexes performed at 37 °C, identified Crysmeb® as the most effective excipient for enhancing CIC solubility. Compared to HPβCD and HPyCD, Crysmeb enabled the formation of a stable solution suitable for atomization. In contrast, HPβCD did not allow sufficient solubilization under similar conditions but was retained due to its previously reported ability to generate deflated microparticles, leading to the development of suspension-based formulations. Additionally, the influence of SC (5 and 10%, w/v) was investigated at fixed API and excipient ratios.

From a physicochemical standpoint, all spray-dried powders exhibited properties suitable for pulmonary delivery, with high process yields (~77%), residual moisture contents between 4.3 and 6.5% and narrow particle size distributions (span < 2). Particle sizes were within or close to the optimal range for deep lung deposition (2-5 μm) and scanning electron microscopy (SEM) confirmed a consistent deflated morphology across all formulations, known to enhance dispersibility and deposition. Increasing solid content reduced residual moisture without significantly affecting morphology.

Despite these similarities, notable differences were observed depending on liquid feed type. Solution-based powders exhibited smaller particle sizes (~3 μm), whereas suspension-derived powders showed significantly larger particles (>4 μm at 10% w/v), likely due to the presence of non-solubilized drug. Drug recovery after drying also differed, with IND recovery remaining close to 100%, whereas CIC recovery was lower in suspension-based formulations (~91% at 5% and ~86% at 10%), likely due to adhesion of non-solubilized drug to the dryer walls rather than thermal degradation.

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All powders met homogeneity criteria ($CV < 5\%$), although solution-based formulations showed superior uniformity. This was confirmed by Raman hyperspectral imaging, which revealed homogeneous API distribution in solution-derived powders, whereas suspensions exhibited distinct CIC-rich domains, indicating less uniformity.

Aerodynamic performance assessed using the NGI highlighted the strong influence of formulation strategy. Solution-based powders showed superior performance, with FPF values exceeding 60% for both drugs, whereas suspension-derived powders exhibited significantly lower FPF, particularly for CIC. This difference was mainly attributed to the higher MMAD of suspensions, promoting upper airway deposition and limiting deep lung penetration. Although ED were high for all formulations ($>90\%$ at 100 L/min), suspensions showed slightly higher emission due to improved flowability, without improving lung delivery due to increased impaction in the upper respiratory tract.

At a reduced flow rate (60 L/min), ED decreased, but FPF values for solution-based powders remained high and comparable to those at 100 L/min, indicating robust performance under clinically relevant conditions. Compared to the commercial product Onbrez[®], solution-derived powders achieved approximately 10-15% higher FPF for IND, attributed to the carrier-free formulation and optimized particle engineering, which reduces drug-carrier interactions and upper airway losses.

The PreciseInhale[®] system was further used to assess aerosolization performance, providing results consistent with NGI findings. The developed powders exhibited significantly higher C_{max} , M_{cas} and aerosolization yield than Onbrez[®], reflecting improved flowability, dispersibility and aerosol generation. FPF values also exceeded 60%, confirming strong pulmonary deposition potential. Importantly, PreciseInhale[®] offers a complementary and emerging approach, enabling both deposition analysis and deeper insight into aerosolization behavior, thereby strengthening the robustness of *in vitro* evaluation.

Finally, a six-month stability study of the optimized formulation (10% solution-based feedstock) demonstrated excellent stability. Particle size, moisture content, drug content, homogeneity and aerodynamic performance remained unchanged under both standard and accelerated International Council for Harmonisation (ICH) conditions. CIC and IND content remained within 95-105% of initial values and FPF showed no

significant decline, indicating good physicochemical and aerodynamic stability despite the potentially hygroscopic nature of spray-dried powders.

Overall, these results demonstrate that powders produced from atomized solutions, enabled by CIC complexation with Crysmeb, are clearly superior to suspension-based formulations. They exhibit optimal particle size, improved homogeneity, higher CIC recovery and significantly enhanced pulmonary deposition. Their performance remains high at clinically relevant inspiratory flow rates and surpasses that of a marketed reference product. Collectively, these findings confirm the potential of SD combined with cyclodextrin complexation as an effective strategy for developing innovative, stable and high-performance inhalable powders.

III.2.2. Publication

Formulation of a Dry Powder for Inhalation Combining Ciclesonide and Indacaterol Maleate Using Spray Drying

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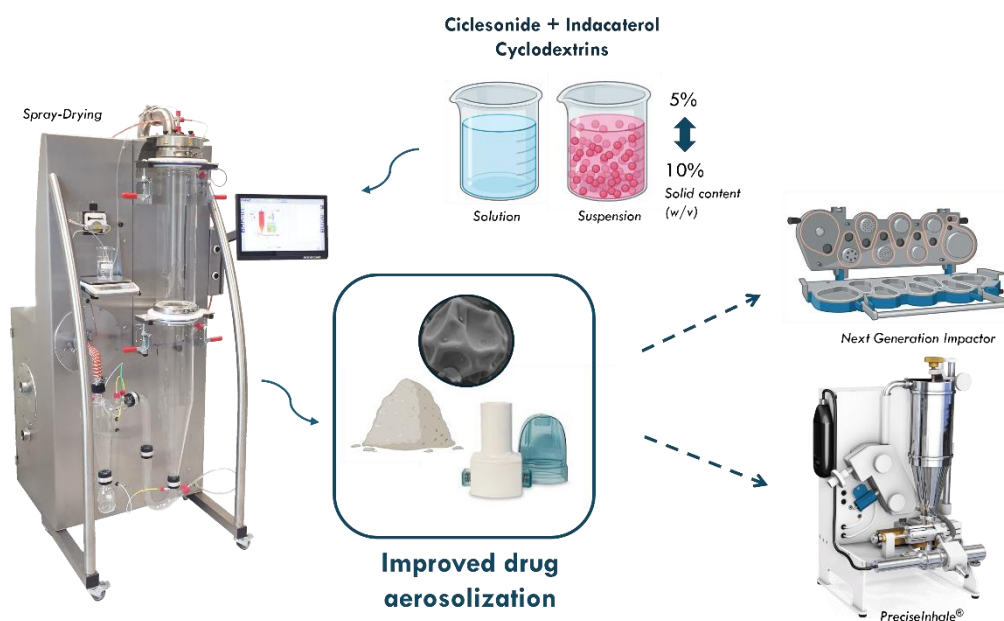
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ABSTRACT

Dry powders for inhalation are evolving to address the challenge of maximizing lung deposition, with growing interest in carrier-free formulations shaping future therapies. This study focuses on developing an inhalation powder with optimal properties, combining ciclesonide and indacaterol, a combination treatment for asthma not yet available on the market. Using spray-drying technology with cyclodextrins, ultra-flying microparticles aim to be produced to enhance aerosolization and therapeutic efficacy. Cyclodextrin screening identified Crysmeb as the most effective for ciclesonide complexation, enabling stable solution atomization, while HP β CD was selected to create deflated particle shapes in a spray-dried suspension.

The impact of active pharmaceutical ingredient solubilization state and solid content on powder properties was investigated, revealing that the solutions provided a more suitable particle size distribution for inhalation. Moreover, the atomized solutions led to fine particle fractions exceeding 60% for both drugs, outperforming commercial products due to this enhanced distribution. Aerodynamic performance was further assessed under reduced flow rates using the Next Generation Impactor, showing no significant reduction in lung deposition at 60 L/min for atomized solutions. The optimized powders also demonstrated higher lung deposition of indacaterol maleate compared to Onbrez[®], with findings confirmed using the PreciseInhale[®] system, providing comprehensive insights into aerosolization behavior. These results highlight the importance of advancing *in vitro* methods to better predict *in vivo* performance and support the development of more effective inhaled therapies.

Overall, this work presents a stable, carrier-free inhalation powder with a novel drug combination that achieves efficient lung deposition and six-month stability.

III.2.2.1. Introduction

Asthma and chronic obstructive pulmonary disease (COPD) represent the foremost chronic inflammatory disorders of the airways, commonly treated via inhaled drug administration [297,298]. Inhalation ensures targeted delivery, rapid therapeutic onset and minimal side effects, using devices like nebulizers, metered-dose inhalers (pMDIs), soft-mist inhalers (SMIs) and dry powder inhalers (DPIs) [17,40,376]. Among them, DPIs stand out due to their lack of propellant gases and the absence of coordination between inhalation and actuation. Most notably, their dry formulation enhances drug stability, making DPIs ideal for delivering sensitive molecules like biotherapeutics, a promising advancement in pulmonary drug delivery [35,377,378]. Beyond the benefits, the main interest in developing DPIs lies in the ability to improve dry powders through particle engineering, ultimately optimizing their performances [306].

Indeed, in the field of DPI, treatment efficacy relies not only on the inhalation device but on specific physicochemical properties of powders that facilitate deep lung deposition [158]. Factors such as the powder's crystalline or amorphous nature, surface charges, residual moisture and particle morphology significantly impact solubility, stability and pulmonary deposition [115,305]. Among the various criteria of powders, the most critical is their aerodynamic diameter (d_{ae}), ideally ranging between 1 and 5 μm , ensuring effective lung deposition and minimizing upper airway impaction [41,152,210]. However, small particle size increases cohesive forces, affecting powder flow and complicating DPI manufacturing [305,379]. To address this, the most widely used technique in current inhalation powder development involves adsorbing micronized active pharmaceutical ingredients (API) onto a sugar-based carrier, typically monohydrated lactose [185,380]. Yet, excessive carrier-API interactions and coarse particle impaction contribute to inefficient delivery, with less than one-third of the medication reaching the deep lung [40,189].

Multiple approaches can be employed to enhance lung deposition, either separately or in combination. These include the optimization of inhalation devices, the refinement of carrier properties, and the development of optimized micronized carrier-free powders [35,381]. As part of these tactics, carrier-free powders development has gained lots of attention in recent years, with techniques like self-agglomeration through granulation or spheronization, as seen in Pulmicort® Turbuhaler® before its market withdrawal in 2021 [232]. Additionally, spray-drying (SD) technology has been pivotal in optimizing carrier-

free powders properties, transforming concentrated liquid feedstocks into dry particles through atomization in a heated gas stream [255,382]. This method is cost-effective, adaptable and reproducible, allowing the creation of engineered DPIs with controlled properties [264]. Modifying parameters like temperature, nozzle pressure and solid content, especially with carbohydrate matrices, enables the development of unique formulations like PulmoSphere™ and PulmoSol™ [194]. This technology employs an emulsion-based SD process to create lightweight, spherical particles, characterized by a high porosity, that reduce surface contact and agglomeration, enhancing flow, dispersion and intrapulmonary deposition [29,194,195]. Furthermore, spherical deflated particles appear to be a very interesting alternative, particularly in terms of increased lung deposition. Dufour *et al.* [205] successfully developed spherical deflated particles by atomizing hydroxypropyl- β -cyclodextrin (HP β CD) with budesonide. Lechanteur *et al.* [177] identified optimal drying parameters that leveraged this excipient to create a DPI combining budesonide and formoterol, ultimately enhancing lung deposition profile due to this specific morphology. Indeed, deflated particles, characterized by lower density, optimized particle size and irregular surfaces that reduce cohesion forces, have demonstrated superior *in vitro* lung deposition compared to traditional lactose-based formulations, thereby significantly enhancing pulmonary delivery efficiency. Gresse *et al.* have recently demonstrated the need to optimize the flow properties of these micronized powders to support industrial-scale production. The blend of coarse-carrier with such spray-dried micronized powder promote accurate capsules filling [181]. These examples underscore the critical but challenging role of particle engineering, where fine-tuning parameters and precise excipient selection can impact multiple properties, requiring careful balance to achieve optimal results [306].

This study aimed to develop an optimized DPI featuring an innovative combination of two anti-asthmatic agents, ciclesonide, an inhaled corticosteroid (ICS) and indacaterol maleate, a long-acting beta agonist (LABA), using spray-drying technology. IND, categorized as an "ultra-LABA" provides rapid bronchodilation within 5 minutes, lasting up to 24 hours [362]. Its once-daily dosing enhances patient adherence and quality of life by combining convenience with sustained therapeutic efficacy. Due to exacerbation risks associated with monotherapy, IND is combined with CIC, an ICS with established therapeutic efficacy but unavailable in DPI formulation and currently only available as a monotherapy (Alvesco®) [383,384]. Furthermore, CIC is a prodrug that, following intracellular uptake within bronchial epithelial cells, is converted into its active

metabolite, desisobutryl-ciclesonide (des-CIC). This targeted activation profile not only enhances its therapeutic efficacy but also contributes to minimizing both local adverse effects and systemic side effects, offering a favorable safety profile within inhaled corticosteroid therapies [363]. This combination could enhance the synergistic therapeutic benefits for asthma and COPD treatment, offering the convenience of a similar posology with a once-daily administration, while benefiting from the advantages of optimized DPIs developed by the SD technology.

The drying outcomes of solution and suspension atomization on the properties of the final developed powders were examined, along with the influence of variations in solid content. Given the high hydrophobicity of CIC and prior research highlighting the advantages of a deflated morphology combined with a promising safety profile for inhalation, cyclodextrins are incorporated to enhance the solubility of the corticosteroid, allowing the atomization of a solution and the comparison to a suspension [177,202,316,385]. Furthermore, the *in vitro* aerodynamic performance of both DPIs was assessed using the Next Generation Impactor (NGI), while the PreciseInhale® system was employed to evaluate aerosolization performance and provide insights into powder dispersibility. Analysis of the trends observed between these two techniques allowed for a better understanding and optimization of dry powder inhalers performance. Overall, this work aimed to develop an innovative DPI formulation that combines a novel API pairing with the creation of spherical, deflated particles to optimize inhalation properties and enhance lung deposition, thereby addressing a gap in current market options.

III.2.2.2. Materials and methods

III.2.2.2.a. Materials

Micronized ciclesonide (CIC) was kindly provided from NewChem spa (Milano, Italy) and indacaterol maleate (IND) was obtained from LEAP Chem CO (Wan Chai, Hong Kong). Hydroxypropyl- β -cyclodextrin (HP β CD), Crysmeb® (a methylated β CD) and hydroxypropyl- γ -cyclodextrin (HP γ CD) were kindly provided by Roquette (Lestrem, France).

HPLC-grade methanol and absolute ethanol were acquired from J.T. Baker (Deventer, The Netherlands) and ThermoFisher Scientific (Geel, Belgium), respectively. Ammonium acetate and 25% ammonia solution were sourced from Sigma-Aldrich (St.

Louis, MO, USA). Water was purified using a Millipore system (18.2 M Ω /cm resistivity, Milli-Q) and subsequently filtered through a 0.22 μ m Millipore Millipak[®] 40 disposable filter units (Millipore Corporation, USA).

Hydroxypropyl methylcellulose capsules, size 3 and suitable for inhalation, were provided by Capsugel[®] (Lonza, Colmar, France) and used with the Onbrez[®] Breezhaler[®] (Novartis Pharma BV, Basel, Switzerland), a low-resistance device with a resistance of 0.0177 (kPa)^{0.5} (min L⁻¹) [144,311].

III.2.2.2.b. Indacaterol maleate and ciclesonide quantification

The quantification of CIC and IND was conducted using an Agilent 1100 Series HPLC system (Santa Clara, USA). The analysis employed a 3 × 50 mm column packed with 3.5 μ m C18 material (X Bridge BEH C18 Column) in conjunction with a VanGuard Cartridge pre-column, 3/PK. Detection was carried out with a UV detector set to 243 nm. The mobile phase comprised an ammonium acetate buffer at pH 10 and methanol, with the following gradient elution program: 0 min – 80/20 (v/v); 1 min – 80/20 (v/v); 10 min – 5/95 (v/v); 13 min – 5/95 (v/v); 13.5 min – 80/20 (v/v); 20 min – 80/20 (v/v). The flow rate was maintained at 0.7 mL/min and the column temperature was set to 30 °C, while the sampler temperature was maintained at 10 °C.

The development and validation of this analytical method, developed at the beginning of this thesis, can be found in Appendix II.

III.2.2.2.c. Determination of the degradation temperature of raw CIC and IND

Thermogravimetric Analysis (TGA) was conducted using a Mettler-Toledo instrument (Schwerzenbach, Switzerland) to assess the degradation temperature of CIC and IND raw material. The analysis involved 5 mg of API placed in aluminum pans, with a heating rate of 20 °C/min, ranging from 25 to 500 °C, under a nitrogen flow of 20 mL/min.

III.2.2.2.d. Phase solubility diagram

The complexation of CIC with cyclodextrins was investigated using the phase solubility diagram method as described by Higuchi and Connors [386]. CIC was added in excess to 4.0 mL of purified water containing increasing concentrations of cyclodextrins ranging from 0 to 200 mM. The vials were then placed in a shaker bath at 37 °C and 150 rpm

for 48 hours where CIC excess was maintained until equilibrium was reached. Afterward, the samples were filtered through a 0.22 μm pore filter, transferred to vials and absolute ethanol was added to prevent any potential APIs precipitation. The vials were subsequently analyzed using HPLC, as described in Section III.2.2.2.b (Chapter 2).

III.2.2.2.e. Feedstocks preparation

Each prepared liquid formulation containing the two active ingredients of interest was prepared exclusively using purified water, with a total volume of 100.0 mL. The formulations had a solid content of either 5 or 10% (w/v), representing the proportion of solid substances in the atomized liquid. As mentioned in the introduction, due to high hydrophobicity of CIC, formulation and atomization of a solution ($\text{SOL}_{\text{Crysmeb}}$) and a suspension ($\text{SUS}_{\text{HP}\beta\text{CD}}$) were investigated. The suspension included $\text{HP}\beta\text{CD}$ and a surfactant agent (Tween[®] 80). Solubilization efficiency was enhanced with Crysmeb, a methyl- β -cyclodextrin, as determined by phase-solubility tests described in Section III.2.2.2.d (Chapter 2). For the preparation of the solutions, once Crysmeb was fully dissolved, CIC was added and allowed to fully complex with the cyclodextrin. IND was subsequently introduced and mixed until complete dissolution. For the formulation of the suspensions, $\text{HP}\beta\text{CD}$ was initially dissolved in water, followed by the addition of IND. Tween[®] 80, at 1% of the total mass of CIC intended for suspension, was then incorporated to stabilize the hydrophobic drug. In both the solution and suspension formulations, IND and CIC were added at concentrations of 0.83% and 0.53%, respectively, corresponding to 125 μg of IND and 80 μg of CIC in a 15 mg powder. These concentrations were selected based on the clinical dosages of the two compounds when administered separately on the market. To achieve this, at a 5% (w/v) solid content with fixed proportions of excipient and API, the preparation of 100.0 mL feedstock requires 41.40 mM of Crysmeb and 35.55 mM of $\text{HP}\beta\text{CD}$. Under these conditions, the targeted amount of CIC for complexation is 26.65 mg.

III.2.2.2.f. Spray-drying of the liquid preparation

Powders were produced by spray-drying $\text{SOL}_{\text{Crysmeb}}$ and $\text{SUS}_{\text{HP}\beta\text{CD}}$ with concentrations of 5 and 10% (w/v) using the Procept 4 M8-Trix Formatrix spray-dryer (Procept, Zelzate, Belgium) equipped with a bi-fluid nozzle. The resulting mixtures were atomized under the following conditions: an inlet temperature of 160 $^{\circ}\text{C}$, a feed flow rate of 3.85 g/min,

a nozzle gas pressure of 3 bar, a cyclone gas pressure of 0.4 bar, an inlet gas flow of 0.4 m³/min and a nozzle diameter of 0.4 mm. These parameters were established based on previous research aimed at engineering ultra-light microparticles, facilitated by the deflated shape of cyclodextrin [177]. The process yield for each formulation was calculated using the equation provided in Equation 9.

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

Equation 9: Drying process yield calculation.

III.2.2.2.g. Characterization of the dried powders

.III.2.2.2.g.1 Particle size distribution

The particle size distributions (PSDs) of the powders were determined by laser diffraction using the Malvern Mastersizer 3000® (Malvern Instruments, Worcestershire, UK), equipped with an AeroS unit. A standard Venturi was utilized to disperse a quantity of powders equivalent to a spatula tip on the micro tray, accommodating the low material quantity. The feed rate was set at 30%, with an air pressure of 4.0 bar, to achieve the required obscuration range of 0.5-8.0%, resulting in a sample measurement duration of 10 seconds. The Malvern Mastersizer software was used to analyze the particle size of the powders. Three replicates of each sample were measured to determine the average PSD.

.III.2.2.2.g.2 Residual moisture content

The residual water content of all powders was assessed rapidly after spray-drying using Thermogravimetric Analysis (TGA; Perkin Elmer, Norwalk, CT). Powder samples ranging from 8 to 13 mg were placed on a platinum sample pan and heated from 25 to 150 °C at a rate of 10 °C/min. TGA is employed to assess the mass loss of a sample when subjected to elevated temperatures that surpass the evaporation point of water. A decrease in the mass of the sample is recorded as water evaporates, enabling the quantification of the moisture content in the analyzed powders.

.III.2.2.2.g.3 Particle morphology

Particulate morphology was analyzed using scanning electron microscopy (SEM) with either a Philips XL30 ESEM or an FEI Quanta 600, after metallizing the samples with

approximately 50 nm of gold. Representative micrographs were captured and approximately ten particles from each powder were randomly selected for detailed morphological analysis. Additionally, the average number of dimples (N_d) was manually counted, assuming that the visible dimples represent approximately half of the total number on a particle. The dimple depth (DepthD) was then estimated by measuring the distance from the deepest point of the dimple to the highest point of the surrounding surface, normalized to the diameter (d) of the particle. This measurement was conducted using image analysis tools, with the depth of each visible dimple carefully quantified and averaged across the sampled particles [202].

III.2.2.2.h. Evaluation of dried powders homogeneity

III.2.2.2.h.1 Relative standard deviation determination

The uniformity of each powder produced was assessed by collecting 10 samples from various locations within the powder bed, covering the entire surface and depth. These powders were stored at room temperature and shielded from light. Each sample, containing approximately 20 ± 1 mg of SD powder, was dissolved in methanol, allowing for the determination of CIC and IND concentrations using HPLC, as presented in Section III.2.2.2.b (Chapter 2). The recovery rate was evaluated using the specified equation below (Equation 10). Blend uniformity was determined by calculating the relative standard deviation (RSD) from the 10 samples. This metric represents the ratio of the standard deviation to the mean, expressed as a percentage. Powders with an RSD of $\leq 5\%$ for the mean CIC and IND recovery rates were considered to exhibit satisfactory content uniformity.

$$\text{Recovery rate (\%)} = \frac{\text{Quantified drug content}}{\text{Theoretical drug content}} \cdot 100$$

Equation 10: Active pharmaceutical ingredient recovery rate upon spray-drying calculation.

III.2.2.2.h.2 Raman microscopy

Raman hyperspectral imaging (R-HSI) was conducted using a Labram HR Evolution (Horiba Scientific) system, which included an EMCCD detector with a 1600×200 -pixel sensor (Andor Technology Ltd.). The setup featured a Leica 50x Fluotar LWD objective, a 300 gr/mm grating and a 785 nm laser (45 mW output) sourced from a Toptica Photonics XTRA II single frequency diode laser. A 25% neutral density filter was used

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to reduce light intensity and avoid sample damage. Spectral data, obtained by averaging two acquisitions of 2 s each, covered the range from 463 to 1853 cm^{-1} .

For consistency, raw materials were characterized under the same conditions as the mapping experiments. Prior to imaging, the powders were compressed using a Specac 5 mm pellet die with a 0.5 T load. Mapping consisted of a 75×75 pixels grid with a 2 μm step size, resulting in a total area of $150 \times 150 \mu\text{m}^2$. Raw spectral data underwent preprocessing via PCA denoising and baseline adjustments with Asymmetric Least Square correction (parameters: $p = 1 \times 10^{-4}$, $\lambda = 1 \times 10^5$).

The number of pure signals in the mapping was estimated using singular value decomposition. Independent component analysis (ICA) with the JADE algorithm was used to separate the previously estimated number of pure signals: three for the suspension-derived powder (MAH17/LAC-50/50) and one for the solution-derived powder (HPBCD-100). These results were validated by comparing the ICA loadings to spectra from pure components. Among the three ICs separated for the suspension data, one IC represented highly noisy pixels and was discarded for subsequent analyses.

III.2.2.2.i. *In vitro* lung deposition determination

III.2.2.2.i.1 *Next Generation Impactor*

The aerosol performance *in vitro* was assessed using a Next Generation Impactor (NGI; Apparatus E, Copley, Nottingham, UK), in accordance with the European Pharmacopoeia guidelines. This impactor, consisting of eight stages, includes a pre-separator and the induction port is connected to the device via an appropriate mouthpiece adapter. Airflow was regulated to 60 or 100 L/min by a flow controller (TPK; Copley, Nottingham, UK), with the airflow maintained for 4.0 and 2.4 seconds, respectively. The study employed the Onbrez® Breezhaler® (Novartis Pharma AG), a device characterized by low resistance, with 12 capsules tested per run. The powder deposited on each stage, including the mouthpiece, capsules and device, was collected using methanol and subsequently analyzed by HPLC after sonication to complete APIs solubilization. The recovery dose (RD) denoted the total amount of API retrieved from the capsules and the device up to the final stage of the NGI. The emitted dose (ED), representing the percentage of the drug released from the inhaler, was calculated from the cumulative quantity of powder from the induction port to the final NGI stage. The fine particle dose (FPD) was defined as the mass of the drug with an aerodynamic

diameter of less than 5 µm. The fine particle fraction (FPF) was calculated based on the emitted dose, as no changes were made to the inhaler used throughout the study and was determined using the following equation (Equation 11).

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

Equation 11: Fine Particle Fraction (FPF) calculated based on the Emitted dose (ED).

The Mass Median Aerodynamic Diameter (MMAD) was derived from the cumulative aerosol mass distribution curve, representing the diameter through which 50% of the total aerosol mass can pass. The Geometric Standard Deviation (GSD) was determined using the ratio of the diameters at the 84th percentile (D84) and the 50th percentile (D50). Additionally, the pulmonary deposition results of our powders, specifically focusing on IND deposition, were compared to those of Onbrez[®], a DPI formulation marketed in Belgium that utilizes lactose as a carrier and contains IND.

.III.2.2.2.i.2 *PreciseInhale[®]*

The PreciseInhale[®], as previously described in detail [387–389], is an innovative inhalation exposure platform which allows to investigate how inhaled particles act in the lungs. This machine enables aerosol exposures to a wide range of *in vitro*, *ex vivo* and *in vivo* models, using a small amount of raw substance to generate aerosols in a free-flowing, fine particulate stream and producing repeatable data [390]. The PreciseInhale[®] system offers several different aerosol sources which can be used according to the study's goal: micronized dry powders [387], solutions (nebulizers) and clinical inhalers, such as DPIs and pMDI [391]. In the current study, the PreciseInhale[®] dry powder inhaler setup was used to analyze the test powders' dispersibility, aerodynamic performance and PSD and to eventually compare these data with those generated with the NGI.

The PreciseInhale[®] dispensing system (Inhalation Sciences AB, Huddinge, Sweden) was used to generate powder aerosols (Supplementary data A, Appendix IV). Powder samples were loaded into empty DPI capsules (Capsugel Zephyr, Lonza, Verviers, Belgium) in carefully weighed doses of around 1 mg, using an analytical balance (Mettler Toledo XP26) with a minimum weight capacity of 0.1 mg. The sample-filled capsules were actuated in the dry powder inhaler of the specialty Onbrez[®] Breezhale[®] (Novartis Pharma BV, Basel, Switzerland), with an inhalation flow of 60 L/min and a

corresponding pressure drop of 4 kPa over the inhaler. The actuation time to ideally place the inhaler puff in the 300 mL holding chamber at the chosen actuation flow rate was calculated by equation B.1 (Supplementary data B, Appendix IV).

Following actuation, the flow direction in the holding chamber was reversed and an exposure air flow of 400 mL/min was employed to draw the aerosol through the Casella and to enable the collection of the test powders' aerosols for analysis. Moreover, a pre-exposure aerosol mixing period of 0.6 s was chosen for all exposures and exposure times of 90 s for the yield determination and 120 s for the particle size determination were applied.

The aim of the aerosol-generation procedure with the PreciseInhale[®] was to assess the flowability, dispersibility and aerodynamic PSD of the spray-dried formulations. The flowability and dispersibility were measured as the Casella maximum concentration ($C_{\max}$) and as the amount of aerosol likely to be deposited on the inhalation filter (M_{cas}) [389,392].

The yield was calculated as the weight difference of the end filter (25 mm GF/A end-filters, Inhalation Sciences, AB, Huddinge, Sweden) before and after aerosol exposure, divided by the powder dose shot in the machine, according to the following equation (Equation 12):

$$\text{Aerosol yield (\%)} = \frac{\text{End filter after exposure (mg)} - \text{End filter before exposure (mg)}}{\text{Powder dose loaded in the capsule (mg)}} \cdot 100\%$$

Equation 12: Aerosol yield calculation, using the PreciseInhale[®] system.

To calculate the $C_{\max}$, the M_{cas} and the yield, five measurements were performed for each formulation.

MMAD, GSD and FPF were measured by cascade impaction analysis with the use of a 9-stage Marple Cascade Impactor (CI) (MSP Corporation, Shoreview, MN, USA), attached to the exposure outlet of the PreciseInhale[®]. FPF was calculated as the ratio of the weight of fine particles with a diameter below 5 μm and the total weight of the powder recovered on all the 9 filters of the Marple cascade impactor, according to the following equation (Equation 13):

$$\text{Fine particle fraction (FPF, \%)} = \frac{\text{Weight of particles} < 5\mu\text{m (mg)}}{\text{Recovery dose (mg)}} \cdot 100$$

Equation 13: Fine Particle Fraction (%) calculation, using the PreciseInhale[®] system.

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The aerodynamic PSD measurements were performed according to the provider's instructions. Following the inhaler actuation cycle, a reversed exposure flow rate of 2000 mL/min was employed to draw the aerosol from the holding chamber through the CI. According to their particle size, the powders impacted on the stages of the CI and were captured on the filters (Cascade Impactor, stage 1-8 filter, GF/A; Cascade Impactor, end-filter, GF/F, Inhalation Sciences, AB, Huddinge, Sweden). The MMAD, GSD and FPF were calculated by gravimetric measurement (Microbalance XP26, Mettler Toledo). The experiments were performed in triplicate for each formulation. The recovery of the PSD measurements was also calculated as the ratio of the weight of the 9 filters in the CI stages before and after exposure and the total weight of powder shot in the CI, according to the following equation (Equation 14):

$$\text{Recovery (\%)} = \frac{9 \text{ CI filters after exposure (mg)} - 9 \text{ CI filters before exposure (mg)}}{\text{Total powder dose loaded in the capsules (mg)}} \cdot 100$$

Equation 14: Recovery (%) calculation, using the PreciseInhale® system.

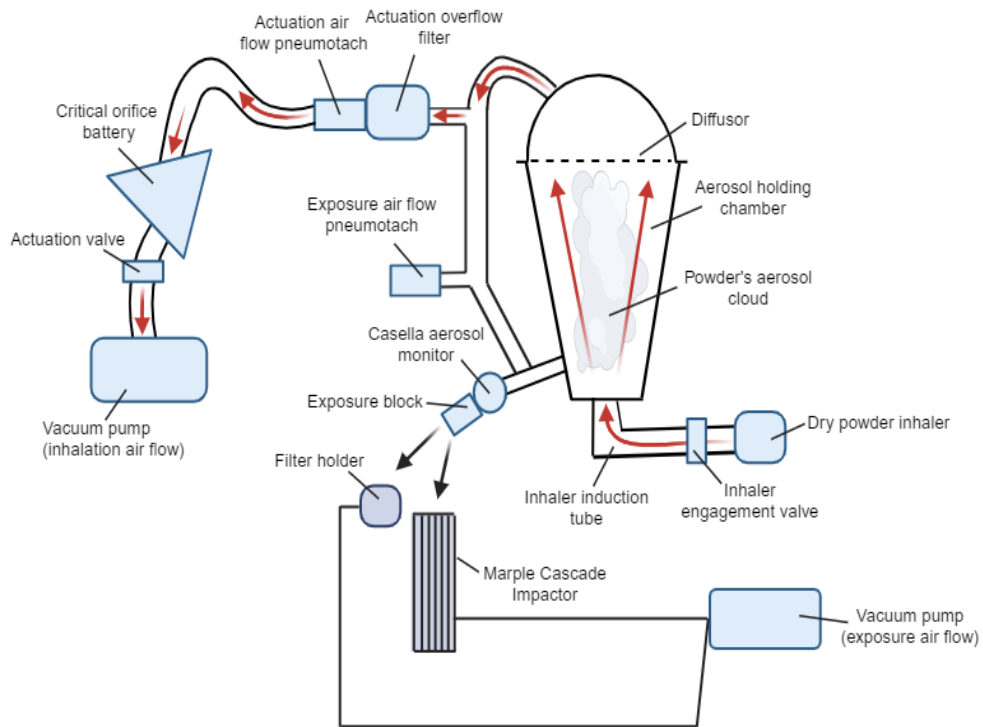
The recovery refers to the collection efficiency of the aerosolized particles after they have been generated by the PreciseInhale® system and it determines how much of the aerosolized material is captured on the Marple Cascade Impactor stages.

A LabVIEW based proprietary software (National Instruments, Austin, TX) of Inhalation Sciences AB is used by the PreciseInhale® platform to manage both execution of aerosol exposure cycle and exposure data acquisition of pressure, airflow and aerosol concentration [393].

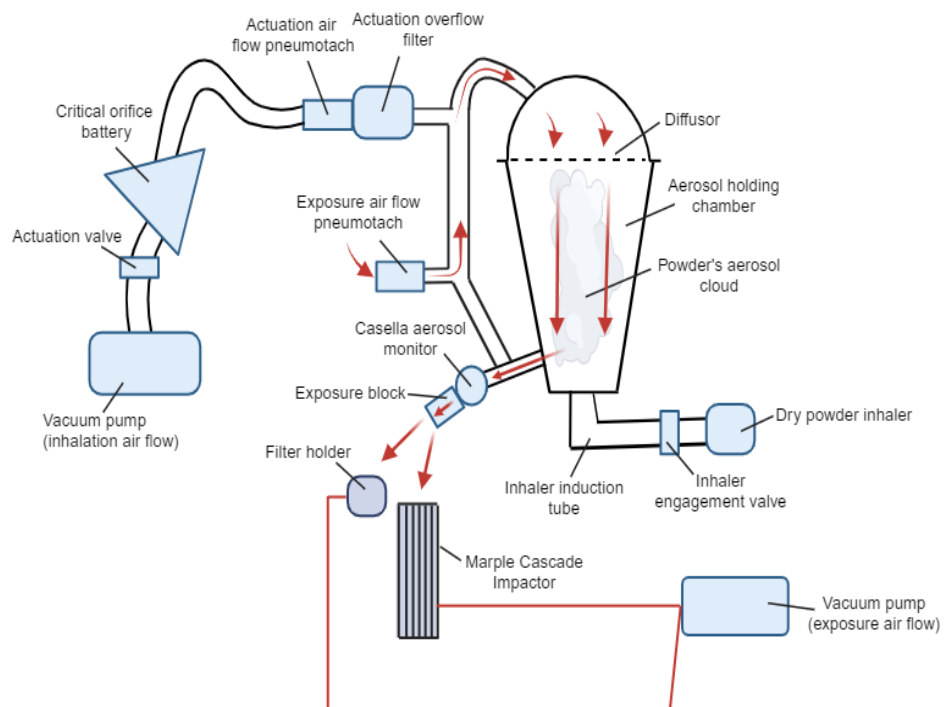
Figure 46A and B provide a schematic representation of the functioning of the PreciseInhale® system in the dry powder inhaler configuration. The PreciseInhale® system in the dry powder inhaler setup is represented in Supplementary data C (Appendix IV).

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A



B



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Figure 46: A schematic overview of how the PreciseInhale® system works in the dry powder inhaler setup. Red arrows show the direction of the inhalation flow (A) or exposure flow (B). 1 mg of powder is loaded in DPI capsules and actuated in the dry powder inhaler coupled to the PreciseInhale®'s inhaler induction tube. The inhalation air flow is provided by the critical orifice battery and its vacuum pump (A). The aerosol settles in the holding chamber and it is drawn towards the exposure target by the air flow supplied by the exposure air flow vacuum pump (B). The Casella Microdust Pro measures the aerosol concentration during the procedure. The exposure modules used in the current project were the filter holder for the yield, C_{max}, M_{cas} determination and the Marple cascade impactor for the particle size distribution analysis (MMAD, GSD, FPF). The PreciseInhale® also has four fast acting pneumatic pinch valves which control pressure build-up and release as well as the correct direction of the airflow. These valves have been omitted for clarity in the overview. A detailed description of the PreciseInhale® system can be found in reference [387].

III.2.2.2.j. Stability studies

Stability studies of the optimized 10% (w/v) powder were conducted at controlled room temperature (25 °C ± 2, 60% ± 5 RH) and under accelerated conditions (40 °C ± 2, 75% ± 5 RH) following ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) guidelines. The powder was stored in sealed vials and subjected to the specified climate conditions. Samples were taken at 1, 2, 3 and 6 months after being placed in the stability chamber. The powders were inspected for signs of caking or discoloration and analyses of drug content, moisture content and particle size were performed periodically. Impaction studies utilizing the NGI were performed after a 6-month period.

III.2.2.2.k. Statistical analysis

All data were statistically analyzed using GraphPad Prism software (version 8.4.3, La Jolla, CA, USA) and are expressed as mean ± standard deviation. Statistical comparisons between multiple-group comparisons were assessed by one-way ANOVA followed by Tukey's post hoc test. Two-way ANOVA with Sidak's post hoc test was used when two experimental factors were analyzed simultaneously. Statistical significance was set at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

III.2.2.3. Results and discussion

III.2.2.3.a. Characterization of the cyclodextrin-ciclesonide complexes

Considering the low aqueous solubility of ciclesonide, a highly hydrophobic ICS with a solubility in water less than 0.5 mg/L at room temperature [366], various cyclodextrins

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were tested to compare the atomization of a feedstock, where the CIC was solubilized on one hand and suspended in the liquid on the other.

According to the phase-solubility diagram classifications by Higuchi and Connors, the solubility diagrams for CIC complexation with all tested cyclodextrins, shown in Figure 47, correspond to A-type profiles [386]. These diagrams demonstrate a proportional increase in CIC solubility with rising cyclodextrin concentration. Within the 0 to 200 mM cyclodextrin range, the apparent water solubility of CIC increases linearly, attributed to the formation of a soluble 1:1 inclusion complex with the tested carbohydrates. Among them, the methylated cyclodextrin Crysmab shows superior encapsulation efficiency for CIC compared to HP β CD and HP γ CD.

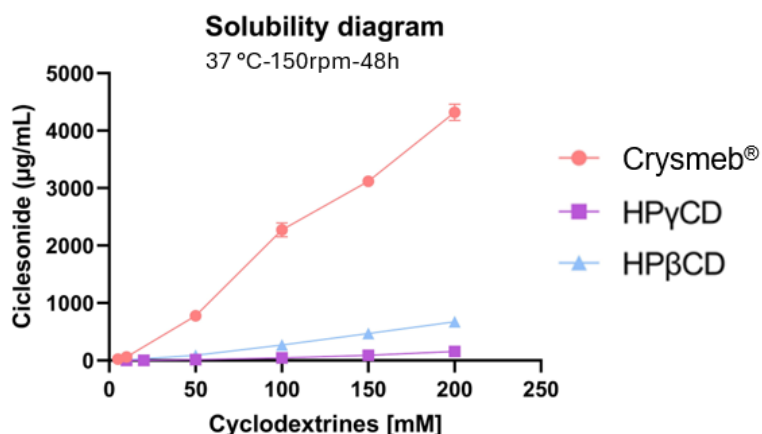


Figure 47: Phase solubility diagram of Ciclesonide in complexation with Crysmab®, HP β CD, HP γ CD. Analysis was performed in triplicate ($n=3$) and values are presented as mean and standard deviation.

The results indicate that, at the cyclodextrin concentrations required to prepare a 5% (w/v) feedstock (35.55 mM Crysmab, 31.29 mM HP γ CD and 31.29 mM HP β CD), Crysmab exhibits the capacity to complex up to 10 times more CIC than HP β CD. This can be explained by the fact that CIC complexation with cyclodextrins is closely related to the hydrophobicity of their cavities. Indeed, Crysmab exhibits the highest hydrophobic cavity, as methyl substitutions increase the apolar character of the cavity by repelling water molecules. However, complete complexation of CIC at high concentrations with HP β CD could not be achieved, as previously observed. This is likely due to the limited stability constant of the CIC-HP β CD complex, related to the more hydrophilic nature of its cavity resulting from hydroxypropyl substitutions, which reduces its ability to

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effectively complex highly lipophilic compounds. As a result, suspension-based spray drying proved less suitable for the development of optimized DPI formulations. In this context, enhancing CIC complexation with HP β CD became essential, requiring further optimization of the formulation strategy.

In contrast, HP β CD, due to its hydroxypropyl substitutions, presents a more hydrophilic cavity that enhances overall aqueous solubility but reduces the ability to effectively complex highly lipophilic drugs, as these groups partially interact with water at the cavity entrance [394,395]. Finally, HP γ CD, with its larger cavity, permits greater water access, making it the least hydrophobic. These structural differences likely account for the superior complexation capacity of Crysmeb towards CIC [395].

Based on these findings, Crysmeb will be used to complex CIC to produce a solution that will subsequently be atomized to generate powders designated as DPI-SOL_{Crysmeb}. Similarly, HP β CD will be employed to create a suspension and upon drying, this powder is named DPI-SUS_{HP β CD}. A recap of the dried formulations investigated in this study is provided in the following table (Table 8):

Table 8: Summary of the composition of the atomized formulations.

	DPI-SOL_{Crysmeb}	DPI-SUS_{HPβCD}
Feedstock nature	Solution	Suspension
Excipient nature	Crysmeb	HP β CD
Other component	/	Tween 80
Excipient (%)	98.63	
CIC (%)	0.53	
IND (%)	0.83	

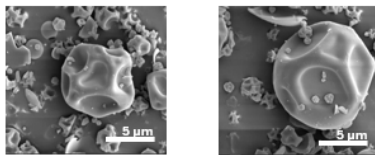
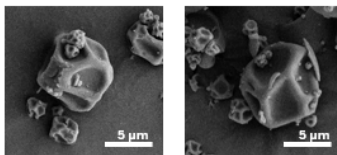
In addition to studying the comparison of the influence of the APIs solubilization state on the properties of the developed powders, the effect of increasing the solid content from 5 to 10% (w/v) will be investigated. The atomization of Crysmeb under optimal drying parameters will also be evaluated to determine whether this cyclodextrin enables the development of a DPI that improves lung deposition, similar to the results obtained with HP β CD in previous studies by Dufour *et al.* [205] and Lechanteur *et al.* [177].

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III.2.2.3.b. Impact of feedstock nature on powder properties

The physicochemical properties of the powders obtained from dried solutions (DPI-SOL_{Crystmeb}) and suspensions (DPI-SUS_{HPβCD}), with solid particle concentrations of 5 and 10% (w/v), are presented in Table 9.

Table 9: Properties of developed powders from atomized solutions (DPI-SOL_{Crystmeb}) and suspensions (DPI-SUS_{HPβCD}), at solid content of 5 and 10% (w/v). Analyses were performed in triplicates (n=3) and results are presented as mean ± standard deviation.

Feedstock nature	DPI-SOL _{Crystmeb}		DPI-SUS _{HPβCD}	
	Solution		Suspension	
Solid content (%) (w/v)	5	10	5	10
Process yield (%)	77.60 ± 1.03	77.29 ± 1.26	77.39 ± 2.82	65.85 ± 10.39
Particle size (μm)				
D10	1.27 ± 0.08	1.30 ± 0.04	1.38 ± 0.04	1.59 ± 0.11
D50	2.94 ± 0.04	2.99 ± 0.12	3.45 ± 0.21	4.12 ± 0.22
D90	6.33 ± 0.11	5.89 ± 1.49	7.73 ± 0.48	10.01 ± 1.36
Span	1.72 ± 0.02	1.70 ± 0.07	1.84 ± 0.05	2.05 ± 0.36
Water content (%)	6.46 ± 0.54	4.74 ± 1.01	4.34 ± 0.21	4.62 ± 0.67
Morphology (FoV 20)				
Number dimples (Nd) (-)	10.57 ± 1.90	11.75 ± 2.91	11.75 ± 2.49	11.5 ± 4.99
Depth dimples/d (Dd) (-)	0.28 ± 0.07	0.19 ± 0.03	0.25 ± 0.07	0.23 ± 0.08

Firstly, regarding the SD process yield, values remained relatively high across most tested conditions, averaging around 77%, emphasizing minimal powder losses during the drying process.

The powders' water content ranged between 4.34 and 6.46% (Figure 48B) which theoretically supports API stability and optimal lung deposition [172]. These residual moisture levels help mitigate issues related to excessive electrostatic charge and particle aggregation, issues already present due to the powder's hygroscopic properties [40]. The results show that an increase in the solid concentration of the matrix decreases the residual moisture content in the resulting particles by raising the ratio of solute to water within each drying droplet, promoting more efficient water removal during drying [396]. This effect led to a significant ($p < 0.05$) decrease in water content, dropping from 6.46 to 4.74% as solid content increased. Interestingly, at a lower solid concentration of 5% (w/v), suspension-derived powders showed ($p < 0.05$) lower water content of 4.34%

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compared to the 6.46% from the solution-derived ones. This may be attributed to the presence of suspended drug particles in the liquid feed prior to atomization, which are consequently present within the generated droplets during drying. These solid particles can reduce water retention during drying, resulting in lower moisture content in the final powders. Yet, at a 10% solid content, no significant differences were observed between dried solutions and suspensions.

As previously highlighted, assessing particle size is crucial for developing inhalable powders. To ensure deep lung deposition, particle sizes should lie from 1 to 5 μm . According to Ou *et al.* [351], particles with a size of approximately 3 μm are considered optimal for enhanced deposition. The powders produced in this study, especially for DPI-SOL_{Crysmeb}, met these criteria, displaying particle sizes close to 3 μm (Figure 48A). Conversely, DPI-SUS_{HP β CD}, particularly at 10% solid content, exhibited considerable ($p < 0.05$) larger particles (4.12 μm) compared to solutions (2.99 μm). Due to the presence of hydrophobic drug solid particles within the droplets during suspension atomization, in contrast to solutions where all components are dissolved, the likelihood of obtaining larger particles upon drying is increased. Moreover, for atomized suspensions, higher solid content further amplified particle sizes, ranging from 3.45 μm at 5% to 4.12 μm at 10%. This is likely due to the increase in solid concentration per droplet under identical drying conditions [275].

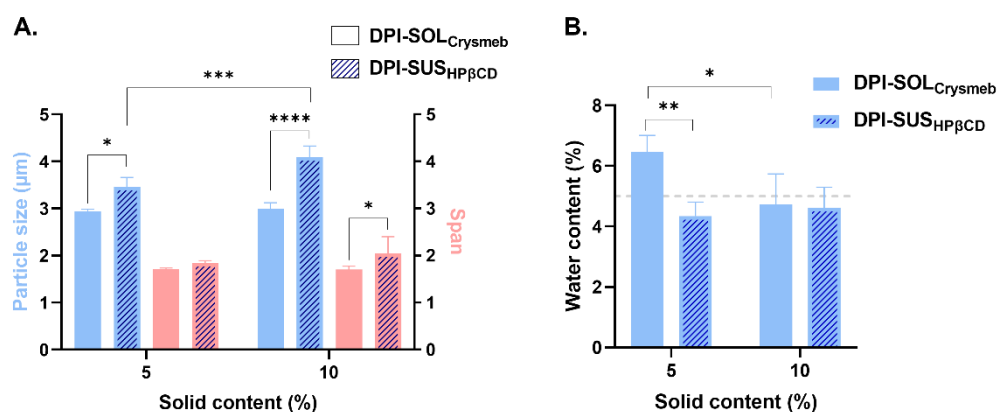


Figure 48: (A) Particle size and (B) water content of all produced powders from atomized solutions and suspensions, at both solid contents of 5 and 10% (w/v). Analyses were performed in triplicates ($n=3$) and values are presented as mean $\pm$ standard deviation.

Finally, regarding particle morphology, deflated structures were observed in all powders, appearing to be suitable for improved DPIs aerosolization efficiency and lung

deposition [258,397]. Focusing on the influence of increased solid content on particle morphology, literature indicates that higher solid generally tends to promote more spherical particles by accelerating crust formation, providing greater resistance to deformation [275]. Additionally, studies highlighted that at higher feed concentrations, formation of SD particles with greater porosity and lower bulk density are obtained, due to reduced solvent in each droplet and faster evaporation times [277]. In this study, no morphological changes were observed whatever the solid content. However, in comparison to typical DPI production atomized with around 1% solid content, the high feed concentrations will result in faster production times which represents a considerable interest from an industrial standpoint.

Furthermore, previous investigations have highlighted the Péclet number (Pe) as a determinant of particle morphology [205,259]. High molecular weight excipients, such as HP β CD (1541.5 g/mol), are associated with higher Pe values and have been shown to promote the formation of deflated particles at optimized drying parameters, in contrast to lower molecular weight excipients like mannitol (182.2 g/mol) [202]. Interestingly, in this study, the nature of the cyclodextrin, whether originating from dried solutions or suspensions, did not significantly influence particle morphology or the measured morphological parameters (Nd and Dd). Crysmeb cyclodextrin was dried for the first time under optimized conditions and the results confirmed that its high molecular weight (1191 g/mol) likely contributed to a high Pe value, leading to the successful formation of deflated, ball-shaped particles [398].

III.2.2.3.c. Impact of feedstock composition on powders homogeneity and drug recovery

Following the drying process and the evaluation of powders properties, the recovery rate of both active substances from the resulting powder was assessed to evaluate any potential degradation of the drug, due to the drying process temperature, or the influence of its solubilization state. Figure 49A shows that most powders unveil recovery rates between 95 and 105%, indicating no significant APIs losses during the drying process [167], independently of the solid content. However, CIC recovery rates in atomized suspensions were significantly lower reaching only 91.30% (± 3.05) and 86.11% (± 4.26) ($p < 0.05$), at 5 and 10% solid content respectively. This observation is hypothesized to be due to its non-solubilized state, which may lead to particle adhesion to the spray dryer and consequently reduced recovery rates. The decrease in the

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recovery rate of the hydrophobic molecule in atomized suspensions is proposed to be attributed to the solubilization state of the feed liquid rather than thermal degradation since the degradation temperature for CIC was 354 °C and the atomization process temperature of 160 °C. The thermal degradation of IND (203 °C) and its soluble state make it unsurprising that no degradation is observed upon drying (thermal degradation TGA curves of CIC and IND are shown in Supplementary data D, Appendix IV).

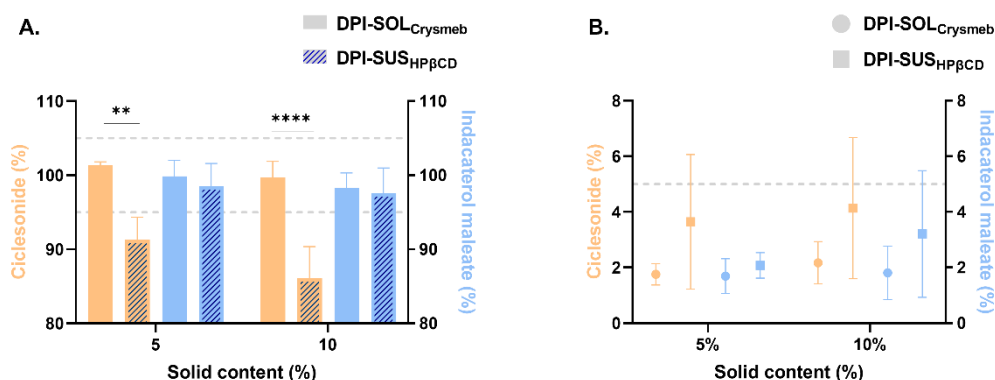


Figure 49: (A) APIs recovery rate post-drying from atomized solutions (DPI-SOL_{Crysmeb}) and suspensions (DPI-SUS_{HPβCD}), at both tested solid content (5 and 10% (w/v)). (B) Coefficients of variation for APIs across 10 samples from atomized solutions and suspensions, at both tested solid contents (5 and 10% (w/v)). Analyses were performed in triplicates (n=3) and values are presented as mean ± standard deviation.

Moreover, in DPIs development, API typically constitutes a small fraction of the total powder mass. To ensure consistent therapeutic efficacy and minimize adverse effects, each dose of the inhaled product must deliver an identical number of active substances [349]. The uniformity of powder homogeneity was evaluated using the RSD calculated based on the API content after drying. Dose uniformity in DPIs is considered acceptable when the RSD is less than 5% [350].

Figure 49B shows CiCs distribution within 10 samples and indicates a RSD of 1.76% (± 0.38) and 2.17% (± 1.94) for atomized solutions (DPI-SOL_{Crysmeb}) with solid contents of 5 and 10%, respectively and 3.65% (± 2.42) and 4.13% (± 4.24) for atomized suspensions (DPI-SUS_{HPβCD}) at the same solid contents. Similarly, INDs distribution exhibited an RSD of 1.69% (± 0.62) and 2.08% (± 0.46) for atomized solutions at 5 and 10% solid content and 2.08% (± 0.45) (5% solid content) and 3.21% (± 3.47) (10% solid content) for atomized suspensions. Homogeneity appears to be maintained when atomizing solutions, regardless of the solid content. However, when non-solubilized

particles are atomized, the RSD tends to increase particularly for hydrophobic particles, which could be attributed to reduced product homogeneity resulting from the random distribution of suspended particles into aerosolized droplets during atomization [399,400].

Further analysis was performed using Raman hyperspectral imaging (R-HSI) to investigate the distribution of the components within the SD powders. For dried solutions, the imaging revealed a single Raman signature for each pixel across the sample, as shown in Figure 50A. This spectral uniformity indicates a homogeneous and intimate distribution of the excipient and API, attributed to their complete solubilization prior to the atomization process. In contrast, the analysis of atomized suspensions showed distinct signatures corresponding to the CIC non solubilized particles (Figure 50B). These particles, with a D0.5 of 1.81 μm , closely match the green dots observed in the imaging data, which are approximately the size of a single pixel (2 $\mu\text{m} \times 2 \mu\text{m}$). These results highlight a less uniform distribution of CIC particles within the final product, likely caused by incomplete solubilization and the uneven distribution of suspended particles into droplets during atomization of the hydrophobic substance before atomization and subsequent drying [399,400].

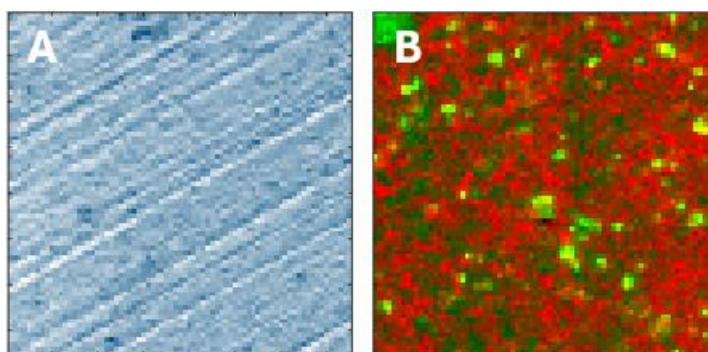


Figure 50: Raman hyperspectral imaging results (following ICA analysis) were obtained for powders produced from an atomized solution (DPI-SOL_{Crysmeb}) (A) and an atomized suspension (DPI-SUS_{HPβCD}) (B), respectively. In the imaging for the solution (A), the blue color represents a single signature observed uniformly across all pixels, indicative of homogeneity. The texture of the image is resulting from sample surface imperfections. In contrast, in the imaging for the suspension (B), red and green colors correspond to distinct signatures attributed to the excipient HPβCD and the hydrophobic CIC, respectively, reflecting a less uniform distribution.

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This difference in API repartition underscores the influence of the initial solubilization state on the final distribution of components within the powder, resulting in distinct impacts on the uniformity of the excipient and API distribution [401].

III.2.2.3.d. *In vitro* aerosolization performance

In this study, the *in vitro* aerodynamic performance of produced powders was evaluated using two distinct impaction methods. First, the cascade impaction method with NGI equipment which is a conventional assay described in the European Pharmacopea and second, the PreciseInhale® system, an innovative and less widely known technique focusing on aerosolization performance.

III.2.2.3.d.1 *Impact of solid content and drugs solubilization state on DPIs in vitro aerodynamic performance*

To further investigate the impact of solid concentration and drugs solubilization state on DPI performances, the lung deposition potential of all produced powders was evaluated using the NGI. All data overviews from the impaction studies on atomized suspensions and solutions, at solid concentrations of 5 and 10% (w/v), are presented in Table 10. The aerodynamic particle size distribution profile of CIC and IND from DPI-SOL_{Crysmeb} and DPI-SUS_{HPβCD}, at both solid contents of 5 and 10% (w/v) are represented in Supplementary data E A-B (Appendix IV).

Table 10: Mean values and standard deviations of Fine Particle Fraction (FPF), Fine Particle Dose (FPD), Emitted Dose (ED), Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) for all powders produced from atomized solutions (SOL) and suspensions (SUS) with solid contents of 5 and 10% (w/v). Analyses were performed in triplicates (n=3) and results are presented as mean ± standard deviation.

DPI		FPF (%)		FPD (μg)		ED (%)		MMAD (μm)		GSD (-)	
		SOL _{Crysmeb}	SUS _{HPβCD}	SOL _{Crysmeb}	SUS _{HPβCD}	SOL _{Crysmeb}	SUS _{HPβCD}	SOL _{Crysmeb}	SUS _{HPβCD}	SOL _{Crysmeb}	SUS _{HPβCD}
5%	CIC	64.43 ± 8.46	37.12 ± 4.10	34.07 ± 1.79	19.81 ± 3.45	90.46 ± 0.82	94.59 ± 2.25	4.13 ± 0.49	5.97 ± 0.21	1.91 ± 0.07	1.64 ± 0.03
	IND	59.68 ± 4.33	46.99 ± 6.06	60.05 ± 9.00	48.35 ± 7.25	91.00 ± 2.08	90.83 ± 5.29	4.24 ± 0.33	5.21 ± 0.30	1.92 ± 0.06	1.66 ± 0.22
	CIC	68.05 ± 8.64	29.66 ± 5.42	34.20 ± 2.91	15.62 ± 3.45	90.62 ± 2.55	96.38 ± 0.85	3.93 ± 0.43	5.79 ± 0.96	1.91 ± 0.05	1.66 ± 0.10
	IND	60.39 ± 4.42	41.05 ± 7.39	63.22 ± 4.61	39.80 ± 7.54	90.45 ± 1.18	94.74 ± 0.42	4.05 ± 0.43	5.11 ± 0.82	1.90 ± 0.05	1.68 ± 0.25

According to the results, atomized solutions produced significantly (p<0.05) higher lung deposition for both APIs compared to atomized suspensions, as indicated by the FPF values. For CIC, the FPF reached 64.43 (± 8.46) and 68.05% (± 8.64) for atomized

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solutions at 5 and 10% (w/v), respectively, compared to 37.12 ($\pm$ 4.10) and 29.66% ($\pm$ 5.42) for atomized suspensions. Similarly, IND showed increased lung deposition with atomized solutions, achieving an FPF of 59.68 ($\pm$ 4.33) and 60.39% ($\pm$ 4.42) for powders at 5 and 10% (w/v), respectively, compared to 46.99 ($\pm$ 6.06) and 41.05% ($\pm$ 7.39) for atomized suspensions.

This difference is primarily attributed to the higher Mass Median Aerodynamic Diameter (MMAD), which considers particle morphology and density, whereas D50 is a value that describes the particle size and is based on the assumption of spherical particle geometry. Both MMAD and D50 indicate that atomized suspensions result in larger particles, as reflected by higher values. Larger particles, as shown in Figure 51A-B, increase impaction in the upper respiratory tract, reducing deposition in deeper lung regions and resulting in lower FPF. Conversely, the Geometric Standard Deviation (GSD), which measures aerodynamic particle size dispersion, remained low for all powders, indicating uniformity in aerodynamic diameter (d_{ae}).

Regarding the Emitted Dose (ED), all powders achieved values exceeding 90%. However, powders produced from atomized suspensions tend to exhibit higher ejection efficiency from the capsule during inhalation. While the nature of the excipients could have been a contributing factor, both cyclodextrins used in this study share similar morphological properties. This phenomenon can then be due to the larger particle size of powders from atomized suspensions, improving powder flowability, thereby leading to increased ED. Nonetheless, this larger size also led to greater API loss in the throat, increasing the risk of undesirable effects.

Furthermore, despite varying API doses, all developed powders demonstrated homogeneous deposition of both APIs across pulmonary stages. However, powders from atomized solutions exhibited more uniform lung deposition with similar FPF values for both APIs across NGI stages, whereas atomized suspensions showed greater variability and more divergent FPF values. This uniformity is linked to the enhanced and even API distribution in powders from dried solutions, as confirmed by R-HSI analysis (Section .III.2.2.2.h.2, Chapter 2). These results highlight the potential of atomized solutions to improve pulmonary drug delivery, ensuring consistent API availability and maximizing therapeutic efficacy.

Finally, based on the data presented in Table 10, increasing solid content from 5 to 10% (w/v) had no significant effect on deposition values for either API, regardless of the initial atomized liquid state. Given the minimal impact of solid concentration variation on powder properties, drug recovery and homogeneity, discussed in previous sections, the 10% solid content was chosen for subsequent studies due to its higher drug content.

.III.2.2.3.d.2 Impact of the flowrate on DPIs in vitro aerodynamic performance

When using a low resistance device, studies typically use an optimal flow rate of 100 L/min to evaluate the *in vitro* aerodynamic performance of their DPIs using the NGI. Indeed, as mentioned in the European Pharmacopoeia guidelines, the flow rate must be adapted to generate a 4 kPa pressure drop across the device, representing the mean pressure drop generated by an adult while breathing quickly and forcefully. For the Breezhaler® used in this study, Abdadelah *et al.* [311] confirmed that a flow rate of ~100 L/min was needed, consistent with its low resistance design [402]. However, evaluating lung deposition at reduced flow rates is crucial to ensure that the FPF remains therapeutically sufficient. This is particularly relevant since patients with asthma and COPD show wide variability in inspiratory capacities due to disease severity and lung function differences [403]. Such variability can hinder the effective use of DPIs, especially in severe cases, where achieving the required flow for optimal drug delivery is challenging, potentially compromising treatment efficacy.

Most DPIs require a minimum flow rate ranging from 30 L/min to more than 60 L/min to function optimally [118,404]. Yet, studies show that the usual flow rate for individuals using Onbrez® Breezhaler®, a DPI containing IND maleate, is around 60 L/min [311].

Therefore, impaction tests were conducted on both produced powders, DPI-SOL_{Crysmeb} and DPI-SUS_{HPβCD}, at 10% solid content, comparing results at 60 and 100 L/min. Based on the aerodynamic particle size distribution of CIC (Figure 51A) and IND (Figure 51B) at flow rates of 60 and 100 L/min, observations indicate increased API retention within the device at lower flow rates for both atomized solutions and suspensions. At 60 L/min, for atomized solutions, ED values of 77.88 (±2.54) and 78.23% (±1.38) were observed for CIC and IND, respectively, while for dried suspensions, the values were 90.77 (±0.34) and 89.30% (±0.46), showing significantly ($p < 0.05$) lower emission doses compared to values at 100 L/min (Table 10). Reduced turbulence within the inhaler at lower inhalation flow rates lead to less efficient powder deagglomeration [150,405].

Consequently, this results in a higher retention of the powder in the device and a lower ED, as represented in Figure 51C.

Conversely, throat impaction was found to be reduced at lower inhalation flow rates, as slower air velocities result in less inertial deposition in the upper airways. Cuinyun *et al.* [351] have shown that lower flow rates decrease throat impaction, allowing the API to reach deeper regions of the lungs. In contrast, higher flow rates increase impaction in the upper respiratory tract. This is particularly relevant since many patients may have limited breathing capacities. An optimal balance must be found between maximizing API emission and minimizing throat impaction to ensure effective pulmonary drug delivery and minimized undesirable side effects.

Furthermore, no major differences in drug deposition were observed across all stages of the NGI, from the preseparator to the final stages, with variations in flow rate, particularly for atomized solutions. No significant difference was found in the FPF values for both CIC and IND in atomized solutions when the flow rate was reduced to 60 L/min, indicating that the DPI design is effectively optimized to maintain performance across varying flow rates. At the optimal flowrate of 100 L/min, the FPF values were 68.05% (± 8.64) for CIC and 60.39% (± 4.42) for IND, compared to 74.79 (± 6.40) and 71.51% (± 6.62) at 60 L/min, as illustrated in Figure 51D.

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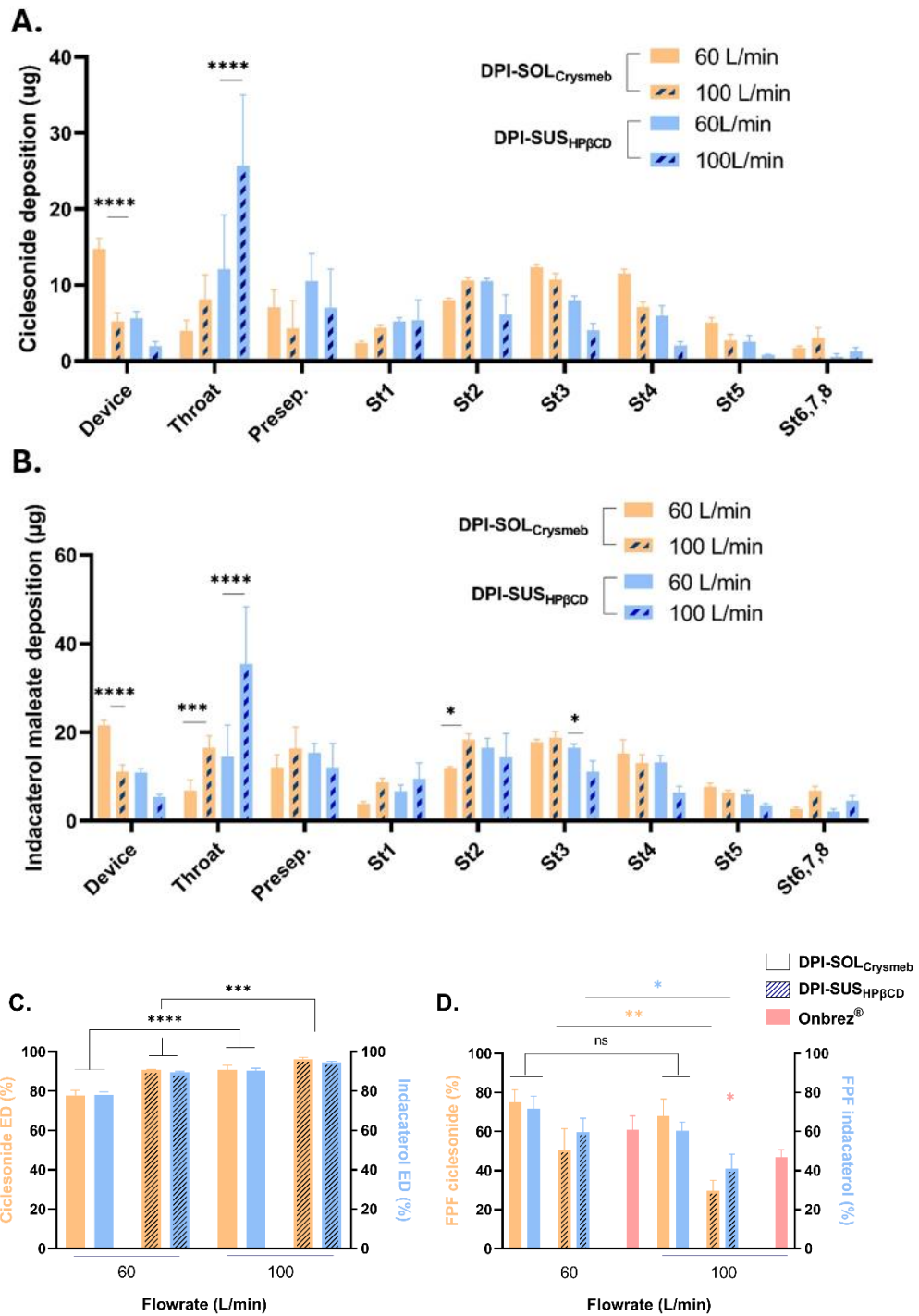


Figure 51 : (A) Aerodynamic particle size distribution profile of ciclesonide (CIC) from atomized solution and suspension, at tested flow rates of 60 and 100 L/min. (B) Aerodynamic particle size distribution profile of indacaterol maleate (IND) from atomized solution and suspension, at tested flow rates of 60 and 100 L/min. (C) Emitted Dose comparison for both API at 60 and 100 L/min. (D) Fine Particle Fraction (FPF) analysis for both API at 60 and 100 L/min. Analyses were performed in triplicates (n=3) and results are presented as mean $\pm$ standard deviation.

The final stage of this NGI aerodynamic performance study compared our laboratory-produced powder (10% solid content), DPI-SOL_{Crysmeb} and DPI-SUS_{HPBCD}, with Onbrez[®], a commercial DPI in the Belgian market containing indacaterol maleate and lactose. As previously noted, studies indicate that the typical flow rate for individuals using this marketed specialty is approximately 60 L/min, leading to IND FPF close to 40% [311,406].

As shown in Figure 51D, our atomized solution powder achieved a 10% higher FPF than the marketed formulation, particularly at 100 L/min. These differences were not due to the inhaler device, as all formulations used the same Breezhaler[®]. This improved performance stems from the absence of lactose in the carrier-free formulation. In the marketed product, large lactose particles cause higher pre-separator impaction, leading to IND losses due to adhesion. Strong API-carrier interactions in the marketed formulation further reduce detachment during impaction [190].

These results highlight the superior aerodynamic performance of powders from atomized solutions compared to current market formulations, highlighting the potential of optimized carrier-free powders for improved asthma and COPD treatment.

The findings also underscore the importance of API solubilization in achieving consistent aerodynamic performance. Powders from atomized solutions show reduced variability in API deposition across flow rates, suggesting that patients with lower inspiratory capacities (e.g., 60 L/min with the Breezhaler[®]) can achieve comparable therapeutic efficacy to those with higher capacities when using optimized dried solutions.

.III.2.2.3.d.3 Additional Characterization of Powder Aerodynamic Properties with the PreciseInhale[®] System compared to NGI

In parallel to NGI, the PreciseInhale[®] dispensing system (Inhalation Sciences AB, Huddinge, Sweden) was used to generate the powders' aerosols.

The aerodynamic performance and dispersibility of the spray-dried formulations (DPI-SOL_{Crysmeb} and DPI-SUS_{HPβCD}) were additionally evaluated with the Preciselnhale® system by measuring the C_{max} and the M_{cas} . The C_{max} refers to the maximum concentration of aerosol particles (µg/L) measured by the Casella device in the aerosol chamber during powder dispersion. Specifically, it represents the highest recorded concentration of particulate matter during an exposure cycle and it evaluate the dispersibility of the test powder, being its ability to be effectively aerosolized and dispersed into the air [389,392]. A higher C_{max} typically reflects better dispersibility of the powder under the given test conditions. The M_{cas} (µg) provides a measure of the total amount of powder that was successfully aerosolized and collected on the downstream filter during the exposure period. It offers an overall indication of the powder's aerosolization efficiency and flowability under airflow conditions, reflecting its performance in a dynamic, application and relevant context. By comparing M_{cas} values across different formulations, it becomes possible to assess which ones are more effectively dispersed into aerosol form and therefore exhibit superior flowability [389,392]. Finally, the aerosol yield relates to the aerosol generation efficiency of the Preciselnhale® system, quantifying the proportion of the initial powder sample that is effectively aerosolized. This parameter is particularly valuable, as a low *in vitro* yield may indicate poor aerosolization performance, which could potentially result in suboptimal lung deposition *in vivo*. Furthermore, the yield (%) was calculated after each run. The aerosol deposition properties were assessed by analyzing the particle size distribution (MMAD and GSD) of the engineered powders and by calculating the FPF (%). The commercial specialty Onbrez® Breezhaler® was used as a comparative reference.

Drawing on NGI results which investigated the influence of flow rates on powder deposition, as well as literature evidence indicating that patients using Onbrez® typically achieve an inhalation flow rate of 60 L/min [311], this same flow rate was selected for the Preciselnhale® experiments. At predefined settings, the powder formulations spray-dried from atomized solutions (DPI-SOL_{Crysmeb}) and suspensions (DPI-SUS_{HPβCD}) displayed significantly higher ($p < 0.0001$) C_{max} , M_{cas} and aerosol yield compared to the reference powder Onbrez®, as represented in the Figure 52A. This superior performance of the DPI-SOL_{Crysmeb} and DPI-SUS_{HPβCD} formulations can be attributed to the use of cyclodextrins as excipients. Unlike the lactose-based carrier used in the commercial Onbrez® formulation, cyclodextrins act as surface-modifying agents,

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enabling fine-tuning of surface morphology and internal structure. This modification reduces interparticle cohesion, thereby enhancing the powders' flowability (M_{cas}) and dispersibility (C_{max}) during actuation, indicating that they aerosolized more rapidly and efficiently, releasing a greater mass of particles over a shorter exposure time compared to Onbrez[®]. Although DPI-SOL_{Crysmeb} exhibited tendentially higher C_{max} , M_{cas} and aerosolization yield, Figure 52B and C show that no significant differences were observed in comparison to powder obtained from DPI-SUS_{HPβCD}.

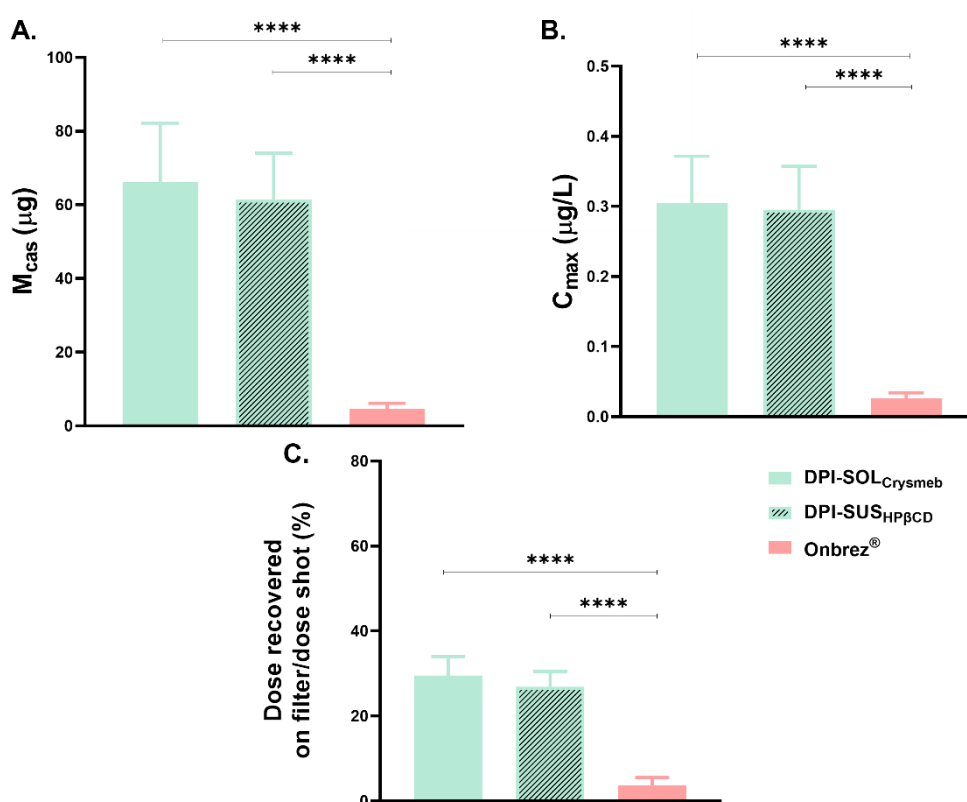


Figure 52: Aerosol performance of powder formulations from spray-dried powder from atomized solution and suspension at 10% and the Onbrez[®] reference with the PreciseInhale[®] system. (A) Maximum concentration in Casella (C_{max}), (B) cumulative dose (M_{cas}) and (C) aerosol yield in the PreciseInhale[®] system for spray-dried powder formulations of indacaterol and ciclesonide tested at PreciseInhale[®] settings of 60 L/min/400 mL/min/4 KPa/168 ms (inhalation flow/ exposure airflow/ pressure drop/ actuation time). Data points represent three independent experiments with exposures $n = 5$ and they display mean values $\pm$ SD. **** $p < 0.0001$ via one-way ANOVA with Tukey's post-test.

Furthermore, the MMAD was evaluated for the two powders using a 9 stage Marple cascade impactor. Onbrez[®] resulted in having a statistically significant larger MMAD, illustrated in Figure 53A, as compared to the IND/CIC developed powders from dried

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solutions and suspensions. Powders from atomized solutions (DPI-SOL_{Crysmeb}) and suspensions (DPI-SUS_{HPβCD}) exhibited comparable MMAD values, both below 5 μm and consistent with MMAD values previously obtained using the NGI. All the three test powders had an adequate GSD within the appropriate range for pharmaceutical heterodisperse aerosol [407], between 1.5-2.5 (Figure 53B).

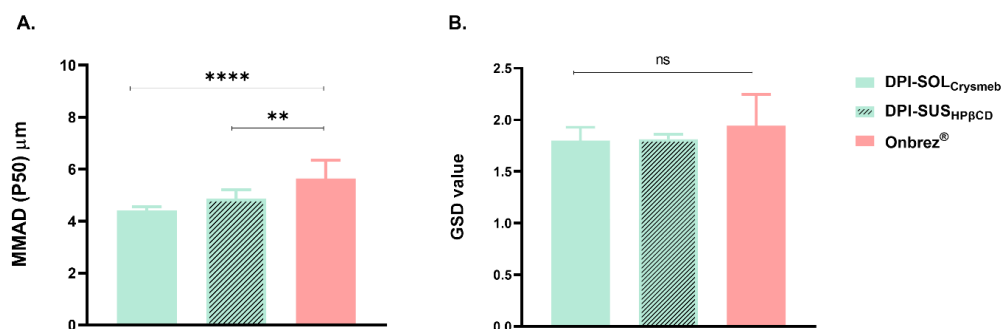


Figure 53: (A) The mass median aerodynamic diameter (MMAD) and (B) geometric standard deviation (GSD) from spray-dried powder from atomized solution and suspension at 10% and the Onbrez® reference with the Preciselnhale® system, based on measurements performed using a 9-stage Marple cascade impactor. Data points represent three independent experiments with exposures $n=3$ and they display mean values $\pm$ SD. ** $p < 0.01$, **** $p < 0.0001$ and ns: not significant via one-way ANOVA with Tukey's post-test.

During the PSD analysis, the aerosol particles travel through the Preciselnhale® system to the impactor after being aerosolized. In this process, losses can occur due to particle deposition on internal surfaces, particles bypassing the impactor stages, particle rebound, or inefficiencies in the collection system [408,409]. Therefore, the aerosol collection efficiency was calculated as the recovery. The recovery, represented on Figure 54A, measures the amount of the aerosolized material captured on the stages of the Marple Cascade Impactor. As for the yield, both engineered powders exhibited comparable recovery rates, both of which were significantly higher than that of Onbrez® ($p < 0.0001$).

Alongside MMAD, the FPF is one of the most common descriptors for the aerosol deposition properties of DPI formulations [392]. All three powders exhibited a relatively high FPF, exceeding 60% (Figure 54B), likely attributable to their favorable inhalation properties and appropriate flow rates. However, Onbrez® displayed a lower FPF compared to developed powders from dried solution and suspension. In contrast, powder from atomized solution also exhibited a significantly higher FPF than powders from dried suspensions, which is partly attributable to its slightly smaller MMAD. As for

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the aerosolization properties, developed engineered powders in this study showed a more favorable lung deposition profile than Onbrez[®], due to higher FPF and lower MMAD.

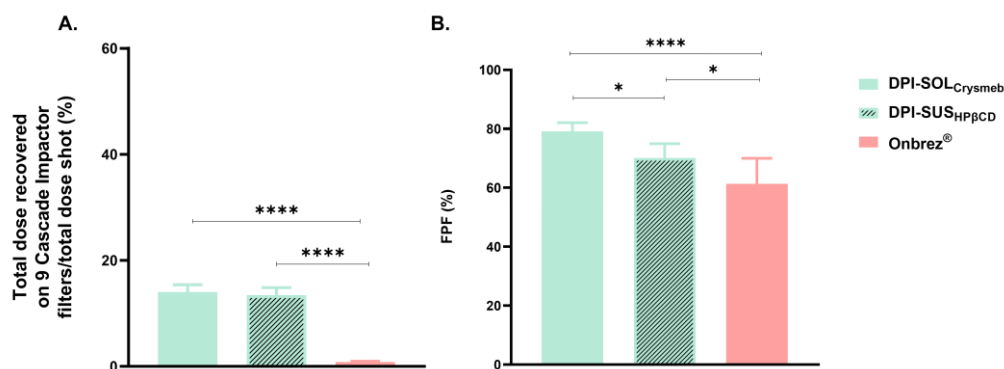


Figure 54: (A) The recovery and (B) fine particle fraction (FPF) from spray-dried powder from atomized solution and suspension at 10% and the Onbrez[®] reference with the Preciselnhale[®] system, based on measurements performed using a 9-stage Marple cascade impactor. Data points represent three independent experiments with exposures $n=3$ and they display mean values $\pm$ SD. * $p < 0.05$ and **** $p < 0.0001$ via one-way ANOVA with Tukey's post-test.

Overall, the Preciselnhale[®] system is an advanced aerosol generation system which enables precision dosing of powders aerosols and is widely used in *in vivo*, *ex vivo* and *in vitro* studies to investigate the solubility, dissolution and pharmacokinetic/pharmacodynamic profiles of airborne particles [36,410]. While its application in these contexts is well established, its potential for characterizing aerosolization properties of powders, particularly in comparison with the NGI, remains less explored.

In this study, the aerosol performance of DPI powders was evaluated using both the NGI and Preciselnhale[®] systems, demonstrating the complementarity between these two techniques. The NGI, a cascade impactor that operates at constant flow rates, provides detailed aerodynamic particle size distribution. On the other hand, the Preciselnhale[®] system primarily functions as an aerosol generation and delivery device, offering precise control over flow rate, aerosol volume and generation duration. This precision allows the system to closely replicate human inhalation dynamics, making it a valuable tool for translational respiratory research and preclinical inhalation studies. When combined with a Marple Cascade Impactor, it facilitates a thorough analysis of aerosolization and deposition profiles, along with real-time monitoring of aerosol concentration during dispersion. The integration of this system allows for the collection

of additional performance metrics (such as $C_{\max}$, M_{cas} and yield) that offer novel insights into powder dispersibility, flowability and aerosol delivery efficiency, metrics that are not typically captured by NGI analysis alone, while still confirming the aerodynamic properties of the powders.

Indeed, despite differences in methodology, both systems revealed consistent trends in aerosolization performance. Specifically, atomized solutions (DPI-SOL_{Crysmeb}) outperformed dried suspensions (DPI-SUS_{HPβCD}) in both systems, demonstrating higher FPF values and lower MMAD. The commercial Onbrez[®] formulation consistently exhibited the lowest performance, likely due to its larger particle size and suboptimal excipient morphology.

In this context, the integration of NGI and PreciseInhale[®] data offers a more comprehensive evaluation of inhalation powders, combining aerodynamic size profiling with aerosol delivery dynamics. While NGI remains the gold standard for aerodynamic characterization, PreciseInhale[®] complements this by providing additional parameters related to aerosolization behavior and lung deposition potential. Together, these *in vitro* approaches offer novel insights into powder dispersibility, flowability and aerosol delivery efficiency, deepening the understanding of inhalation product performance and supporting the optimization of formulations prior to *in vivo* studies. Ultimately, this combination of complementary techniques enhances the reliability of performance trends and establishes a robust foundation for selecting and optimizing formulations for further development.

III.2.2.3.e. Powders stability

While many studies suggest that dry formulations improve stability [31], others highlight the potential instability of SD powders, primarily due to high humidity sensitivity and to their hygroscopic properties [153,411]. Given these concerns, a stability study was conducted on the most promising formulation. Among the powders developed, those produced from atomized solutions (10% solid content), DPI-SOL_{Crysmeb}, demonstrated significantly enhanced aerodynamic properties and aerosolization efficiency, highlighting their strong potential for inhalation applications.

Regarding the physicochemical properties over time, the powder's particle size remained unchanged after 6 months under both tested conditions. Powder's moisture content, which is around 6% for both storage conditions, remained stable after 6 months

(Supplementary data F A, Appendix IV). A small increase in water content may lead to slight agglomeration of the powder (Supplementary data F B, Appendix IV). Monitoring the moisture content over time is crucial to ensure proper aerosolization and aerodynamic performance while preventing potential API degradation and maintaining the therapeutic efficacy of the powder [153,190]. To minimize moisture uptake for further investigation of this developed powder, solutions such as incorporating desiccant tablets or optimizing the packaging with moisture-resistant materials can be considered.

About the API content in powders over time results, presented in Figure 55A, show that both active substance contents were not impacted over time, whatever the temperature or RH tested, because API content remained between 95 and 105% of the initial content. Furthermore, Figure 55B shows that the optimized powder from the atomized solution maintained API homogeneity after 6 months under both ICH conditions, suggesting that the slight moisture increase did not cause agglomeration. Finally, *in vitro* aerodynamics profiles after 3 and 6 months remained similar for both API, at both storage conditions and this is proven by identical and FPF results for both active substances, as shown by Figure 55C, with similar MMAD and GSD values. This stability is further enhanced by the use of non-reducing sugars, specifically cyclodextrin Crysmeb in the optimized powder, which exhibit higher T_g, leading to improved API stability due to their amorphous state at room temperature [40]. This underscores the critical importance of excipient selection in the production of DPIs.

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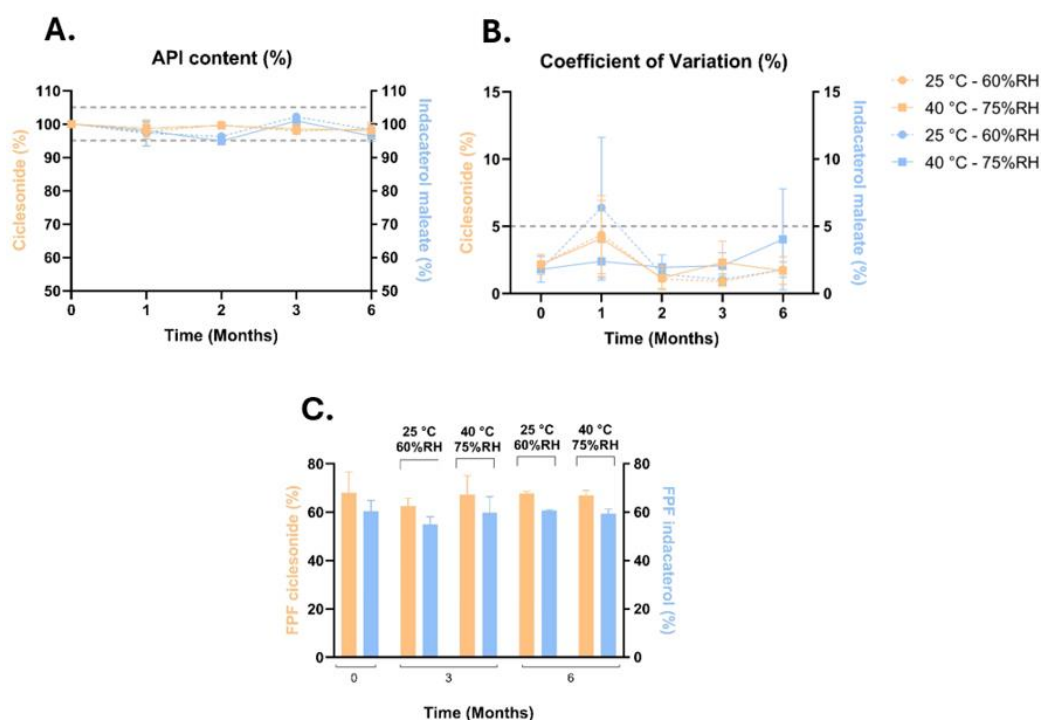


Figure 55: (A) CIC and IND content from powders from atomized solutions (DPI-SOL_{Crysmeb}) with 10% solid content (w/v), monitored over six months under ICH stability conditions. (B) Coefficients of variation for APIs across five samples produced from powders from atomized solutions with 10% solid content (w/v), monitored over six months under ICH stability conditions. (C) CIC and IND FPF values from powders from atomized solutions with 10% solid content (w/v), monitored over six months under ICH stability conditions. Analyses were performed in triplicates (n=3) and results are presented as mean $\pm$ standard deviation.

III.2.2.4. Conclusion

Limited lung deposition reduces the efficacy of many commercial inhalation powders. Addressing this requires improved formulation strategies, better understanding of inhalation dynamics, exploration of new API combinations and enhanced evaluation methods to develop more effective treatments for pulmonary diseases.

To address this, in this study, a successful combination developed a dry powder inhaler combining ciclesonide and indacaterol maleate for asthma and COPD treatment. Optimized spray-dried powders from atomized solutions demonstrated superior aerosolization, lung deposition and physicochemical properties compared to those from suspensions. The solubilization of APIs in the atomized liquid, facilitated by cyclodextrin

excipients, played a key role in obtaining favorable particle size, recovery and stability in multi-API formulations.

Additionally, consistent and complementary results obtained from both the NGI and the PreciseInhale® system validated the *in vitro* aerosolization performance of the developed formulation, reinforcing the reliability of the findings. This strong agreement highlights the potential of the new carrier-free DPI to enhance therapeutic efficacy across a range of patient profiles. However, evaluating the *in vivo* performance of inhaled powders remains a significant challenge [412]. Bridging this gap calls for novel *in vitro* methods and advanced equipment capable of better predicting *in vivo* outcomes. These findings lay a solid foundation for future *in vivo* studies and the continued development of optimized inhalable therapies for respiratory diseases.

III.3. Development of a non-liposomal reference DPI matched to the optimized liposome-based formulation

In addition to the optimized CIC-IND formulation using Crysmeb, referred to as DPI-SOL_{Crysmeb}, another non-liposome-based DPI was developed as a reference formulation to match the composition and API content of the liposome-based DPI developed in Part I of this second chapter.

The initial DPI-SOL_{Crysmeb} formulation was designed to deliver clinically relevant doses (125 µg of IND and 80 µg of CIC per 15 mg of powder, corresponding to 0.83% and 0.53% w/w, respectively). In contrast, the optimized liposome-based DPI contained lower API levels (0.27% w/w for both CIC and IND), dictated by the drug loading achieved in the liposomal system and the L/C of 1:9 selected during DoE optimization. Accordingly, a non-liposome-based DPI with aligned composition and API content was developed as a reference formulation for direct comparison with the liposomal system in terms of powder properties and performance (Part III).

However, complete complexation of CIC at high concentrations with HPβCD could not be achieved, as previously observed in the published paper. This limitation is likely due to the relatively low stability constant of the CIC-HPβCD complex, related to the more hydrophilic nature of its cavity resulting from hydroxypropyl substitutions, which reduces its ability to effectively complex highly lipophilic compounds [394,413]. In addition, suspension-based SD was previously shown to be less suitable for the development of optimized DPI formulations, further emphasizing the need for a solution-based system.

Based on the phase-solubility diagram established earlier in this work (Section III.2.2.2.d, Chapter 2), the amount of CIC required to reach the target composition (0.27% w/w) could theoretically be fully complexed in highly concentrated HP β CD solutions (50% w/v), where sufficient cyclodextrin is available to accommodate the lipophilic drug. Accordingly, a two-step complexation strategy was implemented. CIC was first complexed in a highly concentrated HP β CD solution (50% w/v) to maximize inclusion complex formation. Once full complexation was achieved, IND was added, and the formulation was subsequently diluted to the target cyclodextrin concentration (5% w/v) prior to SD. This approach preserved CIC in a solubilized state upon dilution, without visible precipitation, yielding a stable and homogeneous feedstock suitable for SD. The final formulation was then processed using the same optimized SD conditions as those applied for DPI-SOL_{Crysmeb} (T_{in} 160 °C, feed flow rate 3.85 g/min, NGP 3 bar, CGP 0.4 bar, inlet gas flow 0.4 m³/min, nozzle diameter 0.4 mm).

As a reminder, these parameters were selected based on previous studies aimed at engineering ultra-light, highly dispersible microparticles, notably through the formation of deflated structures induced by cyclodextrins [177]. Such particle characteristics are known to enhance aerosolization performance and improve lung deposition. Importantly, these SD conditions were deliberately chosen to obtain an optimized non-liposome-based DPI formulation serving as a reference to the optimized liposome-based DPI. This approach ensures that the reference powder exhibits optimal inhalation performance, thereby enabling a meaningful and unbiased comparison with the liposome-based formulation, which was processed using its own optimized drying conditions.

Powders were produced with a drying yield of $80.15 \pm 1.82\%$, enabling the generation of dry powders suitable for inhalation. The resulting particles exhibited a particle size of $3.17 \pm 0.29 \mu\text{m}$, with a span of 1.72 ± 0.03 and a residual water content of $3.88 \pm 0.52\%$, all of which fall within the acceptable range for pulmonary delivery. The API content was $0.28 \pm 0.01\%$ for CIC and $0.26 \pm 0.01\%$ for IND, closely matching the target content of 0.27% for each API, as defined to match the composition of the liposomal DPI developed in Part I of this chapter. A homogeneous distribution of both APIs within the powders was confirmed, with CV below 5%, specifically $2.03 \pm 0.31\%$ for CIC and $2.25 \pm 0.23\%$ for IND, indicating good content uniformity. Following SD, the recovery of both APIs was evaluated to assess potential degradation during processing. Recovery rates

ranged between 95% and 105%, demonstrating the absence of significant API loss. More specifically, recovery values of $102.12 \pm 2.61\%$ and $96.50 \pm 0.86\%$ were obtained for CIC and IND, respectively.

Overall, the developed powder exhibited physicochemical properties consistent with the requirements for pulmonary delivery, including appropriate particle size, low residual moisture, high content uniformity and consistent API recovery, supporting efficient lung deposition and adequate stability.

In line with the objectives of this part, a non-liposome-based DPI with matched composition and API content was successfully established as a robust reference formulation for subsequent comparison with the liposomal system in terms of powder properties and performance. Additionally, the implementation of a dedicated complexation strategy using highly concentrated HP β CD solutions proved critical to overcoming the limitations associated with CIC solubilization, enabling the generation of a stable and homogeneous liquid feed suitable for SD. This HP β CD-based solution system also supported subsequent *in vivo* investigations within the LNPulmo project, contributing to the selection of the final liposome formulation for asthma management.

III.4. Conclusion

In line with the initial objectives of this part, the feasibility of **developing optimized non-liposome-based DPIs co-containing CIC and IND** was successfully demonstrated. More specifically, this work led to the development of two distinct non-liposome-based DPI formulations, each designed to address a specific objective within the overall strategy of this chapter.

First, an optimized carrier-free DPI based on Crysmeb (DPI-SOL_{Crysmeb}) was developed to deliver CIC and IND at clinically relevant doses, consistent with marketed inhalation products. This formulation represents a novel therapeutic approach, as no DPI combining both APIs is currently available. Its development underscored the critical importance of CIC solubilization prior to SD by demonstrating that solution-based feedstocks produce powders with superior overall performance compared to suspension-based formulations. The resulting powders, DPI-SOL_{Crysmeb}, exhibited an appropriate particle size distribution, good homogeneity, satisfactory residual moisture content, high FPF and good stability over time under ICH conditions.

Second, a complementary non-liposome-based reference DPI based on HP β CD was successfully developed with matched composition and API content relative to the optimized liposome-based DPI established in Part I. The implementation of a dedicated complexation strategy using highly concentrated HP β CD solutions proved essential to overcome the limitations associated with CIC solubilization and enabled the generation of a stable and homogeneous feedstock suitable for SD. The resulting powder exhibited properties compatible with pulmonary delivery and therefore constitutes a robust reference system for subsequent comparison with the liposomal formulation.

Taken together, these results not only confirm the feasibility of producing efficient non-liposome-based DPIs co-delivering the innovative CIC-IND combination but also highlight the critical role of API solubilization prior to SD in ensuring optimal physicochemical properties of the resulting powders. Although different cyclodextrin derivatives could be considered as solubilization enhancers, their use is particularly relevant in this context due to their combined ability to improve drug solubility and generate particles with properties suitable for pulmonary delivery.

IV. Part III. Comparative evaluation of liposome-based and non-liposome-based DPI formulations containing CIC and IND

IV.1. Context

Following the development of two optimized inhalable dry powder formulations, one liposome-based (Part I) and one non-liposome-based reference (Part II), this Part III is dedicated to a direct comparative evaluation of these two DPI systems. The primary objective of this part is thus to elucidate the impact of liposome incorporation on aerosol performance and pulmonary deposition under clinically relevant inhalation conditions, as well as in physicochemical stability during storage.

To address these objectives, a first section investigates the aerodynamic performance of both optimized formulations through impaction studies. The influence of inhalation flow rate is evaluated by comparing optimal (100 L/min) with clinically relevant inspiratory flows (~60 L/min). In addition, two inhalation devices with distinct intrinsic resistances are used to assess the impact of device-dependent airflow dynamics on powder emission and fine particle delivery. This approach allows assessment of the robustness of each formulation across realistic inhalation scenarios and determination of the influence of liposome incorporation on key performance parameters.

Following, a second section examines the stability of both powders under refrigerated conditions and under the two ICH-recommended storage environments. The objective is to determine whether liposomal incorporation influences the long-term physicochemical stability of the powders. This includes the evolution of particle characteristics, moisture content, API chemical stability and, for the liposomal formulation, the preservation of liposomal integrity upon rehydration. The results of this stability study allow identification of the most suitable storage conditions for each formulation and help anticipate potential limitations associated with the lipid-based nature of liposomes.

IV.2. Materials and methods

The materials and methods are largely consistent with those described in Section II.2.2 (Chapter 1) of the publication “*Optimizing Spray-Dried Liposomes for Pulmonary Delivery: Impact of Lipid Composition and Drying Parameters Using Experimental Design*”, presented in this manuscript. Additional materials and methods are described

in Section III.2.2.2 of Part II of this chapter and further methodological details are therefore provided in the present section.

IV.2.1. In vitro lung deposition study

The *in vitro* lung deposition methodology using the NGI at flow rates of 60 and 100 L/min, along with the corresponding results obtained with the Breezhaler® device, are described in Section II.2.2.8 (Chapter 1). Additionally, impaction tests were performed using a HR device, namely the RS01, operated at a flow rate of 44 L/min, corresponding to a pressure drop of 4 kPa. For these experiments, 10 size 3 HPMC capsules were actuated. Impaction tests using the HR device were conducted by the industrial partner Aquilon Pharma.

IV.2.2. Powder flowability evaluation

Bulk and tapped density measurements were performed in accordance with the Ph. Eur. 2.9.34, with minor adaptations previously reported in the literature [361,414]. Bulk density, defined as the ratio of the powder mass to the volume it occupies in its loose, uncompressed state, reflects the packing efficiency and the amount of interparticulate voids within the powder bed. To determine this parameter, a known quantity of powder was gently poured into a 10 mL graduated cylinder, and the apparent bulk volume was recorded directly. The bulk density (g/cm^3) corresponded to the mass-to-volume ratio obtained from this initial measurement.

Tapped density represents the mass of the powder divided by its volume after mechanical consolidation through repeated tapping, providing insight into the powder's compressibility and its tendency to densify under external stress. For this measurement, the same cylinder was subjected to mechanical tapping, and the tapped volume was recorded after 10 and 500 taps at a tapping height of 3 mm. Tapped density (g/cm^3) was calculated from the powder mass and the final tapped volume.

Powder flow characteristics were subsequently evaluated through the Hausner ratio, calculated as the ratio of tapped density to bulk density. This index is widely used to estimate flowability, with values closer to 1 indicating good flow and higher values reflecting increasing cohesiveness and poor flow behavior.

IV.2.3. *Stability study*

An initial stability study was performed on both the optimized powder formulations, with and without liposomes. The formulations were stored under different conditions: refrigerated storage (5 ± 5 °C), controlled room temperature (25 ± 2 °C, $60 \pm 5\%$ RH), and accelerated conditions (40 ± 2 °C, $75 \pm 5\%$ RH). The latter two conditions were established in accordance with ICH stability guidelines. The powders were stored in sealed vials and maintained under the specified climatic conditions for 3 months. Samples were collected at the end of the storage period for subsequent analyses.

Both powders were evaluated for general physicochemical properties, including particle size distribution, moisture content and API content. In addition, the liposome-based powder was specifically assessed for liposomal integrity through measurements of liposome size, Pdl and ZP, as well as the amount of API encapsulated within the liposomes. API degradation products were quantified for NLP_{opt} by the industrial partner Aquilon Pharma (the impurity quantification method is detailed in Appendix V).

IV.3. Results and discussion

A comparative evaluation of both optimized powders, with and without liposomes, was conducted to assess their pulmonary deposition and stability. For clarity, the optimized non-liposome-based and liposome-based powders are hereafter referred to as NLP_{opt} and LP_{opt}, respectively.

To provide a comprehensive overview of these formulations, the key characteristics of their corresponding feed liquids, resulting powders and SD conditions are summarized in Table 11. LP_{opt} liposomal integrity results are reported in the previous section (Table 7).

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Table 11: Comparative summary of feed suspension characteristics, spray-drying (SD) parameters, and resulting powder properties for the optimized non-liposome-based (NLP_{opt}) and liposome-based (LP_{opt}) DPI formulations. Analyses were performed in triplicates (n=3) and results are presented as mean ± standard deviation.

		Optimal formulations	
		NLP _{opt} (w/o liposomes)	LP _{opt} (w/ liposomes DLPC/CHOL/DSPE-PEG ₂₀₀₀ 55/30/15, molar ratio)
Liquid feed properties	CIC content (%)	~ 0.27	
	IND content (%)	~ 0.27	
	Sugar-based excipient	HPβCD	
	Solid content (%)	5	
	Lipid-to-carbohydrate ratio (%)	/	1:9
Spray-drying parameters	Inlet temperature (°C)	160	120
	Outlet temperature (°C)	~ 81	~ 75
	Feed flowrate (g/min)	~ 3.85	~ 1.56
	Nozzle gas pressure (bar)	3.0	3.4
	Cyclone gas pressure (bar)	0.4	0.3
Dry powders properties	Drying yield (%)	80.15 ± 2.82	77.92 ± 4.48
	Particle size (D50) (μm)	3.13 ± 0.29	2.65 ± 0.25
	Moisture content (%)	3.88 ± 0.52	3.66 ± 0.41
	CIC content (%)	0.28 ± 0.01	0.27 ± 0.03
	IND content (%)	0.26 ± 0.01	0.27 ± 0.02

IV.3.1. In vitro aerodynamic performance of optimal powders

IV.3.1.1. Aerodynamic performance under varying flowrates

The *in vitro* pulmonary deposition of the two optimized powders (LP_{opt} and NLP_{opt}) was evaluated using the NGI with the LR Breezhaler® device. Two inspiratory flow rates were investigated: the recommended flow rate of 100 L/min, corresponding to a 4 kPa pressure drop across the device, and a lower, clinically relevant flow rate (~60 L/min) reflecting typical patient use. This approach enables the evaluation of aerodynamic performance under both optimal and more realistic inhalation conditions, which is particularly relevant given the variability in inspiratory capacities among patients with asthma and COPD, with values around 60 L/min commonly reported for Breezhaler® users [311,403].

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At the optimal inspiratory flow rate (100 L/min), the deposition profiles of both powders (Figure 56) show that a substantial fraction of the APIs reaches the deep lung regions, with FPF values approaching 70% and no significant difference between the two formulations. An d_{ae} around 3 μm is generally considered optimal for deposition in the lower airways [351], in agreement with the small particle sizes observed for both formulations, with D50 values of 2.65 ± 0.25 and 3.13 ± 0.29 μm for NLP_{opt} and LP_{opt} , respectively. This likely contributes to their high aerosolization efficiency, as the FPF is inversely related to particle size, with smaller particles leading to higher respirable fractions. Furthermore, these results are particularly encouraging, considering that, as previously mentioned, most commercially available powders typically achieve low lung deposition values [40]. Additionally, regardless of the powder formulation, both APIs exhibited similar deposition profiles, as evidenced by comparable FPF values and a uniform distribution across the different NGI stages (Figure 57). This homogeneous deposition is important as it indicates consistent co-delivery of both drugs throughout the respiratory tract, which is essential to ensure synchronized therapeutic action and optimal efficacy in combination therapies [415].

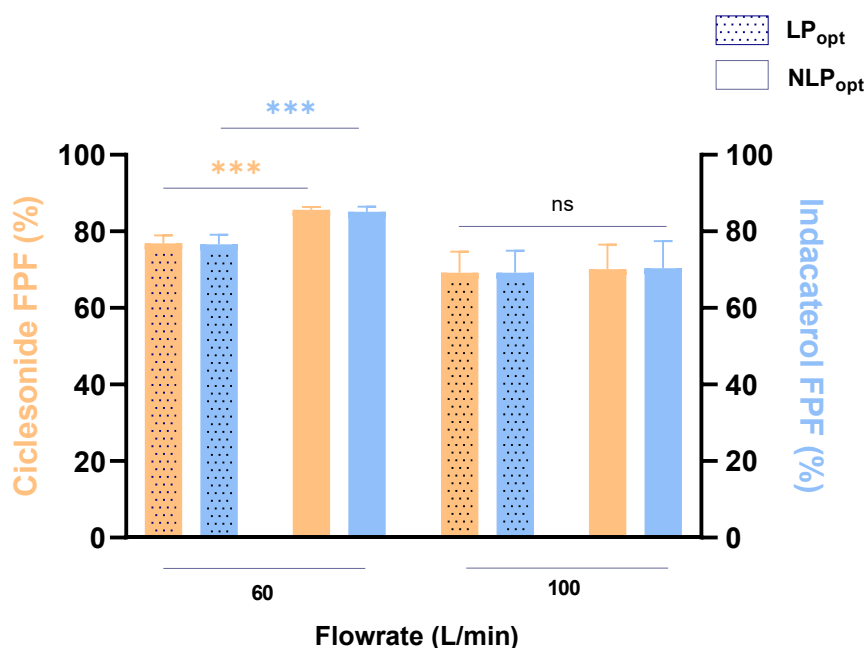


Figure 56: Fine particle fraction (FPF) of ciclesonide and indacaterol measured for the optimized liposome-based and non-liposome-based powders at 60 and 100 L/min using the Next Generation Impactor (NGI) with the Breezhaler® device. Analyses were performed in triplicates ($n=3$) and results are presented as mean $\pm$ standard deviation.

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At a lower clinically relevant inspiratory flow rate (60 L/min), both formulations maintained high lung deposition profiles. For LP_{opt}, FPF values reached $76.91 \pm 2.06\%$ for CIC and $76.59 \pm 2.53\%$ for IND, while NLP_{opt} exhibited even higher values of $86.53 \pm 1.57\%$ for CIC and $85.07 \pm 1.57\%$ for IND. These results confirm that both powders preserve strong aerodynamic performance across different inspiratory flow rates.

Notably, FPF values were even higher at the reduced flow rate for both powders, especially for NLP_{opt} ($p < 0.05$), indicating improved lung deposition under these conditions. This behavior can be attributed to reduced inertial impaction at lower flow rates, leading to decreased deposition in the throat and upper NGI stages, as already reported in the literature [351]. This trend is consistent with the stage-by-stage deposition profiles (Figure 57), which show lower deposition in the upper stages at 60 L/min (Figure 57A) compared to 100 L/min (Figure 57B).

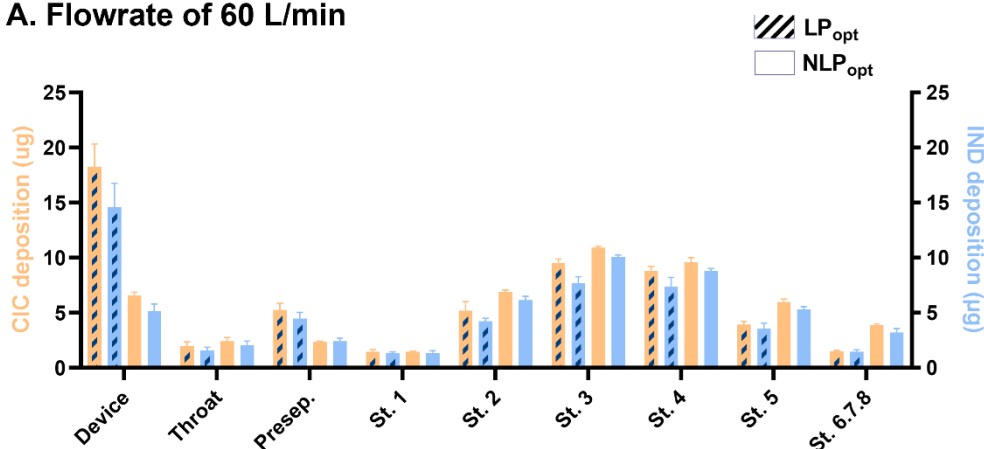
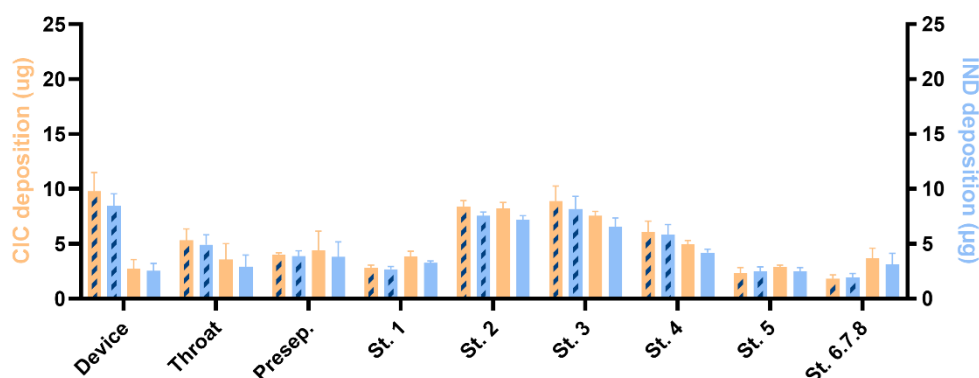
A. Flowrate of 60 L/min**B. Flowrate of 100 L/min**

Figure 57: Aerodynamic particle size distribution profiles of ciclesonide and indacaterol measured for the optimized liposome-based and non-liposome-based powders using the Next Generation Impactor (NGI) at (A) 60 L/min and (B) 100 L/min with the Breezhaler® device. Analyses were performed in triplicates ($n=3$) and results are presented as mean $\pm$ standard deviation.

However, at 60 L/min, a clearer difference between the two formulations becomes apparent. Although both powders benefit from reduced inertial impaction, the improvement in FPF is more pronounced for NLP_{opt} ($p < 0.0005$), thereby revealing formulation-related limitations associated with the liposome-based powder. Building on this observation, the ED analysis further confirms a formulation-dependent limitation for LP_{opt}, as its ED values remain consistently lower than those of NLP_{opt} at both inspiratory flow rates (Figure 58).

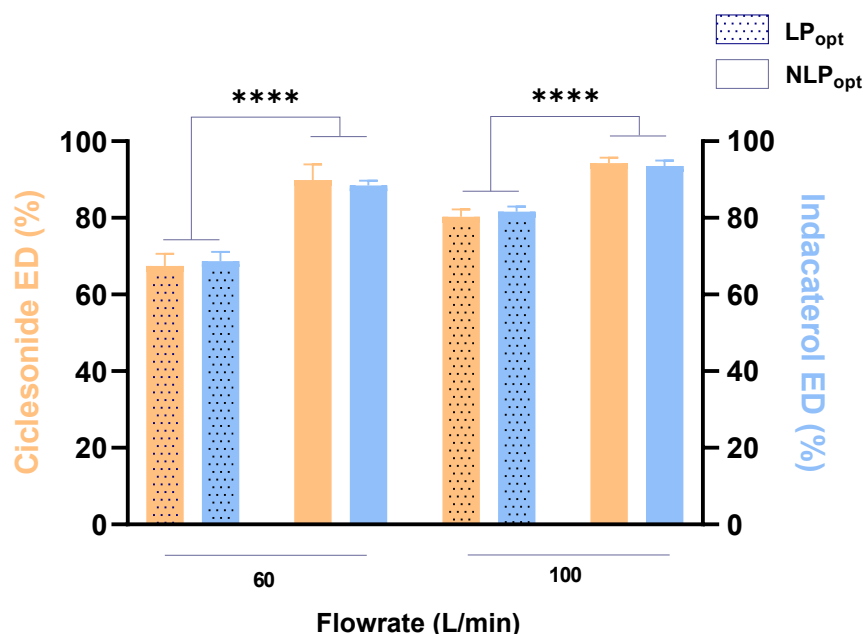


Figure 58: Emitted dose (ED) of ciclesonide and indacaterol measured for the optimized liposome-based and non-liposome-based powders at 100 and 60 L/min using the Next Generation Impactor (NGI) with the Breezhaler® device. Analyses were performed in triplicates ($n=3$) and results are presented as mean $\pm$ standard deviation.

More specifically, while NLP_{opt} exhibited stable ED values of approximately 90% with no significant variation between flow rates, LP_{opt} showed a significant decrease ($p < 0.0001$) in ED at 60 L/min for both APIs, with values decreasing from $80.29 \pm 1.90\%$ for CIC and $81.64 \pm 6.24\%$ for IND at 100 L/min to $67.43 \pm 3.21\%$ and $68.69 \pm 2.43\%$ at 60 L/min, respectively. These findings suggest that, despite satisfactory aerosolization once dispersed, the liposomal formulation delivers a smaller fraction of the nominal dose from the device.

This reduced emission is further supported by visual inspection of the capsules after impaction testing, which revealed a substantially higher amount of powder remaining in both the capsule and the inhaler for LP_{opt} compared to NLP_{opt}. As reported in the literature, powder retention within capsules is strongly influenced by powder characteristics, in addition to capsule shell properties and inhaler performance [84]. Since the capsule and device were identical for both formulations, these observations further indicate that the differences primarily arise from powder properties.

To better understand the origin of this behavior and determine whether it stems from intrinsic formulation properties or processing conditions, two potential factors were investigated: the presence of liposomes and the influence of SD parameters. To specifically isolate the effect of SD conditions, the non-liposomal formulation was re-produced using the optimized parameters established for liposome SD in Chapter 1, and its aerodynamic performance was subsequently evaluated using impaction studies.

This rationale is supported by the fact that LP_{opt} and NLP_{opt} were initially produced under different SD conditions (Table 11), introducing potential process-related variability in particle attributes. Although both powders exhibited comparable physicochemical characteristics, particularly in terms of particle size and residual moisture, differences in parameters such as lower T_{in} or reduced feed rate may have modified particle structure or surface properties [177,416]. Such process-induced variations can enhance interparticulate cohesion independently of liposome content, thereby contributing to the differences observed in ED.

However, as shown in Figure 59, the non-liposomal formulation produced under the liposome-optimized SD conditions exhibited no significant change in ED compared with the original NLP_{opt} at 100 L/min, with ED values of $94.41 \pm 1.04\%$ and $94.29 \pm 0.89\%$ for CIC and IND, respectively, resulting in similarly high FPF values ($72.29 \pm 2.59\%$ and $71.00 \pm 3.84\%$). These findings indicate that SD-related variations did not affect powder emission or aerosolization for the non-liposomal formulation, thereby reinforcing that the reduced ED observed for LP_{opt} is attributable to intrinsic formulation characteristics associated with the presence of liposomes.

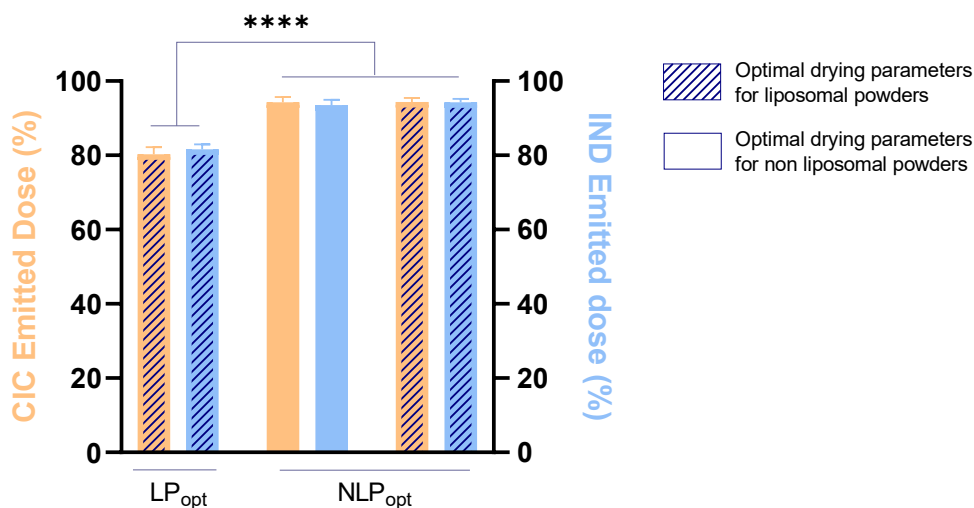


Figure 59: Emitted dose (ED) of both APIs measured at 100 L/min using the Next Generation Impactor (NGI) with the Breezhaler® device. Comparison between liposome-based and non-liposome-based powders as a function of spray-drying conditions. Analyses were performed in triplicates ($n=3$) and results are presented as mean $\pm$ standard deviation.

Since SD-related variations were ruled out as the source of the reduced ED, the focus shifts to intrinsic formulation characteristics. To better understand this effect, we conducted a preliminary evaluation of powder flow properties, as these parameters are closely linked to interparticulate cohesion and powder retention inside the device. Interestingly, the Hausner ratio (Figure 60A) suggested better flowability for LP_{opt} compared with NLP_{opt}. This behavior may be related to the presence of the lipid fraction, as LP_{opt} showed a greater tendency to form visible, weak agglomerates. The 10% w/w lipid content may promote interparticulate interactions and the formation of more structured aggregates. While the lipid-based system and lipid content differ, Wang *et al.* reported that increasing lipid content in DPI formulations resulted in reduced ED, likely due to enhanced interparticulate cohesion and aggregate formation, despite good flowability under tapping conditions [417]. These observations support the hypothesis that lipids can promote particle-particle interactions and the formation of more cohesive aggregates, which may improve bulk flowability but also lead to denser packing and stronger interparticulate interactions.

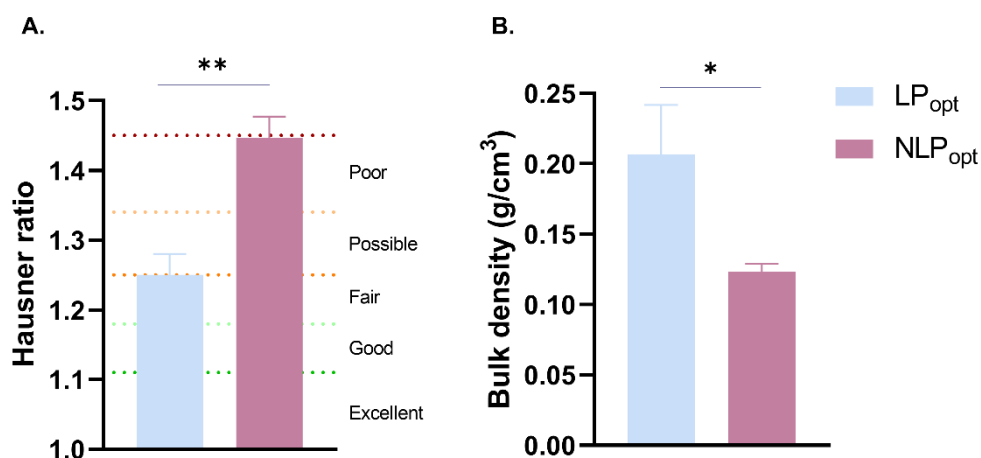


Figure 60: (A) Hausner ratio and (B) bulk density of optimized liposome-based and non-liposome-based powders. Analyses were performed in triplicates ($n=3$) and results are presented as mean $\pm$ standard deviation.

Consistently, LP_{opt} also exhibited a higher bulk density (Figure 60B), indicative of a more compact powder bed. Such increased packing within the capsule can hinder powder release during inhalation, contributing to the lower ED observed for the liposomal formulation (Figure 59). Consistent with this interpretation, Ki Ku *et al.* showed that powders with lower bulk density tend to be more readily dispersed, whereas higher bulk density is associated with reduced dispersibility, which may limit efficient aerosolization during inhalation [418]. Additionally, Charasiya *et al.* reported that DPIs with an MMAD between 1 and 5 μm and a bulk density below 0.4 g/cm³ are more suitable for aerosol formation and deep lung deposition, highlighting the importance of low bulk density for optimal aerosol performance [115]. In the present case, the presence of liposomes appears to be associated with a higher bulk density, likely due to increased interparticulate interactions, which may in turn reduce powder emission and limit dispersion efficiency under low-flow conditions compared to NLP_{opt}.

More broadly, the role of lipid incorporation in governing DPI powder performance remains largely unexplored. In particular, the influence of lipid components on interparticulate interactions and pulmonary deposition is still poorly characterized. The present findings point toward a potentially critical formulation-related limitation, highlighting the need for further studies to elucidate the underlying mechanisms.

The overall results of this study, including lung deposition values, MMAD and GSD at both flow rates, are summarized in Table 12 below. Notably, the eFPF, corresponding

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to the fraction of particles with a d_{ae} below 2 μm that are more likely to penetrate and deposit in the small airways [355,358], remained high for both powders at both flow rates, exceeding 25% at 100 L/min and 35% at 60 L/min. As a reminder, this parameter is particularly relevant for COPD, as the disease primarily affects these peripheral regions [356,419]. In addition, MMAD values fell within the optimal range of 1-5 μm , and the relatively low GSD indicates a narrow particle size distribution, supporting efficient and consistent pulmonary deposition. Despite the observed formulation-related limitation, the overall impact on pulmonary deposition performance appears limited, as both formulations still achieved high lung deposition.

Table 12: Summary of aerodynamic performance results of optimized powders, with and without liposomes, from studies conducted at 60 and 100 L/min using the Breezhaler®. The fine particle fraction is expressed relative to both the recovered dose (RD) and the emitted dose (ED). Analyses were performed in triplicates (n=3) and results are presented as mean $\pm$ standard deviation.

Flowrate (L/min)	NLP _{opt}		LP _{opt}	
	60	100	60	100
<i>Aerodynamic Performance of CIC</i>				
Recovery dose (%)	86.33 $\pm$ 4.58	79.18 $\pm$ 4.12	91.95 $\pm$ 4.75	80.33 $\pm$ 8.94
Emitted dose (%)	87.85 $\pm$ 1.62	93.59 $\pm$ 1.29	67.43 $\pm$ 3.21	80.29 $\pm$ 1.90
Fine particle fraction/RD (%)	76.03 $\pm$ 2.78	65.69 $\pm$ 6.88	51.83 $\pm$ 1.73	55.47 $\pm$ 3.23
Fine particle fraction/ED (%)	86.53 $\pm$ 1.57	70.13 $\pm$ 6.43	76.91 $\pm$ 2.06	69.17 $\pm$ 5.52
eFPF (%)	39.45 $\pm$ 1.33	27.83 $\pm$ 4.53	37.89 $\pm$ 2.86	25.65 $\pm$ 3.21
MMAD (μm)	2.89 $\pm$ 0.04	3.65 $\pm$ 0.44	3.58 $\pm$ 0.25	4.01 $\pm$ 0.30
GSD	2.13 $\pm$ 0.01	2.01 $\pm$ 0.11	1.93 $\pm$ 0.05	1.86 $\pm$ 0.03
<i>Aerodynamic Performance of IND</i>				
Recovery dose (%)	86.78 $\pm$ 1.60	77.08 $\pm$ 6.31	83.47 $\pm$ 8.24	81.64 $\pm$ 6.24
Emitted dose (%)	88.99 $\pm$ 0.83	92.97 $\pm$ 1.62	68.69 $\pm$ 2.43	81.58 $\pm$ 1.40
Fine particle fraction/RD (%)	75.71 $\pm$ 2.10	65.47 $\pm$ 7.70	52.58 $\pm$ 1.16	56.44 $\pm$ 3.78
Fine particle fraction/ED (%)	85.07 $\pm$ 1.57	70.34 $\pm$ 7.12	76.59 $\pm$ 2.53	69.24 $\pm$ 5.69
eFPF (%)	39.29 $\pm$ 1.44	27.27 $\pm$ 4.79	39.03 $\pm$ 3.10	27.33 $\pm$ 3.35
MMAD (μm)	3.35 $\pm$ 0.12	3.66 $\pm$ 0.47	2.94 $\pm$ 0.05	3.94 $\pm$ 0.31
GSD	1.99 $\pm$ 0.02	2.00 $\pm$ 0.11	2.07 $\pm$ 0.04	1.89 $\pm$ 0.04

IV.3.2. *Aerosolization performance across devices with different intrinsic resistances*

The aerosolization performance of DPIs is governed by the interplay between the patient's inspiratory flow, the physicochemical properties of the formulation and the intrinsic resistance of the device [13,144]. While the influence of formulation properties has been extensively characterized, this part of the study specifically focuses on evaluating the impact of device-related factors. To this end, two inhalers with distinct resistance classes, the LR Breezhaler® and the HR RS01, were selected to assess how device resistance influences the performance of the optimized powders (LP_{opt} and NLP_{opt}).

Device resistance is commonly characterized by the inspiratory flow required to generate a standardized pressure drop (ΔP) of 4 kPa. The relationship between pressure drop, device resistance, and inspiratory flow rate can be described by the following equation (Equation 15):

$$R = \frac{\sqrt{\Delta P}}{Q}$$

Equation 15: Relationship between device resistance, pressure drop, and inspiratory flow rate.

where R is the device resistance, ΔP is the pressure drop and Q is the inspiratory flow rate. Accordingly, LR devices necessitate higher inspiratory flow rates to generate a given pressure drop, while HR devices achieve the same pressure drop at lower flow rates [420,421]. In this study, a PIF of approximately 100 L/min is required for the Breezhaler®, compared to about 44 L/min for the HR RS01 [150,422]. Applying device-specific flow rates to achieve an equivalent pressure drop ensures a meaningful comparison between inhalers under comparable aerodynamic conditions [423].

Results show that when FPF is expressed relative to the ED, no significant differences are observed between LR and HR devices for either formulation, as both LP_{opt} and NLP_{opt} show similar values across resistance conditions (Figure 61). This indicates that, under the tested conditions, both devices provide sufficient energy to ensure effective dispersion of the emitted powder. This finding is consistent with the fact that FPF normalized to the ED reflects the intrinsic dispersibility of the formulation, independently of device-specific emission effects.

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However, when isolating the results obtained with the HR device, a significant difference emerges between formulations, with improved aerosolization observed for NLP_{opt} compared to LP_{opt}. This finding is consistent with the trend previously observed using the LR Breezhaler® at 60 L/min, where NLP_{opt} also demonstrated more reliable dispersibility. Together, these observations indicate that NLP_{opt} maintains efficient aerosolization across devices with different resistance classes, whereas LP_{opt} is more sensitive to device-related airflow dynamics.

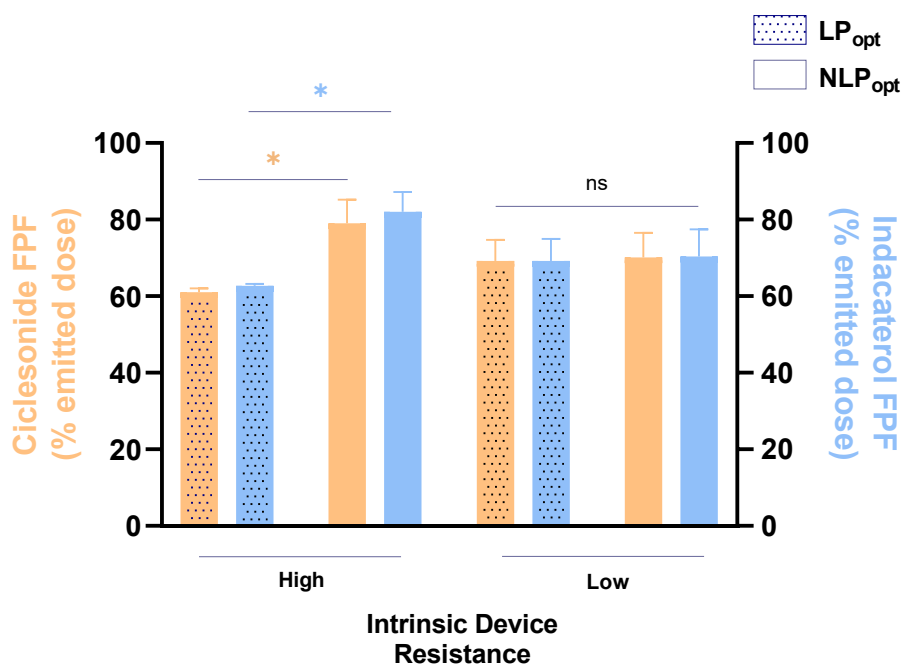


Figure 61: Fine particle fraction (FPF) of ciclesonide and indacaterol calculated based on the emitted dose (ED) and measured for the optimized liposome-based and non-liposome-based powders at a pressure drop of 4 kPa using the Next Generation Impactor (NGI) with high-resistance (RS01) and low-resistance (Breezhaler®) devices. Analyses were performed in triplicates (n=3) and results are presented as mean $\pm$ standard deviation.

In contrast, when FPF is expressed relative to the total dose, thereby accounting for both emission and dispersion and reflecting the overall performance of the formulation-device combination, a different trend is observed. This indicates that while dispersion of the ED is comparable, the devices differ in their ability to effectively emit the powder.

For both devices, although non-significant, NLP_{opt} shows a tendency toward higher FPF values compared to LP_{opt} (Figure 62). For CIC and IND, FPF values reached $53.07 \pm 7.29\%$ and $52.03 \pm 7.02\%$ with the HR device, and $65.69 \pm 6.88\%$ and $65.47 \pm 7.70\%$

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with the LR device for NLP_{opt}, compared to $35.54 \pm 2.55\%$ and $36.36 \pm 2.00\%$ (HR) and $55.47 \pm 3.23\%$ and $56.44 \pm 3.78\%$ (LR) for LP_{opt}. These results indicate that NLP_{opt} achieves more efficient emission irrespective of the inhaler, consistent with earlier observations.

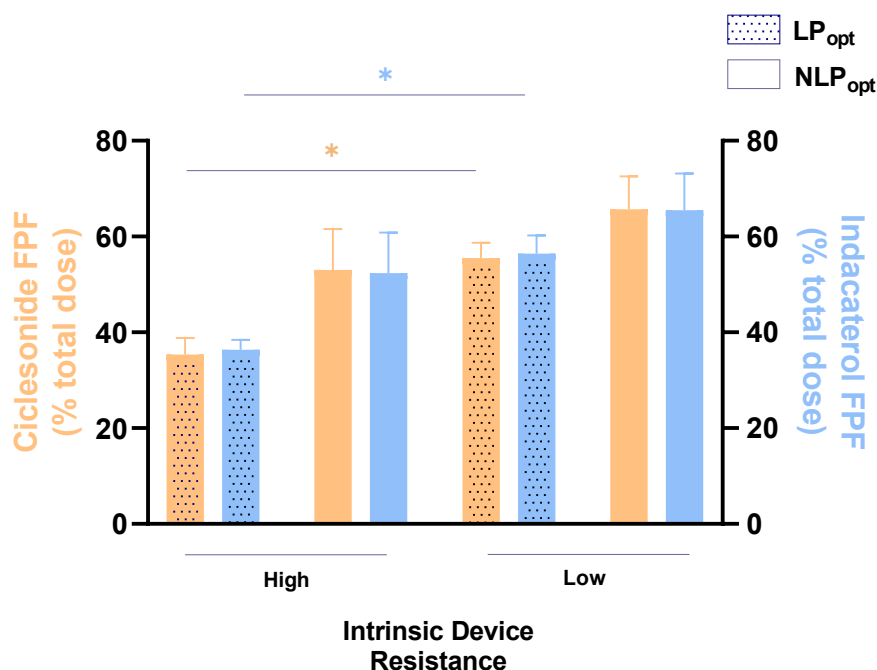


Figure 62: Fine particle fraction (FPF) of ciclesonide and indacaterol calculated based on the total dose and measured for the optimized liposome-based and non-liposome-based powders at a pressure drop of 4 kPa using the Next Generation Impactor (NGI) with high-resistance (RS01) and low-resistance (Breezhaler®) devices. Analyses were performed in triplicates ($n=3$) and results are presented as mean $\pm$ standard deviation.

More importantly, a significant difference was observed between LR and HR devices for LP_{opt}, with improved performance obtained using the LR Breezhaler®. This indicates that, despite achieving similar pressure drops, the higher internal turbulence characteristic of HR devices may be detrimental for such cohesive formulations. Increased turbulence can intensify particle-particle and particle-surface interactions, promoting re-agglomeration or impaction within the capsule and device [424]. In this context, Almeida *et al.* reported that higher device resistance generates faster tangential airflow along the device walls, which enhances particle impaction within the inhaler [425]. This mechanism likely contributes to the emission limitations observed for liposome-based powders, which are more pronounced with the HR device. These effects are likely exacerbated for denser and more cohesive liposome-based powders,

resulting in greater retention and a reduced fraction of powder effectively emitted and recovered as fine particles.

Overall, these results indicate that the performance of LP_{opt} is more sensitive to device resistance, whereas NLP_{opt} maintains efficient aerosolization across both inhalers. For liposome-based powders, LR devices operating at higher inspiratory flows appear to better preserve emission and reduce retention, thereby enhancing pulmonary deposition. In contrast, for NLP_{opt}, both devices perform similarly, making the choice of inhaler largely dependent on the patient's inspiratory capacity, which remains a key determinant of effective drug delivery. Importantly, despite these differences, both formulations demonstrate effective pulmonary deposition across devices with different resistance profiles, confirming their suitability for inhalation therapy.

From a practical standpoint, the observation that HR devices can also achieve good aerosolization performance is noteworthy, particularly given the known handling limitations of micronized powders, which often exhibit poor flowability and can complicate industrial development. In this context, formulation strategies such as carrier-based systems (e.g., lactose) or carrier-free approaches relying on engineered auto-agglomerates may improve powder handling and dispersion. Within this framework, using engineered auto-agglomerated structures, Gresse *et al.* demonstrated that HR devices can effectively promote deagglomeration of such structures, achieving FPF above 60% at low inspiratory flow rates (30 L/min) [175]. Accordingly, HR inhalers may become advantageous depending on the formulation design.

IV.3.3. Stability study of optimized powders

An initial stability study was conducted on both optimized powders, LP_{opt} and NLP_{opt}, for three months. Both ICH-recommended storage conditions were evaluated, together with a refrigerated condition.

The stability of the physicochemical properties of the different powders during storage is presented in Figure 63. As shown in Figure 63A, the moisture content of all powders remained stable over time, with values consistently below the recommended limit of 5%, even after three months of storage under high humidity conditions [172]. Maintaining a stable moisture content is critical, as even small increases in water content may promote

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powder agglomeration, alter aerosolization performance, induce API degradation and potentially affect liposomal integrity, thereby impacting the overall therapeutic performance of the formulation [40,158].

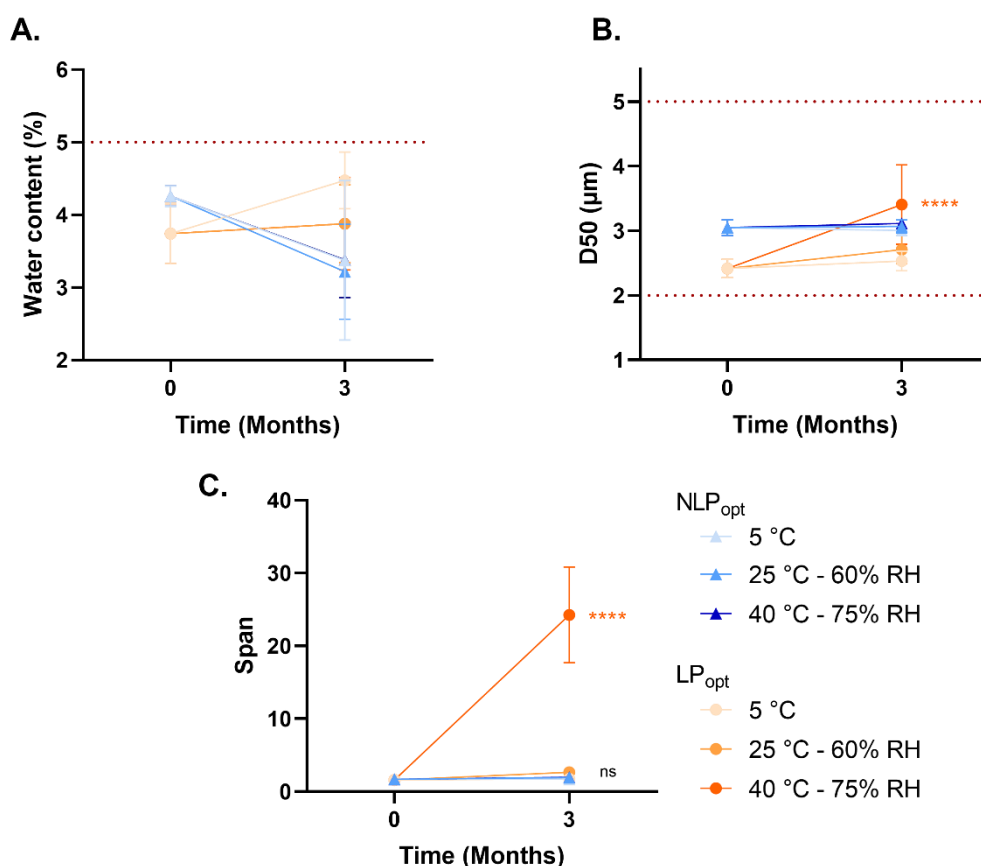


Figure 63: Physicochemical properties of optimized powders without liposomes (blue) and with liposomes (orange): (A) water content, (B) mean dry particle size, and (C) particle size heterodispersity (Span), measured after 3 months of storage under the three tested conditions: refrigerated storage and the two ICH-recommended conditions. Analyses were performed in triplicate ($n=3$), and results are presented as mean $\pm$ standard deviation.

In line with this stable moisture content, the dry particle size distribution remained essentially unchanged for all powders, except for LP_{opt} stored under accelerated conditions, which showed a significant increase in particle size (D50) (Figure 63B). This effect was even more pronounced when considering the span, which increased markedly from 1.65 ± 0.14 to 24.26 ± 6.58 , indicating a substantial broadening of the particle size distribution and reflecting the formation of relatively solid auto-agglomerates observed during powder handling (Figure 63C). Notably, this

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phenomenon occurred despite the moisture content remaining essentially unchanged during storage.

Since powders without liposomes did not exhibit similar changes, the increase in particle size observed for LP_{opt} under accelerated conditions is likely attributable to the presence of lipids. In particular, DLPC-based liposomes, characterized by a very low T_m, may display increased molecular mobility at elevated temperatures. Owusu-Ware *et al.* further reported that DLPC already shows enhanced mobility near 40 °C [426], supporting temperature-driven lipid migration, increased interparticle cohesion and consequent agglomerate formation during storage [346].

In addition, the chemical stability of the APIs was evaluated during storage relative to the initial content measured immediately after SD, normalized to 100%. For all optimized powders without liposomes (NLP_{opt}), CIC (Figure 64A) and IND (Figure 64B) contents remained close to 100% of the initial normalized value under all storage conditions throughout the study, indicating no significant API degradation.

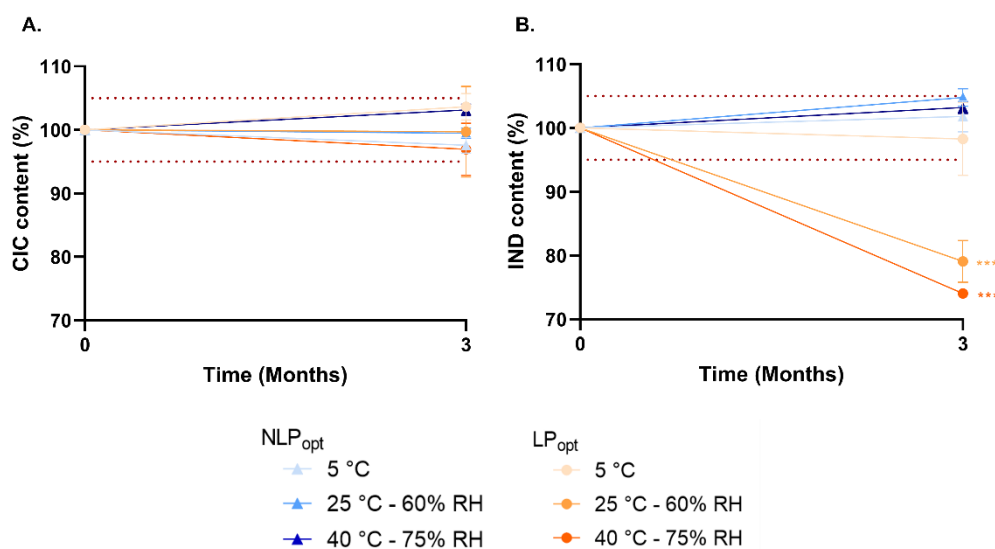


Figure 64: Ciclesonide (A) and indacaterol maleate (B) contents in optimized powders without liposomes (blue) and with liposomes (orange), measured after 3 months of storage under the three tested conditions: refrigerated storage and the two ICH-recommended conditions. Analyses were performed in triplicate ($n=3$), and results are presented as mean $\pm$ standard deviation.

However, API content alone does not fully capture chemical stability, as degradation processes may occur without a marked decrease in total drug content but result in the

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formation of degradation-related impurities. Therefore, total impurities were further investigated as an additional indicator of chemical stability. This preliminary impurity assessment was performed by the industrial partner of the project (Aquilon Pharma), using a dedicated analytical method, on CIC and IND-containing optimized NLP_{opt} immediately after SD (T0) and following storage under all tested conditions. The results are summarized in Table 13.

Table 13: Total impurities (%) of ciclesonide (CIC) and indacaterol (IND) in NLP_{opt} powder at T0 and after 3 months (T3) of storage under different conditions.

Storage condition		Total CIC impurities (%)	Total IND impurities (%)
T0		0.26 ± 0.20	0.25 ± 0.00
T3	Refrigerated	0.60 ± 0.20	0.16 ± 0.06
	25 °C - 60% RH	0.90 ± 0.13	0.20 ± 0.00
	40 °C - 75% RH	1.20 ± 0.20	0.33 ± 0.00

Overall, total impurity levels remained low and generally compliant with ICH guidelines. Although total impurities exceeded the reporting threshold (0.1%) under all storage conditions, they remained below the identification and qualification thresholds (1.0%), as defined by the guideline ICH Q3B (R2) for new drug products, under both refrigerated storage and controlled room temperature (25 °C-60% RH). Under accelerated conditions, a moderate increase in total impurities was observed for CIC, exceeding the identification and qualification thresholds and therefore warranting further investigation. Importantly, when individual impurities were considered separately, their levels remained below the identification and qualification thresholds across all storage conditions. Overall, the formation of degradation-related impurities remained limited over the three-month storage period, with only a slight increase observed under elevated temperature and humidity. Detailed information related to impurity analysis is provided in Appendix V.

It should be noted that reliable evaluation of degradation-related impurities in LP_{opt} was not possible, as the analytical method was not developed for liposome-based DPIs and chromatographic interferences from liposomal components hindered accurate identification and quantification (Appendix V). This limitation is particularly noteworthy, as LP_{opt} formulations exhibited clear signs of chemical instability during storage, as evidenced by changes in API content (Figure 64). In fact, while both APIs remained stable under refrigerated conditions, storage at controlled room temperature and under

accelerated conditions led to a pronounced decrease in IND content, reaching reductions of $20.91 \pm 3.28\%$ and $25.97 \pm 0.47\%$, respectively, whereas CIC content remained consistently close to 100% across all storage conditions. These observations highlight the need for impurity investigations using analytical methods adapted to liposomal matrices.

To better understand the observed differences in API content stability during storage, two main observations can be drawn from these results. First, only NLP_{opt} was able to maintain stable API content throughout the storage period. Given the relatively lipophilic character of both APIs, interactions with the cyclodextrin present in the powder matrix through inclusion complex formation can be expected. Such complexation may contribute to protecting the drug molecules from environmental degradation during storage, thereby helping to preserve their initial concentration over time. Indeed, numerous studies have shown that cyclodextrin complexation enhances drug stability by forming inclusion complexes that partially accommodate hydrophobic regions of the drug molecule within the cyclodextrin cavity, thereby limiting degradation pathways [427,428].

Second, in contrast to NLP_{opt}, the APIs in LP_{opt} are primarily associated with liposomal structures rather than complexed within the cyclodextrin cavity, which may reduce the protective effect provided by cyclodextrin complexation and increase their exposure to environmental degradation factors during storage. This behavior was reflected in the lower stability of IND compared with CIC in liposome-based powders, as a decrease in IND content was observed under all storage conditions except refrigeration, while CIC remained stable throughout the study. This difference in stability may be attributed to the distinct molecular structures of the two APIs. IND contains phenolic and amine functional groups that are susceptible to oxidative and hydrolytic degradation, particularly under elevated temperature conditions and in the presence of residual moisture [429,430]. In contrast, CIC is a highly hydrophobic steroid characterized by a rigid molecular structure and limited reactive functionalities, which confers greater solid-state stability. This structural difference likely explains why IND degradation was observed under stressed storage conditions, whereas CIC remained stable throughout the study in liposome-based powders.

Furthermore, as previously mentioned in published results in Section III.2.2.3.c (Chapter 2), the decrease in IND content is unlikely to result from degradation during SD, as the

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process temperature (120 and 160 °C, for LP_{opt} and NLP_{opt} production, respectively) remained well below the thermal degradation temperatures of IND (203 °C) and CIC (354 °C), while the short residence time during drying further minimizes thermal exposure.

Regarding API homogeneity in both optimized powders, the results show that despite the decrease in IND content in LP_{opt} at ambient and accelerated temperatures, as well as the increased particle size heterodispersity observed under accelerated conditions, the distribution of APIs within the powders remained uniform. Indeed, the CV of both CIC (Figure 65A) and IND (Figure 65B) remained below 5% on average, indicating a homogeneous API distribution that is expected to support reproducible dosing upon inhalation.

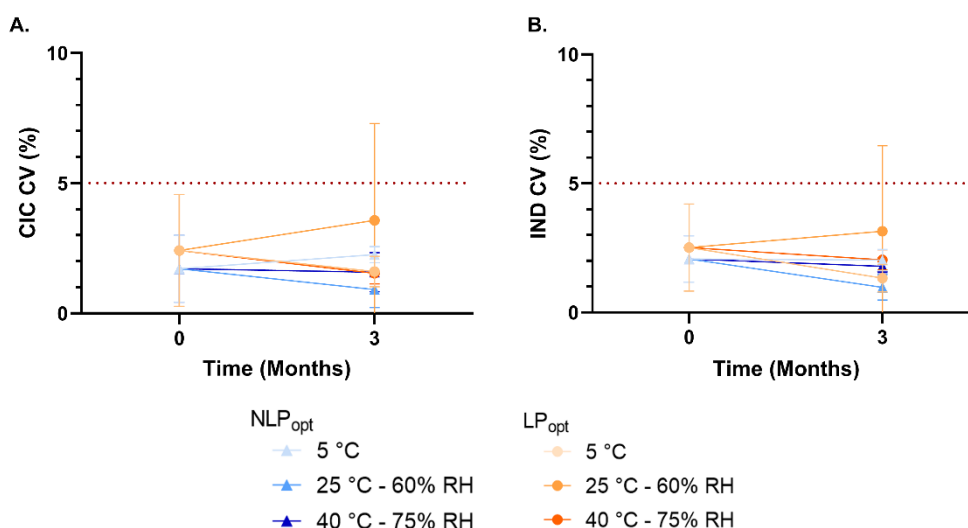


Figure 65: Coefficients of variation for ciclesonide (A) and indacaterol maleate (B) determined across five samples of optimized powders without liposomes (blue) and with liposomes (orange) after 3 months of storage under the three tested conditions: refrigerated storage and the two ICH-recommended conditions. Data represent mean $\pm$ standard deviation (n=3).

Beyond powder properties and API stability, liposomal integrity during storage was also investigated by analyzing the physicochemical properties of LP_{opt} after powder rehydration under the different storage conditions. The results indicate that liposomes remained stable only under refrigerated storage, with a size index of 0.93 ± 0.05 , reflecting minimal size variation ($p < 0.0064$), and a Pdl of 0.15 ± 0.01 , consistent with a monodisperse particle size distribution.

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In contrast, higher storage temperatures led to a marked decrease in liposomal size ($p < 0.0001$) (Figure 66A), with the mean diameter decreasing from 111.13 ± 3.55 nm initially to 58.87 ± 0.27 nm and 65.64 ± 5.50 nm under ambient (25°C - 60% RH) and accelerated (40°C - 75% RH) conditions, respectively. This apparent reduction in size was accompanied by an increase in Pdl ($p < 0.05$) from an initial value of 0.22 ± 0.01 to 0.32 ± 0.05 and 0.31 ± 0.04 under the same storage conditions, indicating a broader particle size distribution (Figure 66B), which suggests a loss of liposomal integrity.

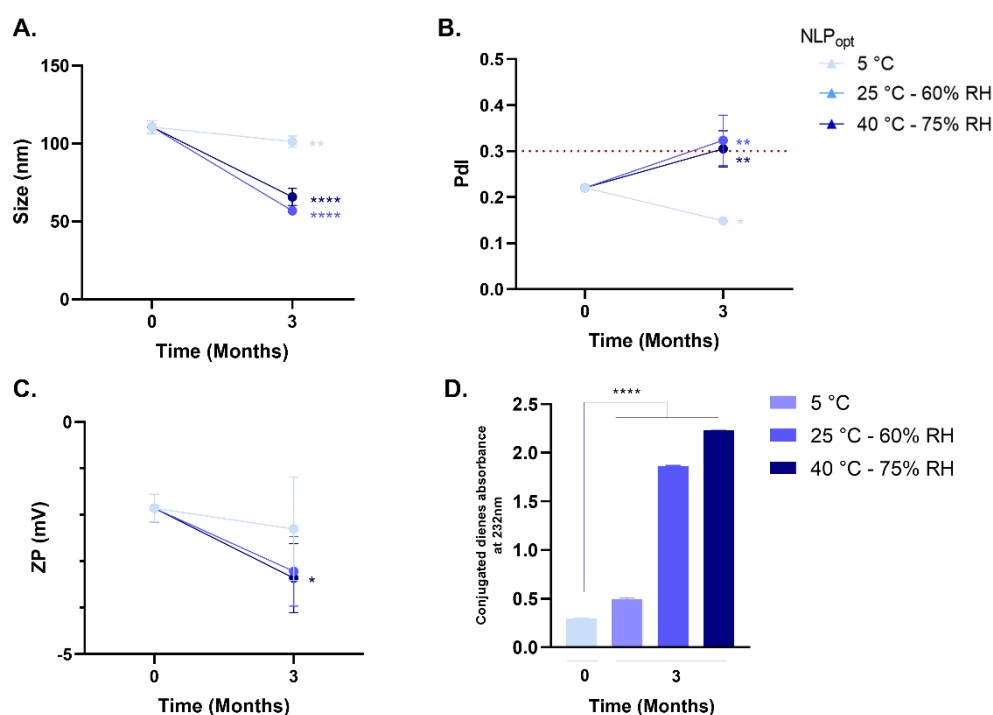


Figure 66: Evaluation of liposomal integrity in optimized liposome-based powders: (A) mean liposomal size, (B) polydispersity index (Pdl), (C) zeta potential (ZP), and (D) conjugated diene absorbance (indicator of lipid oxidation), measured after 3 months of storage under the three tested conditions: refrigerated storage and the two ICH-recommended conditions. Analyses were performed in triplicate ($n=3$), and results are presented as mean $\pm$ standard deviation.

Such changes may result from increased molecular mobility of the lipid bilayer at elevated temperature and humidity, promoting membrane rearrangements and partial liposome disruption. Although not investigated in a dry powder context, Anderson *et al.* demonstrated that liposome stability is strongly governed by the phospholipid T_m , with saturated lipids exhibiting reduced membrane permeability and improved drug retention, whereas low- T_m lipids display increased bilayer fluidity and rapid drug leakage [346]. Based on these findings, it is hypothesized that this behavior also applies

to spray-dried systems, where the very low T_m of DLPC confers high intrinsic bilayer fluidity, further amplified at elevated storage temperatures, thereby promoting molecular mobility, membrane destabilization and drug leakage. Upon rehydration, this could lead to the formation of smaller vesicular fragments or lipid aggregates, while the increased Pdl reflects the coexistence of heterogeneous particle populations, including intact vesicles, fragmented liposomes and newly formed smaller structures [70].

Similar trends have been reported for SD liposomal formulations, such as ciprofloxacin nanocrystal-containing liposomes, which remained stable for several months under low-humidity conditions (5% RH) while maintaining aerosolization performance, but exhibited marked instability at higher humidity levels (20% RH) [431]. In that study, liposome destabilization was attributed to sucrose recrystallization, leading to the loss of the protective amorphous matrix. In the present case, however, such a mechanism is unlikely, as HP β CD exhibits a substantially higher T_g (~250 °C) compared with sucrose (~62 °C) and is therefore not expected to recrystallize under the tested storage conditions, suggesting enhanced physical stability of the matrix [179,322]. High- T_g excipients are generally expected to form rigid amorphous matrices that limit molecular mobility and moisture-induced softening, thereby enhancing the stability of lipid-based systems during storage [171,286]. Despite the stabilizing role of a high- T_g matrix, the destabilization observed under both ICH storage conditions suggests that lipid properties, particularly low T_m -driven molecular mobility, substantially contribute to liposomal instability.

Additionally, this loss of liposomal integrity was accompanied by clear evidence of lipid oxidation. A significant increase ($p < 0.0001$) in conjugated diene absorbance was observed under all tested conditions. However, the increase measured under refrigerated storage remained markedly lower than that observed at higher temperatures (Figure 66D). This pattern indicates that phospholipid oxidation progresses predominantly under warmer storage conditions. Such oxidation is likely promoted by the increased molecular mobility of lipid chains at elevated temperatures, which facilitates the interaction of unsaturated fatty acids with O_2 [432]. These oxidative processes may further destabilize the bilayer, contributing to the structural disruption observed under stressed storage conditions [63,433].

Another indication of liposomal destabilization was the increased release of APIs from liposomes during storage. To assess this effect, encapsulation at the initial time point

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was normalized to 100%. The results indicate that only the powder stored under refrigerated conditions maintained full encapsulation of both APIs, with values remaining close to this reference level (Figure 67). In contrast, API release was observed under ambient and accelerated conditions, reaching $4.56 \pm 4.26\%$ and $9.04 \pm 3.61\%$ for IND under ambient and accelerated storage, respectively (Figure 67A), and $11.98 \pm 10.99\%$ and $20.04 \pm 8.11\%$ for CIC under the same conditions (Figure 67B).

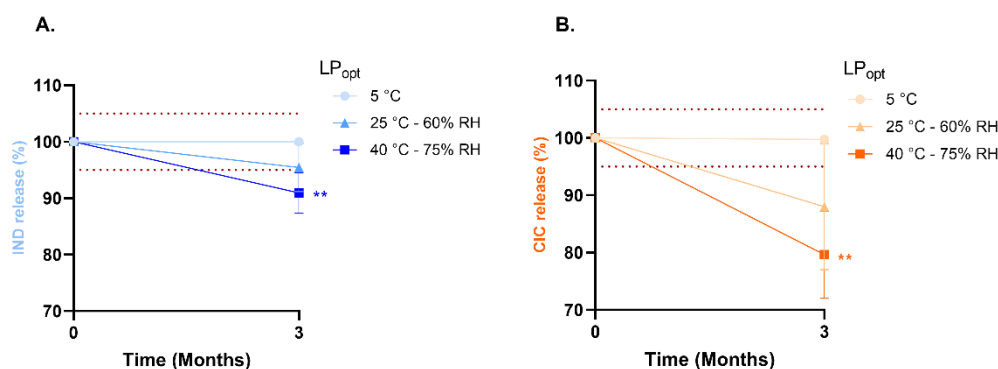


Figure 67: Release of indacaterol maleate (A) and ciclesonide (B) from liposomes in optimized liposome-based powders after 3 months of storage under the three tested conditions: refrigerated storage and the two ICH-recommended conditions. Data represent mean ($n=3$) $\pm$ standard deviation.

This increase in API leakage further confirms the destabilization of the vesicular structures and can therefore be considered a direct consequence of liposomal loss of integrity during storage [170]. Notably, CIC exhibited a higher release than IND, which may be related to differences in their localization within the lipid bilayer. Due to its amphiphilic nature, IND is expected to preferentially locate at the interface between the polar headgroups and the hydrophobic acyl chains, as previously reported by Lombardi *et al.*, who showed that IND resides within the acyl-chain region without disrupting lipid organization [434]. In contrast, the highly lipophilic steroid CIC is more likely to partition deeper into the hydrophobic core of the bilayer. Although CIC is expected to partition within the lipid bilayer, bilayer localization does not necessarily imply stronger retention. In highly fluid DLPC membranes, CIC may remain relatively mobile and diffuse more readily through the bilayer. Conversely, IND, despite being more interfacially located, may establish stabilizing polar interactions with phospholipid headgroups and interfacial components, resulting in lower leakage. Therefore, drug retention likely depends not only on localization but also on the strength of drug-membrane interactions and bilayer fluidity.

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Overall, NLP_{opt} remained stable under all storage conditions after three months, whereas LP_{opt} showed clear instability at ambient and accelerated temperatures, with particle agglomeration, partial API loss and loss of liposomal integrity. From a formulation perspective, these results suggest that the use of more saturated phospholipids or lipids with higher T_m values could improve stability under ambient conditions. Moreover, these results were obtained on bulk powders and do not account for the potential stabilizing effects of final packaging systems (e.g., capsules or moisture-protective packaging), highlighting the need to extend stability assessments to the final packaged product to better reflect clinically relevant storage conditions.

Notably, LP_{opt} stored under refrigerated conditions maintained its stability, representing a significant improvement compared to the initial liposomal formulation produced by PGSS, which exhibited substantial API leakage after only eight weeks under the same conditions (Figure 68). Indeed, despite relatively stable liposomal physicochemical properties for the aqueous formulation, with size remaining comparable (108.38 ± 4.25 nm initially vs. 113.37 ± 12.21 nm after eight weeks), as well as Pdl (0.25 ± 0.04 vs. 0.23 ± 0.05) and ZP (-4.14 ± 2.19 mV vs. -2.55 ± 0.69 mV), a significant decrease in encapsulated drug concentration was observed for both APIs. Specifically, IND concentration decreased from 1088.54 ± 46.69 µg/mL to 681.40 ± 53.96 µg/mL (~37% reduction, $p < 0.0001$), while CIC decreased from 1027.86 ± 65.40 µg/mL to 667.53 ± 73.90 µg/mL (~35% reduction, $p < 0.002$), after eight weeks of storage under refrigerated conditions.

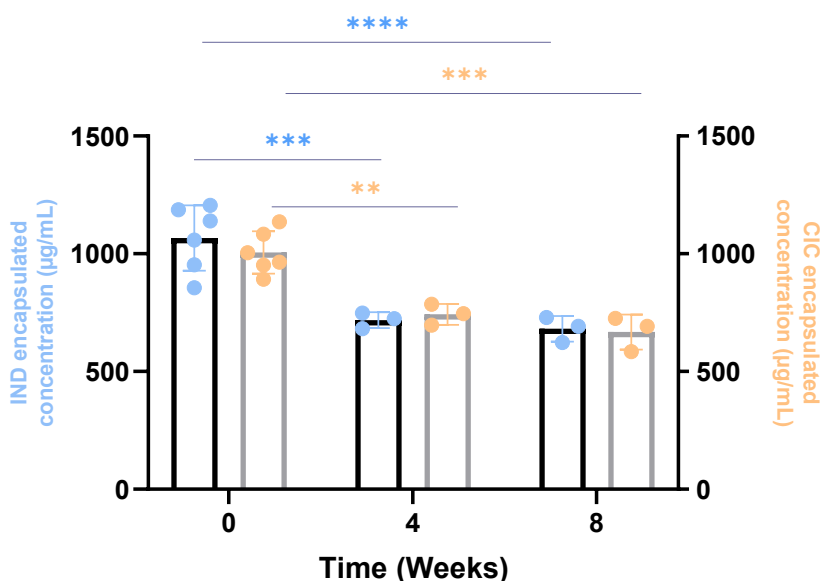


Figure 68: Time-dependent evolution of encapsulated CIC and IND concentrations in optimal liposomal formulations (suspension) stored under refrigerated conditions. Analyses were performed in triplicates ($n=3$) and values are presented as mean $\pm$ standard deviation.

This highlights the potential of the powder formulation to enhance liposomal stability, albeit still requiring refrigerated storage. These results emphasize the critical role of storage temperature in preserving the integrity and therapeutic performance of liposome-based dry powder formulations.

IV.4. Conclusion

The comparative evaluation of the optimized non-liposome-based (NLP_{opt}) and liposome-based (LP_{opt}) dry powder formulations fulfilled the objective of elucidating the impact of liposomal incorporation on aerodynamic performance, pulmonary deposition and physicochemical stability.

Aerodynamic characterization showed that both formulations achieved high lung deposition under optimal conditions, with FPF values close to or exceeding 70%. At clinically relevant inspiratory flow rates, lung delivery improved for both systems due to reduced inertial impaction. However, NLP_{opt} consistently exhibited superior dispersibility, contrasting with LP_{opt} that showed more limited improvement, reflecting increased interparticulate cohesion and the presence of denser lipid-based aggregates. Device-dependent effects further highlighted this behavior, as LP_{opt} exhibited a reduced

ED, particularly with HR devices, indicating greater sensitivity to airflow dynamics, whereas NLP_{opt} maintained more consistent emission across conditions.

Stability studies further emphasized the differences between the two formulations. NLP_{opt} remained stable under all storage conditions, maintaining consistent physicochemical properties and API content, probably due to the protective effect of HP β CD through inclusion complexation. In contrast, LP_{opt} exhibited reduced stability at ambient and accelerated conditions, with evidence of particle agglomeration, IND degradation and loss of liposomal integrity. These effects can be attributed to increased lipid molecular mobility at elevated temperatures, leading to bilayer destabilization, oxidation and API leakage. Notably, refrigerated storage effectively preserved liposomal structure and formulation performance, highlighting the critical role of storage conditions for lipid-based systems.

Overall, these results demonstrate that liposomal incorporation markedly influences the aerodynamic behavior and stability profile of DPI formulations, while still allowing efficient pulmonary delivery. Both LP_{opt} and NLP_{opt} achieved high lung deposition under appropriate inhalation conditions, highlighting their potential suitability for inhalation therapy. Although formulation-dependent differences were observed, this comparative evaluation provides valuable insight into how liposomal incorporation modulates powder performance and stability. These findings support the rational optimization of liposome-based dry powder formulations and contribute to a deeper understanding of formulation and processing parameters governing inhaled lipid-based drug delivery systems.

V. Conclusion

This chapter aimed to perform a comparative evaluation of inhalable dry powder formulations with and without liposomal carriers, co-containing the novel asthma drug combination CIC and IND. To this end, optimized liposome-based and non-liposome-based DPI formulations were rationally developed and characterized using a common strategy, enabling the systematic assessment of the impact of liposomal incorporation on powder properties and inhalation-relevant performance.

In the first part of the chapter, an inhalation-tailored liposomal formulation encapsulating CIC and IND was successfully transformed into a DPI by SD using the optimized conditions established in Chapter 1. These conditions demonstrated good reliability, preserving the structural and physicochemical integrity of the liposomes despite the high PEGylated lipid content required for mucus penetration, while maintaining powder characteristics suitable for pulmonary administration.

In parallel, optimized non-liposome-based DPI formulations co-containing CIC and IND were developed to serve as a reference system for comparison. This work demonstrated that, when appropriate formulation strategies were applied, particularly drug solubilization prior to SD, non-liposomal formulations could achieve excellent homogeneity, aerodynamic performance and stability. Importantly, the development of this reference formulation enabled a direct and meaningful head-to-head comparison with the liposome-based DPI, ensuring that observed differences could be attributed to the presence of liposomes.

The comparative evaluation revealed that both systems achieved high pulmonary deposition and suitable aerodynamic performance. Incorporation of liposomes did not compromise powder dispersibility, confirming that liposome-based DPIs can meet the fundamental requirements for efficient inhalation. However, differences were observed in terms of powder emission out of the device and storage stability, highlighting that liposomal incorporation introduces additional formulation constraints that must be carefully managed. These findings underline that while liposomal encapsulation offers added functionality, including drug retention, potential sustained release and mucus-penetrating properties, it also increases formulation complexity.

Overall, this chapter demonstrates that liposome-based and non-liposome-based DPI formulations each present distinct advantages and limitations.

DISCUSSION

DISCUSSION

Pulmonary drug delivery represents a promising strategy for the treatment of chronic respiratory diseases, as it enables direct targeting of therapeutics to the lungs. However, its clinical effectiveness remains limited by physiological barriers that hinder drug deposition and reduce overall therapeutic efficiency. In this context, liposomes have emerged as attractive drug delivery carriers due to their ability to encapsulate and protect APIs, enhance pulmonary retention and modulate drug release. Nevertheless, the liquid nature and nanometric size of conventional liposomal formulations limit their physicochemical stability, aerosolization efficiency and deep lung deposition. Within this framework, dry powder inhalers (DPIs), which predominantly rely on spray-drying (SD) to generate respirable particles, represent a relevant technological platform for the development of liposome-based inhalable formulations.

Accordingly, **the main objective of this work was to assess the feasibility of developing optimized inhalable liposome-based dry powder formulations for pulmonary administration.** To address this challenge, a comprehensive and integrated approach was adopted, combining rational formulation design, optimization of SD conditions and systematic evaluation of powder performance, liposome integrity and aerosolization behavior.

Overall, this work addresses four fundamental questions:

- 1) Is spray-drying a suitable approach to produce efficient liposome-based DPIs, and under which conditions?
- 2) How transposable is the developed integrated spray-drying framework to other APIs, systems and pulmonary indications?
- 3) What is the clinical relevance of the developed formulations in today's therapeutic and technological landscape?
- 4) What are the key drivers and barriers for the industrial translation of the liposome-based DPI platform developed in this work?

The following discussion adopts a translational perspective (Figure 69), integrating formulation design, process optimization, performance evaluation and broader applicability, while critically assessing the strengths, limitations and future perspectives of the proposed approach.

DISCUSSION



Figure 69: Translational framework of the discussion. Refined using ChatGPT.

I. Is spray-drying a suitable approach to produce efficient liposome-based DPIs, and under which conditions?

To be suitable for pulmonary delivery, DPIs must exhibit critical attributes such as a particle d_{ae} within the respirable range, low residual moisture content, limited cohesiveness and a morphology favorable to aerosolization. In carrier-free systems, these properties are essential to ensure efficient dispersion, deep lung deposition and reproducible performance [40]. Particle engineering, defined as the control of physicochemical and aerodynamic properties through formulation and processing, is therefore central to DPI development, particularly using FD and SD [158].

While FD is widely used to stabilize liposomal formulations for parenteral applications, as exemplified by AmBisome®, its relevance for inhalation is limited by poor dispersibility and suboptimal aerosol performance [241,435]. In contrast, SD offers greater control over particle properties, enabling the production of inhalable powders with suitable aerodynamic characteristics. Its potential is illustrated by commercialized technologies such as PulmoSphere™, used in products like TOBI Podhaler® [436].

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Nevertheless, the application of SD to liposomal systems remains challenging, as thermal and mechanical stresses may compromise liposome integrity and drug retention. Consequently, despite the demonstrated clinical interest of liposomal pulmonary delivery, the absence of commercially available liposome-based DPIs raises a key question: can such systems be engineered to achieve both high aerosol performance and preserved liposome integrity? To address this, a QbD approach supported by DoE was used to investigate the influence of formulation and process parameters. The resulting designs, covering more than 42 liposome-based powders, enabled the identification of the key factors governing powder performance and liposome stability, while providing mechanistic insight into their interplay during drying. These parameters are summarized in Figure 70.

This framework highlights the hierarchical contribution of API physicochemical properties, lipid bilayer composition, excipient selection and drying conditions in determining the final product quality. In particular, API lipophilicity emerged as a primary determinant of drug retention during SD, while lipid composition and carbohydrate matrix properties modulate membrane stability and response to drying-induced stress. Process parameters further influence these outcomes through their impact on drying kinetics and particle formation.

Building on this integrated overview, the following sections provide a detailed mechanistic analysis of the interplay between formulation and process parameters governing liposome stability and DPI performance.

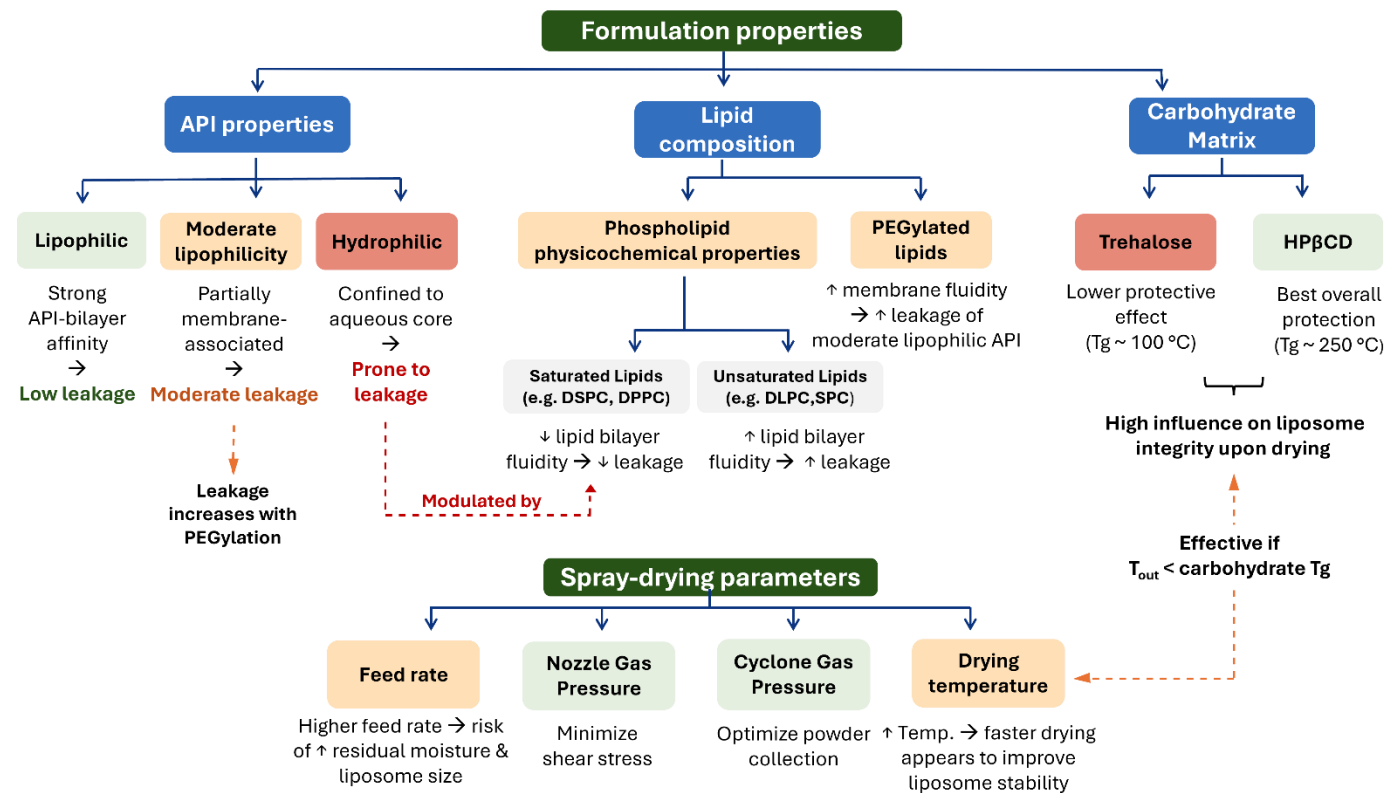


Figure 70 : Schematic, hypothesis-driven representation of the influence of formulation properties and spray-drying parameters on liposomal integrity and drug leakage, based on the results of this thesis. Color coding indicates the relative impact of each factor: green = low effect, orange = moderate effect and red = critical effect.

DISCUSSION

I.1. Impact of formulation and spray-drying parameters on liposome-based DPI properties

I.1.1. *Interplay between API properties and liposomal composition*

Liposome stability upon drying is not governed by a single formulation parameter but rather arises from a complex and interdependent interplay between API physicochemical properties, lipid membrane characteristics and the nature of the protective excipient. Understanding and controlling this multifactorial balance is therefore essential to rationally design liposome-based DPIs with preserved integrity and clinically relevant aerosol performance.

Within this framework, **API lipophilicity** emerged as a key determinant of drug retention during SD, reflecting differences in drug localization within the liposomal structure. Drug leakage decreased with increasing lipophilicity (logP), following a strong exponential correlation ($R^2 = 0.98$) under identical optimized drying conditions. Lipophilic APIs, strongly associated with the lipid bilayer, remained largely preserved despite drying stresses, whereas hydrophilic (low logP) compounds confined to the aqueous core were more prone to leakage due to membrane reorganization.

This increased leakage of hydrophilic API during SD is in line with earlier observations reported by Gulati *et al.*, who showed that drug lipophilicity governs encapsulation, release and stability in liposomal systems [437]. In this conceptual model, lipophilic compounds predominantly residing within the lipid bilayer exhibit high entrapment and slow release, whereas hydrophilic drugs confined to the aqueous core display lower encapsulation and faster leakage. Although these observations were not originally discussed in the context of SD, they provide a useful mechanistic framework for interpreting the present results. Specifically, the findings of this work suggest that API localization within the liposome similarly governs drug leakage during SD, as illustrated in Figure 71.

DISCUSSION

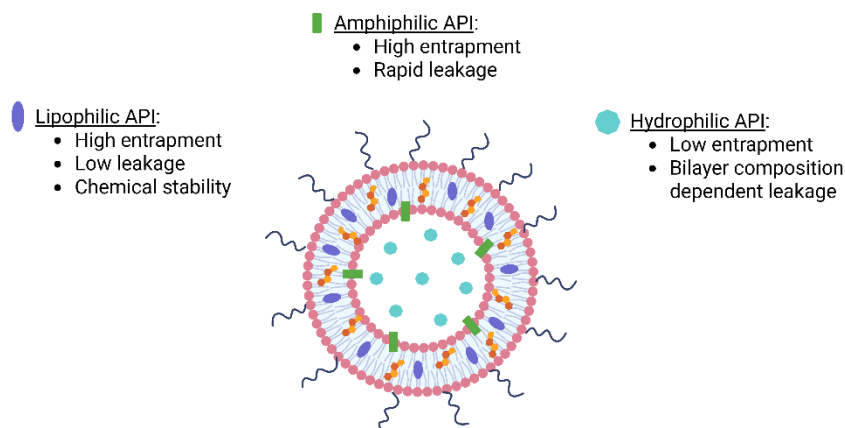


Figure 71: Drug localization in liposomes as a function of lipophilicity and its influence on retention and susceptibility to leakage. Created with BioRender and inspired by [437].

These results also confirm the central role of API lipophilicity in drug retention under SD conditions, while highlighting potential limitations for hydrophilic compounds unless additional stabilization strategies are implemented.

Beyond API lipophilicity, drug retention during SD is strongly governed by **membrane fluidity**, which is intrinsically linked to lipid composition and phospholipid phase transition temperature (T_m) [56]. Consistent with this concept, Khan *et al.* demonstrated, although outside of the context of SD, that membrane rigidity plays a critical role in drug leakage from liposomes [438]. In their study, liposomes composed of high- T_m saturated lipids (DSPC) exhibited, after prolonged storage, markedly lower leakage than more fluid DPPC-based systems. This behavior is attributed to reduced acyl-chain mobility and decreased membrane permeability. Importantly, as further supported by our results, these observations reveal an intrinsic relationship between API physicochemical properties and membrane composition, whereby API-bilayer interactions critically modulate the impact of membrane fluidity on drug retention. Although not originally discussed in the context of SD, this framework provides a coherent basis for interpreting drying-induced API leakage in liposomal systems.

In the context of liposomal SD, saturated phospholipids with high T_m values (in the range of 40-50 °C) form rigid and thermally stable bilayers, whereas unsaturated lipids exhibit increased membrane fluidity under drying conditions. While Rahim *et al.* reported that saturated phospholipids (e.g., DPPC or DSPC) are often considered favorable for liposome SD [439,440], their use has been associated with reduced encapsulation

DISCUSSION

efficiency of hydrophobic drugs [441]. In this work, the reliance on unsaturated phospholipids (SPC or DLPC) likely contributed to the increased leakage observed for hydrophilic compounds. Nevertheless, despite the higher membrane fluidity expected at the optimized T_{out} (~75 °C), the retention of highly lipophilic APIs remained largely unaffected, with drug leakage remaining below 15% across all formulations. This observation suggests that strong API-bilayer interactions can effectively compensate for increased membrane fluidity during drying.

In contrast, although not investigated in this thesis, increasing membrane rigidity, for instance by using saturated phospholipids with higher T_m , may improve hydrophilic drug retention during drying. This is supported by the work of Van den Hoven *et al.*, who reported minimal drug leakage and preserved liposome integrity after SD using DPPC-based liposomes, under similar drying conditions (T_{out} ~68 °C) and with a comparable carbohydrate matrix (HP β CD) [286]. Notably, nearly complete retention was achieved for a highly water-soluble drug, prednisolone disodium phosphate, encapsulated in the aqueous core. Taken together, these results emphasize lipid composition as a critical and tunable formulation parameter that must be tailored to API-specific physicochemical properties, underscoring that liposome design for SD cannot rely on a universal formulation strategy.

Notably, the **PEGylated lipid content** was the main compositional parameter systematically investigated in this thesis, allowing a focused analysis on its influence during SD. PEGylation is widely recognized for providing a hydrophilic steric barrier that enhances colloidal stability, reduces aggregation and improves mucopenetration while limiting macrophage uptake, making a minimal PEG content essential in pulmonary liposomal formulations [74,99,101]. However, the present results highlight a more complex and context-dependent role of PEGylation during drying. Increasing DSPE-PEG₂₀₀₀ content promoted the release of moderately lipophilic drugs, suggesting that PEG-induced surface hydrophilicity, as reported by Giakoumatos *et al.* [333], combined with thermal stresses, may destabilize the lipid bilayer and increase its fluidity. This effect is particularly pronounced for drugs located within the membrane, whose retention depends on bilayer integrity, as observed with BUD. By contrast, PEGylation had a negligible effect on highly lipophilic APIs such as IND and CIC, even at elevated PEG contents (15%), indicating that strong API-bilayer interactions dominate over PEG-induced membrane perturbations beyond a certain lipophilicity threshold. Overall,

DISCUSSION

these findings demonstrate that the influence of PEGylation during SD cannot be generalized across APIs, but is primarily governed by drug localization and membrane affinity, and must therefore be carefully considered when designing liposomal formulations for pulmonary SD.

Taken together, these findings highlight the dual role of PEGylation in liposomal systems: while essential for stability and biological performance, it can also influence membrane behavior during drying in a manner that is highly dependent on API properties. To the best of our knowledge, the influence of PEGylated lipids on API leakage during SD has not yet been reported. This observation highlights the importance of carefully tailoring PEG content to the physicochemical properties of the encapsulated drug, rather than considering PEGylation solely as a stabilizing formulation parameter.

Beyond lipid composition, another parameter that has emerged as a significant determinant of API retention during SD, and that has not yet been reported in the liposomal SD literature, is the **amount of liposomes in the feed liquid**, expressed as the **lipid-to-carbohydrate (L/C) ratio**. Increasing lipid content was associated with reduced drug leakage, irrespective of API lipophilicity, indicating that this parameter governs drug retention through a mechanism distinct from API-bilayer affinity. This behavior can be rationalized by considering the spatial distribution of liposomes within atomized droplets. At higher L/C ratios, a larger number of liposomes is present in each droplet, resulting in a shielding effect whereby liposomes located in the inner regions are less exposed to thermal, interfacial and mechanical stresses generated during drying. This reduced exposure promotes membrane preservation and limits API leakage, particularly for compounds that are intrinsically prone to release.

From a formulation perspective, these findings suggest that increasing lipid content in the feedstock may be an effective strategy to enhance API loading and retention, particularly for leakage prone compounds. However, this approach requires careful optimization, as excessive lipid content may promote vesicle crowding, increased heterogeneity and could negatively impact powder properties and formulation robustness [372].

Collectively, these findings show that liposome integrity during drying is governed by the combined influence of lipid composition, liposome concentration and API properties,

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reflecting the intrinsic complexity of liposomal systems, which should therefore be approached as integrated systems when designing robust spray-dried formulations.

1.1.2. Role of carbohydrate matrix in liposome stability and DPI performance

Alongside API and liposomal composition, the selection of the **protective carbohydrate matrix** plays a dual and critical role in liposomal SD, as it contributes both to liposome stabilization during drying and to the bulk properties and aerodynamic performance of the resulting powder. Accordingly, two carbohydrate matrices were investigated in this work: trehalose, for its well-established protective effect on liposomes during drying [314,315], and HP β CD. The latter was selected for its ability to stabilize liposomal systems while promoting the formation of highly dispersible, carrier-free powders with favorable particle morphologies, as consistently demonstrated in previous work from our laboratory [177,202,205,286]. Results from the DoE identified HP β CD as the most effective excipient for preserving liposome integrity upon drying, as reflected by maintained vesicle size and low Pdl.

The superior performance of HP β CD can be explained by its physicochemical properties and by the two main mechanisms commonly proposed for carbohydrate-mediated liposome stabilization during drying, namely vitrification and water replacement. Through the formation of a rigid amorphous matrix, vitrification limits molecular mobility and mechanically stabilizes the lipid bilayer, while hydrogen bonding between carbohydrates and phospholipid head groups replaces water molecules during dehydration, thereby preserving membrane structure [280,286,321]. In this context, the higher glass transition temperature (T_g) of HP β CD (~ 250 °C) compared to trehalose (~ 100 °C), as measured in this work, may contribute to maintaining a glassy state during drying and limiting matrix plasticization. In addition, its high density of hydrogen bond donors and acceptors could further strengthen interactions with phospholipid head groups, acting synergistically to reduce membrane disruption. However, these interpretations remain primarily theoretical within the scope of this study, as direct experimental verification of these mechanisms remains challenging.

Consistently, Van den Hoven *et al.*, demonstrated that HP β CD outperforms conventional carbohydrates in preserving liposome size, drug retention and long-term stability in both FD and SD processes [286]. Nevertheless, their performance strongly

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depends on processing conditions, as illustrated by Dattani *et al.*, who showed that trehalose effectively stabilizes liposomes during SD when the T_{out} remains below its T_g , thereby preserving nanocarrier integrity [170].

This highlights the importance of aligning process conditions with excipient properties. In liposomal SD, system behavior is governed by the interplay between the flexibility of liposome membrane (governed by T_m) and carbohydrate T_g , which together control membrane fluidity and matrix rigidity during drying. However, although HP β CD effectively preserved liposome structure (size and Pdl), this did not fully translate into retention of hydrophilic compounds such as SAL, as drug diffusion remains largely governed by membrane fluidity. Accordingly, optimal stabilization requires not only appropriate excipient selection but also careful tuning of lipid composition, as demonstrated by Van den Hoven *et al.*, who achieved improved retention by combining HP β CD with high- T_m phospholipids [286].

Beyond stabilization, carbohydrate selection also influences final powder properties. As the excipient constitutes most of the DPI mass, it largely dictates particle morphology and aerosol performance. HP β CD enables the formation of low-density, deflated particles with improved dispersibility and *in vitro* lung deposition, as reported by previous studies [177,202,205,361]. The results of this work showed that, under the optimized SD conditions developed for liposomal drying, a characteristic cyclodextrin deflated particle morphology was obtained, contributing to improved lung deposition.

1.1.3. Influence of spray-drying parameters on powder characteristics and liposome integrity

The influence of SD parameters on the physicochemical properties and aerosol performance of inhalable powders has been extensively studied and is now well established. Parameters such as drying temperature, feed rate or atomization conditions are known to directly impact particle size, morphology, density and residual moisture, thereby governing aerosolization efficiency and lung deposition. However, in the context of liposomal systems, the impact of these parameters remains far less well documented. Building on the limited number of studies investigating liposomal DPIs produced by SD, a more detailed analysis of process parameters highlights the importance of their interactions in governing both powder characteristics and liposome integrity.

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In particular, DoE analysis showed a strong interaction between feed rate and drying temperature in maintaining liposome integrity during SD, indicating that stability depends on the balance between atomization conditions and drying kinetics rather than on any single parameter. Indeed, while higher flow rates did not significantly affect powder properties within the tested range, they were associated with a reduction in liposome size after rehydration, likely due to altered drying kinetics and increased residual moisture in larger droplets, promoting membrane reorganization. This is consistent with previous studies reporting reduced atomization efficiency at higher flow rates, leading to larger droplets, higher residual moisture, and decreased stability of lipid-based systems [442]. Conversely, increasing T_{in} appeared to mitigate these effects, as higher drying temperatures promote faster solvent evaporation, thereby shortening membrane exposure to destabilizing conditions and enabling rapid matrix formation that limits lipid rearrangement.

Drying temperature is typically regarded as a critical parameter for lipid-based systems, as excessive thermal stress can induce membrane disruption and lipid loss during SD. In this regard, Zimmermann *et al.* reported a pronounced temperature sensitivity of siRNA-loaded LNPs spray-dried using lactose as excipient. While processing at a T_{out} of approximately 62 °C preserved particle integrity, increasing T_{out} to around 72 °C resulted in severe structural degradation, visible melting and CHOL loss exceeding 80%, highlighting the strong sensitivity of LNPs to thermal stress [171]. In contrast, in the present work, increasing drying temperature did not result in comparable liposomal degradation when HP β CD was employed as the stabilizing excipient. This suggests that the strong vitrification and water-replacement properties of HP β CD effectively mitigated thermal stress during SD, preserving liposomal integrity even at higher T_{out} of 75 °C. Overall, these observations indicate that although drying temperature is generally a decisive parameter for lipid-based systems, its impact can be markedly attenuated when an appropriate excipient ensures effective vesicle stabilization during drying.

Regarding other SD parameters, trends from the DoE profiler suggest that **nozzle gas pressure** (NGP) had a limited impact on liposome integrity. This contrasts with reports of shear-induced liposome disruption [170,442,443], and may indicate effective protection of liposomes within droplets, likely due to the combined effect of HP β CD and the relatively high solid content (5% w/v), which may have increased viscosity and attenuated shear stress transmission. Similarly, **cyclone gas pressure** (CGP) had

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minimal influence on overall powder properties, although a slight trend toward improved liposome integrity at higher values suggests that downstream handling conditions may still affect liposomal organization and should therefore not be overlooked.

I.1.4. Integrated framework governing liposome stability during spray-drying

Taken together, these findings establish a mechanistic and predictive framework (Figure 70) describing liposome behavior during SD, integrating API properties, formulation composition and process conditions. Beyond rationalizing the results obtained in this work, this model provides a basis for anticipating formulation performance and guiding the design of liposome-based DPIs.

Importantly, the central role of API physicochemical properties, particularly lipophilicity, suggests that the transposability of this framework to other systems is primarily API-driven. This raises the question of how broadly the developed SD strategy can be applied to other drug candidates, NP systems and pulmonary indications, which is addressed in the following section.

II. How transposable is the developed integrated spray-drying framework to other APIs, systems and pulmonary indications?

The QbD approach implemented in this thesis enabled the identification of a robust and validated integrated SD framework, combining optimized liposomal composition with process-formulation conditions, as summarized in Table 14. Under these conditions, liposomal integrity was consistently preserved while generating dry powders with suitable aerodynamic properties for pulmonary delivery.

As discussed in the previous section, the insights gained into the influence of SD parameters and liquid feed formulation provide a solid foundation for extending this approach to other systems, while addressing the feasibility of SD liposomal formulations. Building on the mechanistic model established in Section I of this discussion, it becomes evident that API physicochemical properties are the primary determinants of framework transposability, with lipophilicity emerging as a key parameter governing drug retention during SD. Within this **API-driven perspective**, the applicability of the developed model can be rationally assessed, with either full implementation or partial adaptation, depending on API physicochemical properties.

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Table 14: Integrated and validated spray-drying framework for liposome-based DPIs identified through QbD.

Integrated spray-drying framework for liposome-based DPIs			
Liposomal composition		DLPC/CHOL/DSPE-PEG ₂₀₀₀	
		55/30/15, molar ratio	
Process-formulation conditions	Liquid feed formulation	Solid content (%)	5
		Lipid-to-carbohydrate ratio (%)	1:9
		Excipient	HPβCD
	Process conditions	Inlet temperature (°C)	120
		Outlet temperature (°C)	~75
		Pump speed (rpm)	42
		Flowrate (g/min)	~1.56
		Nozzle gas pressure (bar)	3.4
		Cyclone gas pressure (bar)	0.3

From a formulation perspective, the transposability of the integrated SD framework for liposome-based DPIs is primarily governed by the physicochemical properties of the encapsulated APIs, with lipophilicity emerging as a key determinant.

In this context, the successful and reproducible application of the integrated SD model to liposomes co-encapsulating CIC and IND, with limited drug leakage (<10%) upon rehydration, supports its applicability to liposomal systems of similar composition encapsulating **highly lipophilic APIs**. This is particularly relevant for asthma and COPD therapies, where many ICS and LABAs exhibit moderate to high lipophilicity (Table 15). Accordingly, combinations such as salmeterol/fluticasone propionate (Seretide®), vilanterol/fluticasone furoate (Relvar®), or IND/mometasone (Atecura®) may represent relevant candidates to evaluate within this framework, as they could similarly benefit from the advantages of liposomal delivery in the pulmonary context [142]. Moreover, the co-encapsulation of alternative or novel API pairs, such as mometasone and salmeterol, could also be envisioned.

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Table 15: Representative APIs used in asthma and COPD therapies available on the Belgian market, with a particular focus on ICS and LABA (salbutamol included as a reference) [142], along with their corresponding logP values [340,444,445]. A theoretical assessment of their leakage behavior during spray drying, based on lipophilicity and sensitivity to PEGylation as interpreted from this study, is provided for illustrative purposes.

Molecule	logP	PEG influence on API leakage upon SD	API leakage upon SD
Salbutamol (SABA)	0.44	None	High
Olodaterol (LABA)	1.84	Expected to be significant	Expected to be moderate
Formoterol fumarate (LABA)	1.91		
Budesonide (ICS)	2.42		
Indacaterol maleate (LABA)	3.31		
Vilanterol (LABA)	3.40	Expected to be negligible	Expected to be low
Fluticasone propionate (ICS)	3.70		
Salmeterol (LABA)	3.82		
Fluticasone furoate (ICS)	3.89		
Ciclesonide (ICS)	4.08		
Beclomethasone dipropionate (ICS)	4.59		
Mometasone furoate (ICS)	4.73		

For **moderate lipophilic compounds**, some degree of leakage during the drying process may occur, as observed in this work with BUD and FOR, and may be further enhanced by the high PEG content required for efficient mucopenetration, a phenomenon previously discussed. However, this does not necessarily represent a limitation if a substantial fraction of the API remains encapsulated after SD. A similar dual distribution has been reported for the clinically approved liposomal formulation Arikayce®, where approximately 30% of the drug is immediately available as a free fraction, while the remainder is gradually released from liposomes, enabling both rapid and sustained effects [446]. By analogy, the relevance of maintaining partial encapsulation in the present system must be pharmacologically evaluated, suggesting that the developed SD framework may also be applicable to compounds with lower logP values, such as olodaterol. This may be particularly relevant in light of the emerging inhalation Biopharmaceutics Classification System (iBCS), within which lipophilic APIs are considered iBCS class II-like compounds, characterized by low solubility and high permeability [37]. In this context, the liposomal strategy developed in this work could help overcome solubility-related limitations by modulating drug release and prolonging

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pulmonary residence time, thereby potentially mitigating some of the biopharmaceutical constraints associated with iBCS class II-like behavior.

In contrast, **highly hydrophilic APIs** remain particularly challenging to process by SD, as they typically exhibit pronounced leakage during drying-induced stresses, as observed for SAL in this work. Consequently, extending the integrated liposomal SD framework becomes increasingly complex, since drug retention is no longer governed primarily by process parameters but by intrinsic limitations related to drug localization within the aqueous core and membrane dynamics. Nevertheless, the process-formulation conditions developed in this study may still be applied, provided that the liposomal formulation is adapted to highly hydrophilic APIs, for instance by increasing membrane rigidity using saturated phospholipids or by implementing alternative encapsulation strategies to limit drug diffusion during drying. One promising approach involves inducing intraliposomal drug crystallization, as reported by Khatib *et al.*, where ciprofloxacin achieves enhanced encapsulation and sustained release through the formation of nanocrystals within the liposomal core during SD, facilitated by rapid solvent evaporation and stabilization by disaccharides such as sucrose [288,447].

More broadly, it is important to note that drug leakage during SD, whether for moderately lipophilic or hydrophilic compounds, does not imply loss of API from the formulation, as the released fraction remains within the powder matrix, as represented in Figure 72. Consequently, the impact of such leakage on therapeutic performance remains uncertain, and it cannot be assumed that non-encapsulated API is necessarily less effective upon pulmonary delivery. From a clinical perspective, the relative contribution of encapsulated versus free API therefore remains to be elucidated.

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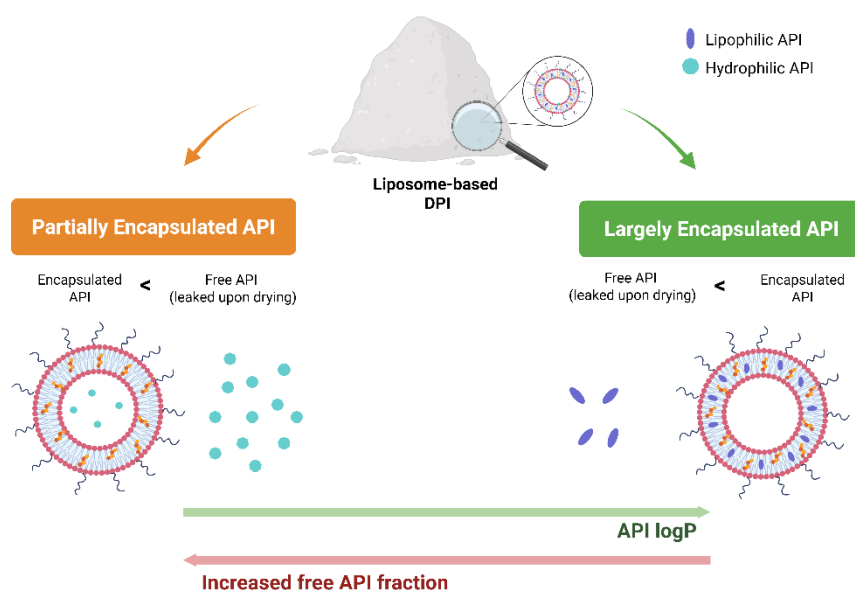


Figure 72: Influence of API lipophilicity on drug distribution in spray-dried liposome-based DPI formulations.

While this API-driven analysis is primarily discussed in the context of asthma and COPD, the same physicochemical rationale supports the extension of the developed framework, either in its entirety or through its process-formulation conditions, to other pulmonary indications. **Lung cancer** represents a particularly relevant example, as illustrated by Borah *et al.*, who demonstrated that FD liposomal paclitaxel can retain liposomal integrity and high entrapment efficiency (>90%) with a favorable FPF (~50%), while Dattani *et al.* showed that liposomes with the same drug can also be processed by SD using trehalose, if the T_{out} remains below its T_g [170,448]. In this context, the high lipophilicity of paclitaxel ($\log P \sim 3.96$) supports the feasibility of a full implementation of the developed framework, as drug leakage during SD is expected to remain limited, even in PEGylated systems designed for mucopenetration [449]. Moreover, the use of high- T_g excipients such as HP β CD may enable higher drying temperatures while maintaining liposomal stability.

In pulmonary indications where mucus penetration is not a primary requirement, such as **infectious lung diseases**, direct application of the liposomal framework developed in this work is not necessarily appropriate. In these contexts, PEGylation can be reduced or omitted, as deep mucus penetration is not essential and the therapeutic objective primarily involves local antimicrobial activity or mucus disruption. Importantly, the results

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of this thesis demonstrate that the validated SD process-formulation conditions remain robust upon reducing the PEG content to 2.5%, as evidenced by preserved liposomal integrity after drying and even reduced leakage of moderately lipophilic APIs such as BUD. This approach is further supported by clinically validated formulations such as liposomal amikacin (e.g., Arikayce®), which are designed for local drug delivery in airway infections where pathogens are embedded in mucus or biofilms, and notably do not rely on PEGylation [104,450]. Previous work on lyophilized liposomal amikacin reported high drug retention (~97%) and moderate aerosol performance (FPF ~30%) [451], suggesting that the application of the validated SD conditions could further improve aerosolization through the development of carrier-free systems. Moreover, the presence of high T_m lipids such as DPPC in Arikayce®, combined with HPβCD, may help limit the leakage of hydrophilic drugs such as amikacin during SD.

Finally, beyond small-molecule drugs, **biotherapeutic APIs** such as peptides, proteins and nucleic acids are increasingly investigated as promising therapeutic agents for local pulmonary applications. However, due to their inherently high hydrophilicity, they are expected to face similar, if not greater, challenges during SD, further exacerbated by their pronounced sensitivity to thermal and mechanical stresses.

Within a local treatment strategy, inhaled delivery of nucleic-acid-based therapeutics, particularly siRNA, represents a promising approach for respiratory diseases, including lung cancer and cystic fibrosis, by enabling localized gene silencing while limiting systemic exposure. However, due to their limited affinity for lipid bilayers, successful formulation of such APIs cannot rely on conventional liposomal encapsulation alone and instead requires structurally adapted carrier systems. In this context, LNPs, typically composed of phospholipids, CHOL and PEGylated lipids, and incorporating ionizable lipids to enable efficient electrostatic complexation of negatively charged nucleic acids, have emerged as the carrier of choice for siRNA delivery [171]. Under these conditions, the complete liposomal platform developed in this work does not appear to be directly applicable. Nevertheless, the process-formulation conditions identified here, including moderate T_{out} and the use of a high T_g carbohydrate matrix, remain highly relevant. For instance, Zimmermann *et al.* showed that SD of siRNA-loaded LNPs at ~62 °C preserved particle integrity and biological activity, while ensuring moderate lung deposition (FPF ~30%), whereas increasing T_{out} to 72 °C induced lipid loss and structural disruption, highlighting their thermal sensitivity [171]. The limited protection

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provided by low Tg excipients such as lactose may contribute to this effect, suggesting that incorporating HP β CD could enhance LNPs stability during drying and potentially further improve aerosol performance. Although this remains to be experimentally validated, it supports the transposability of these process-level considerations beyond liposomal systems.

Overall, these findings suggest that the applicability of the liposomal SD framework is largely influenced by API physicochemical properties, with lipophilic compounds enabling more straightforward implementation, whereas hydrophilic and biotherapeutic payloads may require increasing formulation adaptation. As such, this platform might offer a versatile basis for further development across a wide range of APIs and pulmonary indications, provided that formulation and particle engineering are carefully optimized and experimentally validated.

III. What is the clinical relevance of the developed formulations in today's therapeutic and technological landscape?

Chronic respiratory diseases represent a major and growing global health challenge, contributing substantially to morbidity, premature mortality and healthcare costs worldwide. Among them, asthma and COPD are the predominant contributors, affecting hundreds of millions of individuals and accounting for millions of deaths each year [12,452,453]. Despite advances in management, their prevalence remains high and is expected to increase with population ageing and continued exposure to environmental and lifestyle risk factors, highlighting the need for more effective and accessible therapeutic strategies [11]. In this context, this thesis integrates three complementary strategies: the development of an **innovative ICS-LABA combination**, its **co-encapsulation into liposomal carriers** and its **formulation as an optimized DPI**. These strategies were explored individually and in combination throughout this work.

For chronic respiratory diseases, inhaled therapies continue to represent the cornerstone of disease management, with ICS-LABA combinations forming the basis of maintenance treatment in asthma and being used in COPD, particularly in patients with frequent exacerbations, often as part of triple therapy with LAMA [296,299]. In this work, the **first strategy** concerns the combination of CIC (ICS) and IND (LABA), leveraging the complementary pharmacological profiles of two well-established yet distinct APIs. IND, already available as a DPI either alone (Onbrez[®]) or in combination (Ultibro[®],

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Aectura® and Enerzair®), is characterized by its ultra-LABA profile, providing a rapid onset of bronchodilation together with sustained airway relaxation over 24 hours [362,368]. CIC, marketed as a pMDI formulation (Alvesco®), is a locally activated prodrug that ensures targeted anti-inflammatory activity while minimizing local and systemic side effects [365,366]. The integration of these two APIs into a once-daily inhaled powder enables simultaneous targeting of bronchoconstriction and inflammation. This approach simplifies treatment, improves patient adherence and convenience while reducing the risk of exacerbations associated with monotherapy [454]. In addition, ICS-LABA combination inhalers have been associated with prescription cost reductions of over 20%, supporting their economic benefit for patients and healthcare systems, while also suggesting potential advantages in manufacturing and development [455,456].

The **second strategy** addresses a major limitation of current inhaled therapies: suboptimal pulmonary deposition. Many marketed inhalation products are carrier-based powders, which are often associated with poor lung deposition efficiencies, potentially limiting therapeutic efficacy and contributing to poor disease control [40,189]. In this work, an optimized carrier-free DPI formulation was successfully developed using the CIC-IND combination. This formulation (DPI-SOL_{Crysmeb}), based on crysmeb as both bulk and complexing agent, contains clinically relevant doses of 125 µg of IND and 80 µg of CIC per 15 mg capsule (or 250 µg of IND and 160 µg of CIC per 30 mg), within the range of marketed products. Moreover, it demonstrated superior aerodynamic performance, with FPF >60% across various inhalation conditions and significantly higher IND deposition compared to the pharmaceutical product Onbrez®, as shown in this work. Additionally, the homogeneous distribution (CV <5%) of both APIs within the powder ensures their simultaneous deposition throughout the respiratory tract, promoting coordinated and complementary pharmacological effects.

Beyond formulation performance, DPIs offer several advantages that are highly relevant in today's clinical and environmental context. Their breath-actuated mechanism simplifies administration and reduces coordination errors compared with pMDIs, thereby facilitating correct use and improving adherence in real-life conditions. In addition, DPIs do not require propellant gases, unlike pMDIs, which are known to significantly contribute to greenhouse gas emissions. In line with the European F-gas Regulation (EU) 2024/573, which aims at a progressive reduction and eventual phase-out of HFCs

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by 2050, increasing regulatory pressure is driving the transition toward more sustainable inhalation technologies [457,458]. In this context, DPIs, and particularly reusable or refillable devices such as Breezhaler® or Aerolizer®, represent environmentally preferable alternatives.

To further enhance therapeutic efficacy beyond the API combination itself, the **third strategy** involves the incorporation of liposomal carriers to optimize pulmonary drug delivery. Liposomes, due to their membrane-like structure, can encapsulate both hydrophilic and lipophilic drugs while enabling controlled release and prolonged retention within the lungs. In addition, they can facilitate penetration through the mucus layer and partially overcome mucociliary clearance, two major barriers to efficient pulmonary drug delivery [23,92]. Consistent with this rationale, the optimized liposomal formulation developed by partners demonstrated these advantages, showing improved mucus penetration, enhanced bronchodilatory effects and increased overall drug activity, together with a reduction in airway inflammation and hyperresponsiveness *in vivo*. These findings support the added value of liposomal systems in enhancing local drug efficacy while limiting systemic exposure.

The successful drying of these liposomes enabled the development of an optimized liposomal powder formulation (LP_{opt}), alongside its non-liposomal counterpart (NLP_{opt}). Importantly, both formulations exhibited robust aerosol performance across clinically relevant inspiratory flow rates and inhalers with different resistance profiles, confirming that appropriate particle engineering allows efficient aerosolization regardless of liposome incorporation. A comprehensive overview of both formulation and process parameters, as well as the key physicochemical properties of LP_{opt} and NLP_{opt}, is summarized in Table 16.

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Table 16: Summary of the key physicochemical, aerodynamic properties and spray-drying parameters of the optimal formulations developed in this work.

Optimal DPI formulations		LP _{opt} (w/ liposomes)	NLP _{opt} (w/o liposomes)
Referred section of the manuscript		Chapter 2 Part I	Chapter 2 Part II
Liquid feed properties	CIC content (%)	~0.27	
	IND content (%)	~0.27	
	Sugar-based excipient	HPβCD	
	Solid content (%)	5	
	Lipid-to-carbohydrate ratio (%)	1:9	/
Spray-drying parameters	Inlet temperature (°C)	120	160
	Outlet temperature (°C)	~75	~81
	Feed flowrate (g/min)	~1.56	~3.85
	Nozzle gas pressure (bar)	3.4	3.0
	Cyclone gas pressure (bar)	0.3	0.4
Dry powder properties	Drying yield (%)	77.92 ± 4.48	80.15 ± 2.82
	Particle size (D50) (μm)	2.65 ± 0.25	3.13 ± 0.29
	Moisture content (%)	3.66 ± 0.41	3.88 ± 0.52
	CIC content (%)	0.27 ± 0.03	0.28 ± 0.01
	IND content (%)	0.27 ± 0.02	0.26 ± 0.01
Liposome integrity	Size post-drying (nm)	108.79 ± 4.35	/
	Size ratio (Sf/Si)	1.02 ± 0.04	
	PdI	0.18 ± 0.01	
	ZP (mV)	-2.60 ± 0.32	
	IND leakage (%)	5.05 ± 5.41	
	CIC leakage (%)	5.38 ± 7.19	
Aerodynamic performance (100 L/min- Breezhaler®)	Emitted dose (%)	80.29 ± 1.90	93.59 ± 1.29
	C FPF/ED (%)	69.17 ± 5.52	70.13 ± 6.43
	I MMAD (μm)	4.01 ± 0.30	3.65 ± 0.44
	C GSD (%)	1.86 ± 0.03	2.01 ± 0.11
	Emitted dose (%)	81.58 ± 1.40	92.97 ± 1.62
	I FPF/ED (%)	69.24 ± 5.69	70.34 ± 7.12
	N MMAD (μm)	3.94 ± 0.31	3.66 ± 0.47
	D GSD (%)	1.89 ± 0.04	2.00 ± 0.11

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This represents a key step toward combining the benefits of advanced drug delivery systems with the practical advantages of DPI formulations, including improved stability compared with liquid liposomal systems. However, some limitations remain, notably the need for refrigerated storage of liposome-based powders, as highlighted by the stability studies conducted in this work, which demonstrated that optimal stability is achieved under refrigerated conditions and may therefore affect patient convenience and adherence.

Importantly, the development of both LP_{opt} and NLP_{opt} provides a relevant and complementary framework for future pharmacokinetic, pharmacodynamic and toxicological evaluations, which will be essential to confirm the added value of liposomal formulations compared to their non-liposomal counterparts.

More broadly, alongside the development of optimized formulations, a key strength of this work lies in the systematic co-encapsulation strategy enabling the co-delivery of multiple APIs within a single inhalation system, consistent with current therapeutic approaches. Combinations such as ICS-LABA, notably BUD-FOR as in the drug product Symbicort®, and ICS-SABA, notably SAL-BUD as in the pharmaceutical product Pulmicort®, were explored, highlighting the versatility and clinical relevance of this platform [142]. Importantly, all developed liposome-based powders, including LP_{opt} , preserved liposome integrity after drying, with physicochemical properties compatible with pulmonary delivery (size < 200 nm, Pdl < 0.3 and ZP ~0 mV). In parallel, they exhibited favorable aerosol performance, with FPF >60% and eFPF >20%, supporting efficient lung deposition. These values further confirm their relevance for asthma and COPD treatment, as eFPF values above 5% are widely reported as indicative of effective deep lung deposition potential. This approach integrates pharmacological synergy with optimized delivery to address key limitations of existing therapies.

IV. What are the key drivers and barriers for the industrial translation of the liposome-based DPI platform developed in this work?

IV.1. Liposome-based DPI produced through the combined use of PGSS and SD: a robust and scalable alternative for industrial translation

DISCUSSION

The industrial translation of liposome-based DPIs remains challenging due to limitations associated with both liposome manufacturing and downstream powder production at scale. In this work, the association of PGSS for liposome preparation and SD for powder engineering is proposed as a reliable and scalable platform addressing key industrial drivers while mitigating common translational barriers.

The PGSS technique using scCO₂ was selected in this work as an alternative to conventional liposome manufacturing methods to overcome key limitations associated with traditional approaches. Techniques such as TFH, reverse-phase evaporation, ethanol injection, and microfluidic technologies typically involve complex, multi-step, and often batch-based processes, which can hinder scalability, reproducibility, and compliance with Good Manufacturing Practice (GMP) requirements, ultimately limiting clinical translation [79,80,459].

Most importantly, these methods rely on organic solvents, which may affect encapsulated compounds during liposome production and require extensive purification, as residual solvent traces in the final product raise potential concerns for patient safety. From an industrial perspective, the use of organic solvents also introduces significant practical constraints. These include high costs associated with solvent purchase, handling and processing, as well as the need for dedicated storage infrastructure due to their flammability and volatility. Their handling requires strict safety measures, often involving explosion-proof equipment and controlled environments, thereby increasing operational complexity [460,461].

In contrast, scCO₂-based processes, such as PGSS, enable single-step, solvent-free liposome production under mild and tunable conditions, making them particularly suitable for sensitive compounds. This approach ensures high reproducibility, reduced particle size and Pdl, high encapsulation efficiency, and even the potential for direct sterile production [85]. Moreover, CO₂ is inexpensive, non-toxic, recyclable and completely removed upon depressurization, supporting a sustainable and scalable manufacturing process [82].

Using the optimized PGSS conditions established by Penoy *et al.* [79], this work further confirmed the robustness and reproducibility of the method across 13 independent batches, which consistently exhibited a mean size of 108.79 ± 4.35 nm, a Pdl of 0.26 ± 0.04 , near-neutral ZP (-4.16 ± 2.62 mV) and high EE (>95%) for both CIC and IND.

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Comparison between TFH and PGSS revealed that both methods yield highly similar liposomes once optimized, and that SD preserved liposomal integrity independently of the production method. Importantly, SD affected both formulations similarly, with preservation of liposomal integrity, limited drug leakage and maintained physicochemical properties, regardless of the production method. Notably, PGSS offers a throughput advantage by enabling larger batch volumes and efficient processing of high-lipid formulations in a single step, unlike TFH which required multiple batches.

These results demonstrate that PGSS is a reliable method for liposome production when properly optimized. Nevertheless, no clinically approved liposomal formulations have yet been produced using scCO₂-based technologies. In the pulmonary field, Arikayce[®], as well as most approved liposome-based products for systemic use, such as Doxil[®], AmBisome[®] and Onivyde[®], rely on conventional multi-step processes [462]. Although effective, these methods require tight control at each stage and may pose challenges at industrial scale, as illustrated by the withdrawal of DepoCyt[®] due to manufacturing issues, emphasizing the need for simpler and more robust production strategies [463,464]. In this context, PGSS represents a promising alternative offering a simplified, scalable and reproducible process compatible with GMP and sustainability requirements, supported by the availability of GMP-compliant large-scale scCO₂ equipment already implemented across multiple industrial sectors [465,466].

Following liposome production, SD was selected as the downstream technology to convert the aqueous liposomal dispersion into DPIs. SD is a well-established and scalable technology widely used in the food and pharmaceutical industries [262]. In pharmaceuticals, which represents over 80% of the global SD market, it is routinely applied for advanced particle engineering, offering precise control over particle size, morphology, crystallinity and moisture content [271]. Its versatility has led to numerous approved products, ranging from the production of ASDs prior to compression, such as in the pharmaceutical product Erleada[®] [467], to the manufacturing of inhalable powders such as Bronchitol[®] [468]. As previously discussed, technologies such as Pulmosphere[™], incorporated in TOBI Podhaler[®], further illustrate how SD enables sophisticated particle engineering for pulmonary delivery [436].

From an energy and environmental perspective, SD is more advantageous than FD, requiring up to threefold less energy, generating lower CO₂ emissions, and enabling much shorter processing times. In contrast, FD is a batch and time-consuming process,

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up to six times more expensive per kilogram of water removed, while SFD is even more energy and resource-intensive [257,469]. These differences make SD particularly attractive for large-scale and cost-efficient production of inhalable powders.

In this work, the optimized SD conditions proved highly effective for liposomes produced by both TFH and PGSS, preserving their integrity (size ratio ~ 1 , Pdl < 0.3 , near-neutral ZP) and ensuring high drug retention ($< 10\%$ loss), while providing appropriate physicochemical and aerodynamic properties for inhalation. Importantly, the ability to apply the same SD condition to both conventional (TFH) and industrial scale method (PGSS) strengthens flexibility for industrial translation, allowing TFH to serve as an alternative if PGSS encounters scale-up or regulatory challenges.

In addition to its compatibility with various liposome production methods, both a tenfold increase in atomized volume (20 to 200 mL) and the relatively high solid content used during SD (5-10% w/v) did not affect powder properties or liposomal integrity. These results highlight the robustness of the process under more scale-relevant conditions and support its industrial scalability by improving productivity, reducing energy consumption, and enhancing overall process robustness.

Finally, another major strength of this work lies in the choice of equipment. While most academic studies rely on benchtop systems such as the Büchi Mini Spray Dryer B-290, which are suitable for formulation screening but not fully representative of industrial constraints [470], this study employed a pilot-scale spray-dryer (ProCepT 4M8-Trix) compatible with GMP-like environments. This enabled controlled process development, improved reproducibility, and the production of representative batches for scale-up, thereby significantly strengthening the translational relevance of the process, with conditions directly applicable to industrial settings.

Overall, although no spray-dried liposome-based DPIs are currently marketed, the combined use of PGSS and SD demonstrated in this work represents a coherent and robust platform for industrial translation. The reproducible and robust results obtained further support the strong translational potential of this integrated approach when appropriately matched to the formulation. However, as a formulation-dependent process, its extension to other systems may require further optimization of key parameters and careful control of thermal and shear stresses [170,251].

DISCUSSION

Figure 73 summarizes the proposed industrial production pathway of liposome-based DPIs, from liposome generation to device filling.

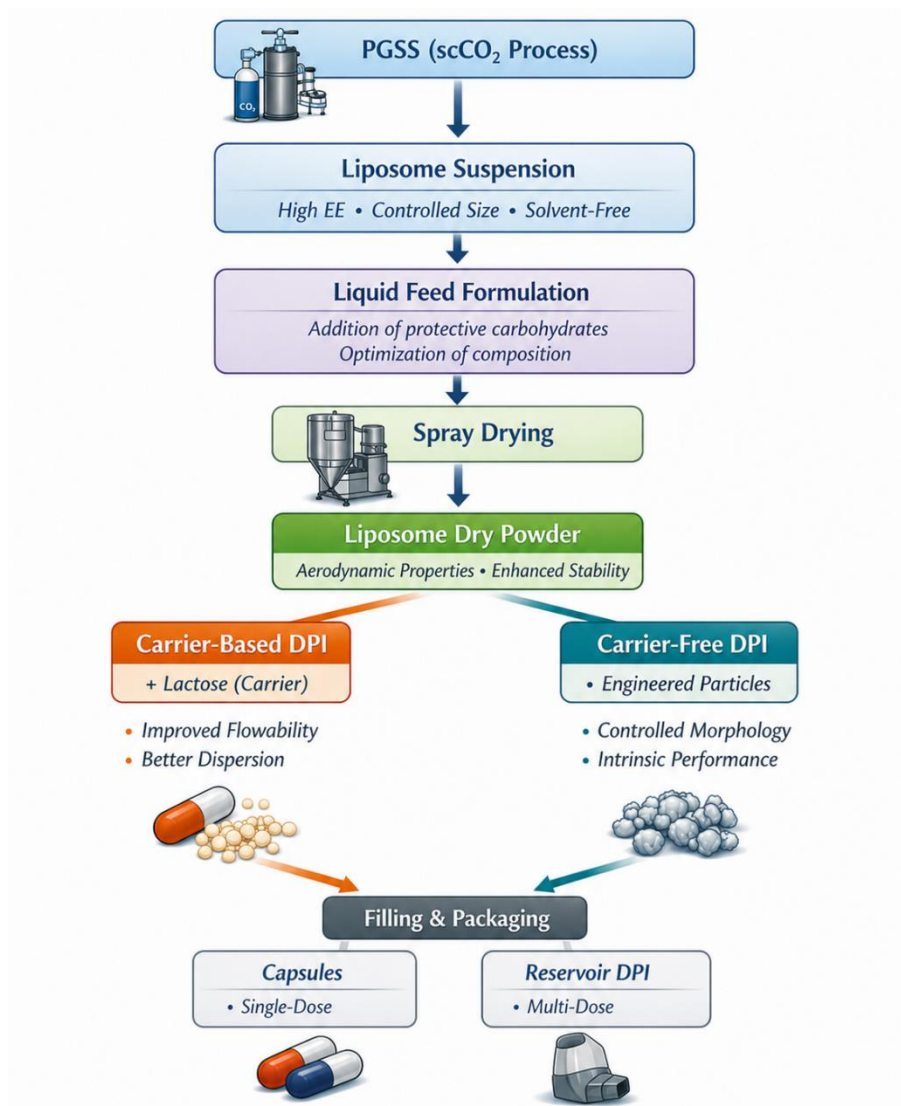


Figure 73: Schematic overview of the proposed industrial pathway for liposome-based DPI production, combining PGSS-based liposome manufacturing, spray-drying powder engineering and final DPI formulation and device filling. Refined using ChatGPT.

IV.2. Formulation and regulatory constraints for the industrial translation of liposome-based DPIs

Despite the strong potential of the developed processes, the industrial translation of the optimized formulations (LP_{opt}, NLP_{opt} and DPI-SOL_{Crysmeb}) remains primarily constrained

DISCUSSION

by the limited availability of excipients approved for pulmonary administration, particularly for DPI applications [84,156]. While commonly approved excipients such as lactose or mannitol are widely used in marketed products, they are mainly associated with carrier-based systems, and due to their relatively low T_g , were not suitable candidates for the present study. In contrast, carrier-free DPIs remain limited, as their performance relies on a delicate interplay between excipient properties and optimized SD parameters [40].

In this study, HP β CD emerged as the most relevant excipient for LP_{opt} and NLP_{opt}. Although not currently approved for inhalation, it is already used in several marketed pharmaceutical products for oral and intravenous administration, including antifungal agents such as Sporanox[®] and Vfend[®], where it enhances the solubility of poorly water-soluble drugs [142]. This established clinical use supports its safety profile and facilitates its potential translation to pulmonary delivery. Consistently, HP β CD has demonstrated good tolerability in inhalation studies, with accumulating evidence indicating low intrinsic toxicity and no significant cytotoxic or functional effects *in vitro* and *in vivo* [205,471,472]. Notably, Lu *et al.* reported no adverse effects at concentrations up to 10% w/v, supporting the safety of the concentration range used in this work (5 or 10% w/v) [473]. In addition, recent industrial safety evaluations have enabled its use in Phase I and II clinical trials following toxicological assessment (Aquilon Pharma). Altogether, these data highlight the strong potential of HP β CD as a promising and translatable excipient for pulmonary drug delivery.

In contrast, while Crysmeb demonstrated favorable functional and safety properties, with limited interaction with membrane lipids, reduced membrane disruption, and low pulmonary toxicity reported *in vivo* [474,475], its lower regulatory maturity may require additional toxicological evaluation for clinical translation.

Beyond excipient selection, the development of liposomal pulmonary formulations is further constrained by the limited number of lipids with established regulatory acceptance for inhalation. While many lipids are considered biocompatible and are widely used in parenteral formulations, only a few, mainly endogenous or biomimetic phospholipids such as DPPC and DSPC, often combined with CHOL, are currently regarded as acceptable for pulmonary delivery due to their similarity to lung surfactant [61,225]. Their use is exemplified by liposomal formulations such as Arikayce[®], while DSPC is also employed in the PulmoSphere[™] technologies [226,227].

DISCUSSION

In this work, except for CHOL, most lipids used in the final formulation (**DLPC/CHOL/DSPE-PEG₂₀₀₀ (55/30/15)**) are not approved for pulmonary administration. Although DSPC and DPPC are FDA-approved and could have facilitated regulatory acceptance, they were not retained by collaborators due to their lower drug encapsulation efficiency and higher leakage compared to unsaturated phospholipids, particularly DLPC, which showed overall favorable results. Regarding DLPC safety profile, previous studies conducted by Waldrep *et al.* supported good pulmonary tolerability in humans and a favorable safety profile [476]. With respect to DSPE-PEG₂₀₀₀, although PEGylated lipids are not currently approved for pulmonary administration despite their widespread use in parenteral formulations such as Doxil®, preclinical studies have consistently reported good tolerability following repeated inhalation exposure [228,332].

Although the number of excipients approved for pulmonary use is limited, existing data on individual components are generally reassuring, a trend further supported by the results of this thesis. Indeed, the optimized liposomal formulations developed by partners showed good tolerability, characterized by low cytotoxicity, absence of pro-inflammatory responses and, when drug-loaded, a reduction in inflammatory markers. In contrast, while Crysmeb demonstrated promising functional properties and an encouraging initial safety profile, its regulatory progression is likely to require additional toxicological evaluation.

Beyond excipients, the choice of APIs also supports translational feasibility, as both CIC and IND are already approved for inhalation with well-established safety and pharmacological profiles. Although the combined dry powder formulation represents a new medicinal product requiring comprehensive evaluation, existing clinical knowledge may facilitate its regulatory development.

Overall, despite regulatory challenges related to excipient and lipid selection, the convergence of favorable safety data, clinically validated APIs, and robust, scalable manufacturing processes supports the feasibility of liposome-based DPIs. This work provides a practical and scientifically grounded framework for the further translation of carrier-free, liposome-based DPI formulations and may be extended to other lipid-based nanocarriers and pulmonary therapies following appropriate formulation optimization.

CONCLUSION AND PERSPECTIVES

CONCLUSION AND PERSPECTIVES

I. Conclusion

The main objective of this thesis was to **evaluate the feasibility of developing optimized liposome-based dry powder inhalers for pulmonary administration**, combining appropriate powder properties for efficient aerosolization with the preservation of liposome integrity and functionality. To achieve this, a comprehensive approach was implemented, integrating formulation design, process optimization and in-depth evaluation of performance and stability.

A major contribution of this work lay in establishing a robust formulation-process framework governing the spray-drying of liposomal systems. Through a QbD approach supported by DoEs, this thesis elucidated the complex interplay between API physicochemical properties, lipid-related properties, excipient selection and drying conditions. In particular, API lipophilicity emerged as a key determinant of drug retention, while PEGylation level, lipid-to-carbohydrate ratio and carbohydrate matrix properties were identified as critical formulation parameters. These findings provided a mechanistic basis for predicting liposome behavior during drying and contributed to the rational design of spray-dried liposome-based formulations, an area that remains relatively underexplored. Additionally, an optimized SD condition was identified and successfully applied to liposomes co-encapsulating clinically relevant APIs for asthma and COPD.

Building on this framework, an optimized liposomal formulation co-encapsulating an innovative association of ciclesonide and indacaterol maleate, specifically tailored for pulmonary delivery, was successfully converted into an inhalable dry powder via SD. Importantly, the optimized drying conditions enabled the preservation of liposomal integrity, high drug retention and the production of powders with physicochemical properties compatible with efficient pulmonary deposition. This supports the feasibility of producing liposome-based DPIs using industrially relevant and scalable processes. Although liposome incorporation introduced constraints in terms of powder emission and stability at ambient temperatures, highly dispersible powders with high aerodynamic performance, high lung deposition and stability over three months under refrigerated conditions were successfully developed. These results further confirm the potential of appropriately engineered liposome-based DPIs produced by SD.

CONCLUSION AND PERSPECTIVES

In parallel, the feasibility of delivering the innovative combination of ciclesonide and indacaterol in a dry powder format was demonstrated through the development of optimized non-liposomal formulations. This represents an innovative approach, as no DPI currently combines these two APIs, enabling the co-delivery of complementary anti-inflammatory and bronchodilator therapies within a single inhalation system, with the potential to improve treatment adherence and therapeutic efficacy.

From a broader perspective, this work highlighted the importance of balancing formulation complexity with performance and stability. While non-liposomal systems provided robustness and stability, liposomal formulations offered access to advanced functionalities that may enhance therapeutic outcomes. The challenge therefore lay in optimizing these systems to fully exploit the advantages of nanocarrier-based delivery while ensuring manufacturability and stability compatible with pharmaceutical development.

Taken together, the findings of this thesis established a strong foundation for the rational design of next-generation inhalable formulations. They provided both practical guidelines for the development of liposome-based DPIs and deeper insight into the parameters governing their performance. This work contributed to advancing pulmonary drug delivery toward more efficient, targeted and clinically relevant therapeutic strategies.

II. Perspectives

At the end of this work, several perspectives can be identified, some of which are already being explored, while others may be addressed in future research.

II.1. Pharmacokinetic and toxicity assessment

Ongoing research, conducted by PhD student Benedetta Bottero, primarily focuses on the evaluation of the pharmacokinetic (PK) and toxicological profiles of the optimized dry powder formulations, with (LP_{opt}) and without liposomal encapsulation (NLP_{opt}). PK studies aim to compare pulmonary and systemic exposure to IND and CIC following dry powder administration, in order to assess the potential impact of liposomal encapsulation on pulmonary retention, release kinetics and systemic exposure. To this end, both formulations are currently being administered intratracheally to rats using the Preciselnhale® system as an aerosol generator, enabling the determination of drug concentrations in lung tissue and plasma.

Preliminary data obtained with LP_{opt} indicate greater pulmonary exposure to IND than systemic exposure, as evidenced by a lung AUC exceeding the plasma AUC. Moreover, systemic exposure appears to be delayed compared with literature data for commercially available non-liposomal formulations.

These preliminary PK results suggest that the liposomal carrier may prolong pulmonary retention of IND and reduce systemic exposure, potentially consistent with a sustained-release behavior. Preliminary findings for CIC appear to support the same trend, although further analysis is still required. Confirmation of these data and analysis of the PK profile of the non-liposomal control are currently ongoing.

A short-term toxicological study will also be conducted to assess the local and systemic safety of LP_{opt} and NLP_{opt} powders following pulmonary administration. A single administration of both formulations was well tolerated, with no evidence of local or systemic toxicity. Based on these reassuring findings, a repeated-dose study over 7 days is planned in mice to further investigate potential cumulative effects. This study will determine whether single or repeated short-term exposure could induce pulmonary inflammation, macrophage activation or tissue injury.

II.2. Application of the optimized liposome-based spray-drying framework to other APIs

The SD framework developed in this work demonstrated robust preservation of liposomal integrity along with efficient pulmonary deposition, suggesting its potential transposability to other liposomal drug delivery systems. This potential transposition appears to be influenced by the physicochemical properties of the encapsulated APIs, with lipophilicity emerging as a key factor governing drug retention during drying. As a result, this approach may be particularly well suited for co-encapsulating lipophilic drug combinations, such as ICS/LABA therapies used in asthma and COPD, where controlled pulmonary retention may offer therapeutic benefits.

Beyond this application, extension of the validated SD conditions to liposomes for other pulmonary indications, or to alternative nanoparticulate carriers, could be envisaged, although further optimization would likely be required to account for formulation and carrier-specific constraints.

II.3. Influence of lipid bilayer composition on spray-drying and DPI stability

A key perspective arising from this work concerns the role of the main phospholipid constituting the lipid bilayer in governing the stability of liposome-based DPIs, both during SD and after drying during storage. Lipid bilayer composition directly controls membrane fluidity through the phospholipid T_m , and thus strongly influences the response of liposomes to the thermal and mechanical stresses imposed during SD. Increasing bilayer rigidity by using more saturated phospholipids with higher T_m values could theoretically represent an effective strategy to improve retention of hydrophilic or moderately lipophilic APIs during drying. Overall, these findings identify lipid composition as a critical and tunable parameter that should be adapted to the physicochemical properties of the encapsulated APIs, and point to the need for further mechanistic investigations into liposomal SD.

In the present formulation, the use of DLPC as the primary phospholipid enabled effective preservation of liposomal integrity immediately after SD, with limited API leakage due to the highly lipophilic nature of both CIC and IND. However, stability studies revealed poor stability at room temperature, indicating that although the drying

process itself was reliable, the intrinsic properties of the lipid bilayer remained a limiting factor for long-term powder stability. Beyond the drying step, lipid bilayer composition thus appears to be a dominant determinant of post-drying behavior. The low T_m of DLPC likely promotes residual lipid mobility in the solid state, which becomes exacerbated under ambient storage conditions and may drive progressive membrane destabilization and drug leakage over time.

Accordingly, replacing DLPC with phospholipids exhibiting higher saturation degrees and higher T_m values, such as DPPC (16:0, $T_m = 41\text{ }^{\circ}\text{C}$) or DSPC (18:0, $T_m = 55\text{ }^{\circ}\text{C}$) [439], could theoretically represent a promising strategy to enhance the long-term stability of spray-dried liposome-based DPIs. Overall, these findings demonstrate that lipid bilayer composition is a central determinant of both resistance to drying-induced stress and long-term powder stability, and that liposomal design for SD cannot rely on a universal formulation approach. Future work should therefore systematically explore alternative lipid compositions to identify optimal trade-offs between encapsulation efficiency, drying robustness and storage stability, ultimately enabling the development of liposome-based DPIs that are both functionally effective and pharmaceutically robust.

II.4. Investigation of powder flowability enhancement

A major challenge associated with carrier-free DPI formulations stems from their intrinsic composition as fine micrometric particles within the respirable size range, which promotes strong interparticulate interactions, including van der Waals forces and electrostatic attractions. As a result, carrier-free powders are often highly cohesive and exhibit poor flowability, which can complicate powder handling, metering and filling operations, and ultimately limit their suitability for large-scale manufacturing. Consequently, improving the flowability of the carrier-free powders developed in this work represents a key formulation challenge for their potential industrialization.

In this context, two main formulation strategies can be considered to address flowability limitations. These approaches could be directly applied to the carrier-free formulations developed in this work. A conventional approach relies on carrier-based systems, typically involving blending with coarse lactose particles to improve powder handling. In this regard, Gresse *et al.* demonstrated that blending micronized spray-dried powders with lactose (50:50) significantly improved flowability and capsule filling homogeneity, while maintaining high aerosolization performance for inhaled drugs [181]. While

effective from a processing perspective, carrier-based formulations generally require higher total powder doses to deliver an equivalent amount of API, which may be undesirable.

Alternatively, approaches that preserve the carrier-free design while improving powder handling may offer a more attractive solution. Among these, powder self-agglomeration strategies represent a promising perspective. In particular, the vibrating inclined plate methodology further described by Gresse *et al.* involves the flow of micronized powders along a vibrating surface, where repeated particle-particle and particle-wall interactions generate triboelectric charging, promoting the formation of so-called soft pellets [175]. These weakly bound agglomerates have been shown to be sufficiently cohesive to enhance flowability and processability, while remaining mechanically fragile and capable of redistributing into primary particles under the aerodynamic shear forces encountered during inhalation. As such, this flowability-enhancement strategy could be explored for the carrier-free powders developed in this work, as illustrated in Figure 74.

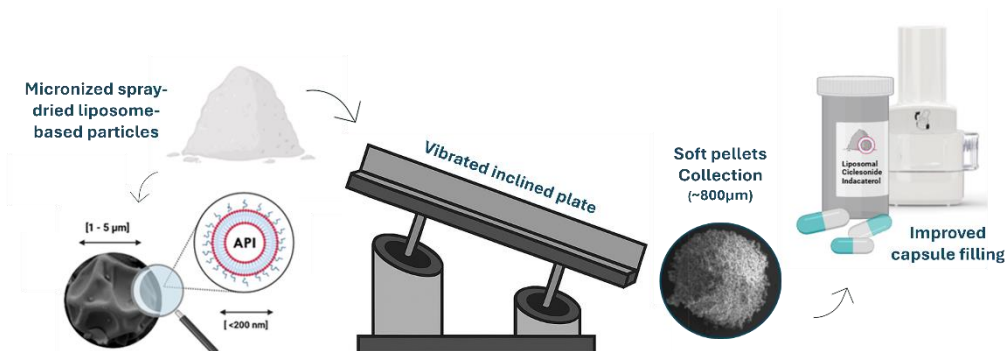


Figure 74: Illustration of the vibrating inclined plate approach, showing the potential of soft pellet formation to enhance flowability of liposome-based dry powders. Inspired from [175].

II.5. Transposability to GMP-Compliant and Scalable Production

A further important perspective of this work concerns the transposability of the optimized liposome-based DPI formulation toward GMP-compliant and industrially scalable manufacturing processes. In the present work, the optimized liposomal formulation has already been successfully produced using GMP-compatible PGSS equipment, including a 500 mL autoclave operated within cleanroom facilities at the University of Liège, yielding promising preliminary results at this scale. This scale-up and GMP-oriented production was carried out by Tuan Nghia Dinh (PhD. Student).

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These findings together with the demonstrated robustness of SD toward liposomes produced by both TFH and PGSS, support the potential feasibility of integrating PGSS for liposome manufacture with SD for powder formation into a coherent and scalable process chain.

Future work should focus on coupling PGSS-based liposome production with industrial-scale SD, in order to fully validate end-to-end manufacturing reproducibility, batch consistency and long-term stability under GMP conditions. Overall, this integrated PGSS-SD approach offers a realistic pathway for the translation of liposome-based DPIs from laboratory development toward industrial production.

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APPENDICES

I. Appendix I: Schematic Representation and Description of the PGSS Process Setup

Based on the principles described above, the PGSS process is implemented using the experimental setup schematically shown in Figure 75. Briefly, the lipid dispersion is loaded into a high-pressure autoclave (G), which is maintained at the desired temperature (H) under continuous stirring (I). CO₂, initially supplied in the gaseous state (A), is liquefied using a cooling unit (D) and subsequently pumped (C) into the autoclave (G). A series of valves (B, E and F) ensures controlled circulation of CO₂ throughout the system. Temperature and pressure are adjusted to maintain CO₂ under supercritical conditions and to promote saturation of the lipid dispersion. The process is completed by a rapid depressurization step using a pneumatic valve (J), during which the saturated mixture is expanded through a nozzle (K) under a decreasing differential pressure (ΔP), leading to liposome formation within an expansion vessel (L). The resulting liposomes suspension is then collected in a receiving container (N), while CO₂ is released from the system (M) [79,89].

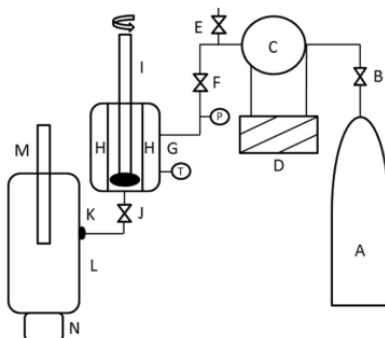


Figure 75: Schematic of the PGSS apparatus, showing the main components: (A) CO₂ bottle, (B, E and F) on/off valves, (C) pump, (D) refrigerant, (G) high-pressure reactor, (H) heating jacket, (I) stirrer, (J) pneumatic valve, (K) nozzle, (L) expansion tank, (M) CO₂ outlet, (N) sample recovery vial, (P) pressure gauge and (T) thermostat.

II. Appendix II: Validation of an HPLC Method for the Quantification of Ciclesonide and Indacaterol Maleate

The aim of validation is to establish that the analytical method is suitable for its intended use and consequently to prove the reliability of the results obtained, within well-defined limits. Several widely-recognized validation criteria should be tested in order to ensure the reliability of the developed method.

The validation criteria presented in this report are:

- response function (calibration curve)
- trueness
- precision (repeatability and intermediate precision)
- accuracy
- uncertainty of measurements
- linearity
- limits of detection (LOD) and of quantitation (LOQ)
- range

The quantification of CIC and IND were assessed by a validated HPLC method. The HPLC equipment consisted of an Agilent 1100 (Santa Clara, USA) with OpenLab CDS LC Chemstation version C.01.05 as the software. 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 (MeOH) (at least 10-fold) to disrupt the liposomal structures and enable accurate quantification of APIs.

CALIBRATION SOLUTIONS

Standard solutions of CIC and IND were prepared at a concentration of 1 mg/mL. For each compound, accurately weighed amounts of 2 mg were quantitatively transferred into separate 2.0 mL volumetric flasks. For CIC, approximately 1.0 mL of absolute ethanol was added, whereas methanol was used as the diluent for indacaterol. The solutions were sonicated for 2 minutes to ensure complete dissolution, allowed to cool

to room temperature, and then diluted to volume with the respective solvent to obtain stock solutions (S1C and S1I, 1 mg/mL).

Seven calibration solutions were subsequently prepared by appropriate dilutions of the stock solutions with MeOH:

- S2 (100 µg/mL): 1 mL of each stock solution (S1C and S1I) into a 10 mL volumetric flask, completed with MeOH.
- S3 (50 µg/mL): 1 mL of S2 completed in a 2 mL volumetric flask with MeOH.
- S4 (25 µg/mL): 1 mL of S3 completed in a 2 mL volumetric flask with MeOH.
- S5 (10 µg/mL): 800 µL of S4 completed in a 2 mL volumetric flask with MeOH.
- S6 (5 µg/mL): 500 µL of S5 in an HPLC completed to 1 mL with 500µL of MeOH.
- S7 (2 µg/mL): 200 µL of S5 in an HPLC completed to 1 mL with 800µL of MeOH.
- S8 (1 µg/mL): 100 µL of S5 in an HPLC completed to 1 mL with 900µL of MeOH.

All solutions were prepared at room temperature and transferred into HPLC vials for analysis.

Figure 76 shows representative chromatographic peaks of IND (retention time: 8.881 min) and CIC (retention time: 11.236 min) at different concentrations of the calibration curve.

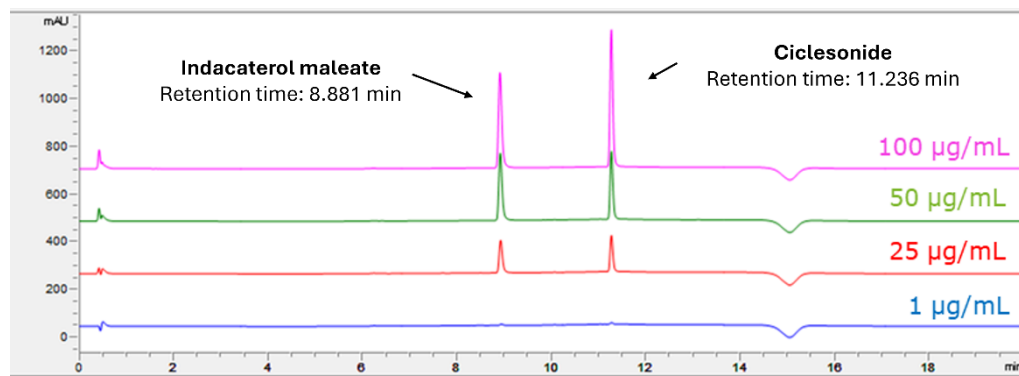


Figure 76: Example of chromatographic peaks for indacaterol maleate (retention time: 8.881 minutes) and ciclesonide (retention time: 11.236 minutes).

II.1. Validation report of ciclesonide

Experimental design:

In order to validate the analytical method, two kinds of samples were prepared in an independent way: calibration standards and validation standards. The calibration standards without matrix, containing known concentrations of the analyte of interest and are only used for calibration.

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Calibration standards consisted of four independent series, each including seven concentration levels (1.0, 2.0, 5.0, 10.0, 25.0, 50.0, and 100.0 µg/mL), with one replicate per level (total n = 28). Validation standards were prepared similarly, with three independent replicates per concentration level (total n = 84).

Response to function:

The response function of an analytical method is, within the range, the existing relationship between the response (signal) and the concentration of the analyte sample. The calibration curve is the most appropriate response function. Table 17 presents all selected regression models that have been sorted according to the Accuracy Index.

Table 17: Sorting of the Calibration Models.

Model	Accuracy Index	Lower and upper limits of quantification (LOQ) (µg/mL)	Dosing range Index	Precision Index	Trueness Index
Weighted (1/X) Linear regression	0.7402	[3.205 , 101.4]	0.9782	0.4295	0.9651

The selected calibration model is: **Weighted (1/X) Linear Regression**. The calibration curves obtained from this regression model (cf. Table 18 and Figure 77) are represented by the following equation: $Y = a + bX$ where Y = Analytical response (in Unit) and X= Introduced concentration (in µg/mL).

Table 18: Regression parameters.

Series	Intercept	Slope	r ²	Residual d.f.	RSS
1	-3.290	23.78	0.9997	5	1551
2	-2.037	24.73	0.9995	5	2413
3	-4.175	23.24	0.9999	5	403.1
4	-0.8191	23.49	0.9989	5	4516

r²= coefficient of determination; d.f.= degrees of freedom; RSS= residual sum of squares

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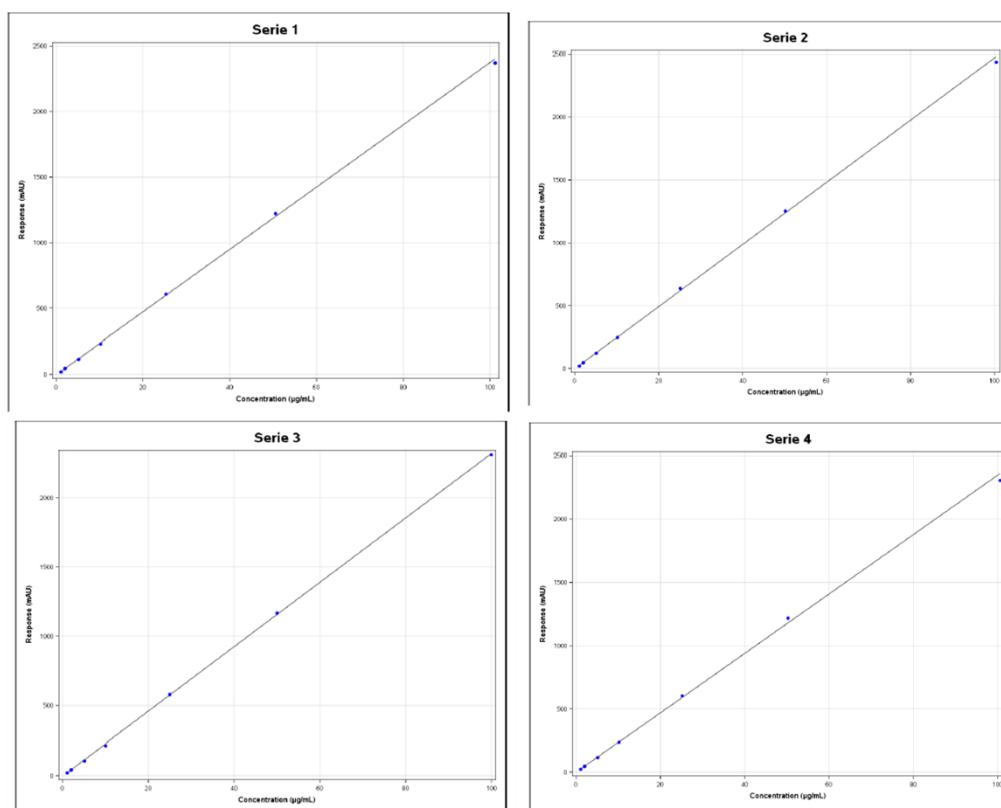


Figure 77: Calibration curves (series 1 to 4). Responses (mAU) are on the y-axis and concentrations (µg/mL) are on the x-axis.

Trueness:

Trueness refers to the closeness of agreement between a conventionally accepted value or reference value, and a mean experimental one. It gives information on systematic error. As shown in Table 19, trueness is expressed in terms of absolute bias (in µg/mL), relative bias (%), or recovery (%) at each concentration level of the validation standards. If, for a concentration level, is the mean of the introduced concentrations and is the estimate of the mean concentration obtained from calculated concentrations then we have:

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$$Absolute\ bias = \bar{x} - \hat{\mu}$$

$$Relative\ bias\ (\%) = 100 \times \frac{\bar{x} - \hat{\mu}}{\hat{\mu}}$$

$$Recovery\ (\%) = 100 \times \frac{\bar{x}}{\hat{\mu}}$$

The 95% confidence interval for the recovery is as follows:

$$L_{recovery} = \left[\frac{lower \cdot 100}{\hat{\mu}}, \frac{upper \cdot 100}{\hat{\mu}} \right]$$

Where *lower* and *upper* are the normal confidence limits for the mean of the concentration as follow:

$$\bar{x} \pm t_{np-1; 1-\frac{\alpha}{2}} \cdot \frac{\hat{\sigma}}{\sqrt{np}}$$

Where:

t is the quantile of a Student t distribution with np-1 degree of freedom, $\alpha = 0.05$, $\hat{\sigma}$ is the empirical standard deviation of the concentrations, n is the number of repetitions per series and p is the number of series.

Table 19: Trueness.

C level (µg/mL)	Mean introduced C (µg/mL)	Mean back- calculated C (µg/mL)	Absolute bias (µg)	Relative bias (%)	Recovery (%)	95 Confidence Interval of Recovery (%)
1.0	1.014	1.023	0.008802	0.8680	100.9	[97.75 , 104.0]
2.0	2.028	2.073	0.04455	2.196	102.2	[99.54 , 104.9]
5.0	5.070	5.055	-0.01515	-0.2987	99.70	[97.00 , 102.4]
10.0	10.14	10.25	0.1127	1.112	101.1	[98.54 , 103.7]
25.0	25.35	26.19	0.8389	3.309	103.3	[101.7 , 105.0]
50.0	50.70	51.83	1.125	2.218	102.2	[100.3 , 104.1]
100.0	101.4	100.1	-1.298	-1.280	98.72	[97.29 , 100.2]

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Precision:

Precision is the closeness of agreement among measurements from multiple sampling of a homogeneous sample under the recommended conditions. It gives information on random errors and can be evaluated at two levels: repeatability and intermediate precision. As can be seen in Table 20 and Table 21, precision is expressed in terms of standard deviation (SD) and RSD values for repeatability and intermediate precision. The estimates of variance components are obtained by the iterative approach of restricted maximum likelihood (REML).

Table 20: Relative Intermediate Precision and Repeatability.

C level (µg/mL)	Mean introduced C (µg/mL)	Repeatability (RSD%)	Intermediate precision (RSD%)
1.0	1.014	2.357	5.315
2.0	2.028	3.074	4.393
5.0	5.070	4.143	4.279
10.0	10.14	4.044	4.053
25.0	25.35	2.594	2.594
50.0	50.70	2.686	3.111
100.0	101.4	2.201	2.266

RSD%= Repeatability and Intermediate precision has been obtained by dividing the corresponding SD by “Mean introduced concentration”

Table 21: Absolute intermediate Precision and Repeatability.

C level (µg/mL)	Mean introduced C (µg/mL)	Repeatability (SD - µg/mL)	Between-series (SD - µg/mL)	Ratio of Variance component (between/within)	Intermediate precision (SD - µg/mL)
1.0	1.014	0.02390	0.04831	4.086	0.05390
2.0	2.028	0.06234	0.06365	1.043	0.08910
5.0	5.070	0.2101	0.05428	0.06678	0.2170
10.0	10.14	0.4101	0.02804	0.004677	0.4110
25.0	25.35	0.6577	0	0	0.6577
50.0	50.70	1.362	0.7964	0.3421	1.577
100.0	101.4	2.232	0.5449	0.05960	2.298

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Table 22 : 95% Upper Confidence Limit.

C level (µg/mL)	Mean introduced C (µg/mL)	95% Upper Confidence Limit Repeatability (SD - µg/mL)	95% Upper Confidence Limit Precision (SD - µg/mL)
1.0	1.014	0.04089	0.1480
2.0	2.028	0.1067	0.2202
5.0	5.070	0.3594	0.4377
10.0	10.14	0.7016	0.8051
25.0	25.35	1.020	1.020
50.0	50.70	2.330	3.494
100.0	101.4	3.819	4.620

In addition, the Recovery of each series as well as for all the series is reported in the Table 23.

Table 23: By Series Recovery of the Samples.

C level (µg/mL)	Series	Mean introduced C (µg/mL)	Back-calculated C (µg/mL)	Recovery (%)
1.0	1	1.004	1.061	104.6
1.0	2	1.005	0.9580	94.47
1.0	3	1.027	1.064	104.9
1.0	4	1.021	1.008	99.43
1.0	Mean of all series	1.014	1.023	100.9
2.0	1	2.008	2.084	102.8
2.0	2	2.010	1.990	98.11
2.0	3	2.054	2.165	106.8
2.0	4	2.041	2.051	101.1
2.0	Mean of all series	2.028	2.073	102.2
5.0	1	5.021	5.076	100.1
5.0	2	5.024	4.874	96.12
5.0	3	5.134	5.079	100.2
5.0	4	5.103	5.193	99.70
5.0	Mean of all series	5.070	5.055	98.97
10.0	1	10.04	10.04	99.51
10.0	2	10.05	10.09	101.9
10.0	3	10.27	10.33	104.1
10.0	4	10.21	10.55	101.1
10.0	Mean of all series	10.14	10.25	103.1
25.0	1	25.10	26.14	101.8
25.0	2	25.12	25.81	103.0

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25.0	3	25.67	26.10	105.4
25.0	4	25.51	26.71	103.3
25.0	Mean of all series	25.35	26.19	100.2
50.0	1	50.21	51.44	101.4
50.0	2	50.24	50.61	99.81
50.0	3	51.34	52.00	102.6
50.0	4	51.03	53.27	105.1
50.0	Mean of all series	50.70	51.83	102.2
100.0	1	100.4	101.8	100.4
100.0	2	100.5	98.95	97.58
100.0	3	100.7	98.96	97.58
100.0	4	102.1	100.8	99.35
100.0	Mean of all series	101.4	100.1	98.72

Uncertainty of measurements:

The uncertainty is a parameter associated with the results of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand (Table 24).

Table 24: Uncertainty.

C level ($\mu\text{g/mL}$)	Mean introduced C ($\mu\text{g/mL}$)	Uncertainty of the bias ($\mu\text{g/mL}$)	Uncertainty ($\mu\text{g/mL}$)	Expanded Uncertainty ($\mu\text{g/mL}$)	Relative Expanded Uncertainty (%)
1.0	1.014	0.02512	0.05947	0.1189	11.73
2.0	2.028	0.03656	0.09631	0.1926	9.497
5.0	5.070	0.06644	0.2269	0.4538	8.950
10.0	10.14	0.1192	0.4280	0.8559	8.440
25.0	25.35	0.1899	0.6846	1.369	5.401
50.0	50.70	0.5595	1.674	3.348	6.602
100.0	101.4	0.6996	2.402	4.804	4.737

Accuracy:

Accuracy refers to the closeness of agreement between the test results and the accepted reference value, namely the conventionally true value. The Accuracy takes into account the total error, i.e. systematic and random errors, related to the test results. It is assessed from the Accuracy Profile illustrated in Figure 78.

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The acceptance limits have been accepted at 7.5%, selected according to the intended use of the analytical procedure. An accuracy profile is obtained by linking on one hand the lower bounds and on the other hand the upper bounds of the β -expectation tolerance intervals calculated at each concentration level. The formula for calculating these β -expectation tolerance intervals is:

$$\text{bias } (\%) \pm kRSD_{IP} (\%)$$

The method is considered as valid within the range for which the Accuracy Profile is within the acceptance limits. This approach gives the guarantee that each further measurement of unknown samples is included within the tolerance limits at the 10.0% level.

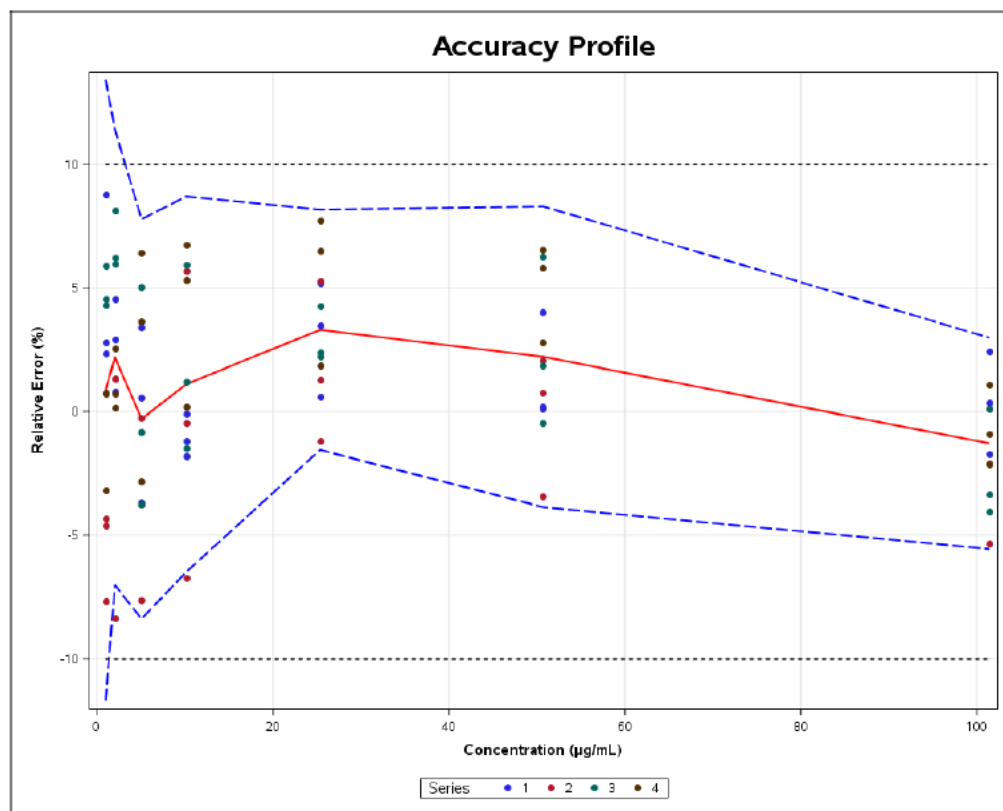


Figure 78: Accuracy Profile Obtained by Considering Weighted (1/X) Linear Regression.

The plan red line is the relative bias, the dashed lines are the β -expectations tolerance limits and the dotted lines represent the acceptance limits. The dots represent the

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relative error of the back-calculated concentrations and are plotted with respect to their targeted concentration.

The upper and lower β -expectation tolerance limits expressed in relative error are also presented in Table 25 as a function of the introduced concentrations. Risk of measurements at each level are only estimated when there are at least two replicates per series (Figure 79).

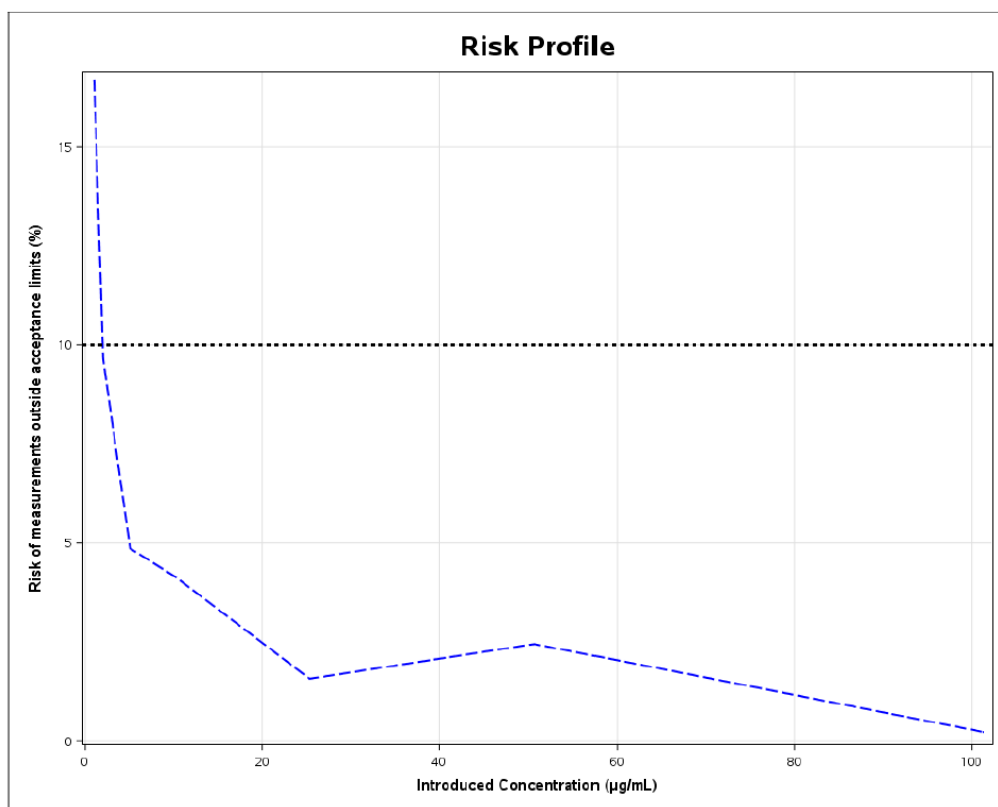


Figure 79: Risk Profile by Considering Weighted (1/X) Linear Regression.

The dotted line represents the maximum risk level chosen: 10.0 %.

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Table 25: Method accuracy obtained by considering Weighted ($1/X^2$) linear regression.

C level (µg/mL)	Mean introduced C (µg/mL)	β-expectation tolerance limits (µg/mL)	Relative β-expectation tolerance limits (%)	Risk (%)
1.0	1.014	[0.8956 , 1.150]	[-11.69 , 13.42]	16.70
2.0	2.028	[1.886 , 2.259]	[-7.010 , 11.40]	9.636
5.0	5.070	[4.646 , 5.465]	[-8.374 , 7.777]	4.867
10.0	10.14	[9.484 , 11.02]	[-6.481 , 8.705]	4.156
25.0	25.35	[24.96 , 27.42]	[-1.549 , 8.167]	1.576
50.0	50.70	[48.75 , 54.91]	[-3.859 , 8.296]	2.451
100.0	101.4	[95.78 , 104.4]	[-5.553 , 2.993]	0.2290

Risk = the risk of having measurements falling outside the acceptance limits.

Linearity of results:

The linearity of an analytical method is the ability within a definite range to obtain results directly proportional to the concentration (quantity) of the analyte in the sample.

A linear regression model (Figure 80) is fitted on the back-calculated concentrations as a function of the introduced concentrations to obtain the following equation: $Y = 0.3735 + 0.9908 X$.

where Y = Back-calculated concentrations (µg/mL) and X = Introduced concentration (µg/mL). The coefficient of determination (r^2) is equal to 0.9987. The coefficient of correlation (r) is equal to 0.9993. The residual sum of squares (RSS) is equal to 125.4.

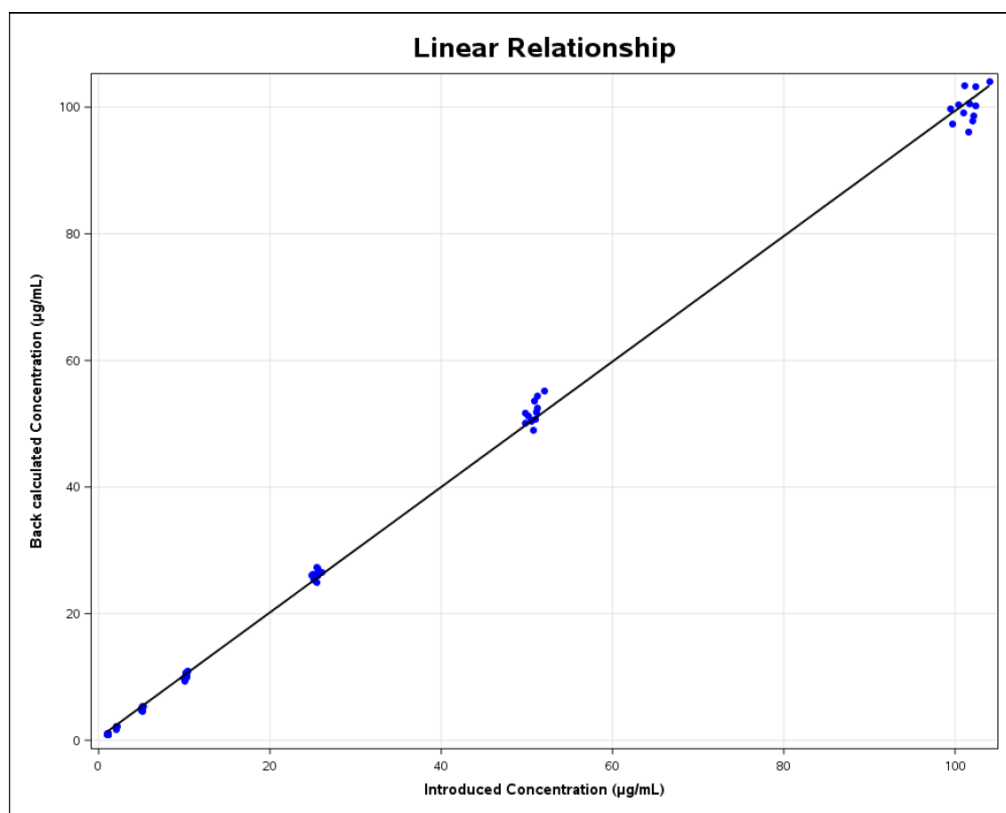


Figure 80: Relationship between the Introduced and the Back-Calculated Concentrations.

In order to demonstrate method linearity, the approach based on the absolute β -expectation tolerance limits as illustrated in Figure 81 can be applied.

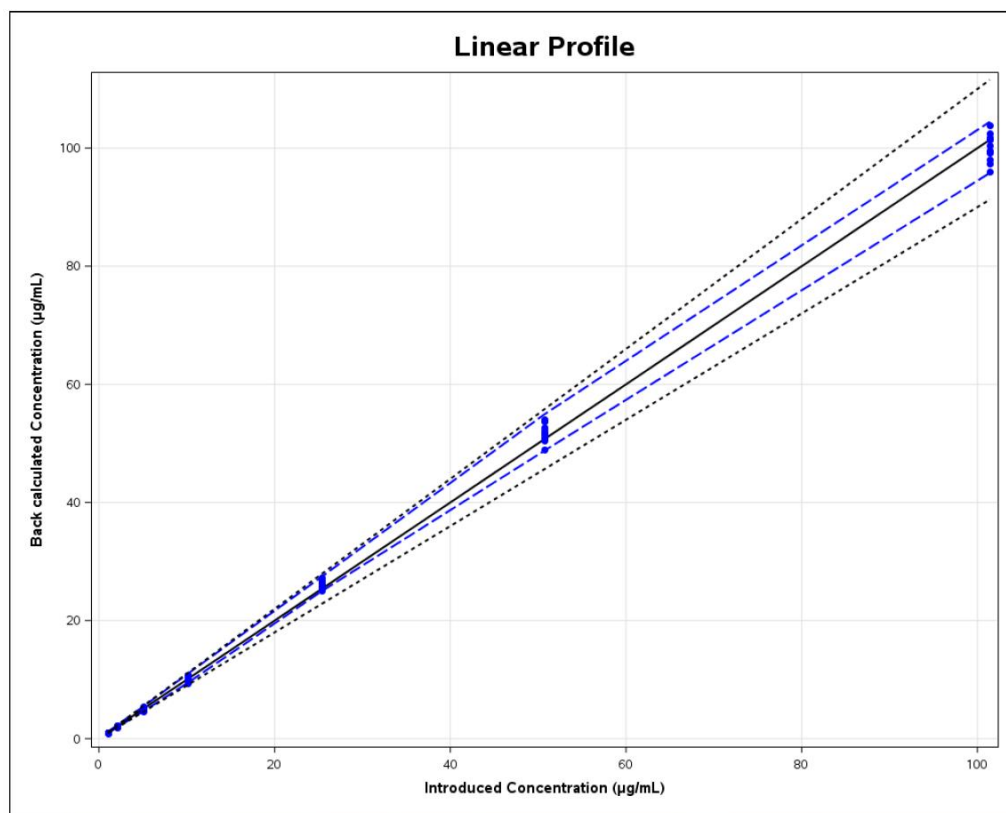


Figure 81: Linearity Graph.

The plain line is the identity line: $Y = X$. The dashed limits on this graph correspond to the Accuracy Profile *i.e.* the β -expectation tolerance limits expressed in absolute values. These limits are calculated as follows:

$$\bar{x} \pm kS_{IP}$$

The dotted curves represent the acceptance limits expressed in the concentration unit. The method is considered as valid within the range for which the dashed curves are within the dotted acceptance limits.

The linearity of the model is demonstrated when the absolute β -expectation tolerance limits are within the absolute acceptance limits.

Limit of detection (LOD), limits of quantification (LOQ) and dosing range:

The limit of detection (LOD) is the smallest quantity of the targeted substance that can be detected, but not accurately quantified in the sample.

There actually two ways of computing the LOD

- $LOD = LOQ/3.3$
- Using Miller ET Miller (not applicable if only one level and no repetition)
- Computation by series of the Y $LOD = \text{Intercept (0 if negative)} + 3 * \text{residual SD}$ obtained by ANOVA. Using the selected regression model, the back-calculation will give the X_{lod} for each series.
- The means of the different back-calculated X_{lod} will give the LOD of the procedure.

The application will choose the lowest value.

LOD ($\mu\text{g/mL}$) = 0.4600

The lower limit of quantification is the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy. The definition can also be applicable to the upper limit of quantification, which is the highest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy. The limits of quantification are obtained by calculation the smallest and highest concentrations beyond which the accuracy limits or b expectation limits go outside the acceptance limits.

The Dosing Range is the interval between the lower and upper limits where the procedure achieves adequate accuracy.

Lower LOQ ($\mu\text{g/mL}$) = 3.205

Upper LOQ ($\mu\text{g/mL}$) = 101.4

II.2. Validation report of indacaterol maleate

Experimental design:

In order to validate the analytical method, two kinds of samples were prepared in an independent way: calibration standards and validations standards. The calibration standards without matrix, containing known concentrations of the analyte of interest and are only used for calibration.

Calibration standards consisted of four independent series, each including five concentration levels (2.0, 5.0, 25.0, 50.0, and 100.0 $\mu\text{g/mL}$), with one replicate per level

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(total n = 20). Validation standards were prepared similarly, with three independent replicates per concentration level (total n = 60).

Response to function:

The response function of an analytical method is, within the range, the existing relationship between the response (signal) and the concentration of the analyte sample. The calibration curve is the most appropriate response function. presents all selected regression models that have been sorted according to the Accuracy Index.

Table 26: Sorting of the Calibration Models.

Model	Accuracy Index	Lower and upper limits of quantification (LOQ) (µg/mL)	Dosing range Index	Precision Index	Trueness Index
Weighted (1/X) Linear regression	0.6477	[2.031 , 101.5]	1.000	0.2746	0.9895

The selected calibration model is: **Weighted (1/X) Linear Regression**. The calibration curves obtained from this regression model (cf. Table 27 and Figure 82) are represented by the following equation: $Y = a + bX$ where Y = Analytical response (in Unit) and X= Introduced concentration (in µg/mL).

Table 27: Regression parameters.

Series	Intercept	Slope	r ²	Residual d.f.	RSS
1	-7.753	16.06	0.9996	3	605.2
2	-7.356	16.67	0.9992	3	1467
3	-8.367	15.77	0.9999	3	204.1
4	-7.130	17.12	0.9987	3	2495

r²= coefficient of determination; d.f.= degrees of freedom; RSS= residual sum of squares

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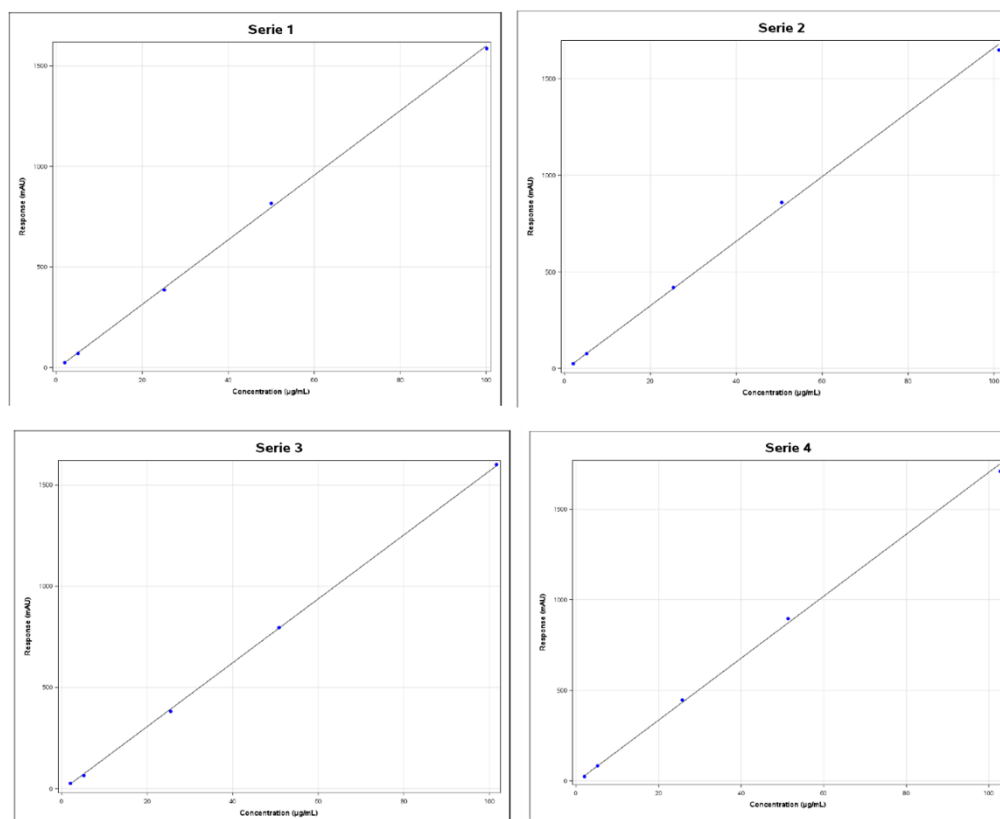


Figure 82: Calibration curves (series 1 to 4). Responses (mAU) are on the y-axis and concentrations ($\mu\text{g/mL}$) are on the x-axis.

Trueness:

Trueness refers to the closeness of agreement between a conventionally accepted value or reference value, and a mean experimental one. It gives information on systematic error. As shown in Table 28, trueness is expressed in terms of absolute bias (in $\mu\text{g/mL}$), relative bias (%), or recovery (%) at each concentration level of the validation standards.

Table 28: Trueness.

C level ($\mu\text{g/mL}$)	Mean introduced C ($\mu\text{g/mL}$)	Mean back- calculated C ($\mu\text{g/mL}$)	Absolute bias (μg)	Relative bias (%)	Recovery (%)	95 Confidence Interval of Recovery (%)
2.0	2.031	2.056	0.02561	1.261	101.3	[99.04 , 103.5]
5.0	5.076	5.089	0.01313	0.2587	100.3	[97.68 , 102.8]
25.0	25.38	25.79	0.4072	1.604	101.6	[99.52 , 103.7]
50.0	50.76	51.28	0.5147	1.014	101.0	[99.14 , 102.9]
100.0	101.5	101.5	-0.03488	-0.03436	99.97	[98.28 , 101.6]

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Precision:

Precision is the closeness of agreement among measurements from multiple sampling of a homogeneous sample under the recommended conditions. It gives information on random errors and can be evaluated at two levels: repeatability and intermediate precision. As can be seen in Table 29 and Table 30, precision is expressed in terms of standard deviation (SD) and RSD values for repeatability and intermediate precision. The estimates of variance components are obtained by the iterative approach of restricted maximum likelihood (REML).

Table 29: Relative Intermediate Precision and Repeatability.

C level (µg/mL)	Mean introduced C (µg/mL)	Repeatability (RSD%)	Intermediate precision (RSD%)
2.0	2.031	2.427	3.697
5.0	5.076	3.870	4.099
25.0	25.38	2.065	3.489
50.0	50.76	1.697	3.163
100.0	101.5	1.325	2.863

RSD%= Repeatability and Intermediate precision has been obtained by dividing the corresponding SD by “Mean introduced concentration”

Table 30: Absolute intermediate Precision and Repeatability.

C level (µg/mL)	Mean introduced C (µg/mL)	Repeatability (SD - µg/mL)	Between-series (SD - µg/mL)	Ratio of Variance component (between/within)	Intermediate precision (SD - µg/mL)
2.0	2.031	0.04927	0.05663	1.321	0.07506
5.0	5.076	0.1965	0.06852	0.1216	0.2081
25.0	25.38	0.5241	0.7137	1.854	0.8855
50.0	50.76	0.8615	1.355	2.474	1.606
100.0	101.5	1.345	2.576	3.667	2.906

Table 31 : 95% Upper Confidence Limit.

C level (µg/mL)	Mean introduced C (µg/mL)	95% Upper Confidence Limit Repeatability (SD - µg/mL)	95% Upper Confidence Limit Precision (SD - µg/mL)
2.0	2.031	0.08431	0.1898
5.0	5.076	0.3362	0.4295
25.0	25.38	0.8968	2.307
50.0	50.76	1.474	4.276
100.0	101.5	2.302	7.937

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In addition, the Recovery of each series as well as for all the series is reported in the Table 32.

Table 32: By Series Recovery of the Samples.

C level (µg/mL)	Series	Mean introduced C (µg/mL)	Back-calculated C (µg/mL)	Recovery (%)
2.0	1	2.009	2.021	99.55
2.0	2	2.019	2.033	100.1
2.0	3	2.027	2.151	105.9
2.0	4	2.068	2.019	99.43
2.0	Mean of all series	2.031	2.056	101.3
5.0	1	5.022	4.895	96.44
5.0	2	5.048	5.123	100.9
5.0	3	5.067	5.147	101.4
5.0	4	5.169	5.192	102.3
5.0	Mean of all series	5.076	5.089	100.3
25.0	1	25.11	25.05	98.71
25.0	2	25.24	25.21	99.32
25.0	3	25.33	26.62	104.9
25.0	4	25.85	26.27	103.5
25.0	Mean of all series	25.38	25.79	101.6
50.0	1	50.22	50.01	98.51
50.0	2	50.48	50.06	98.62
50.0	3	50.67	52.70	103.8
50.0	4	51.69	52.34	103.1
50.0	Mean of all series	50.76	51.28	101.0
100.0	1	100.4	102.1	100.6
100.0	2	101.0	98.71	97.23
100.0	3	101.3	104.9	103.4
100.0	4	103.44	100.2	98.68
100.0	Mean of all series	101.5	101.5	99.97

Uncertainty of measurements:

The uncertainty is a parameter associated with the results of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand (Table 33).

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Table 33: Uncertainty.

C level (µg/mL)	Mean introduced C (µg/mL)	Uncertainty of the bias (µg/mL)	Uncertainty (µg/mL)	Expanded Uncertainty (µg/mL)	Relative Expanded Uncertainty (%)
2.0	2.031	0.03169	0.08148	0.1630	8.025
5.0	5.076	0.06626	0.2184	0.4368	8.604
25.0	25.38	0.3876	0.9666	1.933	7.616
50.0	50.76	0.7217	1.760	3.521	6.936
100.0	101.5	1.345	3.203	6.405	6.309

Accuracy:

Accuracy refers to the closeness of agreement between the test results and the accepted reference value, namely the conventionally true value. The Accuracy takes into account the total error, i.e. systematic and random errors, related to the test results. It is assessed from the Accuracy Profile illustrated in Figure 83.

The acceptance limits have been accepted at 7.5%, selected according to the intended use of the analytical procedure. An accuracy profile is obtained by linking on one hand the lower bounds and on the other hand the upper bounds of the β -expectation tolerance intervals calculated at each concentration level. The formula for calculating these β -expectation tolerance intervals is:

$$bias (\%) \pm kRSD_{IP} (\%)$$

The method is considered as valid within the range for which the Accuracy Profile is within the acceptance limits. This approach gives the guarantee that each further measurement of unknown samples is included within the tolerance limits at the 10.0% level.

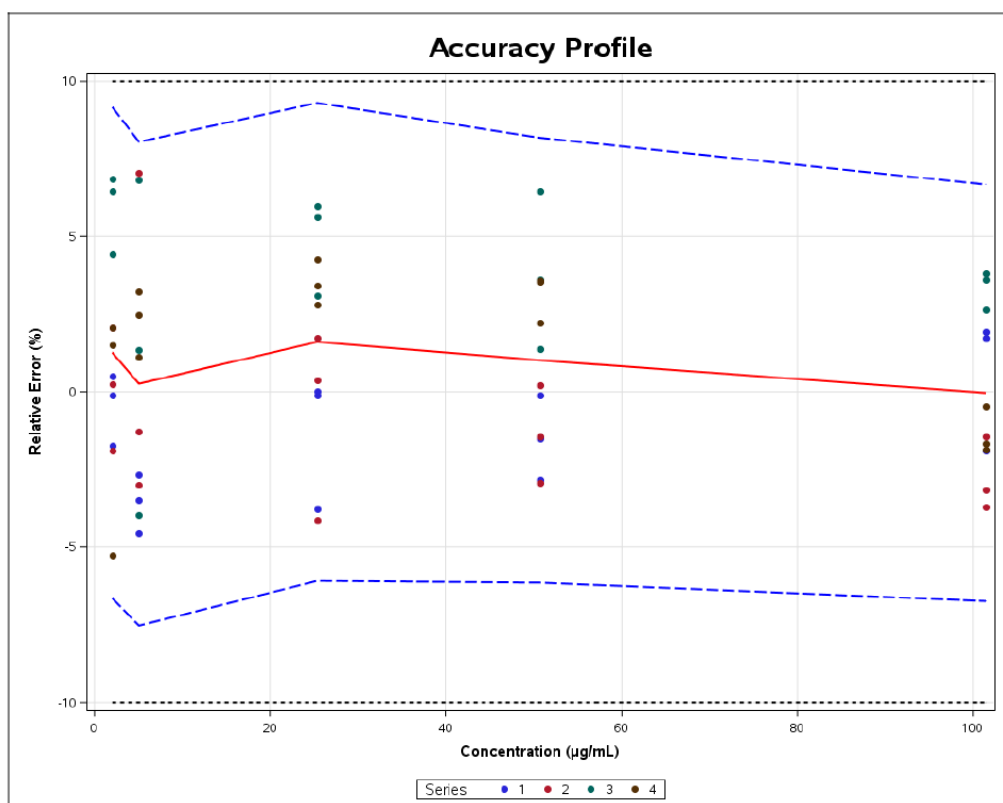


Figure 83: Accuracy Profile Obtained by Considering Weighted ($1/X$) Linear Regression.

The plan red line is the relative bias, the dashed lines are the b-expectations tolerance limits and the dotted lines represent the acceptance limits. The dots represent the relative error of the back-calculated concentrations and are plotted with respect to their targeted concentration.

The upper and lower β -expectation tolerance limits expressed in relative error are also presented in Table 34: Method accuracy obtained by considering Weighted ($1/X^2$) linear regression. as a function of the introduced concentrations. Risk of measurements at each level are only estimated when there are at least two replicates per series (Figure 84).

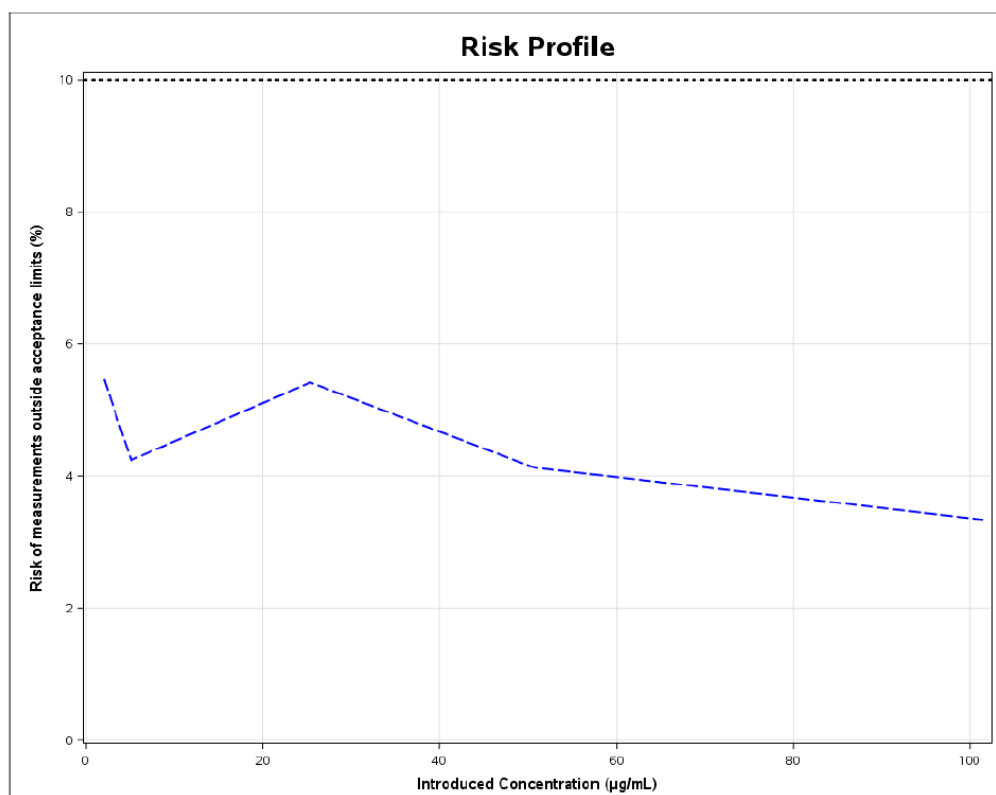


Figure 84: Risk Profile by Considering Weighted ($1/X$) Linear Regression.

The dotted line represents the maximum risk level chosen: 10.0%.

Table 34: Method accuracy obtained by considering Weighted ($1/X^2$) linear regression.

C level (µg/mL)	Mean introduced C (µg/mL)	β -expectation tolerance limits (µg/mL)	Relative β -expectation tolerance limits (%)	Risk (%)
2.0	2.031	[1.896 , 2.217]	[-6.646 , 9.169]	5.466
5.0	5.076	[4.694 , 5.485]	[-7.530 , 8.048]	4.242
25.0	25.38	[23.84 , 27.74]	[-6.092 , 9.300]	5.417
50.0	50.76	[47.64 , 54.91]	[-6.150 , 8.178]	4.128
100.0	101.5	[94.68 , 108.3]	[-6.740 , 6.671]	3.338

Risk = the risk of having measurements falling outside the acceptance limits.

Linearity of results:

The linearity of an analytical method is the ability within a definite range to obtain results directly proportional to the concentration (quantity) of the analyte in the sample.

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A linear regression model (Figure 80) is fitted on the back-calculated concentrations as a function of the introduced concentrations to obtain the following equation: $Y = 0.1907 + 0.9998 X$.

where Y = Back-calculated concentrations ($\mu\text{g/mL}$) and X = Introduced concentration ($\mu\text{g/mL}$). The coefficient of determination (r^2) is equal to 0.9986. The coefficient of correlation (r) is equal to 0.9993. The residual sum of squares (RSS) is equal to 115.6.

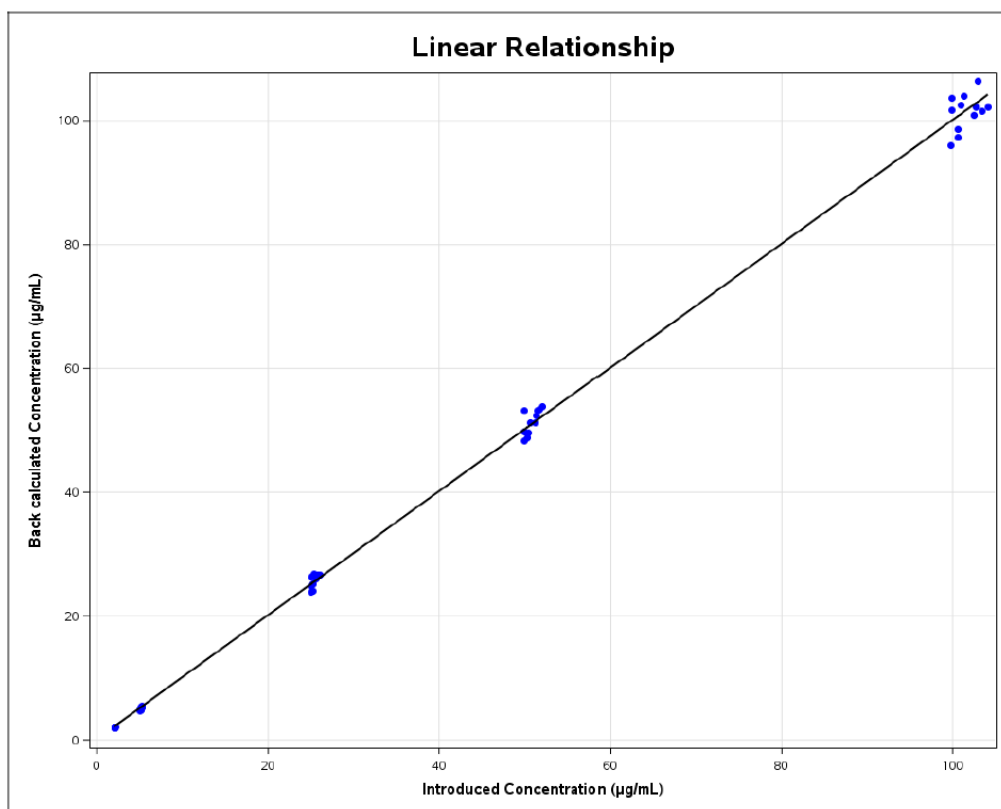


Figure 85: Relationship between the Introduced and the Back-Calculated Concentrations.

In order to demonstrate method linearity, the approach based on the absolute β -expectation tolerance limits as illustrated in Figure 86 can be applied.

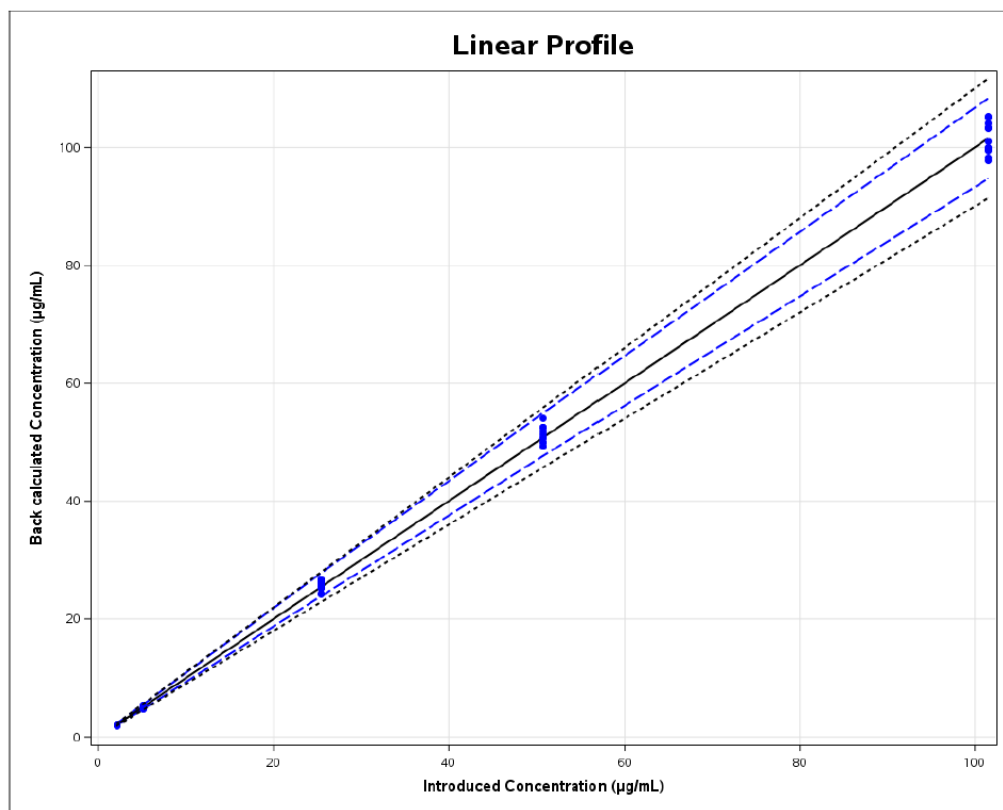


Figure 86: Linearity Graph.

The plain line is the identity line: $Y = X$. The dashed limits on this graph correspond to the Accuracy Profile *i.e.* the β -expectation tolerance limits expressed in absolute values. These limits are calculated as follows:

$$\bar{x} \pm kS_{IP}$$

The dotted curves represent the acceptance limits expressed in the concentration unit. The method is considered as valid within the range for which the dashed curves are within the dotted acceptance limits.

The linearity of the model is demonstrated when the absolute β -expectation tolerance limits are within the absolute acceptance limits.

Limit of detection (LOD), limits of quantification (LOQ) and dosing range:

The limit of detection (LOD) is the smallest quantity of the targeted substance that can be detected, but not accurately quantified in the sample.

There actually two ways of computing the LOD

- $LOD = LOQ/3.3$
- Using Miller ET Miller (not applicable if only one level and no repetition)
- Computation by series of the Y $LOD = \text{Intercept (0 if negative)} + 3 * \text{residual SD}$ obtained by ANOVA. Using the selected regression model, the back-calculation will give the X_{lod} for each series.
- The means of the different back-calculated X_{lod} will give the LOD of the procedure.

The application will choose the lowest value.

LOD ($\mu\text{g/mL}$) = 0.6153

The lower limit of quantification is the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy. The definition can also be applicable to the upper limit of quantification, which is the highest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy. The limits of quantification are obtained by calculation the smallest and highest concentrations beyond which the accuracy limits or b expectation limits go outside the acceptance limits.

The Dosing Range is the interval between the lower and upper limits where the procedure achieves adequate accuracy.

Lower LOQ ($\mu\text{g/mL}$) = 2.031

Upper LOQ ($\mu\text{g/mL}$) = 101.5

III. Appendix III: Supplementary Data for the results presented in Chapter 1

This section presents supplementary data supporting the published results reported in Chapter 1 of the article entitled “Optimizing Spray-Dried Liposomes for Pulmonary Delivery: Impact of Lipids Composition and of Drying Parameters using experimental design”.

Supplementary Data A:

Table 35: 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	Pdl 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

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Supplementary Data B:

Table 36: Summary of drying parameters showing significant effects on response variables (dry powder 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 ($p < 0.05$)
	Pdl	/

Supplementary Data C:

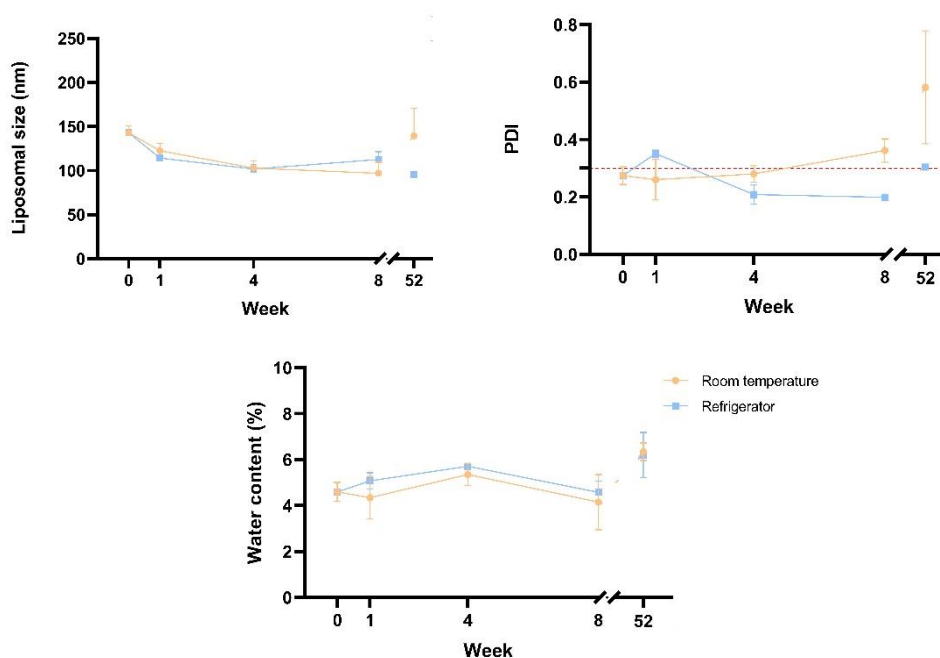


Figure 87: Long-term stability of DoE_A processed empty liposomal powders.

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Long-term stability was evaluated for empty liposomal powders produced by DoE_A under optimal processing conditions using HP β CD, 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. Pdl values remained stable during refrigerated storage, whereas a gradual increase in Pdl 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.

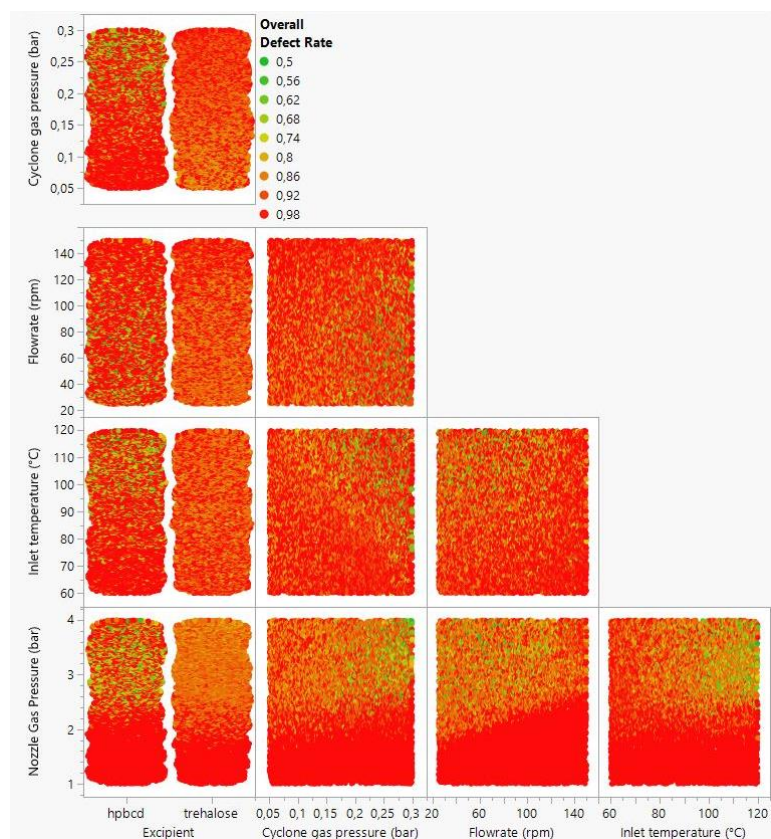
Supplementary Data D:

Figure 88: 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.

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Supplementary Data E:

Table 37: 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	Pdl
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:

Table 38: 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)
	Pdl	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 [89]. When examining the results, Figure below shows that, for non-dried liposomes, after three weeks, the release reached 93 ± 0.20 for SAL and $19.74 \pm 1.77\%$ for BUD. In contrast, in the spray-dried powders, encapsulation remained stable between time zero and three

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weeks. In addition to its drying protective effect, HP β CD also serves as an effective stabilizing agent after drying. Unlike other carbohydrates matrix such as trehalose or sucrose, HP β CD possesses a high T_g above 200 °C 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 [325,477–479].

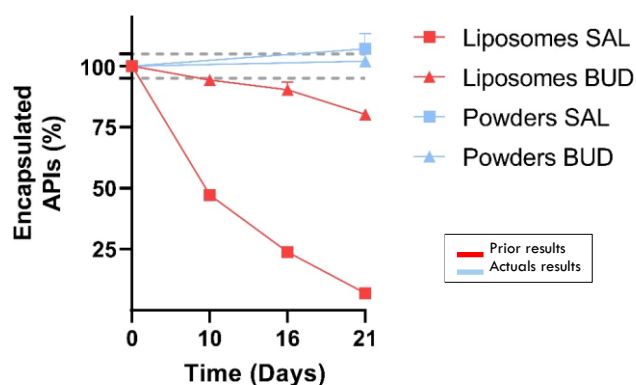


Figure 89 : Stability of liposomal formulations over time, showing the percentage of encapsulated API for PGSS-processed suspensions versus spray-dried powders under BUD-optimized conditions. Values are normalized to 100% at time zero. Analyses were performed in triplicates (n=3) and results are presented as mean $\pm$ standard deviation.

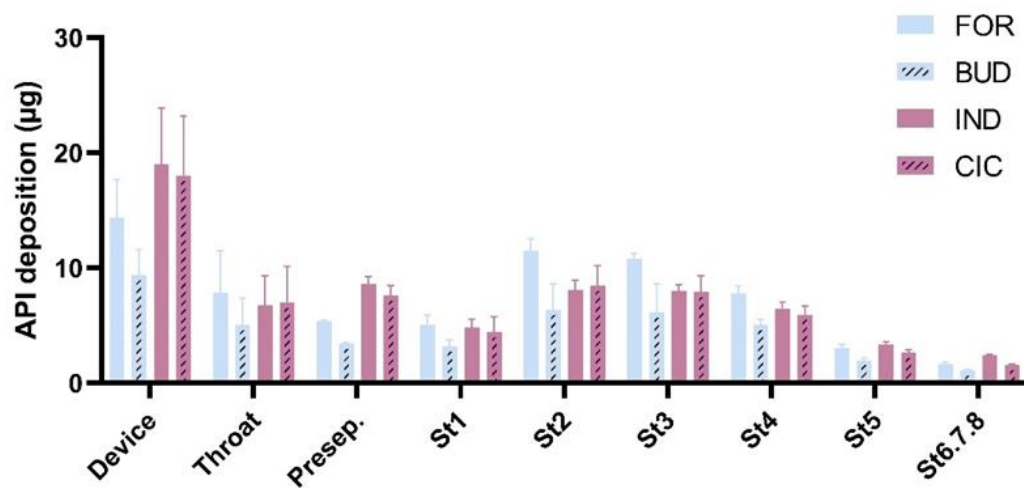
Supplementary Data H:

Figure 90: Stage-by-stage NGI deposition profiles of the different APIs at 100 L/min.

IV. Appendix IV: Supplementary Data for the results presented in Chapter 2, Part II.

This section presents supplementary data supporting the published results reported in Chapter 2, Part II of the article entitled “Formulation of a Dry Powder for Inhalation Combining Ciclesonide and Indacaterol Maleate Using Spray Drying”.

Supplementary A: PreciseInhale® DPI aerosol generator

The DPI aerosol generator of the PreciseInhale® follows a two-steps working cycle: controlled actuation and controlled exposure with monitored dosing of the generated aerosol. In the controlled actuation, the firing of the inhaler is immediately followed by the aspiration of the aerosol puff through the induction port, which is based on the design of the Andersen induction port in the US Pharmacopeia [480]. Afterwards, the aerosol rises vertically into the aerosol holding chamber (~300 mL) drawn by an inhalation air flow rate provided by a critical orifice battery and a rapidly acting pneumatic valve synchronised with the pneumatic firing of the DPI [481]. This step is crucial to enable the pre-separation of bigger particles (>5µm) in the throat-like induction port and to produce aerosols with comparable yields and particle size [393]. Once the generated pre-separated aerosol settles in the holding chamber, it can be dispensed downwards to the study subject or it can be collected on filters for analysis, after passing through the active aerosol dosing system Casella Microdust Pro (Casella CEL, Inc., Buffalo, NY, USA). The Casella is a real-time light-dispersion aerosol concentration measurement instrument, which tracks and displays graphically the aerosol levels throughout the analysis.

Supplementary B: Calculation of the Optimal Actuation Time using the PreciseInhale®

The actuation time to ideally place the inhaler puff in the 300 mL holding chamber at the chosen actuation flow rate was calculated as follows (Eq. (B.1)):

$$AT (min) = (VIT + EV) / (IF) \quad (B.1)$$

Where AT is the Actuation Time, IF is the Inhalation flow that gives 4 kPa in pressure drop (L/min), VIT is the Volume of the inductor tube (VIT = 68 mL = 0.068 L), and EJ is the Ejected volume (L), which is the volume of the PreciseInhale® holding chamber to be filled with aerosol during actuation (the ejected volume (EV, mL) needs to be 1/3 of the holding chamber volume).

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For the current experiments, the actuation time was calculated according to the over-mentioned equation (Eq. (B.2)):

$$AT (min) = (0.068 L + (1/3 * 0,3 L)) / 60 L/min = 0,168 L / (60 L/min) = 0,0028 min = 0,168 s = 168 ms$$

(B.2)

Supplementary data C:



Figure 91: The PreciseInhale® system in the dry powder inhaler setup.

Supplementary data D:**1. Ciclesonide**

Onset 354.02 °C
Endset 412.22 °C

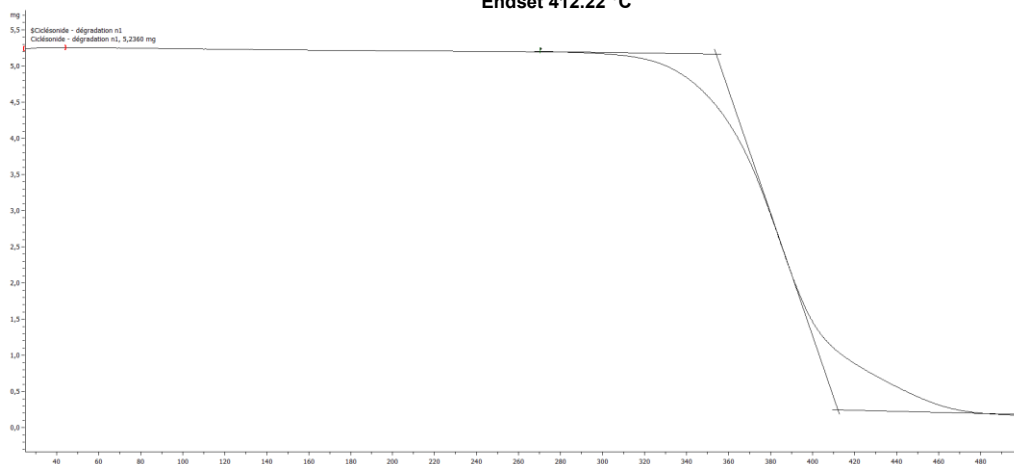


Figure 92: Degradation temperature determination of ciclesonide under increasing temperature, as assessed by the TGA system.

2. Indacaterol maleate

Onset 203.40 °C
Endset 214.22 °C

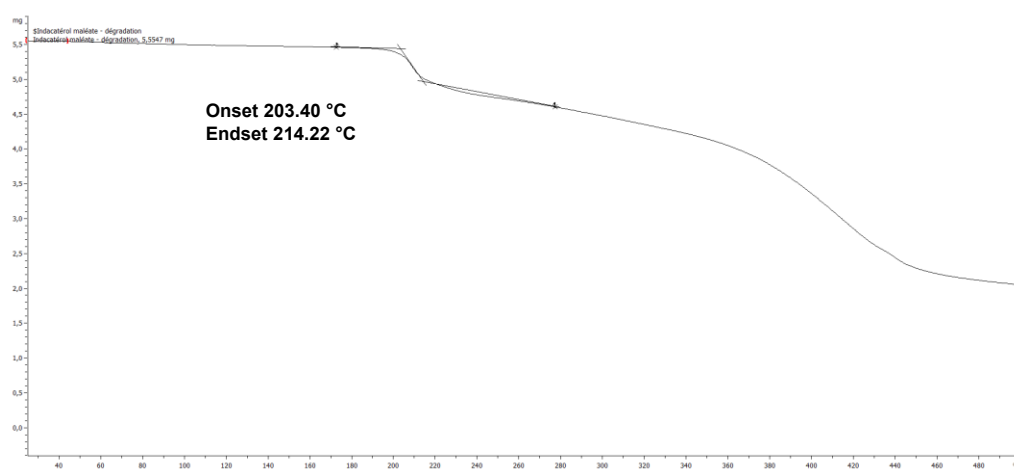


Figure 93: Degradation temperature determination of indacaterol maleate under increasing temperature, as assessed by the TGA system.

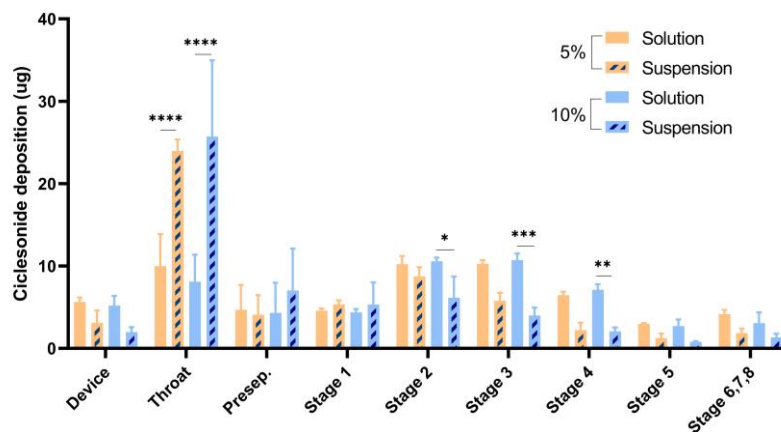
Supplementary data E:

Figure 94: Aerodynamic particle size distribution profile of ciclesonide from atomized solution and suspension, at 5 and 10% (w/v) solid content tested at optimal flow rate of 100 L/min.

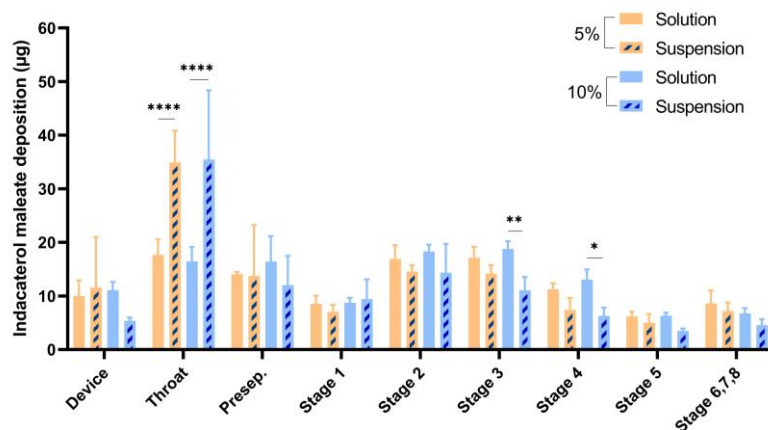


Figure 95: Aerodynamic particle size distribution profile of indacaterol maleate from atomized solution and suspension, at 5 and 10% (w/v) solid content tested at optimal flow rate of 100 L/min.

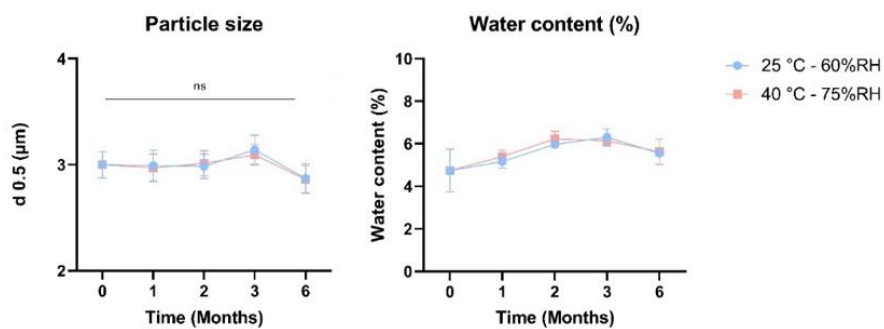
Supplementary data F:

Figure 96: Stability results in terms of particle size and moisture content of powders from the atomization of a solution (DPI-SOL_{Crysmeb}) conducted over 6 months of stability under ICH conditions. On the left, particle size stability of DPI-SOL_{Crysmeb} over 6 months under ICH conditions is presented, while on the right, the corresponding water content stability over the same period and conditions is shown.

V. Appendix V: Impurity quantification Method and Supplementary Information

Quantification method:

The analytical method described below was developed by Aquilon Pharma (industrial project partner). The evaluation of process and degradation-related impurities of CIC and IND was performed using reversed-phase high-performance liquid chromatography (RP-HPLC). Chromatographic separation was achieved on a Waters Acquity HSS T3 C18 column (2.1 × 100 mm, 1.8 µm). The column temperature was maintained at 45 °C, while the autosampler temperature was set to 20 °C. Detection was carried out using UV absorption at 254 nm. The mobile phase flow rate was 0.400 mL/min, with an injection volume of 50 µL and a total run time of 20 minutes.

The separation of the APIs from their associated impurities was achieved using a gradient elution program. Mobile phase A consisted of LC-MS grade water containing 0.1% formic acid and 0.1% triethylamine, and mobile phase B consisted of LC-MS grade acetonitrile supplemented with 0.1% formic acid and 0.1% triethylamine. The gradient was initiated at 70% A / 30% B and held for 5 minutes, followed by a linear gradient to 25% A / 75% B between 5 and 7 minutes. These conditions were maintained until 15 minutes to allow elution of late-eluting impurities, after which the system was returned to the initial conditions between 15 and 17 minutes and equilibrated until 20 minutes.

Under these conditions, the approximate retention times of the main components were 2.8 minutes for CIC and 11.3 minutes for IND, while related impurities were adequately resolved across the chromatographic window. The method demonstrated sufficient chromatographic selectivity to separate the APIs from their potential impurities and degradation products.

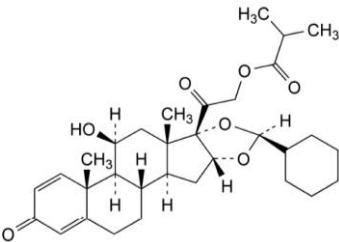
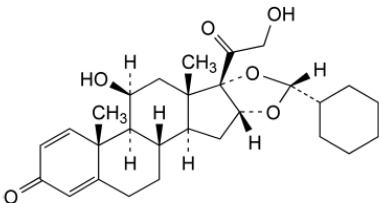
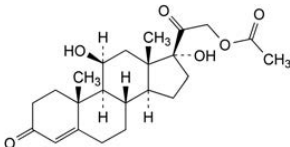
Additional Data on Impurity Studies:

Regarding impurities detection, as no monograph is currently available in either the USP or the Ph.Eur for IND (maleate), no official pharmacopoeial monograph is currently available. In contrast, the Ph. Eur monograph for CIC provides a detailed description of its related impurities, which were therefore used for the identification of CIC impurities in the present study. Among these, CIC impurity A (corresponding to the S-epimer of CIC) is classified as a process-related impurity rather than a degradation product. Consequently, its concentration is not expected to increase over time or under different storage conditions. Furthermore, due to its structural similarity to the parent compound,

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CIC and its impurity A are expected to exhibit very similar, if not identical, retention times under the chromatographic conditions employed. The impurities listed in the Ph. Eur monograph for CIC are summarized in Table 39.

Table 39: Impurities of ciclesonide (CIC) as described in the European Pharmacopoeia (Ph. Eur.) monograph.

Letter	Impurity name	Structure
Impurity A	2-methylpropanoate de (2'S)-2'-cyclohexyl-11 β -hydroxy-3,20-dioxo-16 β H-[1,3]dioxolo[4',5':16,17]pregna-1,4-dien-21-yle Synthesis impurity (S-epimer of Ciclesonide)	
Impurity B	(2'R)-2'-cyclohexyl-11 β ,21-dihydroxy-16 β H-[1,3]dioxolo[4',5':16,17]pregna-1,4-diene-3,20-dione	
Impurity C	2-methylpropanoate de (2'R)-2'-[(1RS)-cyclohex-3-enyl]-11 β -hydroxy-3,20-dioxo-16 β H-[1,3]dioxolo[4',5':16,17]pregna-1,4-dien-21-yle.	

In addition to the total impurity levels reported previously, the detected impurities of IND were characterized based on their relative retention time (RRT), defined as the ratio between the retention time of the impurity and that of the reference API. In this study, the retention time of each corresponding API was used as the reference for the calculation of all RRT values.

These impurities were quantified and reported for both APIs. However, their levels remained below 1% and therefore below the identification threshold defined by the ICH guidelines (ICH Q3B(R2)), depending on the maximum daily dose. As a result, structural identification was not required. Nevertheless, all impurities exceeding the reporting threshold (0.1%) were systematically reported and are presented in Table 40 for CIC and Table 41 for IND.

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Table 40: Impurities of ciclesonide (CIC) detected during stability studies, reported as relative retention time (RRT) and percentage content (%), in accordance with ICH Q3B(R2) reporting thresholds.

Time - 3 mois	Impurities	Conc. (%)
Refrigerated condition	Cic. Imp. B	0.43 ± 0.17
	Cic. Imp C	0.10 ± 0.00
	RRT ~0.792	0.09 ± 0.02
	RRT ~0.808	0.12 ± 0.04
	RRT ~0.830	0.05 ± 0.00
25 °C – 60% RH	Cic. Imp. B	0.46 ± 0.10
	Cic. Imp C	0.29 ± 0.11
	RRT ~0.711	0.05 ± 0.00
	RRT ~0.753	0.05 ± 0.00
	RRT ~0.792	0.08 ± 0.00
	RRT ~0.808	0.01 ± 0.01
40 °C – 75% RH	RRT ~0.830	0.59 ± 0.01
	Cic. Imp. B	0.45 ± 0.14
	Cic. Imp C	0.31 ± 0.13
	RRT~0.753	0.06 ± 0.00
	RRT ~0.783	0.05 ± 0.00
	RRT ~0.792	0.08 ± 0.02
	RRT ~0.808	0.12 ± 0.02
	RRT ~0.830	0.07 ± 0.00
	RRT ~0.847	0.06 ± 0.00
	RRT ~1.308	0.21 ± 0.19
	RRT ~1.317	0.23 ± 0.03

Table 41: Impurities of indacaterol maleate (IND) detected during stability studies, reported as relative retention time (RRT) and percentage content (%), in accordance with ICH Q3B(R2) reporting thresholds.

Time - 3 mois	Impurities	Conc. (%)
Refrigerated condition	RRT~ 0.381	0.11 ± 0.01
	RRT~ 0.399	0.08 ± 0.00
25 °C – 60% RH	RRT~ 0.378	0.18 ± 0.07
	RRT~ 0.385	0.05 ± 0.00
	RRT~ 0.398	0.11 ± 0.01
40 °C – 75% RH	RRT~ 0.382	0.20 ± 0.08
	RRT~ 0.387	0.26 ± 0.00

Additionally, comparison of chromatograms from powders with and without liposomes reveals significant interferences in liposome-containing samples, demonstrating that the analytical method is not suitable for reliable impurity detection in these formulations.

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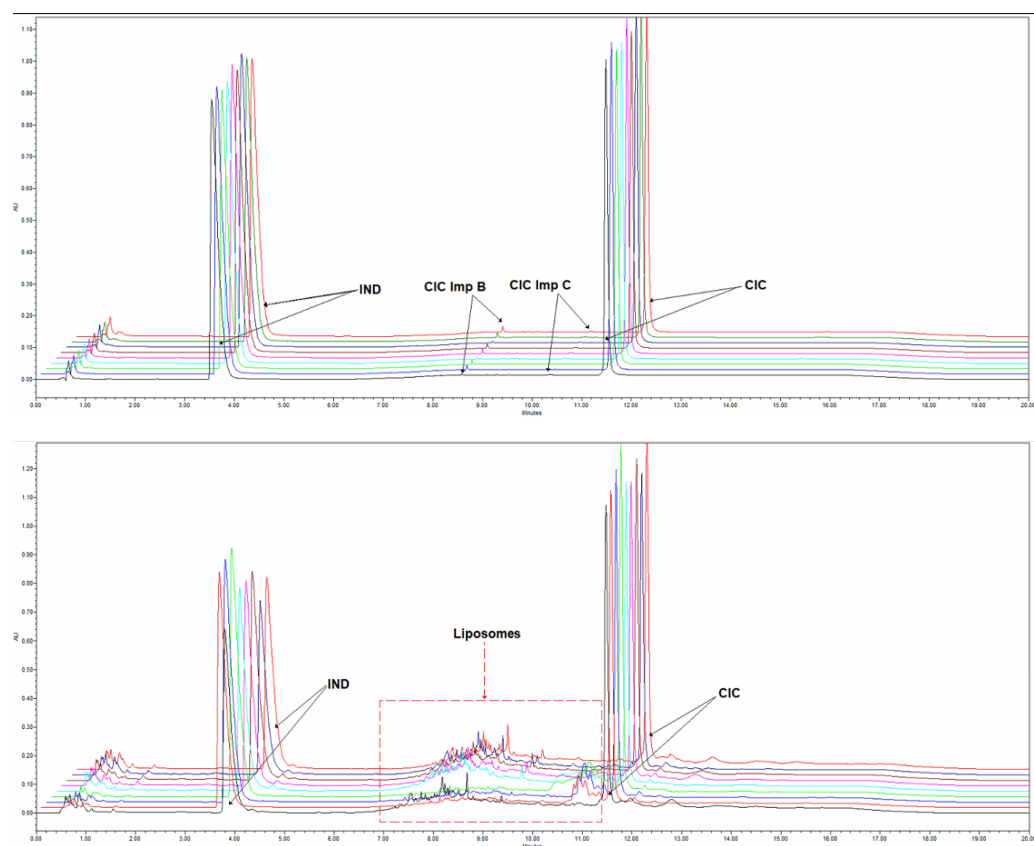


Figure 97: Chromatograms obtained from powders with and without liposomes.

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Pulmonary drug delivery is a promising strategy for treating chronic respiratory diseases, as it enables high local drug concentrations while minimizing systemic side effects. However, its effectiveness is often limited by physiological barriers that hinder drug deposition and therapeutic efficiency. To address this, liposomes have emerged as attractive carriers, enabling the encapsulation of active pharmaceutical ingredients, their protection from degradation and improved bioavailability, although their limited aqueous stability remains a major challenge.

In this context, formulating liposomes as dry powders for inhalation represents a relevant approach to enable efficient pulmonary delivery and improve stability. Spray-drying, a widely used technique to produce respirable particles, allows control over powder properties but may also compromise liposome integrity and induce premature drug leakage. This thesis therefore aimed to evaluate the feasibility of developing optimized inhalable liposome-based dry powder formulations.

A Quality by Design approach supported by Design of Experiments was implemented to assess the feasibility of spray-drying liposomes and to identify critical formulation and process parameters. This work provided fundamental insights into the impact of drying conditions and liquid feed composition on liposome integrity and powder properties. Liposome stability during spray-drying was shown to depend on a complex interplay of factors, where active ingredient properties influence susceptibility to leakage, while lipid composition, excipient environment and process conditions govern membrane response to stress and powder properties. These findings highlight the need to consider liposomal systems as integrated formulations. Additionally, optimized spray-drying conditions were identified and validated, enabling the production of inhalable powders with preserved liposome integrity, limited drug leakage and suitable aerodynamic properties, resulting in high in vitro lung deposition (FPF ~70%).

Subsequently, optimized inhalable dry powder formulations were developed, incorporating an innovative combination of ciclesonide and indacaterol for asthma treatment, which is not currently available on the pharmaceutical market in this form. The spray-drying conditions identified were successfully applied to liposomes designed to co-encapsulate this combination for pulmonary delivery, yielding inhalable powders with preserved liposome integrity, enhanced lung deposition and satisfactory stability under refrigerated conditions. In parallel, the same drug combination was formulated into non-liposomal inhalable dry powders, including a clinically relevant optimized formulation and a reference formulation matched in composition and drug content to the optimized liposome-based DPI, enabling assessment of the added value of liposomal incorporation.

Overall, this work demonstrates the feasibility of developing liposome-based inhalable dry powders, highlights the challenges associated with their formulation and provides a better understanding of the parameters governing their performance. These findings establish a solid foundation for the design of next-generation inhalable formulations and open new perspectives for the development of more efficient and targeted therapeutic strategies for the treatment of respiratory diseases.

